Sickle Cell Anaemia in Cameroon: Co-Inheritance of α-Thalassemia, *HBB* Gene Haplotypes, Clinical & Haematological Characterisations

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DECLARATION

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// ACKNOWLEDGEMENTS

"Smooth seas do not make skilful sailors."

- African Proverb

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Table of Contents

ABBREVIATIONS **10**

SUMMARY **11**

CHAPTER 1: INTRODUCTION **13**

CHAPTER 2: LITERATURE REVIEW **15**

2.1 SICKLE CELL ANAEMIA (SCA) **15**
- 2.1.1 EPIDEMIOLOGY 15
- 2.1.2 MOLECULAR AND CLINICAL MANIFESTATIONS OF SCA 16
- 2.1.4 HAPLOTYPES OF THE *HBB* GENE 22

2.2 ALPHA-THALASSEMIA (α-thalassemia) **26**
- 2.2.1 EPIDEMIOLOGY 26
- 2.2.2 MOLECULAR AND CLINICAL MANIFESTATIONS OF α-THALASSEMIA 27
- 2.2.4 ALPHA-THALASSEMIA: A GENETIC MODIFIER OF SCA 29

2.3 ROLE OF MALARIA **30**

2.4 STUDY RATIONALE: **33**

2.5 AIM AND OBJECTIVES: **34**

CHAPTER 3: MATERIALS AND METHODS **35**

3.1 ETHICAL CONSIDERATION **35**

3.2 STUDY DESIGN **35**

3.3 RESEARCHER'S ROLE **35**

3.4 RESEARCH SETTING AND CLINICAL DATA COLLECTION **36**

3.5 SAMPLE/PATIENT INFORMATION **36**

3.6 LEVEL OF PATIENT CARE **38**

3.7 HbF DETECTION **38**
- 3.7.1. ALKALI DENATURATION TEST (ADT) 39
- 3.7.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) 40

3.8 DNA EXTRACTION **40**
- 3.8.1 Rationale 40
- 3.8.2 Method 41

3.8.3 Principle 41

3.9 PHASE I: MOLECULAR DIAGNOSTIC TESTING FOR SCA (HbSS) 43
3.9.1 Rationale 43
3.9.2 Method 43
3.9.3 Principle 43
3.9.4 Expected fragment sizes 45

3.10 PHASE II: HAPLOTYPING OF THE *HBB* GENE 45
3.10.1 Rationale 45
3.10.2 Method 45
3.10.3 Principle 45
3.10.4: Expected fragment sizes 50
3.10.5: Haplotype determination 50

3.11 PHASE III: α-THALASSEMIA DELETION SCREENING 51
3.11.1 Rationale 51
3.11.2 Method 51
3.11.3 Principle 51
3.11.4: Expected fragment sizes 53

3.12 STATISTICAL ANALYSIS USING SPSS (V21, IBM, USA) 53

CHAPTER 4: RESULTS 56

4.1 MOLECULAR CHARACTERISATION OF SCA (HbSS) 56

4.2 SOCIO-DEMOGRAPHIC CHARACTERISATION OF THE STUDY COHORT 57
4.2.1: Age and gender 57
4.2.2 Ethnicity 59

4.3 CLINICAL PROFILE 60
4.3.1 Haematological parameters 60
4.3.2 Gender-based haematological indices 65
4.3.3 Clinical indices 65

4.4 HAPLOTYPES OF THE *HBB* GENE CLUSTER 66

4.5 CO-INHERITANCE: SCA-α-THALASSEMIA 73
4.5.3: Socio-demographic characterisation for the Control and Patient cohorts with (αα/αα), (αα/α3.7) and (α3.7/α3.7) 77
4.5.4: Haematological characterisation for the Control and Patient cohorts with (αα/αα), (αα/α3.7) and (α3.7/α3.7) 79

4.6 SNP GENOTYPING IN THE *HMIP*, *BCL11A, HGB XmnI-158*&*OR51B5/6* LOCI 87

CHAPTER 5: DISCUSSION 89

5.1 PRIMARY FINDINGS 89

5.2 HAEMATOLOGICAL INDICES: PATIENTS vs. CONTROLS 90

5.3 CLINICAL SEVERITY IN SCA PATIENTS 92

5.4 HAPLOTYPES OF THE *HBB* GENE 93

5.5 CO-INHERITANCE: SCA and α-THALASSEMIA 95

5.6. LIMITATIONS AND RESEARCH RECOMMENDATIONS 98

5.7. POLICY AND PRACTICAL IMPLICATIONS 100

CHAPTER 6: CONCLUSION AND PERSPECTIVES 101

APPENDIX 103

A1: INTRODUCTORY EXPLAINATION 103

A2: TEMPLATE OF THE INFORMED CONSENT DOCUMENT 105

A3: TEMPLATE OF THE QUESTIONAIRE 106

A4: CLINICAL AND EXPERIMENTAL DATA OF INDIVIDUALS IN THE STUDY COHORT 109

A5: PREPARATION OF 1XTE BUFFER 116

A6: PREPARATION OF AN AGAROSE GEL 116

A7: PREPARATION OF EtBr (0.5µg/ml) 116

A8: PREPARATION OF 1XTBE BUFFER 117

A9: PREPARATION OF LOADING DYE 117

A10: PREPARATION OF RBC LYSIS BUFFER 117

A11: PUBLICATIONS 118

REFERENCE LIST: 130

List of Figures

Figure 1: Structure of HbA 18
Figure 2: Structure of HbS 19
Figure 3: The production of HbF 20
Figure 5: The global distribution of the HBB gene haplotypes 21
Figure 6: The global distribution of the HBB gene haplotypes 24
Figure 7: The *HBB* gene region 46
Figure 8: The chromosomal arrangement of the α-globin gene cluster located on chromosome 16 . 52
Figure 9: Gel image of a diagnostic test for the detection of HbSS 56
Figure 10: Distribution curve for the age variable for the unaffected individuals (HbAA) in the study cohort 58
Figure 11: Distribution curve for the age variable for carriers (HbAS) in the study cohort 58
Figure 12: Distribution curve for the age variable for the patient cohort 59
Figure 13: Gel electrophoresis images of the restriction enzyme profiles for *HBB* gene haplotyping. 67
Figure 14: *HBB* gene haplotype distribution in SCA patients 68
Figure 15: Gel image depicting the molecular analysis of the α-thalassemia deletion screening process, post electrophoresis 73
Figure 16: Prevalence and allele frequency of the 3.7kb deletion among patients and controls 74
Figure 17: Age of diagnosis for the co-inheritance of SCA and α-thalassemia 78
Figure 18: Age of diagnosis of HbSS patients with no, one and two α-globin gene deletions at an age less than and greater than five years 79

List of Tables

Table 1: Methods of HbF detection for the study cohort 40
Table 2: *HBB* haplotyping primers 47
Table 3: Restriction endonuclease digest specifications for *HBB* gene haplotyping 49
Table 4: Restriction endonuclease cutting patterns that represent each of the five *HBB* gene haplotypes 51
Table 5: Primers for the detection of the α-globin gene deletions by multiplex gap PCR 52
Table 6: Socio-demographics; a comparison of age and gender between unaffected individuals (HbAA), carriers (HbAS) & patients (HbSS). 57
Table 7: Ethnicities of the patients in the study cohort 60
Table 8: Reference values for the haematological parameters investigated 60
Table 9: Haematological profile for the study cohort: a comparison between HbAA, HbAS, and HbSS 61
Table 10: Significance levels for the haematological profile for the study cohort: a comparison between HbAA, HbAS and HbSS 62
Table 11: Literature derived haematological profile for HbSS 64
Table 12: Demographic data for the SCA patients in the study cohort 65
Table 13: Clinical characterisation for the SCA patients in the study cohort 66
Table 14: Frequencies of the *HBB* gene haplotypes in SCA patients 68
Table 15: *HBB* gene haplotype distribution in SCA patients according to ethnicity 69
Table 16: Haematological profile for the frequently occurring haplotype combinations in the patient cohort 70
Table 17: Clinical characterisation for the frequently occurring haplotypes in the patient cohort 72
Table 18: Frequency of α-globin chain deletions in the world 76
Table 19: Haematological characterisation for the Patient cohort (HbSS): HbSS-(αα/αα), HbSS-(αα/α3.7) and HbSS-(α3.7/α3.7) 81
Table 20: Significance values for the haematological characterisation for the patient cohort (HbSS): HbSS-(αα/αα), HbSS-(αα/α3.7), and HbSS-(α3.7/α3.7) 82
Table 21: Literature-derived haematological parameters for SCA patients with α-thalassemia 84
Table 22: Clinical characterisation for the patient cohort with (αα/αα), (αα/α3.7) and (α3.7/α3.7) 86
Table 23:Effects of α-thalassemia on key clinical and haematological indices, in generalised linear regression models, adjusted for age, gender and five SNPs* that influence HbF level. 87
Table 24: Characterisation of patient information, clinical and experimental data 109

PUBLICATIONS

The following publications have emerged from this study (Appendix A11):

1. Rumaney MB, Ngo Bitoungui VJ, Vorster AA, Ramesar R, Kengne AP, Ngogang J, Wonkam A (2014). The co-inheritance of alpha-thalassemia and sickle cell anemia is associated with better hematological indices and lower consultations rate in Cameroonian patients and could improve their survival. *PLOS ONE,* 9(6):1-10. doi: 10.1371/journal.pone.0100516.
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2. Wonkam A, Rumaney MB, Ngo Bitoungui VJ, Vorster AA, Ramesar R, Ngogang J (2014). Co-inheritance of sickle cell anemia and α-thalassemia delays disease onset and could improve survival in Cameroonian's patients (Sub-Saharan Africa). *American Journal of Hematology*. 89(6):664-665. doi: 10.1002/ajh.23711.
   http://onlinelibrary.wiley.com/doi/10.1002/ajh.23711/abstract

ABBREVIATIONS

ANOVA	Analysis of variance
BMI	Body mass index (kg/m^2)
BP	Blood pressure
DBP	Diastolic blood pressure (mmHg)
F	Female
HbA	Haemoglobin (g/dl)
HbA2	Haemoglobin alpha type-2 (%)
HbF	Foetal haemoglobin (%)
HbS	Sickle haemoglobin (%)
HWE	Hardy-Weinberg Equilibrium
IMF	Intraerythrocytic multiplication factor
M	Male
Max.	Maximum
MCHC	Mean corpuscular haemoglobin concentration (g/dl)
MCV	Mean corpuscular volume (fL)
Mean	Average
Min.	Minimum
N	Number of cases
P.falciparum	*Plasmodium falciparum*
RBC	Red blood cell count (million cells/µl)
SBP	Systolic blood pressure (mmHg)
SCA	Sickle cell anaemia
SD	Standard deviation
SE	Standard error

VOC Vaso-occlusive pain crises

WBC White blood cell count $X(10)^9/L$

SUMMARY

Background: Although sickle cell anaemia (SCA) is genetically characterised by a single point mutation, patients can manifest varying degrees of clinical severity due to various genetic modulators that affect the phenotype of this disease. The co-inheritance of alpha-thalassemia (α-thalassemia) has been associated with a milder phenotype in SCA patients (e.g. lower stoke rate), but could also result in the increase of vaso-occlusive (VOC) pain episodes. There is a scarcity of data on the co-inheritance of α-thalassemia and SCA in Cameroon. The present study explored the correlation between α-thalassemia, haematological indices, and clinical events in Cameroonian SCA patients.

Materials and Methods: For this cross-sectional study, a full blood count and clinical phenotype profile was collected for 262 Cameroonian individuals. Restriction fragment length polymorphism - polymerase chain reaction (RFLP-PCR) was performed for the molecular diagnosis of SCA and for the study of the β-globin (*HBB)* gene cluster haplotypes. Multiplex Gap-PCR was performed to investigate the 3.7kb and 4.2kb α-thalassemia gene deletions.

Results: There were 178 SCA patients (HbSS), 32 carriers (HbAS) and 52 unaffected individuals (HbAA), with median ages of 18, 23 and 26 years, respectively. Among patients, 57% (101) had less than three vaso-occlusive pain crises (VOCs) per year. The median haemoglobin (HbA) level was 7.8g/dl for patients, 12.7g/dl for carriers and 13g/dl for unaffected individuals. *HBB* gene-haplotype analysis revealed the following: 42.2% (71) Benin, 14% (22) Benin/atypical, 26.8% (42) Benin/Cameroon and 5.1% (8) Cameroon haplotypes among patients and carriers.

Up to 37.1% (66) of SCA patients (HbSS) co-inherited α-globin gene deletions, compared to the 20% (10) prevalence of these gene deletions in the unaffected (HbAA) and carrier (HbAS) cohorts. Among patients, the genotype distribution was 30.3% (54) αα/α-3.7 (one 3.7kb α-globin gene deletion), 6.8% (12) α-3.7/α-3.7 (two 3.7kb α-globin gene deletions), and none had the 4.2kb deletion.

Among patients, the median red blood cell count (RBC) increased with the number of 3.7kb deletions [2.6, 3.0 and 3.4 million cells/dl, with no, one and two deletions, respectively ($p=0.01$)]. The median mean corpuscular volume (MCV) [86, 80 and 68fL, with no, one and two deletions, respectively ($p<0.0001$)] and the median white blood cell count (WBC) [13.2, 10.5 and 9.8 X 109/L with no, one and two deletions, respectively ($p<0.0001$)] decreased with an increase in the number of 3.7kb deletions. An analysis of the affect of the co-inheritance of α-thalassemia and SCA on the haematological parameters revealed a significantly lower lymphocyte and monocyte count, which is known to be associated with a better clinical phenotype. In addition, the co-inheritance of α-thalassemia was significantly associated with a delayed age of disease onset among Cameroonian SCA patients. Furthermore, after performing linear logistic regression analysis, the co-inheritance of α-thalassemia was associated with a lower consultation rate ($p=0.038$).

Conclusion: The co-inheritance of α-thalassemia with SCA was associated with an improved haematological profile, with an increase in the number of α-globin gene deletions. The possible positive effect of the co-inheritance of α-thalassemia on SCA patients' survival could explain the high proportion of α-thalassemia among SCA patients when compared to the unaffected controls. These results have implications for disease management in Cameroon in terms of genetic counselling and the detection of SCA.

CHAPTER 1: INTRODUCTION

Sickle cell anaemia (SCA) is the most common form of sickle cell disease and is a life-long genetic disease that begins during childhood (Weatherall and Clegg 2001a). A mutation in the *HBB* gene leads to the formation of aberrant haemoglobin (HbS), which causes the erythrocyte to ‘sickle’ (Weatherall and Clegg 2001b). These 'sickle cells' are rigid, which hinders their movement through blood vessels (Weatherall and Clegg 2001b). This increases the possibility of infection and leads to the blockage of blood vessels, thereby resulting in organ damage, which in turn may lead to death (Weatherall and Clegg 2001b).

On the global scale, SCA affects the tropical regions of the world, with 305,800 neonates affected annually (Modell and Darlison 2008; Weatherall 2010). The majority of cases occur in Africa, making this disease one of the biggest public health problems on the continent (Modell and Darlison 2008; Weatherall 2010; Piel et al. 2013). The dearth of early detection and treatment initiatives has resulted in an extremely high (92%) mortality rate (Piel et al. 2013). The distribution of SCA is growing due to the migration of individuals from sub-Saharan Africa to other parts of the world, like Europe and South Africa, placing a greater burden on the healthcare systems of these countries (De la Iglesia Iñigoet al. 2012; Piel et al. 2013). Treatment and management initiatives aimed at the most severely affected nations of the world could drastically reduce the SCA-induced mortality rate, thereby improving the lives of millions of children (Piel et al. 2013).

SCA is genetically characterised by a single point mutation (Pauling et al. 1949; Ingram 1957; Shelton and Schroeder 1960). However, various genetic modulators affect the phenotype, resulting in varying degrees of clinical severity manifesting in patients with the SCA (Weatherall and Clegg 2001b; Belisário et al. 2010). Increased levels of fetal haemoglobin (HbF) and the genetic loci associated with this trait influence the clinical severity of SCA (Lettre et al. 2008).

Alpha-thalassemia (α-thalassemia) is a heritable haemoglobinopathy that decreases the amount of HbA via α-globin gene deletions or a reduction in α-globin, thereby affecting the processes of oxygen transportation, resulting in anaemia, fatigue and serious, life-threatening complications (Weatherall and Clegg 1981). The co-inheritance of α-thalassemia and SCA has

been associated with a milder phenotype in SCA patients, thereby affording a protective effect against the more severe complications associated with SCA (Steinberg and Nagel 2009).

The present study aimed at exploring the prevalence of α-thalassemia and its correlation with the haematological indices and clinical manifestations in a cohort of Cameroonian SCA patients and an unaffected control cohort.

CHAPTER 2: LITERATURE REVIEW

2.1 SICKLE CELL ANAEMIA (SCA)

2.1.1 EPIDEMIOLOGY

2.1.1.1 SCA WITHIN THE GLOBAL CONTEXT

SCA is thought to have occured in humans for thousands of years (Weatherall and Clegg 2001b), with the first sickle cells found in a patient from the West Indies in the early 1900s (Herrick 1910). It is the most frequently occurring genetic disease in Africa, the Caribbean, India, the Middle East, North America, and South America (Lorey et al. 1996; Solovieff et al. 2011).

SCA is also the most frequent haemoglobinopathy present in the Middle East and Asia, specifically in the Eastern and South Western regions of Saudi Arabia (Akinsheye et al. 2011). In Bahrain, the incidence is 40.3% (Mohammad et al. 1995) and 49.4% in Jordan (Al-Rimawi et al. 2006). In the Chhattisgarh and Madhya Pradesh regions of India, the prevalence of SCA ranges from 15% to 20% (Gupta 2006). In Western Nepal, SCA has a prevalence of 5% (Modiano et al. 1991).

In Europe, SCA occurs at low frequencies (Howard and Davies 2007). In Turkey, a frequency of 3.2% was reported (Koçak et al. 1995). Most occurrences in Europe are as the result of immigrant populations from tropical regions, particularly Africa. Kéclard et al. (1996) reported a high incidence of SCA in the African immigrant French population (72.7%), which Neonato et al. (2000) confirmed. In Italy, the frequency is reported as 21.4% (Russo-Mancuso et al. 1998) and in Norway, the frequency is 16.8% (Graesdal et al. 2001).

2.1.1.2 SCA WITHIN THE AFRICAN CONTEXT

The frequency of SCA in Africa differs depending on the region under investigation (Angastiniotis and Modell 1998). In equatorial Africa, the carrier frequency is 20% of the population, whereas in North Africa, it is less than 1% of the population (Angastiniotis and Modell 1998). In sub-Saharan Africa, 240,000 neonates are affected by SCA each year (Modell and Darlison 2008; Weatherall 2010), with the occurrence of the sickle cell trait in 5% - 40% of the population (Weatherall 2008). Sudan has a prevalence of SCA that varies from region

to region (4% - 24%), with a carrier frequency of 54% (Bayoumi et al. 1985; Elderdery et al. 2008; Osman and Alfadni 2010; Munsoor and Alabid 2011). In Tanzania, 8,000 neonates are born with the sickle trait annually (Modell and Darlison 2008; Weatherall 2010). Nigeria has a carrier frequency of 2% of the population (Smith 1943; Afolayan and Jolayemi 2011; Piel et al. 2013). SCA also occurs in Senegal (Pagnier et al. 1984), Burkina Faso (Labie et al. 1984), Mauritius (Kotea et al. 1995), the Democratic Republic of Congo (DRC) (Tshilolo et al. 1996; Agasa et al. 2010; Aloni and Nkee 2014), Zambia (Beet 1947), Kenya (Foy et al. 1951; Suchdev et al. 2014), Ghana (Edington 1953), Algeria (Pagnier et al. 1984), Benin (Pagnier et al. 1984), Togo (Segbena et al. 2002) and Malawi (Brabin et al. 2004).

There is a low occurrence of SCA in South Africa (Bird et al. 1982, Ramsay et al. 1984; Bird et al. 1987). However, a new trend in the disease prevalence pattern in South Africa, specifically in Cape Town, as a result of immigrants from sub-Saharan Africa using the local healthcare facilities (Wonkam et al. 2012). There has been an increase in the number of SCA patients accessing clinical services, with three patients being seen in 2001 and 12 patients in 2009, which were seen at Red Cross Children's War Memorial Hospital (Wonkam et al. 2012). In Johannesburg, SCA occurs at a low frequency among South Africans of Indian descent (Krause et al. 2013).

2.1.2 MOLECULAR AND CLINICAL MANIFESTATIONS OF SCA

2.1.2.1 SIGNS AND SYMPTOMS

SCA manifests in several ways. A common manifestation is vaso-occlusive pain crises (VOCs), which are episodic bouts of intense pain (Darbari et al. 2012) caused by the inability of the sickled erythrocytes to navigate through the body's smaller blood vessels effectively due to their altered shape (Smith et al. 2008). Another common manisfestation is anaemia. The sickled erythrocytes have a decreased lifespan (10 to 20 days) which results in an insufficient amount of erythrocytes to transport oxygen throughout the body (Platt et al. 1994; Smith et al. 2008). This leads to lethargy, a very pale complexion, and shortness of breath (Smith et al. 2008). Anaemia can in turn hinder growth. The growth rate decreases as the body does not receive the optimal amount of oxygen for regular growth and development (Clarke and Higgins 2000). SCA patients have a lower BMI as opposed to healthy individuals of the same age and gender (Smith et al. 2008).

SCA can also cause eye irregularities, susceptibility to infection, strokes and acute chest syndrome. Eye irregularities occur because low cellular oxygen concentrations can result in damage to the eye and blindness (Elagouz et al. 2010). Infections occur because SCA patients are immune-compromised due to the damage of certain immune protecting cells. This increases their susceptibility to a host of different infections (Platt et al. 1994). Strokes are due to the sickled erythrocytes compromising the blood flow to the brain (Gladwin et al. 2004). Finally, SCA can cause acute chest syndrome, manifesting in severe chest pains and high fevers, which may lead to death (Platt et al. 1994).

2.1.2.2 MOLECULAR PATHOLOGY

2.1.2.2.1 Normal haemoglobin (HbA) (α2β2)

HbA is found in the erythrocytes of healthy adults (Engelhard 1825; Adair 1925), and is of the metalloprotein/globulin protein type (Perutz et al. 1960). The HbA tetramer (Figure 1) consists of two α-globin proteins (141 amino acids in length) and two β-globin proteins (146 amino acids in length) (Perutz et al. 1960). Attached to each chain, is an iron component called "haem'' (Engelhard 1825; Adair 1925). HbA is responsible for the transport of oxygen in the blood (Hünefeld 1840). The Hbα1 protein is the product of the *HBA1* gene, which is located on chromosome 16p13.3 (Perutz et al. 1960). The Hbα2 protein is the product of the *HBA2* gene, which is located on chromosome 16p13.3 (Perutz et al. 1960). The Hbβ protein is the product of the *HBB* gene, which is located on chromosome 11p15.5 (Perutz et al. 1960).

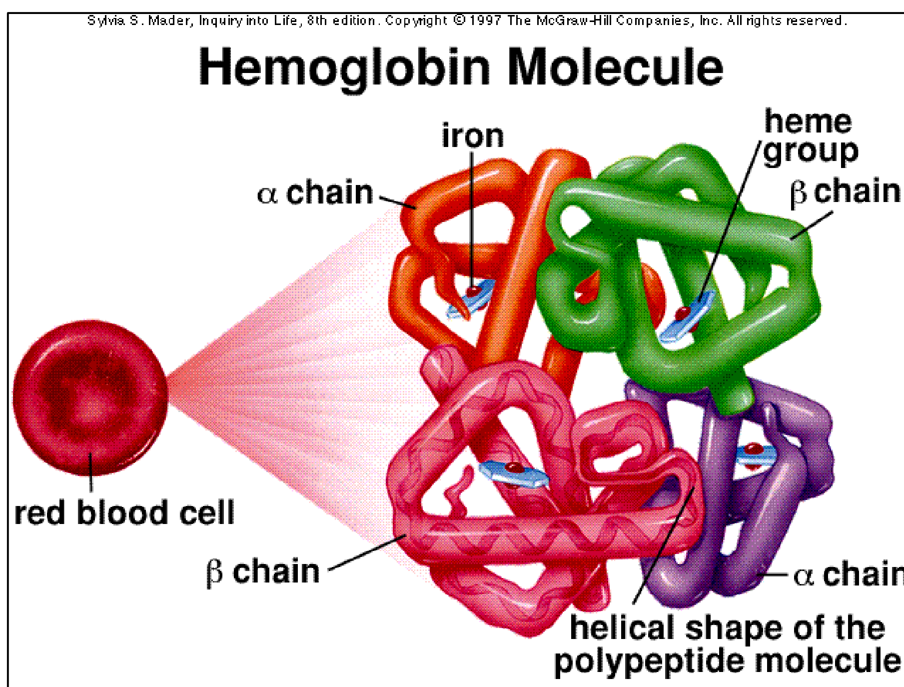


Figure 1: Structure of HbA

The healthy red blood cell contains HbA, which is a three dimentional, quaternary protein with a prosthetic group. It is composed of four polypeptide chains, two α and two β polypeptides. Each polypeptide is associated with an iron-containing haem group. The whole molecule consists of 574 amino acids. In terms of function, HbA aids the transfer of oxygen from the lungs to the rest of the body (Mader 1997).

2.1.2.2.2 Sickle Haemoglobin (HbS) (α2β^S2)

SCA is one of the first heritable, Mendelian diseases for which the fundamental genetic foundation has long been understood (Herrick 1910; Pauling et al. 1949). SCA is an autosomal recessive, monogenic condition due to a single point mutation (Figure 2; Clark and Higgins 2000; Rees et al. 2010; Alpert and Chen 2011; Rees and Gibson 2012). The transition mutation (*rs334*; c.20A>T) substitutes an Adenine with a Thymine at position 20 of the consensus coding sequence for the *HBB* gene (NM_000518.4) (NCBI 2014). This nucleotide substitution leads to an altered amino acid sequence at the sixth codon (Protein: NP_000509.1:p.Glu6Val) (NCBI 2014) of the β chain of the HbA molecule, thereby forming sickle haemoglobin (HbS; Figure 2; Clark and Higgins 2000; Rees et al. 2010; Alpert and Chen 2011; Rees and Gibson 2012; Steinberg and Sebastiani 2012). In SCA, the RBC has an elevated response to oxidative stress, which results in an abnormal haemorheological profile (Hierso et al. 2014).

Heterozygotes (HbAS) are asymptomatic and homozygotes (HbSS) are symptomatic, because the manifestation of symptoms depends on the amount of HbS. Carriers (HbA**S**) have half the amount of HbS as compared to patients (Hb**SS**; Clarke and Higgins 2000).

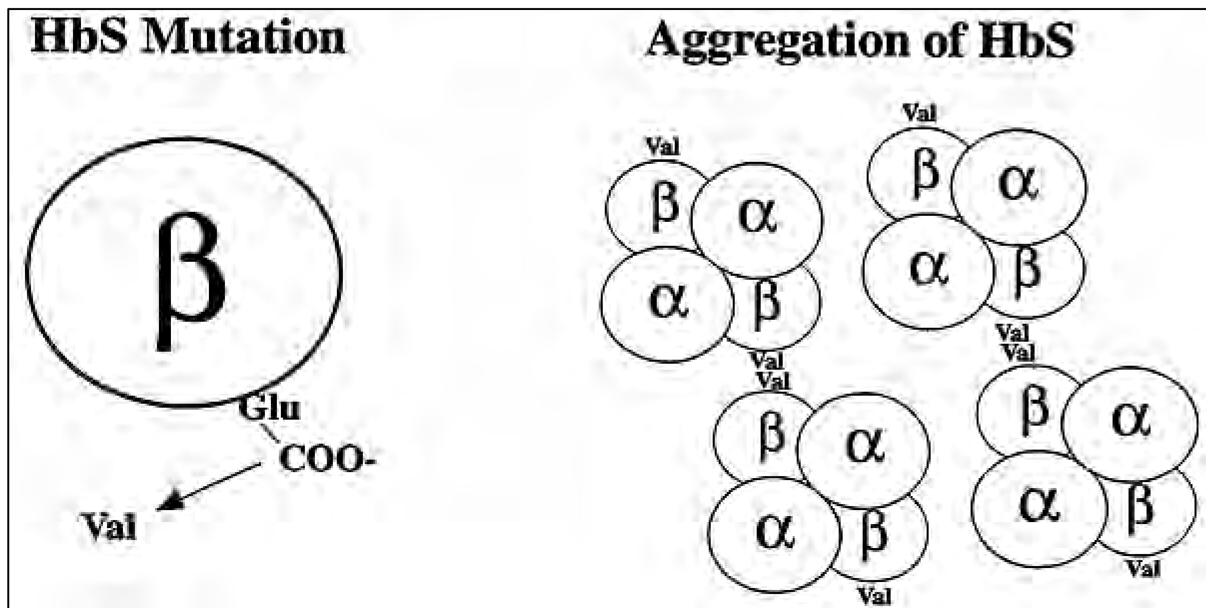


Figure 2: Structure of HbS

HbS is a mutational form of HbA, which occurs due to a point mutation, where Adenine replaces Thymine at position 20 of the nucleotide sequence (Nucleotide: NM_000518.4: c.20A>T) (NCBI 2014). This nucleotide substitution leads to an altered amino acid sequence in the sixth codon (Protein: NP_000509.1:p.Glu6Val) (NCBI 2014) of the β chain of the HbA molecule, thereby forming sickle haemoglobin (HbS). Two β^S molecules are formed, hence the structural nomenclature of α2β^S2 (Adapted from Odièvre et al. 2011; Steinberg and Sebastiani 2012).

2.1.2.2.3 Foetal Haemoglobin (HbF) (α2y2)

Due to its high affinity to bind oxygen molecules, HbF is mainly expressed in the erythrocytes during foetal life, to meet the huge metabolic demand of the developing foetus (Huisman et al. 1977; Steinberg and Sebastiani 2012). The HbF molecule contains a tetramer that is composed of two α-globin and two y-globin protein units (Huisman et al. 1977). There are two genes located on chromosome 11p15.5, *HBG1* and *HBG2,* and on chromosome 16p13.3, *HBA1* and *HBA2,* which together code for the tetrameric HbF (Figure 3; Steinberg et al. 2001).

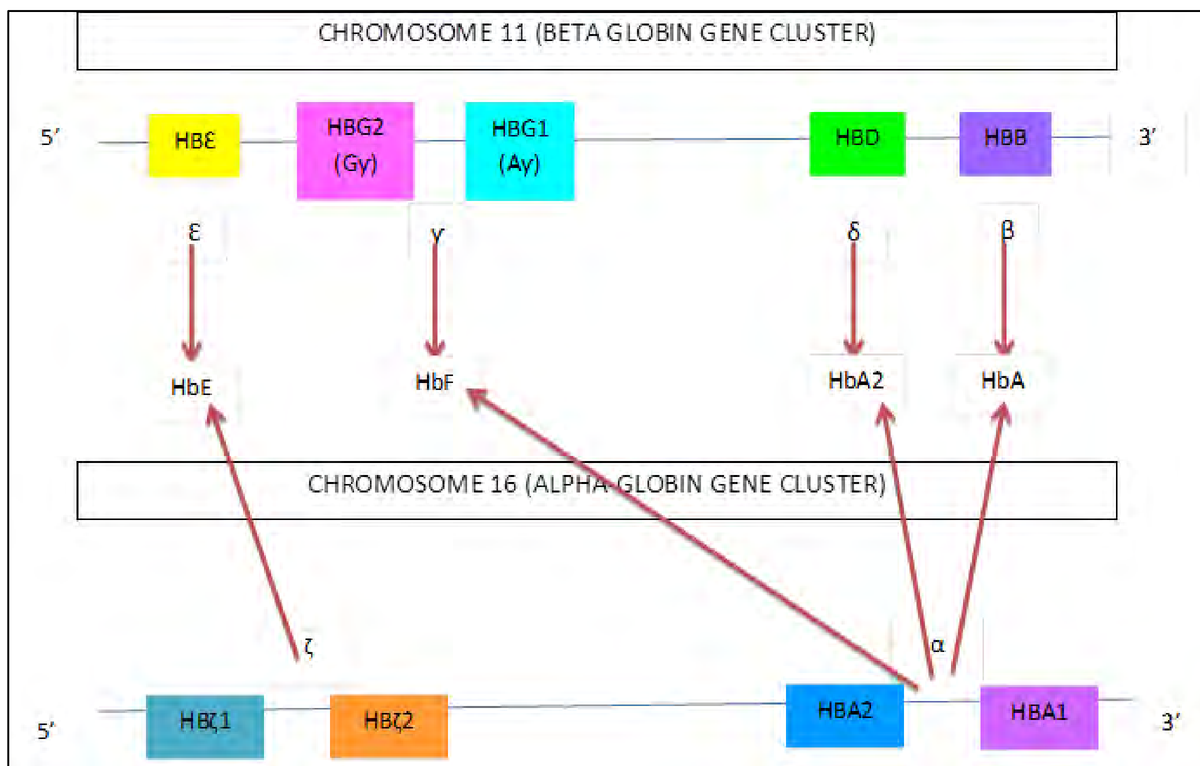
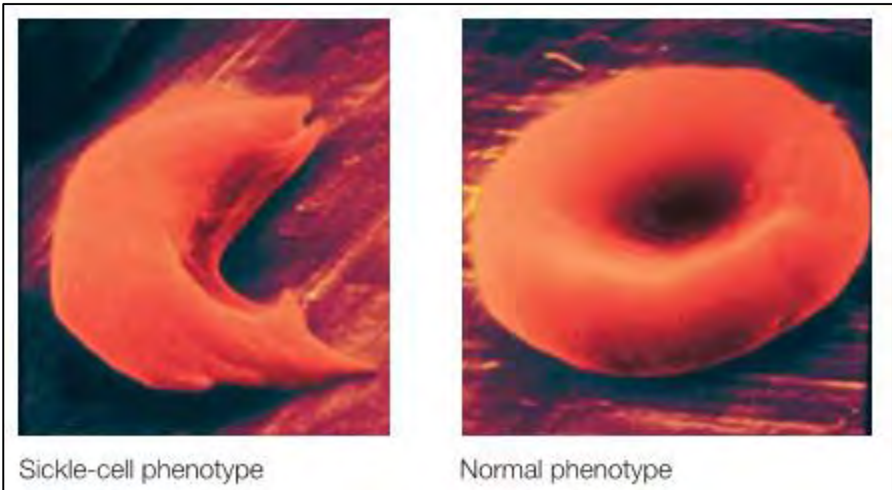


Figure 3: The production of HbF

The short arm of chromosome 11 is contains the following genes: HBE (epsilon), HBG2 and HBG1 (gamma globin cluster), HBD (delta) and HBB (beta) producing HbE, HbF, HbA2 and HbA. Chromosome 16 carries the following genes: HBζ1 and HBζ2 (zeta globin cluster), and HBA2 and HBA1 (α globin cluster), producing HbE and, HbA2 and HbA. The arrows indicate protein products from their relevant genes.

2.1.3.3 PATHOPHYSIOLOGY OF SCA

SCA occurs due to a sickle mutation (NM_000518.4: c.20A>T; NP_000509.1:p.Glu6Val) (NCBI 2014) which leads to the HbA molecule becoming HbS and losing a negative charge, thereby becoming more hydrophobic as a result of the Valine substitution (Bunn et al. 1977; Samuel et al. 1990). There is no alteration in the oxygen affinity, as the mutation exposes a hydrophobic patch in deoxy-HbA, which leads to the build-up and formation of fibres running through the entire length of the erythrocyte, leading to sickling (Figure 4; Bunn et al. 1977; Wilson et al. 1979; Samuel et al. 1990). At the outset, this process is reversible. However, with continued sickling, the erythrocytes ultimately lose their membrane plasticity, and the sickled structure remains (Bunn et al. 1977; Wilson et al. 1979; Samuel et al. 1990). Sickling results in a shortened erythrocyte lifespan (one to two days) and hinders the flow of erythrocytes throughblood vessels and tissues (Bunn et al. 1977; Wilson et al. 1979; Samuel et al. 1990). SCA leads to various clinical complications illustrated in Figure 5.



A healthy red blood cell is biconcave. As the result of SCA, the erythrocyte forms a c-shape (sickle cell) (Gabriel and Pryzbylski 2010).

Figure 4:

rise

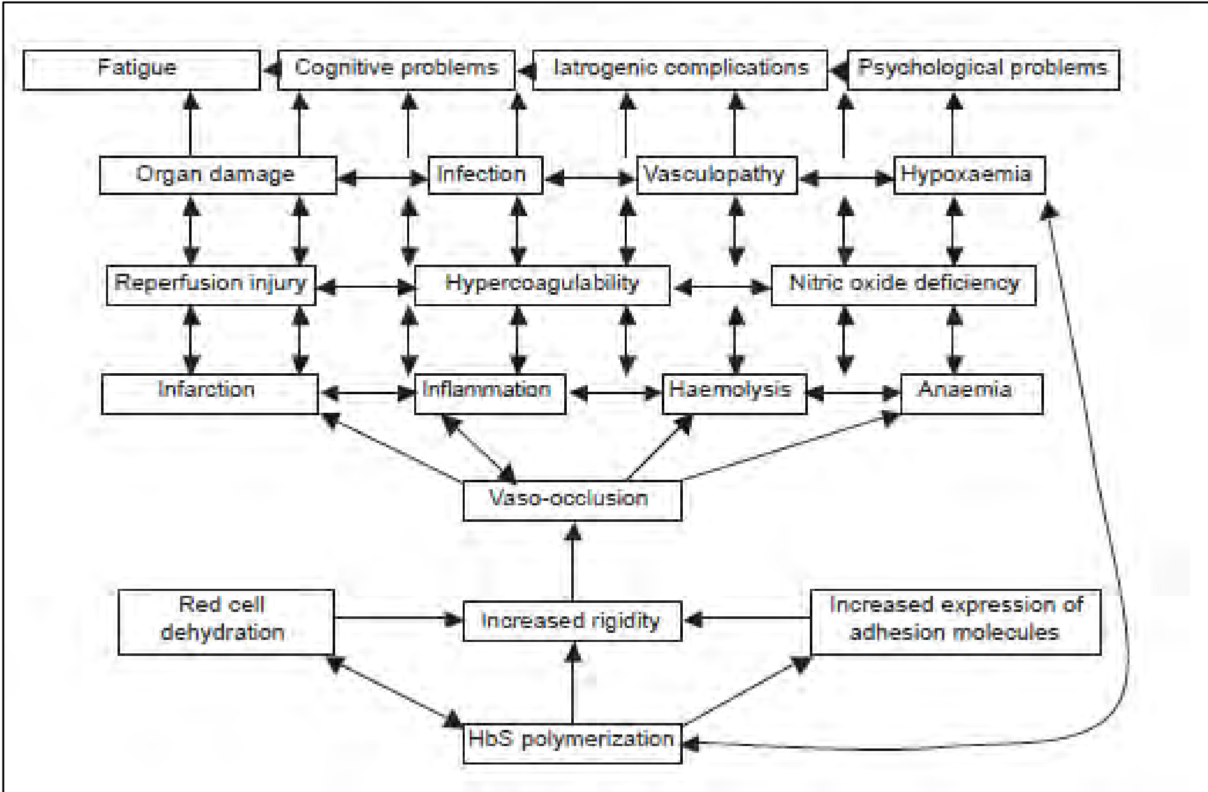


Figure 4: Complications caused by SCA

This figure depicts the multifaceted nature of SCA as a pathway of many varying complications. The polymerisation of sickle haemoglobin (HbS) leads to erythrocytic dehydration and an elevation in the manifestation of the adhesion molecules which results in an elevation in erythrocytic inflexibility. This leads to vaso-occlusion. Vaso-occlusion may lead to infarction, inflammation, haemolysis, and anaemia. Infarction may lead to reperfusion injury, organ destruction and thereby cause cognitive abnormalities and fatigue. Inflammation may lead to hypercoagulability and infection, which may also lead to cognitive abnormalities. Haemolysis leads to vasculopathy, resulting in latrogenic complications. Anaemia leads to a deficiency in nitric oxide, which leads to an abnormally low concentration of oxygen in the blood, leading to psychological

problems. Each eventuality may lead to death. The intricate network displays the complexity of the pathophysiology of SCA (Rees and Gibson 2012).

2.1.3.4 INHERITANCE PATTERN

SCA inheritance takes on an autosomal recessive pattern (Neel 1949). The disease (HbSS) will manifest in the offspring if both parents carry the “sickle trait” or are heterozygous (HbAS; Neel 1949). If each parent is a carrier, there is a 25% chance of conceiving a healthy child, a 25% chance of producing a child with SCA (HbSS), and 50% chance of having a child with the sickle cell trait (HbAS), who would be asymptomatic (Neel 1949).

2.1.4 HAPLOTYPES OF THE *HBB* GENE

The five haplotypes of clinical relevance to SCA (which were identified across the β-globin gene cluster) are the (1) Arab-/Saudi-Indian, (2) Bantu/Central African, (3) Benin, (4) Cameroon, and (5) Senegal haplotypes (Figure 6; Pagnier et al. 1984; Kulozik et al. 1986; Lapoumeroulie et al. 1992). The Bantu/Central African, Benin, Cameroon and Senegal haplotypes originated in Africa, while the Arab-/Saudi-Indian haplotype originated in Asia (Figure 6; Lie-Injo et al. 1987; Labie et al. 1989). Each haplotype confers a varying degree of clinical severity in SCA patients (Labie et al. 1985; Nagel et al. 1985; Nagel et al. 1987; Labie et al. 1989; Green et al. 1993; Nagel 1994).

2.1.4.1 The global distribution of the HBB gene haplotypes

How severely the phenotype manifests can be predicted by determining which haplotype is present, since genetic variants have an impact on the production of HbF (Steinberg and Sebastiani 2012). The Arab-/Saudi-Indian haplotype has the highest associated levels of HbF (Labie et al. 1989), followed by the Senegal and Benin haplotypes (Green et al. 1993; Nagel 1994), while the Bantu haplotype has the lowest concentration of HbF (Labie et al. 1985; Nagel et al. 1985; Nagel et al. 1987).

A study by Hattori et al. (1986), investigating a population from Georgia (USA), reported that 15% of the patients studied, carried the Senegal haplotype, 27% carried the Bantu haplotype and 54% carried the Benin haplotype. Magaña et al. (2005) reported that the Bantu (78.8%) and the Benin (18.2%) haplotypes were most prevalent in Mexico. In a later study, the occurrence of the Senegal (2.5%) and Cameroon (1.2%) haplotypes were reported in the same

population (Guzmán et al. 2010). Jones-Lecointe et al. (2008) reported that the Benin (61.8%), Bantu (17.3%), Senegal (8.5%), Cameroon (3.5%), and Arab-Indian (3.2%) haplotypes occurred in the Trinidad population. In Jamaica, the Benin haplotype is most prevalent (Ndugwa et al. 2012).

The Uruguayan population is made up of individuals of African, European, and Native American origin (Da Luz et al. 2006). Over 30% of the Uruguayan population is of African descent, more specifically from Angola, Benin, Congo, Mozambique, and Gambia (Da Luz et al. 2006). The most common haplotypes found in the Uruguayan population are the Bantu and Benin haplotypes (Da Luz et al. 2006). A study by Pante-de-Sousa et al. (1999) reported that 67% of the Brazilian cohort carried the Bantu haplotype, 30% of the cohort carried the Benin haplotype and the remaining 3% carried the Senegal haplotype. A later study by Cardoso and Guerreiro (2006), confirmed that the Bantu and the Benin haplotypes were most prevalent, and the Senegal and Cameroon haplotypes were the least frequently occurring types in the Brazilian population. Arends et al. (2000) reported that the Benin (51%), Bantu (29.5%), Senegal (12.5%), and Cameroon (2.5%) haplotypes occur in Venezuela. In Colombia, the Bantu, Benin, Senegal, and Cameroon haplotypes were found at the following respective frequencies: 55.5%, 34.8%, 4.3%, and 5.4 % (Cuéllar-Ambrosi et al. 2000).

In Southern India, the Arab-Indian haplotype is most common (91%; Labie et al. 1989). Mukherjee et al. (2004) reported that the Arab-Indian haplotype (91.5%) was most common, with the rare occurrence of the Cameroon haplotype (0.7%) in West India.

In Congo, the Bantu and Senegal haplotypes are most common (Mouélé et al. 2000). In Sudan, the Cameroon, Benin, Bantu, and Senegal haplotypes were most prevalent (Mohammed et al. 2006; Elderdery et al. 2012). Veten et al. (2012), reported that the Senegal (77.8%), Benin (8.8%), Arab-Indian (5.5%) and Bantu (4.4%) haplotypes occurred in the Mauritanian population. In Uganda, the Bantu haplotype is most prevalent (Ndugwa et al. 2012).

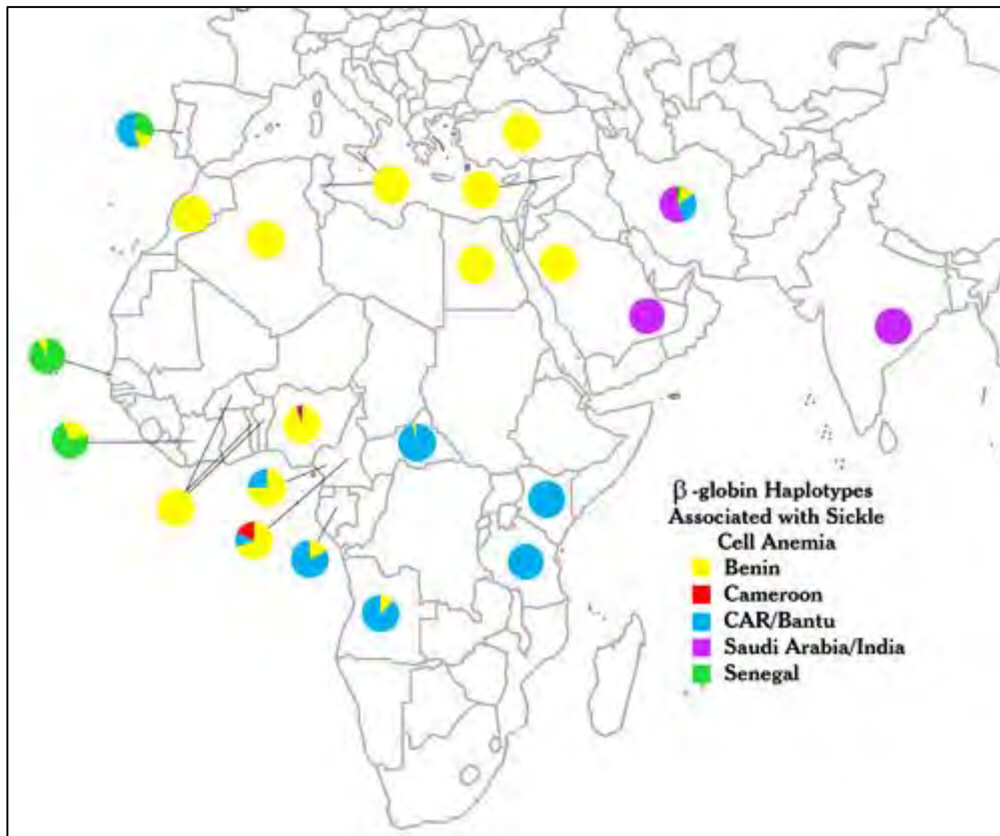


Figure 5: The global distribution of the HBB gene haplotypes

Five distinctive *HBB* gene haplotypes are linked to SCA (designated by specific colours). Each colour depicts a particular haplotype, named after its geographic region of origin. These five haplotypes are the Benin (yellow), Cameroon (red), Bantu/Central African (blue), Saudi-/Arab-Indian (purple) and Senegal (green) types. The haplotype distribution is not restricted to their countries of origin, with a broad distribution across the globe, as the result of migration (Gabriel and Pryzbylski 2010).

2.1.4.2 Genetic modifiers of SCA: HbF, haplotypes and clinical severity

HbF is a genetic modifier for SCA (Steinberg and Sebastiani 2012). The difference in HbF levels for each haplotype can be attributed to the variance in the regulation of the *HBG1* and the *HBG2* genes (Ngo et al. 2013; Lettre et al. 2008; Steinberg and Nagel 2009; Steinberg and Sebastiani 2012). High levels of HbF have a pain-preventing effect as it decreases the polymerisation of HbS, which leads to a decrease in erythrocytic sickling and vaso-occlusion in SCA patients (Darbari et al. 2012).

2.1.4.2.1 ARAB-/SAUDI-INDIAN HAPLOTYPE

The Arab-/Saudi-Indian haplotype occurs most frequently in people of Indian and Arab descent (Pagnier et al. 1984; Kulozik et al. 1986; Labie et al. 1989; Lapoumeroulie et al. 1992; Pandey et al. 2012; Alsultan 2014). The nomenclature for this SNP is NM_000184.2:c.-211C>T, (*rs7482144*), (NCBI 2014) digested by the *Xmn1* restriction endonuclease, which recognises

the T-allele (Gilman and Huisman 1985; Nagel 1994). This causes the elevated expression of *HBG2,* thereby increasing the production of HbF (Ngo et al. 2013). The *rs7482144* SNP significantly influences the phenotypic representation of SCA, where patients have high levels of HbF and present with a milder phenotype (Labie et al. 1989). A recent study by Colah et al. (2014) found that Indian SCA patients with the Arab-/Saudi-Indian haplotype do not manifest a mild form of the disease, despite their high HbF levels.

2.1.4.2.2 BANTU/CENTRAL AFRICAN HAPLOTYPE

The Bantu haplotype occurs mainly in individuals originating from Angola, the Congo, and Mozambique (Pagnier et al. 1984; Lavinha et al. 1992; Öner et al. 1992; Da Luz et al. 2006; Carvalho dos Santos et al. 2012). This haplotype is most frequent in Brazil and Tanzania (Labie et al. 1985; Nagel et al. 1985). The SNP associated with this haplotype is NM_000559.2:c.-264C>T (*rs63750033*), (NCBI 2014) recognised by the T-allele by *HincII* restriction endonuclease digest (Bouhassiera et al. 1989; Month et al. 1990). Individuals with this haplotype have low levels of HbF, which results in a severe form of SCA (Labie et al. 1985; Nagel et al. 1985; Nagel et al. 1987; Steinberg et al. 2001; Leite et al. 2011).

2.1.4.2.3 BENIN HAPLOTYPE

The Benin haplotype originated in West Africa and occurs most frequently in the Caribbean, North America, South America, Algeria, Brazil, and in the Bamileke, Bassa, Boulou, Ewondo, and Yambassa African population groups (Pagnier et al. 1984; Lapoumeroulie et al. 1992; Öner et al. 1992; Da Luz et al. 2006). NM_000184.2:c.327T>A (*rs281860625*) (NCBI 2014) is the SNP associated with the Benin haplotype, recognised by the A-allele, and digested by *HindIII* (Pagnier et al. 1984). The Benin haplotype is associated with intermediary levels of HbF where the clinical features common to this haplotype include leg ulcers and priapism (Green et al. 1993; Alsultan et al. 2012).

2.1.4.2.4 CAMEROON HAPLOTYPE

The first discovery of the Cameroon haplotype was in the Eton population located in the East of Cameroon (Lapoumeroulie et al. 1992). NM_000559.2:c.316-73G>A (*rs112599588*) (NCBI 2014) is the SNP associated with the Cameroon haplotype, recognised by the A-allele and digested by *HindIII* (Lapoumeroulie et al. 1992). Patients with the Cameroon haplotype have low HbF levels (Green et al. 1993). Infants with SCA and the Cameroon haplotype are at an

elevated risk for the development of cerebrovascular disease (Sarnaik and Ballas 2001; Lyra et al. 2005).

2.1.4.2.5 SENEGAL HAPLOTYPE

The Senegal haplotype occurs most frequently in West Africa (Lavinha et al. 1992; Öner et al. 1992). NM_000184.2:c.-211C>T, (rs7482144) (NCBI 2014) is the SNP associated with the Senegal Haplotype, which is recognised by the *HinfI* restriction endonuclease at the T allele (Month et al. 1990; Nagel 1994; Steinberg and Sebastiani 2012). Patients with this haplotype have elevated HbF levels (Green et al. 1993; Nagel 1994). High HbF levels lengthen the survival of SCA patients who have been left untreated (not on Hydroxyurea; Steinberg and Sebastiani 2012). High concentrations of HbF are linked to: (1) a decrease in the frequency of severe painful incidences, (2) fewer leg ulcers, (3) a reduction in the occurrence of severe chest disorders, (4) a protective effect against osteonecrosis, splenic sequestration and cholelithiasis, (5) a prolonged life expectancy for erythrocytes, (6) an elevation in the amount of haemoglobin, (7) increased longevity and (8) a lower risk of peri-natal death during pregnancy (Steinberg and Sebastiani 2012). A low level of HbF elevates the patient's risk of splenic sequestration, which can lead to a loss in splenic function and an increase in capillary occlusion, thereby pre-disposing the patient to retinopathy (Steinberg and Sebastiani 2012).

2.2 ALPHA-THALASSEMIA (α-thalassemia)

2.2.1 EPIDEMIOLOGY

Alpha-thalassemia affects 5% of the global population (Chui 2005; Vichinsky 2010). The global distribution of α-thalassemia corresponds to the tropical and sub-tropical regions of the world: sub-Saharan Africa, the Mediterranean (Italy and Greece), the Middle East, and parts of Asia, such as East India, South East Asia and China (Weatherall and Clegg 2001a).

2.2.1.1 Alpha-thalassemia within a global context

Alpha-thalassemia was introduced to America because of the slave trade and mainly affects African Americans (Weatherall and Clegg 2001a). Europe has a low incidence of α-thalassemia (Affronti et al. 2011; Calleri et al. 2011). High frequencies have been reported in Central and Western Nepal (83% and 73%; Modiano et al. 1991), Melanesia (68%; Flint et al. 1985), Saudi-Arabia (55%; El-Hazmi and Warsy 2000), India (50.84%; Purohit et al. 2014), France (44%;

Kéclard et al. 1996) and Venezuela (proportion not available; Arends et al. 2000). Relatively lower frequencies have been reported in Spain (1.24%; Pellicer 1969), Norway (4.7%; Graesdal et al. 2001), Portugal (5%; Peres et al. 1995), Egypt (8%; Novelletto et al. 1989) and Trinidad (17%; Jones-Lecointe et al. 2008).

2.2.1.2 Alpha-thalassemia within the African context

Alpha-thalassemia is highly prevalent in Nigeria (21%; Kotila 2012), the Congo (29%; Mouélé et al. 2000), Togo (47%; Segbena et al. 2002), Kenya (48.1%; Foote et al. 2013; Suchdev et al. 2014), Malawi (49.8%; Brabin et al. 2004), Senegal (proportion not available; Tine et al. 2012) and Burkina Faso (proportion not available Labie et al. 1984). Alpha-thalassemia occurs at low frequencies in Tunisia (2%; Zorai et al. 2002), Zimbabwe (3.3%; Chidoori et al. 1989), Gambia (12%; Allen et al. 1993), Rwanda (15.1%; Gahutu et al. 2012) and South Africa (Matthew et al. 1983; Rousseau et al. 1985). In South Africa, the 3.7kb α-globin gene deletion is most commonly reported (Krause et al. 2013). For Cameroon, there is no data available for the incidence of α-thalassemia (as per a PubMed search using the key words: “Alpha-thalassemia Cameroon”).

2.2.2 MOLECULAR AND CLINICAL MANIFESTATIONS OF α-THALASSEMIA

2.2.2.1 SIGNS AND SYMPTOMS

Patients with α-thalassemia may manifest symptoms such as a pale facial appearance, mild anaemia, constant weakness, fatigue, slowed growth, delayed puberty, poor appetite, dark urine, jaundice and osteoporosis (Higgs and Weatherall 2009).

2.2.2.2 MOLECULAR PATHOLOGY

The α-globin gene cluster is composed of genes *HBA1* and *HBA2*, and pseudo-genes *HBZP*, *HBAP1* and *HBM* located on chromosome 16p13.3 (Figure 3; Weatherall et al. 1981; Galanello and Cao 1993). Genes *HBA1* and *HBA2* are responsible for the production of α-globin, forming HbA (Weatherall et al. 1981). In a healthy human (diploid), there are four α-globin chains (Weatherall et al. 1981).

Alpha-thalassemia is categorised by a reduced production of the α-globin chain due to mutations in the *HBA1* or *HBA2* genes, which in turn leads to a decrease in the production of HbA (Weatherall and Clegg 2001; Cao and Galanello 2010). In child patients, there is an

increase in the β-globin subunits, forming haemoglobin H (HbH), and in affected infants there is an increase in the γ-globin subunits, resulting in the inability of oxygen to bind to the HbA molecule, which leads to inefficient oxygen transport throughout the body (Weatherall and Clegg 2001; Cao and Galanello 2010). Alpha-thalassemia also occurs due to a mutation where one (αα/α-, silent carrier), two (αα/- -), three (α-/- -) or four (- -/ - -) copies/alleles of the α-globin chains are deleted (Weatherall et al. 1981). There are two forms of the single-gene mutation types: α-thalassemia type-1, which is the result of a cis deletion of both α-globin genes on the same chromosome (- -/αα), and α-thalassemia type-2, a trans deletion of α-globin genes on opposite chromosomes (-α/-α; Bowden et al. 1987).

The two α-thalassemia mutations that occur most commonly are the 3.7kb and the 4.2kb α-globin gene deletions (Tan et al. 2001). These deletions are located in the *HBAP1* pseudogene (Genbank ID: 3041), affecting the *HBA2* gene (Galanello and Cao 1993).

2.2.2.3 PATHOPHYSIOLOGY

The noteworthy variety in phenotypes manifested by patients with SCA is linked to the co-inheritance of α-thalassemia, where the patient inherits two HbS mutations on both HBB genes, HbSS, along with one or more deletions of the α-globin chain (Cao et al. 1994; Ashraf 2008; Akinsheye et al. 2011).

Alpha-thalassemia is due to the quantitative haemoglobinopathies, as a result of the reduction in the α-chain production. In most cases, this is as the result of a deletion of one or more of the four α-globin genes (Culligan and Watson 2009). The consequence of these mutations and reduction in α-globin proteins, is an unbalanced excess of the β-globin chains that form tetramers which crystallise in the red blood cell, and damage them; enhancing a massive haemolysis of the developing and mature erythrocytes (Culligan and Watson 2009). Haematologically, α-thalassemia manifests as microcytic or hypochromic anaemia (Culligan and Watson 2009). Clinically, α-thalassemia may range from being asymptomatic to resulting in intra-uterine death due to severe anaemia or heart failure (Culligan and Watson 2009). The heart problems associated with α-thalassemia are myocardial infarction, ischaemia and arrhythmias (Higgs and Weatherall 2009). If these complications are not sufficiently treated, it may lead to death (Higgs and Weatherall 2009).

One or two α-globin gene deletions are classified as "α-thalassemia trait". Individuals with one deletion have a normal haematological profile and slightly lower mean corpuscular volume (MCV; Murphy 1999; Culligan and Watson 2009). Individuals with two deletions have low MCV, low mean corpuscular haemoglobin concentration (MCHC), microcytosis and, in some cases, anaemia (Murphy 1999; Culligan and Watson 2009). Individuals with three deletions may present with moderate anaemia (HbA = 7-10g/dl), splenomegaly and varying HbA2 levels (Murphy 1999). Individuals with four deleted genes do not produce α-globin and are unable to transport oxygen (Murphy 1999). In this case, infants are stillborn between 28-40 weeks or die shortly after birth (Murphy 1999). These infants are pale, oedematous and have an enlarged spleen and liver (Murphy 1999).

2.2.2.4 INHERITANCE PATTERN

Alpha-thalassemia is heritable. If both parents are heterozygous (α-thalassemia trait, having one or two α-globin gene deletions) for this condition, there will be a 25% chance of conceiving a healthy or diseased child, or a 50% chance to conceive a child that may be an asymptomatic carrier of the disease (Weatherall et al. 1981). If only one parent carries a single gene deletion and the other has no alpha-globin gene deletion, all the offspring will be asymptomatic. Here there is a 50% chance to conceive a child carrying the single gene copy deletion, while there is a 50% chance to conceive a child with no α-globin gene deletions (Weatherall et al. 1981). Varying genotypes give rise to varying phenotypes based on the amount of α-globin genes inherited (Bianchi et al. 1986; Higgs et al. 1989). The inheritance of none of the four α-globin genes (- -/- -) results in HbA Barts syndrome which leads to severe intra-uterine anaemia (Bianchi et al. 1986; Higgs et al. 1989). When one α-globin gene is inherited (- -/- α), HbH disease develops, resulting in moderately severe haemolytic anaemia (Bianchi et al. 1986; Higgs et al. 1989).

2.2.4 ALPHA-THALASSEMIA: A GENETIC MODIFIER OF SCA

Alpha-thalassemia is a genetic modifier of SCA that diminishes the severity of the disease by reducing the amount of sickled erythrocytes, increasing the HbF level, and decreasing the intracellular HbS level (Embury et al. 1982a; Embury et al. 1982b; Steinberg and Embury 1986; Galanello and Cao 1998; Belisário et al. 2010). This results in a reduction in HbS-prompted cellular destruction, thereby reducing haemolysis (Embury et al. 1982a; Embury et al. 1982b; Steinberg and Embury 1986; Galanello and Cao 1998; Belisário et al. 2010). When the mean MCV and MCHC are low, there is a reduction in intravascular sickling (Condon et al. 1983).

These patients manifest an improved clinical phenotype in the form of milder acute chest syndrome, and leg ulceration (Condon et al. 1983). Patients display a decrease in the bilirubin concentration, a lower lactate dehydrogenase (LDH) concentration, less sickled erythrocytes, a prolonged lifespan of the erythrocytes, and an increase in HbA and packed cell volume (PCV) (Steinberg and Embury 1986; Belisário et al. 2010). The extent of the protective effect depends on the amount of α-globin genes that are deleted (Steinberg and Embury 1986; Belisário et al. 2010). The presence of α-thalassemia in a SCA patient reduces the risk of stroke, silent infarction, priapism, leg ulcers, splenic sequestration, cholelithiasis, cardiovascular disease; it also plays a role in the protection of splenic and renal functioning (Belisário et al. 2010; Steinberg and Sebastiani 2012). In children, the presence of α-thalassemia lessens the risk of acute chest syndrome (Steinberg and Sebastiani 2012). The co-inheritance of α-thalassemia has also been associated with decreasing the risk of abnormal cerebral blood flow in Tanzanian SCA patients (Cox et al. 2014).

However, the findings that α-thalassemia improves the SCA phenotype remain controversial (Condon et al. 1983; Steinberg 2005; Joly et al. 2011). It has been reported that the presence of α-thalassemia in SCA patients can also have adverse effects (Steinberg and Sebastiani 2012). Alpha-thalassemia has been linked to a heightened the risk of VOCs and an elevated the risk of osteonecrosis due to higher PCV levels and red blood cell adhesion (Steinberg and Sebastiani 2012). Condon et al. (1983) reported that α-thalassemia had no effect on the more severe phenotypic characteristics such as the VOCs. In addition, Steinberg (2005) reported that α-thalassemia has little or no effect on survival and the number of VOCs. Furthermore, Joly et al. (2011) reported no positive relationship between the occurrence of α-thalassemia and a decrease in the clinical severity of the phenotypes associated with SCA in HbSS patients, which could be attributed to the length of data compilation, availability of neonatal screening, use of transcranial Doppler ultrasound to prevent vasculopathies, and the kind of *HBB* gene haplotype. These studies show that the coinheritance of α-thalassemia does not improve the SCA phenotype in all cases.

2.3 ROLE OF MALARIA

Malaria occurs in 90% of Cameroon (WHO, 2014). SCA and α-thalassemia is prevalent in areas where malaria occurs, and widely distributed throughout sub-Saharan Africa, the Middle East

and parts of India (Alison 1964; Bayouni 1987; Marsh 1992; Saraf et al. 2014). The significant overlapping in the geographical distribution of malaria, SCA and α-thalassemia, results in frequent co-inheritance (Flint et al. 1998). A survival advantage against severe malaria explains the occurrence of HbAS and α-thalassemia at high frequencies in malaria-endemic populations (Purohit et al. 2014). These disorders in the heterozygous state act as malaria resistance genes and influence the susceptibility to the malaria parasite (Purohit et al. 2014). More recently, migration patterns have led to the distribution of SCA into non-endemic malaria regions (Saraf et al. 2014). Malaria protective polymorphisms have reached high frequencies in tropical areas despite the spectrum of deleterious consequences linked to their homozygous state (Beaudry et al. 2014). Furthermore, little is known about the epidemiological and clinical consequences of the co-inheritance of SCA and α-thalassemia, which are common occurences in many parts of the malaria-endemic world. More specifically, it is the SCA trait (HbAS) that is protective against malaria (Alison 1964; Ringelhann et al. 1976, Bayouni 1987; Ojwang et al. 1987; Marsh 1992; Aluoch 1997; Albiti and Nsiah 2014; Suchdev et al. 2014). HbAS offers more protection when compared to other traits (Amoake et al. 2014). Sickle haemogobin offers a selective advantage against malaria (Amoake et al. 2014). This protective effect by the heterozygote is due to a 'balanced polymorphism', where a heterozygote has a selective advantage over a homozygote genotype (Arif 2012). The risk of contracting malaria decreases by 90% for individuals with the sickle cell trait (HbAS) (Taylor et al. 2012). This beneficial effect can be attributed to the malaria parasite exerting selection pressure on human populations to increase the presence of the HbAS gene (Liddell et al. 2014). More specifically, higher malaria mortality rates can exert selective pressure to increase the prevalence of the SCA trait (Liddell et al. 2014). Parasite density decreases in HbAS with malaria co-infection, when compared to patients with Malaria only (no SCA) (Albiti and Nsiah 2014). The mean levels of HbA, PCV, reticulocytes, platelet count, lymphocytes, eosinophils and serum iron were significantly decreased in malaria only patients when compared to HbAS indiviudals co-infected with Malaria (Albiti and Nsiah 2014). Total leukocytes, immature granulocytes, monocytes, erythrocyte sedimentation rate, transferrin saturation and serum ferritin were significantly increased in patients with Malaria only, when compared to HbAS indivuduals who were co-infected with Malaria (Albiti and Nsiah 2014).

The mechanism of protective action is not fully understood. Upon infection, the malaria parasite, *Plasmodium falciparum (P.falciparum),* is prematurely deprived of its growth medium due to the red blood cell membrane becoming instantaneously impermeable to the sporozite and/or merozite (Luzzatto et al. 1970; Aluoch 1997; Ayi et al. 2004). An increase in phagocytosis (by the monocytes) results in a breach of the red blood cell membrane with erythrocyte sickling and fragmentation; therefore, trophozoiteosis is rarely accomplished (Luzzatto et al. 1970; Roth et al. 1978; Aluoch 1997; Ayi et al. 2004). The sickled erythrocytes are detached from circulation and destroyed in the reticuloendothelial system, thereby decreasing the effect of malaria in SCA carriers (Luzzatto et al. 1970; Roth et al. 1978; Aluoch 1997; Ayi et al. 2004). *P.falciparum* may also be killed in the sickled erythrocytes due to low oxygen tensions and resultant metabolic destruction (Friedman 1978; Friedman 1979). Oxygen radical development in the erythrocytes of carriers also inhibits the growth of *P.falciparum* (Anastasi 1984).

The host immune system plays a role in the pathogenicity of the malaria parasite (Cornille-Brogger et al. 1979; Fleming et al. 1979; Fleming 1989). For a foetus, maternal antibodies provide the protection against malaria, which ceases during infancy, where the infant's immune system begins to provide the protection (Cornille-Brogger et al. 1979; Fleming et al. 1979; Fleming 1989).

Alpha-thalassemia also confers a resistance to malaria, in patients with SCA (HbSS; Flint et al. 1985), specifically protecting against severe and fatal malaria in both the heterozygous and homozygous forms (Williams et al. 2005a; Taylor et al. 2012). However, the co-inheritance of α-thalassemia in SCA carriers (HbAS) results in a 10% protection against malaria (Williams et al. 2005; May et al. 2007). Opoku-Okrah et al. (2013), reported that α-thalassemia, specifically the 3.7kb deletion, had no protective effect against malaria. The protective mechanism in this instance was due to the impaired ability of infected erythrocytes to adhere to the microvascular endothelial cells and the monocytes (Krause et al. 2012). In a more recent study, Glushakova et al (2014), showed that haemoglobin erythrocytes reduce the intraerythrocytic multiplication of *P.falciparum* thereby delaying the development of the parasite. These authors used a mathematical model to assess the effect of intraerythrocytic multiplication factor (IMF) (an in vitro assay that quantifies the number of merozites released

from an individual schizont) on malaria progression (Glushakova et al 2014). This lower IMF limits parasite density and offers a lesser clinical severity against anaemia over the first two weeks of *P.falciparum* replication (Glushakova et al 2014). However, for future research and perspective, IMF has yet to be examined in other low-index erythrocytes (Glushakova et al 2014). The mechanism by which α-thalassemia on its own protects against severe malaria remains unresolved (Opi et al. 2014).

Opi et al. (2014), reports that the negative epistasis between HbAS and α-thalassemia can be explained by host genotype-specific changes in the parasitized RBC's adhesion properties that contribute to parasite sequestration and disease pathogenesis *in vivo*. However, rather than conferring an additive protective advantage, the co-inheritance of both HbAS and α-thalassemia is associated with the loss of the malaria shield that is ordinarily afforded by each when co-inherited individually, through an unknown mechanism (Williams et al. 2005b; May et al. 2007; Crompton et al. 2008).

Despite the extraordinary protection that HbAS affords against malaria, there are few geographic regions places where the carrier frequency exceeds 25%. This is seemingly due to the rise of the mutation in populations, above 25%, being kept in check by the morbidity/mortality-disadvantage as a result of homozygosity (Grosse et al. 2011).

2.4 STUDY RATIONALE:

Despite the fact that more than 70% of SCA sufferers live in Africa (which amounts to about 13 million people; Piel et al. 2013), the understanding and management of SCA has been based on investigations in the north of Africa and in other parts of the world (Smith 1943; Embury et al. 1982a; Pagnier et al. 1984; Bayoumi et al. 1985; Steinberg and Embury 1986; Guasch et al. 1999; Da Luz et al. 2006; Belisário et al. 2010; Pandey et al. 2011). Thus, there is a dearth in data on the incidence of SCA in places like Cameroon.

The co-inheritance of α-thalassemia in Africa is also poorly understood. Alpha-thalassemia-2 is the most commonly occurring type in Africa, where it is present in 30% of individuals, with the 3.7kb α-globin gene deletion as the most frequently observed disease-causing mutation (Baysal and Huisman 1994; Da Luz et al. 2006). It has been suspected that co-inheritance of α-thalassemia and SCA correlates to a reduced morbidity and mortality rate of SCA patients

(Embury et al. 1982; Embury et al. 1982b; Steinberg and Embury 1986; Galanello and Cao 1998; Belisário et al. 2010; Steinberg and Sebastiani 2012). However, little is known about the prevalence of α-thalassemia and its effect on the clinical phenotypes in Cameroonian SCA patients.

This study will provide new data for understanding the genotype to phenotype correlation of SCA in Africa, specifically contributing towards a better understanding of the occurrence of this disease within the Cameroonian population. It will further positively contribute towards the anticipatory guidance in the healthcare of patients such as establishing and following early diagnosis, neonatal screening, protocols for optimal disease management, faster treatment initiation, and appropriate comprehensive medical care before the onset of symptoms. In addition, this study will serve as an initial attempt to gauge the influence of α-thalassemia on the clinical severity of SCA in Cameroonian patients.

2.5 AIM AND OBJECTIVES:

The aim of this project is to study the prevalence of α-thalassemia in a sample of Cameroonian SCA patients and to study the correlation between the α-thalassemia genotypes and phenotypes in SCA patients in order to:

1. Confirm the molecular diagnosis of SCA (HbSS type).
2. Describe the *HBB* gene haplotypes in the patient cohort.
3. Study the deletions of the α-thalassemia genes in SCA patients and unaffected individuals.
4. Study the correlation between the α-thalassemia genotypes, the haematological indices and the resultant clinical phenotypes.

CHAPTER 3: MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATION

The study was performed in accordance with the guidelines of the Helsinki Declaration (WMA, 2008). Ethical approval was given by the National Ethical Committee Ministry of Public Health, Republic of Cameroon N°033/CNE/DNM/07 on 23 April 2007, and by the University of Cape Town's Faculty of Health Sciences Human Research Ethics Committee (HREC; RE: 132/2010) on 19 January 2010. Written and signed informed consent was obtained from participants who were 18 years or older, while assent was obtained from the parents/guardians of the children under the age of 18 years.

3.2 STUDY DESIGN

This study was a cross-sectional, descriptive, and analytical based-study.

3.3 RESEARCHER'S ROLE

The project's journey began with the formulation of a research question. The project was designed by Professor Ambroise Wonkam, the principle supervisor. Under his supervision, individuals were recruited in Cameroon by the staff at Yaoundé Central and Laquitine Douala Hospitals, and controls from the areas surrounding these hospitals. DNA was extracted at the molecular diagnosis laboratory of the Gyneco-Obstetric and Paediatric units at Yaoundé Central Hospital. The DNA was stored and brought to Cape Town, South Africa, by Miss Valentina Josiane Ngo Bitoungui, a PhD candidate at The University of Yaoundé. All of the experimental analysis, trouble-shooting, data analysis, interpretation of the data, presented in this dissertation, and the writing up of this dissertation, was performed by Miss Maryam Bibi Rumaney, the MSc candidate working on this project towards a MSc degree in Human Genetics from the University of Cape Town. The project was co-supervised by Miss Anna Alvera Vorster, a Scientific Officer at the Division of Human Genetics at the University of Cape Town, who provided assistance on the experimental and data analysis, and Professor Raj Ramesar, the Head of the Division of Human Genetics at the University of Cape Town, who provided input on the writing of this dissertation.

3.4 RESEARCH SETTING AND CLINICAL DATA COLLECTION

The study was conducted at the Yaoundé Central Hospital, and Laquitine Douala Hospital, in Cameroon. An introductory explanation (Appendix A1) informed patients, parents, and guardians of the purpose of the study. Signed, informed consent (Appendix A2) was obtained at this stage. Socio-demographic and clinical data was collected by means of a structured questionnaire (Appendix A3). The socio-demographic questionnaire enquired about the age, gender, level of formal education, employment, household income, marital status, and residence of each participating individual. Patients' medical records were reviewed to delineate their clinical features, specifically for the confirmation of the number of hospital attendances, admissions, blood transfusions and any administration of hydroxyurea. Haemoglobin electrophoresis and the complete routine blood count of participants were performed upon arrival at the hospital. Patients were previously diagnosed as having the HbSS form of SCA, which was determined based on their medical records.

3.5 SAMPLE/PATIENT INFORMATION

The sample size was mathematically determined. The estimated prevalence of the variable of interest, the desired level of confidence and the acceptable margin of error was considered. The sample size required was calculated according to the following formula (Listen Data 2013):

Calculation:

$$n = \frac{t^2 \times p(1-p)}{m^2}$$

Where 'n' refers to the required sample size, 't'the confidence level at 95% (standard value of 1.96), 'p'the estimated prevalence in the project area and 'm' the margin of error at 5% (standard value of 0.05).

Approximately 20% of the Cameroonian population are heterozygous carriers of the α-thalassemia trait (Weatherall 2010). Thus the mutation allele frequency is estimated to be p = 0.2.

Calculation:

$$n = \frac{1.96^2 \times 0.2(1-0.2)}{0.05^2}$$
$$= 245\ individuals\ (approximately)$$

The study was composed of 262 individuals, with 52 (20%) HbAA, 32 (12%) HbAS and 178 (68%) HbSS individuals.

The control cohort (HbAA and HbAS) consisted of randomly selected individuals who were apparently healthy blood donors, and who volunteered their participation in the study. A complete haematological profile (full haematological indices and haemoglobin electrophoresis results) and socio-demographic data (age, gender, and ethnic background) was collected for these individuals.

The inclusion criteria for SCA patients consisted of (1) the confirmed molecular diagnosis of SCA by the HbSS (homozygous) variant only, (2) a complete haematological profile (full blood count and haemoglobin electrophoresis results) and (3) the availablility of clinical data (frequency of VOCs, stroke, hospital consultations and hospitalisations).

The exclusion criteria consisted of: (1) patients that have had a blood transfusion in the past six months, (2) patients who have had a clinical event requiring hospitalisation and (3) patients who have been subjected to hydroxyurea treatment (Hydroxyurea is the treatment offered to SCA patients to help prevent the formation of sickled erythrocytes. For the purpose of this study, it is important to consider patients without factors that could modify the disease.).

Individuals who were found to have any haemoglobinopathies other than the HbSS form of SCA (upon High Performance Liquid Chromatography (HPLC) screening at Haematology Laboratory of the Centre Pasteur in Yaoundé, Cameroon) were excluded from the study. One haemoglobin C (HbSC) individual and 3.5% of the individuals screened had β-thalassemia. The remaining individuals were HbAA, HbAS, or HbSS.

Education-related data was only available for 98 of the patients in the study cohort. The education levels of patients was documented, which revealed that the majority had been educated at secondary school level (N=37), followed by primary school education (N=37),

university education (N=18), and a few patients with no formal education (N=6). The Appendix contains all the information and data on the subjects that participated in the study (Appendix A4; Table 24).

3.6 LEVEL OF PATIENT CARE

The management of SCA is multidisciplinary, affecting the patient and their family members (Asnani 2010). Comprehensive care requires patient and parent data, genetic counselling, social services, infection prevention, dietary evaluation and suggestions, psychotherapy, specialist medical care, and maternal and child health care (Okpala et al. 2002).

Care is provided mainly by the public health care system in rural areas, which is composed of primary level medical facilities, district hospitals and community clinics (Echouffo-Tcheugui and Kengne 2011). Private medical care is slowly growing in the urban areas (Echouffo-Tcheugui and Kengne 2011). However, medical insurance is not widely used due to its high cost (Echouffo-Tcheugui and Kengne 2011; Wonkam et al. 2014a). The healthcare system is composed of two physicians and 16 nurses and/or midwives for every 100 000 people (Echouffo- Tcheugui and Kengne 2011). In addition, the general resource shortages result in major care being the resposibility of caregivers and family members of patients (Echouffo-Tcheugui and Kengne 2011). Late diagnosis is common due to high laboratory costs. Transport costs are a factor in determining whether consultations were upheld and hospitalisation depends on the severity of the disease (Echouffo-Tcheuguiand and Kengne 2011).

Patients were selected because of their presence at the SCA clinics from which recruitment was performed. Adults have two consultations per year and children have them more frequently.

3.7 HbF DETECTION

Two methods for HbF detection were employed in this study: (i) an Alkali denaturation test (ADT) (Betke et al. 1959; Perutz 1974), and (ii) high performance liquid chromatography (HPLC) (Shelton et al. 1979; Tietz 1990; Fucharoen et al. 1998; Guideline 1998). Both procedures were performed at the Haematology Laboratory of the Centre Pasteur in Yaoundé, Cameroon.

3.7.1. ALKALI DENATURATION TEST (ADT)

3.7.1.1 Sample preparation

The blood sample in the EDTA tube (Greiner Bio-One, Chonburi, Thailand) was rinsed thoroughly with 0.9% saline and lysed by adding 2 volumes of distilled water with 1 volume of toluene or carbon tetrachloride (CCl_4) (Sigma Aldrich, Munich, Germany). The erythrocytes were mixed using a vortex (Vortex Genie 2, New York, USA) and centrifuged for 30 minutes at 1200x*g*. The remaining clear haemoglobin solution (haemolysate) was pipetted off and retained.

3.7.1.2 Components

The following reagents and solutions were required: (1) Drabkin's solution [200mg of $K_3Fe(CN)_6$ (Sigma Aldrich, Munich, Germany) and 200mg of KCN (Sigma Aldrich, Munich, Germany) in 1L of distilled water], (2) 1.2M NaOH (4.8g in 100ml distilled water) (Sigma Aldrich, Munich, Germany), and (3) $(NH_4)_2SO_4$ saturated solution (706g in 1L distilled water, which was heated to dissolve, and then slowly cooled to room temperature - 22°C).

3.7.1.3 Method

The haemolysate (0.6ml) was added to the Drabkin's solution (10ml), which produced a cyanmet solution. Then, 2.8ml of this solution was added to 0.2ml of the alkali solution (NaOH) and mixed using a vortex (Vortex Genie 2, New York, USA). After 2 minutes, 2ml of the $(NH_4)_2SO_4$ solution was added. The solution was mixed using a vortex (Vortex Genie 2, New York, USA) and left to precipitate for 10 minutes. The precipitate was removed by filtration, by using a double layer of filter paper (Whatman no. 6 or 42, GE, Connecticut, USA) and the optical density of the filtrate at 415nm was determined using a Nanodrop (ND-1000 Spectrophotometer, Thermo-fischer Scientific, Delaware, USA).

A control solution was prepared by mixing 1.4ml of the cyanmet solution, 1.6ml of distilled water and 2ml of saturated $(NH_4)_2SO_4$. This solution was diluted, in a 1:10 ratio, with distilled water to obtain a suitable optical density.

The following calculation was performed to obtain the percentage of HbF:

$$\%HbF = \frac{\text{OD TEST SAMPLE AT 415MM X 100}}{\text{OD CONTROL SAMPLE AT 415MM X 20}}$$

3.7.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is an automated process. The CLHP Bio-Rad D-10 analyser (Bio-Rad, California, USA) was used to establish the types of haemoglobin present in the study cohort. A blood sample from the EDTA collection tube (Greiner Bio-One, Chonburi, Thailand) was loaded onto the cartridge and results were produced within 6 minutes. The CLHP Bio-Rad D-10 analyser functions on the principle that samples are automatically diluted and injected into the analytical cartridge. A gradient is established, where haemoglobin types are separated according to their ionic correlation, to the material of the cartridge. The separated haemoglobins are measured for changes in absorbance at 415nm. The built-in software generates a report.

Table 1: Methods of HbF detection for the study cohort

	ADT		HPLC		TOTAL
	N (%)	**Mean**	**N (%)**	**Mean**	**N (%)**
HbAA	62 (87.3)	7.9	9 (12.7)	1	71 (100)
HbAS	18 (66.7)	7.6	9 (33.3)	1	27 (100)
HbSS	100 (72)	15.9	39 (28)	9.5	139 (100)

The two methods of HbF detection that were employed in this study were the Alkali denaturation test (ADT) and high performance liquid chromatography (HPLC). The number of cases (N) as a percentage and the mean HbF level (%) is shown for controls (HbAA and HbAS) and patients (HbSS). HbF data was available for 237 individuals. The ADT method of detection (haemoglobin electrophoresis acid or basic media) was most frequently used.

3.8 DNA EXTRACTION

3.8.1 Rationale

DNA was extracted from peripheral blood lymphocytes. High yield and good quality allows for easy experimental analysis.

3.8.2 Method

DNA extraction was performed using two methods – the Gentra Purgene Blood Kit Plus (Qiagen, California, USA) and the salting out method (Miller et al. 1988) used by the laboratory where the experimental analysis was performed (University of Cape Town, Division of Human Genetics, Cape Town, RSA).

3.8.3 Principle

The general principle for DNA extraction is through the separation of white blood cells from whole blood by way of partial erythrocytic destruction. In an anionic environment, the white blood cells are broken down and the proteins are removed by precipitation. The purified DNA is recovered by ethanol precipitation. The resultant DNA falls within A260/A280 ratio between 1.7 and 1.9. The DNA can be safely stored at 2°C, -8°C, -20°C or -80°C.

3.8.3.1 GENTRA PURGENE BLOOD KIT PLUS (QIAGEN, CALIFORNIA, USA)

Blood samples were collected in 10ml Vacuette EDTA tubes (Greiner Bio-One, Chonburi, Thailand). The DNA was isolated according to the manufacturer's protocol (Qiagen 2011).

Briefly, 30ml of the RBC Lysis Solution (Qiagen, California, USA) was dispensed into a 50ml centrifuge tube (Corning, New York, USA). Then 10ml of whole blood was added and mixed via inversion (10 times). The mixture was incubated for 5 minutes at room temperature (22°C), with one mixing by inversion during the incubation period. The mixture was centrifuged (Eppendorf, Hamburg, Germany) for 2 minutes at 2000x*g* to obtain a white blood cell pellet. The liquid was discarded, keeping 200µl with the suspended pellet. The pellet was vigorously mixed, using a vortex (Vortex Genie 2, New York, USA), to completely re-suspend the pellet. After that, 10ml of Cell Lysis Solution was added (Qiagen, California, USA) and mixed using a vortex (Vortex Genie 2, New York, USA) for 10 seconds. Then 3.3ml of Protein Precipitation Solution (Qiagen, California, USA) was added and mixed vigorously, using a vortex (Vortex Genie 2, New York, USA), for 20 seconds. The mixture was centrifuged (Eppendorf, Hamburg, Germany) for 5 minutes at 2000x*g*. Isopropanol (Sigma-Aldrich, Munich, Germany) was added (10ml) to a new 50ml centrifuge tube (Corning, New York, USA) and the supernatant from the former step was added. The mixture was mixed by inversion (50 times). Thereafter, the mixture was centrifuged (Eppendorf, Hamburg, Germany) for 3

minutes at 2000 x g. After the supernatant was removed and the pellet left to dry, 10ml of 70% ethanol (Sigma-Aldrich, Munich, Germany) was added to the pellet and mixed by inversion (20 times), and thereafter centrifuged (Eppendorf, Hamburg, Germany) for 1 minute at 2000 x g. The supernatant was drained and the pellet left to dry for 10min, after which 1ml of DNA Hydration Solution (Qiagen, California, USA) was added to the pellet and mixed using a vortex (Vortex Genie 2, New York, USA) for 5 seconds. Thereafter, the mixture was incubated at 65°C for 1 hour. Samples were stored at -20°C.

3.8.3.2 Salting out method (Miller et al. 1988)

Blood samples were collected in 10ml Vacuette EDTA tubes (Greiner Bio-One, Chonburi, Thailand). From the sample, 3ml were transferred to a 15ml centrifuge tube (Corning, New York, USA). Then 9ml of RBC lysis buffer (Appendix A10) was added, mixed and incubated at room temperature (22°C) for 30 minutes. The lysate was mixed every five minutes for the duration of the incubation period. This was followed by centrifugation (Eppendorf, Hamburg, Germany) for 10 minutes at 2000rpm. The supernatant was discarded and 1ml of RBC lysis solution (Appendix A10) was added to the pellet to remove excess red blood cell debries (Vortex Genie 2, New York, USA). For the lysis of the WBCs, 3ml of cell lysis solution (Appendix A10) was added to the WBC pellet and mixed using a vortex (Vortex Genie 2, New York, USA). Then 12.5µl of 20mg/ml Proteinase K (Sigma-Aldrich, Munich, Germany) and 100µl of 20% SDS (Sigma-Aldrich, Munich, Germany) was added and mixed, using a vortex (Vortex Genie 2, New York, USA). The mixture was incubated at 37°C for 3 days. For the precipitation of proteins from the lysate, 1ml of saturated 6M NaCl (Sigma-Aldrich, Munich, Germany) was added and mixed, using a vortex (Vortex Genie 2, New York, USA). This was followed by centrifugation (Eppendorf, Hamburg, Germany) for 20 minutes at 2000rpm. After that, 5ml of the supernatant was transferred to a 15ml centrifuge tube (Corning, New York, USA) containing two volumes of absolute ethanol (Sigma-Aldrich, Munich, Germany) was added, mixed by inversion (50 times) and centrifuged (Eppendorf, Hamburg, Germany) for 10 minutes at 2000rpm to precipitate the DNA from solution. The supernatant was discarded and 2ml of ice-cold 70% ethanol (Sigma-Aldrich, Munich, Germany) was added to remove excess NaCl from the DNA pellet. It was then centrifuged (Eppendorf, Hamburg, Germany) for

10 minutes at 2000rpm. The DNA pellet was air dried overnight and re-suspended in 50µl of 1 x TE buffer (Appendix A6).

3.9 PHASE I: MOLECULAR DIAGNOSTIC TESTING FOR SCA (HbSS)

3.9.1 Rationale

The HbSS genotype relating to SCA is of relevance to this study because the homozygous state results in the most common and most severe form of the disease (Kan and Dozy, 1987).

3.9.2 Method

For the molecular diagnostic testing for SCA, Restriction Fragment Length Polymorphism (RFLP) – PCR method as used by Saiki et al. (1985) was implemented with a few modifications.

3.9.3 Principle

RFLP is a method of experimental inquiry that is performed to detect an alteration in the genetic sequence arrangement that is found at a position where a restriction endonuclease *DdeI* (Promega, Madison, USA) nicks at the C▼TNA G/G ANT▲Crecognition site (Saiki et al. 1985). These restriction endonucleases are proteins that are found in bacteria (Saiki et al. 1985). These proteins are able to identify particular short DNA sequences and then cut at the selected site or sites (Saiki et al. 1985). Varying lengths of fragments are produced thereby allowing for differential analysis (Saiki et al. 1985).

3.9.3.1 PCR

Each DNA sample (re-suspended in 1XTE Buffer; Appendix A5) was measured spectrophotometrically using a Nanodrop (ND-1000 Spectrophotometer, Thermo-fischer Scientific, Delaware, USA) to obtain the concentration and purity readings of each sample. A 100ng/µl dilution of each DNA sample was generated.

A total PCR reaction volume of 25µl was prepared from sterile distilled water, 5X Colourless GoTaq Buffer (1.5mM MgCl_2; Promega, Madison, USA), dNTPs (0.2mM each; Thermo Scientific, California, USA), Forward primer (5`-ATAGACCAATAGGCAGAGAGAG-3`; 0.4µM; IDT, California, USA; Saiki et al. 1985), Reverse primer (5`-TGTCATCACTTAGACCTCACC-3`; 0.4µM; IDT, California, USA; Saiki et al. 1985), GoTaq DNA Polymerase (0.5U; Promega,

Madison, USA), and 1µl of DNA (100ng/µl). The PCR was performed using a thermal cycler (BIO-RAD T100, California, USA), under the following cycling conditions: an initial DNA denaturation step at 95°C for 5min, followed by 35 cycles, composed of a DNA denaturation step for 30sec at 95°C, a primer annealing step at 59°C for 30sec and an extension step for 2min at 72°C. This was followed by a final extension step of 7min at 72°C.

Gel electrophoresis was performed to confirm that the correct fragment sizes have been amplified (380bp). A 1.5% (w/v) agarose gel (Seakem Lonza, Basel, Switzerland; Appendix A6), with Ethidium Bromide (EtBr; 0.5ug/ml; Sigma-Aldrich, Munich, Germany; Appendix A7) as the nucleic acid stain, was prepared. The process of loading was performed as follows: 5µl of 5X loading dye (Appendix A9) was utilised for each component - 10µl of GeneRuler 100bp plus DNA ladder (0.5ug/µl; Thermo Scientific, California, USA), 25µl of the no-template control (NTC; a PCR sample that has no DNA, to detect contamination), 25µl of the positive control (a PCR sample that has a known HbSS status) and 25µl of the sample, mixed and loaded into separate wells. The electrophoretic run was performed in 1XTBE buffer (Appendix A8) for 45min at 160V. Thereafter, the gel was viewed under UV light using a protected imaging capture system (UVITech; Bath, UK) and a gel image was captured (Uvipro Gold transilluminator, Bath, UK).

3.9.3.2 RESTRICTION ENDONUCLEASE DIGEST

The purpose of the restriction endonuclease digest was to confirm the presence of the HbSS variant in the study cohort.

A restriction endonuclease digest was performed using the restriction endonuclease *DdeI* (10U/µl; Promega, Madison, USA). A total reaction mixture of 20µl was prepared using sterile distilled water, 10X Buffer D (Promega, Madison, USA), acetylated BSA (0.1ug/µl; Promega, Madison, USA), *DdeI* enzyme (0.25U; Promega, Madison, USA) and 1µl of DNA (PCR product). The mixture was incubated overnight, at 37°C, using a heating block (BIO TBD-100 BOECO, Hamburg, Germany).

Gel electrophoresis was performed by preparing a 1.5% (w/v) agarose gel (Seakem Lonza, Basel, Switzerland; Appendix A6), with EtBr (0.5ug/ml; Sigma-Aldrich, Munich, Germany; Appendix A7) as the nucleic acid stain. The process of loading was performed as follows: 5µl

of 5X loading dye (Appendix A9) was utilised for each component - 10µlof GeneRuler 100bp plus DNA ladder (0.5ug/µl; Thermo Scientific, California, USA), 20µl of the no-template control (NTC; a digested sample that has no DNA, to detect contamination), 20µl of each positive control (confirmed HbAA, HbAS and HbSS samples) and 20µl of the digested PCR sample, mixed and loaded into separate wells. The electrophoretic run was performed in 1XTBE buffer (Appendix A8) for 45min at 160V. Thereafter, the gel was viewed under UV light using a protected imaging capture system (UVITech, Bath, UK) and a gel image generated (Uvipro Gold transilluminator, Bath, UK).

3.9.4 Expected fragment sizes

Expected fragment sizes for HbAA was one band of 192bp, for HbAS two bands of 192bp and 380bp, and for HbSS one band of 380bp. Three fragments are produced which are 10bp, 188bp and 192bp. The 10bp fragment is too small to detect via agarose gel electrophoresis. The 188bp and 192bp fragments are close in size and were therefore seen as one band on the gel.

3.10 PHASE II: HAPLOTYPING OF THE *HBB* GENE

3.10.1 Rationale

The *HBB* gene haplotypes may be used as genetic markers to infer phenotypic variations in SCA patients by correlating the genotypic data with the phenotypic data (Clarke and Higgins 2000). Since there is little data available on the frequency of these variable haplotypes in African SCA patients, more specifically, from Cameroon (Clarke and Higgins 2000), this aspect of the study will help to exclude the specific confounding genetic modifiers related to the *HBB* gene when comparing the α-thalassemia genotypes to the clinical phenotypes associated with SCA.

3.10.2 Method

The method used was RFLP-PCR by Sutton et al. (1989), with a few modifications.

3.10.3 Principle

RFLP-defined detection of the *HBB* gene haplotypes is a proven method that operates effectively in determining and analysing genetic variations that occur in the region under study. Additionally, it provides the platform for the investigation of genotype/phenotype correlations in the Cameroonian study cohort (Sutton et al. 1989).

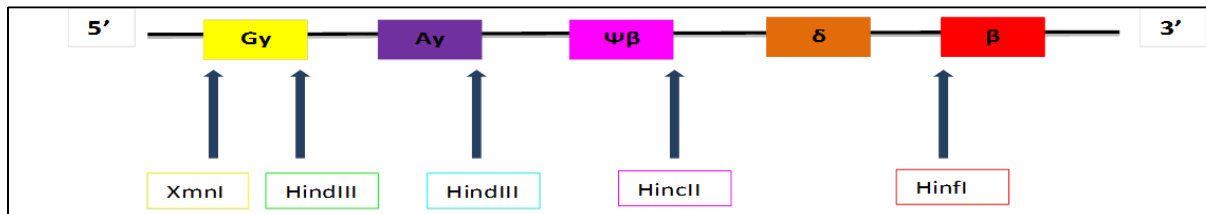


Figure 6: The *HBB* gene region

Chromosome 11 is composed of the following gene regions: *Gy (HBG2), Ay (HBG1), ψβ, δ* and *β (HBB)*. The restriction enzyme utilised on each section of the gene to determine a specific cutting pattern for haplotype characterisation is depicted where arrows indicate the cutting site. *Xmn*I cuts the 5’Gy region, *Hind*III cuts the 3’ *Gy* region and the 3’ *Ay*, *Hinc*II cuts the 3’ *ψβ* region and *Hinf*I cuts the *5’β* region (Adapted from Sutton et al. 1989).

Table 2: *HBB* haplotyping primers

GENE REGION	PRIMER SEQUENCE (5` to 3`)		PCR THERMAL CYCLING CONDITIONS	FRAGMENT SIZE (bp)
	FORWARD	REVERSE		
5'Gy	AACTGTTGCTTTATAGGATTTT	AGGAGCTTATTGATAACTCAGAC	1 cycle of 95 °C for 5min 35 cycles of 94°C for 30sec, 55°C for 30sec, 72°C for 40sec 1 cycle of 72°C for 7min	657
Gy/Ay1	TGCTGCTAATGCTTCATTACAA	AAGTGTGGAGTGTGCACATGA	1 cycle of 95 °C for 5min 35 cycles of 94°C for 30sec, 53°C for 30sec, 72°C for 40sec 1 cycle of 72°C for 7min	780
Gy/Ay2	TGCTGCTAATGCTTCATTACAA	TAAATGAGGAGCATGCACACAC	1 cycle of 95 °C for 5min 35 cycles of 94°C for 30sec, 53°C for 30sec, 72°C for 40sec 1 cycle of 72°C for 7min	780
3'ψβ	TCTGCATTTGACTCTGTTAGC	GGACCCTAACTGATATAACTA	1 cycle of 95 °C for 5min 35 cycles of 94°C for 30sec, 55°C for 30sec, 72°C for 40sec 1 cycle of 72°C for 7min	614
5'β	CTACGCTGACCTCATAAATG	CTAATCTGCAAGAGTGTCT	1 cycle of 95 °C for 5min 35 cycles of 94°C for 30sec, 55°C for 30sec, 72°C for 40sec 1 cycle of 72°C for 7min	386

3.10.3.1 5’Gγ, Gγ/Aγ1, Gγ/Aγ2, 3’ψβ, 5’β Amplification

A total PCR reaction volume of 25µl was prepared, using sterile distilled water, 5X GoTaq green buffer (1.5Mm MgCl_2; Promega, Madison, USA), dNTPs (0.2mM each; Thermo Scientific, California, USA), Forward primer (Table 2; 0.4µM; University of Cape Town’s Department of Molecular and Cell Biology, Cape Town, RSA), Reverse primer (Table 2; 0.4µM; University of Cape Town’s Department of Molecular and Cell Biology, Cape Town, RSA), GoTaq DNA polymerase (0.5U; Promega, Madison, USA) and 1µl of DNA (100ng/µl). Thermal cycling was performed on a Bio-Rad T100 thermal cycler (Table 2; Bio-Rad, California, USA).

Gel electrophoresis was performed by preparing a 2% (w/v) agarose gel (Seakem Lonza, Basel, Switzerland; Appendix A6), with EtBr (0.5µg/ml; Sigma-Aldrich, Munich, Germany; Appendix A7) as the nucleic acid stain. The process of loading was performed as follows: 5µl of 5X loading dye (Appendix A9) was utilised for each component - 10µl of GeneRuler 100bp plus DNA ladder (0.5ug/µl; Thermo Scientific, California, USA), 25µl of the no-template control (NTC; a PCR sample that has no DNA, to detect contamination), 25µl of the positive control (a PCR sample that has a known status) and 25µl of the PCR sample, mixed and loaded into separate wells. The electrophoretic run was performed in 1XTBE buffer (Appendix A8) for 45min at 160V. Thereafter, the gel was viewed under UV light using a protected imaging capture system (UVITech; Bath, UK) and a gel image was generated (Uvipro Gold transilluminator, Bath, UK).

3.10.3.2 Restriction enzyme digestion

Each PCR fragment was digested using a specific restriction endonuclease: (1) 5’Gγ using *Xmn*I, (2) Gγ/Aγ1 and Gγ/Aγ2 using *Hind*III, (3) 3’ψβ using *HincII* and (4) 5’β using *Hinf*I.

Table 3: Restriction endonuclease digest specifications for *HBB* gene haplotyping

HBB GENE REGION	RESTRICTION ENDONUCLEASE	RECOGNITION SEQUENCE	INCUBATION TEMPERATURE (°C)	TIME	Expected fragment/s size/s (bp)
5'Gγ	*XmnI*	5'...G A A N N↓N N T T C...3' 3'...C T T N N↑N N A A G...5'	37°C	16hrs - overnight	657
Gγ/Aγ1	*HindIII*	5'...A↓A G C T T...3' 3'...T T C G A↑A...5'	37°C	20min	340; 430
Gγ/Aγ2	*HindIII*	5'...A↓A G C T T...3' 3'...T T C G A↑A...5'	37°C	20min	340; 430
3'ψβ	*HincII*	5'...G T Y↓R A C...3' 3'...C A R↑Y T G...5'	37°C	20min	470; 590
5'β	*HinfI*	5'...G↓A N T C...3' 3'...C T N A↑G...5'	37°C	20min	240

3.10.3.3.1 5'Gγ

A total reaction mixture with of 16µl was prepared consisting of sterile distilled water, 10X Buffer Tango (Thermo Scientific, California, USA), *Xmn*I enzyme (Thermo Scientific, California, USA; 0.625U) and 1µl of PCR product. The mixture was incubated according to the conditions shown in Table 3, using a thermal cycler (Bio-Rad T100, California, USA).

3.10.3.3.2 Gγ/Aγ1, Gγ/Aγ2, 3'ψβ and 5'β

A total reaction mixture of 15µl (Gγ/Aγ1, Gγ/Aγ2, 3'ψβ and 5'β) was prepared using sterile distilled water, 10X Fast Digest Green Buffer (Thermo Scientific, California, USA), a restriction endonuclease (*Xmn*I,*Hind*III, *Hinc*II and *Hinf*I enzyme, respectively; Figure 7; Thermo Scientific, California, USA; 0.333U) and 1µl of the PCR product. The mixture was incubated according to the conditions shown in Table 3, using a thermal cycler (Bio-Rad T100, California, USA).

Gel electrophoresis was performed by preparing a 2% (w/v) agarose gel (Seakem Lonza, Basel, Switzerland; Appendix A6), with EtBr (0.5µg/ml; Sigma-Aldrich, Munich, Germany; Appendix A7) as the nucleic acid stain. The process of loading was performed as follows: 5µl of 5X loading dye (Appendix A9) was utilised for each component - 10µl of GeneRuler 100bp plus DNA ladder (0.5µg/µl; Thermo Scientific, California, USA), 25µl of the no-template control (NTC; a digested sample that has no DNA, to detect contamination), 25µl of the positive control (an uncut PCR sample) and 25µl of the digested sample, mixed and loaded into separate wells. The electrophoretic run was performed in 1XTBE buffer (Appendix A8) for 45min at 160V. Thereafter, the gel was viewed under UV light using a protected imaging

capture system (UVITech, Bath, UK) and a gel image was generated (Uvipro Gold transilluminator, Bath, UK).

3.10.4: Expected fragment sizes

The amplification of the 5'Gy region produced a 657bp fragment, Gy/Ay a 780bp fragment, 3'ψβ a 614bp fragment, and 5'β a 386bp fragment. The endonuclease digestion of Gy/Ay by *Hind*III (Thermo Scientific, California, USA) resulted in fragments of 340bp and 430bp in size, 3'ψβ by *Hinc*II (Thermo Scientific, California, USA) resulted in fragments of 470bp and 590bp, and 5'β by *Hinf*I (Thermo Scientific, California, USA) resulted in a 240bp fragment.

3.10.5: Haplotype determination

Haplotypes were determined based on the cutting pattern specified by Sutton et al. 1989. A profile of pluses (+) and minuses (-) were recorded based on the cutting pattern and compared to the haplotype profile table. The (+) signifies that the site was cut by the specific restriction enzyme, and the (-) signifies that it was not cut. Haplotypes were thus determined based on the 'best fit' principle.

Table 4: Restriction endonuclease cutting patterns that represent each of the five *HBB* gene haplotypes

Enzymes / Haplotypes	*XmnI* (5'Gγ)	*HindIII* (Gγ)	*HindIII* (Aγ)	*HincII* (3'Ψβ)	*HinfI* (5'β)
Senegal	+	+	-	+	+
Bantu/Central African	-	+	-	-	-
Cameroon	-	+	+	+	-
Benin	-	-	-	+	-
Arab-Indian	+	+	-	+	-

(+) = Cut by a specific restriction endonuclease; (-) = Not cut by that specific restriction endonuclease

3.11 PHASE III: α-THALASSEMIA DELETION SCREENING

3.11.1 Rationale

This aspect of the study dealt with the α-globin chain deletions, their association with the haematological indices and the clinical phenotypeof SCA patients. This is known as the co-inheritance of α-thalassemia by SCA patients.

3.11.2 Method

The method used was Multiplex Gap-PCR by Chong et al. (2000), with a few modifications.

3.11.3 Principle

The identification of the deletions found in the α-globin gene cluster on chromosome 16 by multiplex PCR is the principle upon which this section of the study was based. Primers were designed to flank the deletion breakpoints (Chong et al. 2000). The deletions were 3.7kb and 4.2kb in size (Tan et al. 2001). These are large deletions and therefore the distance between

the flanking primers were too big to amplify the "normal" allele. The product obtained was therefore much smaller than the “normal” allele, thereby indicating the presence of a deletion. The 3.7kb and 4.2kb deletions can be detected in one assay, using five different primers.

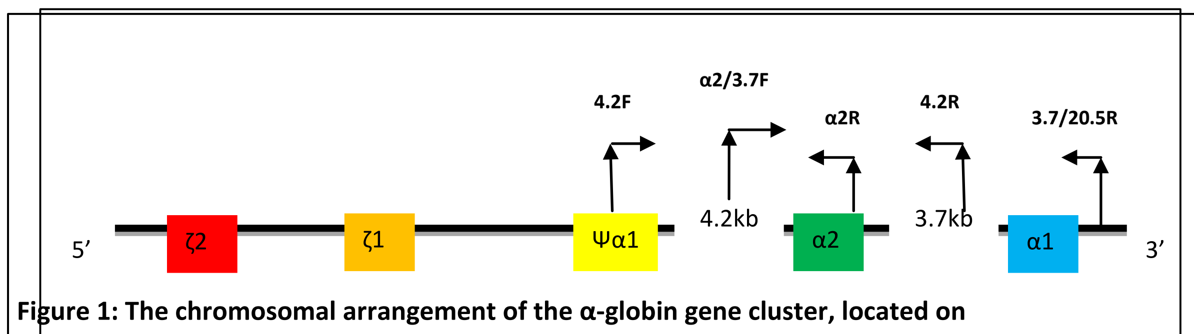


Figure 7: The chromosomal arrangement of the α-globin gene cluster located on chromosome 16

The α-globin gene cluster is located on chromosome 16 and composed of the following regions ζ2, ζ1, ψα1, α2 and α1. The primers for the detection of the 3.7kb and 4.2kb deletions are indicated by arrows. The following combinations may occur: (1) Normal/unaffected (αα/αα) produced by α2/3.7F and α2R, (2) 3.7kb homozygous deletion (α3.7/α3.7) produced by α2/3.7F and 3.7/20.5R, (3) 4.2Kb homozygous deletion (α4.2/α4.2) produced by 4.2F and 4.2R, (4) 3.7kb heterozygous deletion (αα/α3.7) produced by the (1) and (2) primer combination, and (5) 4.2kb heterozygous deletion (αα/α4.2) produced by the (1) and (3) primer combination.

3.11.3.1 Multiplex gap-PCR

Multiplex gap PCR was used to detect the α-globin gene deletion mutations present in the study population. This technique is based upon amplification using five oligo-primers flanking known deletion breakpoints, thereby generating a unique amplicon that will be smaller in the mutant sequence compared with the wild type. The presence or absence of a PCR product is detected by electrophoresis.

Table 5: Primers for the detection of the α-globin gene deletions by multiplex gap PCR

NUMBER	PRIMER	SEQUENCE (5` to 3`)
1	α2/3.7-F	CCCCTCGCCAAGTCCACCC
2	3.7/20.5-R	AAAGCACTCTAGGGTCCAGCG
3	α2-R	AGACCAGGAAGGGCCGGTG
4	4.2-R	CCCGTTGGATCTTCTCATTTCCC
5	4.2-F	GGTTTACCCATGTGGTGCCTC

A total reaction mixture of 25µl was prepared using 10X Roche Expand Long Template Buffer 3 (Roche, Mannheim, Germany), dNTPs (0.2mM each; Thermo Scientific, California, USA),

Betaine (1M; Sigma-Aldrich, Munich Germany), Expand Long Template Taq (1.875U; Roche, Mannheim, Germany), primers (0.8µM each) – (1) α2/3.7-F, (2) 3.7/20.5-R, (3) α2-R, (4) 4.2-F, and (5) 4.2-R (Table 5; University of Cape Town's Department of Molecular and Cell Biology,Cape Town, RSA), 1µl of DNA, DMSO (1.5%; Thermo Scientific, California, USA), and sterile distilled water. The PCR was performed using a thermal cycler (Bio-Rad T100, California, USA) under these cycling conditions: 1 cycle of 95°C for 5min, 35 cycles of 94°C for 45sec, 62.8°C for 1min and 15sec, 72°C for 2min and 30sec, and 1 cycle of 72°C for 5min.

Gel electrophoresis was performed by preparing a 1% (w/v) agarose gel (Seakem Lonza, Basel, Switzerland; Appendix A6), with EtBr (0.5µg/ml; Sigma-Aldrich, Munich, Germany; Appendix A7) as the nucleic acid stain. The process of loading was performed as follows: 5µl of 5X loading dye (Appendix A9) was utilised for each component: 10µl of GeneRuler 100bp plus DNA ladder (0.5µg/µl; Thermo Scientific, California, USA), 25µl of the no-template control (NTC; a PCR sample that has no DNA, to detect contamination), 25µl of the positive control (a PCR sample of known deletion status) and 25µl of the PCR sample, mixed and loaded into separate wells. The electrophoretic run was performed in 1XTBE BUFFER (Appendix A8) for 1hr to 2hrs at 160V. Thereafter, the gel was viewed under UV light using a protected imaging capture system (UVITech; Bath, UK) and a gel image generated (Uvipro Gold transilluminator, Bath, UK).

3.11.4: Expected fragment sizes

A homozygous 3.7kb deletion presents with one 2020bp band, a homozygous 4.2kb deletion presents with one 1628bp band and no deletion presents a with one 1800bp band. Heterozygous combinations may occur; 2020bp and 1800bp represents a 3.7kb heterozygote and 1628bp and 1800bp represents a 4.2kb heterozygote.

3.12 STATISTICAL ANALYSIS USING SPSS (V21, IBM, USA)

Descriptive statistics were performed for all quantitative data. Numerical data was expressed in the form of the mean, median, minimum, maximum and standard deviation (SD). Quantitative data was expressed in the form of frequencies and percentages. Distribution plots were constructed for all the qualitative variables, to assess the normality of the data (parametric vs. non-parametric). The mean was expressed for parametric data and the

median for non-parametric data. Normality was confirmed by the Shapiro-Wilk Test. The following parametric tests were used to determine the p-value: Chi-squared test for categorical variables and the t-test for scale variables. The following non-parametric tests were used to determine the p-value: Mann-Whitney U test for two samples or the Kruskal-Wallis one-way analysis of variance (ANOVA) for more than two samples. Significance was set at the 5% level, p<0.05, for an association to be deemed significant. A one-way ANOVA Tukey's Post-hoc Test was performed to assess the level of significance between and within groups. In addition, an Additive model per copy of the α-globin gene deletions was performed, as well as multinomial or linear logistic regression analysis, incorporating the SCA and α-thalassemia genotypes, age, gender, and clinical events. To correct for the skewness of the HbF distribution, a log10-transformation was applied, which normalised the data to obtain the quantitative trait used in the association analysis (after correcting for age, gender, and the electrophoresis technique). The effects of α-thalassemia on key clinical and haematological indices were investigated in generalised linear regression models, adjusted for age, sex and six SNP genotypes (always assuming log-additive genetic effects) using the R statistical package version 3.0.3 (The R Foundation for statistical computing, Vienna, Austria).

A Hardy-Weinberg Equilibrium (HWE) test was performed for the genotype results of the 3.7kb α-globin gene deletions and the ten selected SNPs. The observed 3.7kb α-globin gene deletion allele frequencies in the control group was consistent with HWE (X^2=2.37; p=0.12), equal to the SCA patients (X^2=1.69; p=0.19).

In a separate study, Wonkam et al. (2014b) reported that sequence variants at the *BCL11A* and *HBS1L-MYB* loci influence HbF levels. In addition to *BCL11A,* the *rs4671393* SNP was associated with a wider range of haematological indices, independently of the HbF levels. In addition, two SNPs in the *HBS1L-MYB* locus were associated with the number of hospitalisations (Wonkam et al., 2014b). Statistical analysis was performed to investigate the effects of α-thalassemia on the clinical and haematological indices, in relation to these above-mentioned variants. Two SNPs were excluded because of a significant violation of the HWE (*rs1188686* in *BCL11A,* HWE p-value = 0.00030; and *rs9389269* in the *HBS1L-MYB locus*, HWE p-value: 0.002876). In addition, two other SNPs were monomorphic (at *rs9376090* in the

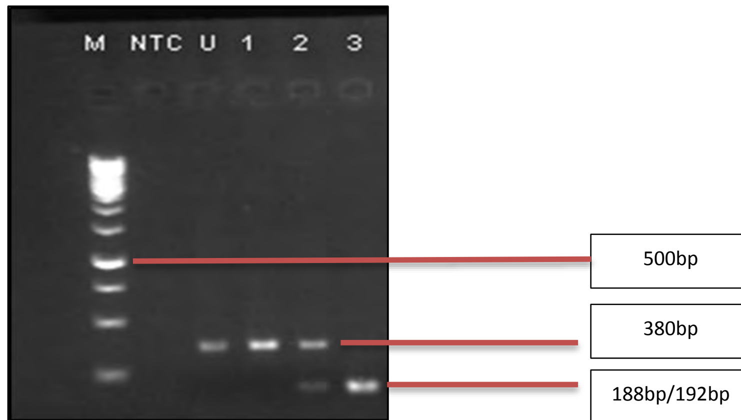
HBS1L-MYB locus, all the patients were T/T homozygous; and at *rs7482144* in the *HBG* loci, where all the patients were G/G homozygous).

CHAPTER 4: RESULTS

4.1 MOLECULAR CHARACTERISATION OF SCA (HbSS)

HbSS is the most severe form of SCA. Genotyping was essential for this study to denote individuals as HbAA (unaffected), HbAS (carriers) and HbSS (patients). The molecular detection of SCA allowed for the foundation of the study to be set and a "working" cohort to be created.

(a) Gel image for the molecular characterisation of SCA by electrophoresis



(b) An illustrataion of the *HBB* gene, digested by the *Dde1* restriction endonuclease and the fragments produced

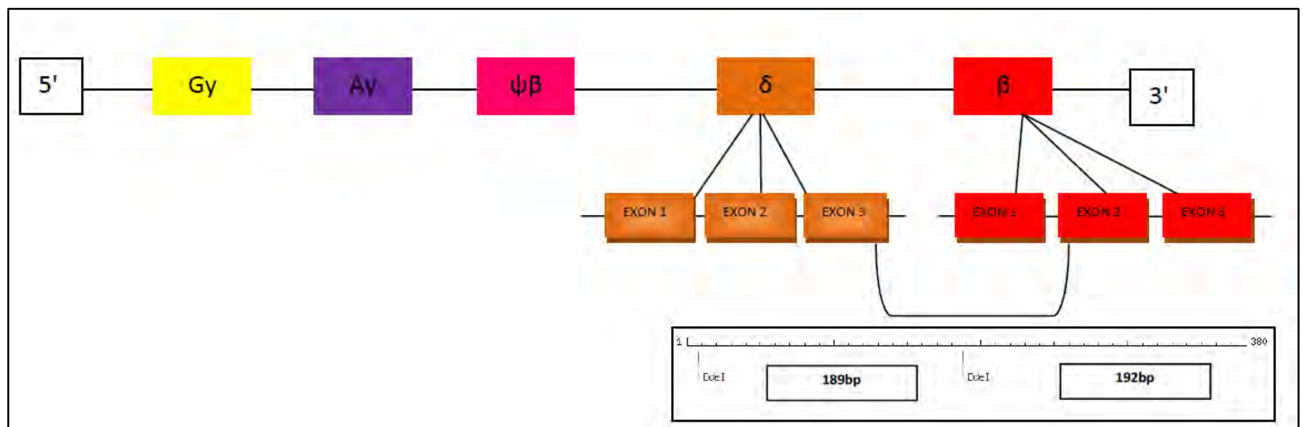


Figure 8: Gel image of a diagnostic test for the detection of HbSS

(a) M= 1Kb molecular weight marker (Bioline, USA); NTC= No-template control: clear, thereby indicating that no contamination had occurred; U= uncut DNA amplified with the diagnostic primer set (White Head Scientific, RSA): 380bp; 1-4= DNA amplified using the diagnostic primer set, then cut using *Dde*I (Promega, Madison, USA): lane 1 – HbSS at 380bp, lane 2 – HbAS at 192bp and 380bp, lane 3 – HbAA at 192bp. (b) The section of the *HBB* gene, digested by *Dde*I and the fragments produced. The gene region under study is 380bp in length, spanning the end of exon 3 of the δ region, exon 1 and the beginning of exon 2 of the β region. *Dde*I cuts twice at the 10bp and 190bp marks. Three fragments are produced which are 10bp, 188bp and 192bp. The 10bp fragment is too small to detect via agarose gel electrophoresis. The 188bp and 192bp fragments are close in size and were therefore seen as one band on the gel.

A single band represented a homozygote and two bands represented a heterozygote. The molecular detection of the banding pattern allowed for the diagnosis of individuals as unaffected (HbAA), carriers (HbAS) or patients (HbSS). Individuals were genotyped by HPLC prior to the RFLP assay, thereby acting as a validation assay. Results from the RFLP-PCR assay were compared to the results generated by HPLC haemoglobin electrophoresis, to eventually confirm the Hb SS status.

4.2 SOCIO-DEMOGRAPHIC CHARACTERISATION OF THE STUDY COHORT

The socio-demographic characterisation of the study cohort illustrated the age, gender and ethnicity of the individuals of the study cohort.

4.2.1: Age and gender

Table 6: Socio-demographics; a comparison of age and gender between unaffected individuals (HbAA), carriers (HbAS) & patients (HbSS).

		HbAA			HbAS			HbSS		
		N	Mean ± SD	Median	N	Mean ± SD	Median	N	Mean ± SD	Median
AGE (years)		47	26.0 ± 7.5	26.5	29	21.5 ± 9.8	24.0	160	19.8 ± 10.7	17.5
GENDER	MALE	14	-	-	11	-	-	75	-	-
	FEMALE	33	-	-	18	-	-	86	-	-

N = Number of cases; SD = Standard deviation

The socio-demographic characterisation for unaffected individuals (HbAA), carriers (HbAS) and patients (HbSS) in terms of age and gender was compared. The mean age for the unaffected individuals was 26.5 years (25th percentile = 23.2 years; 75th percentile = 30 years), carriers 24 years (25th percentile = 17.5 years; 75th percentile = 26 years) and 17.5 years for patients (25th percentile = 11 years; 75th percentile = 24 years). Females formed the bulk of the study cohort (70%). Post-Hoc analysis revealed the following p-values between groups for the age variable: (1) HbAA vs. HbAS p= 0.057, (2) HbAA vs. HbSS **p<0.0001** and (3) HbAS vs. HbSS p= 0.437. For the gender variable, the following p-values were revealed: (1) HbAA vs. HbAS = 0.487, 2) HbAA vs. HbSS = **0.043** and (3) HbAS vs. HbSS = 0.835. There is thus a significant difference between the age and gender distribution of unaffected individuals and patients.

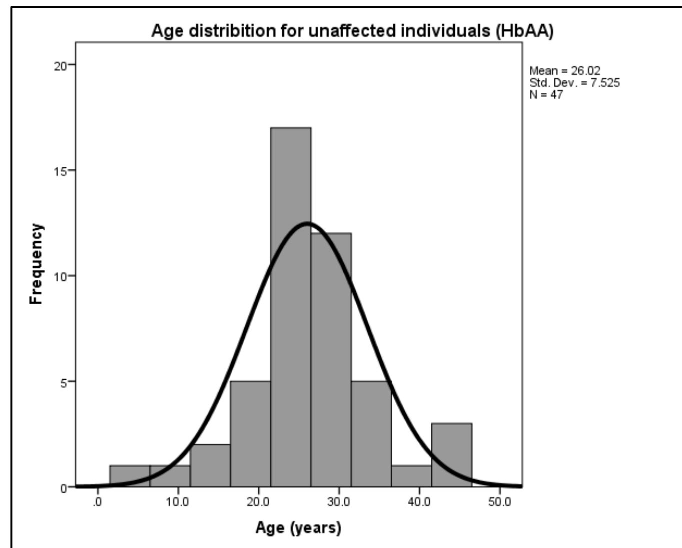


Figure 9: Distribution curve for the age variable for the unaffected individuals (HbAA) in the study cohort

The age variable for the unaffected cohort (HbAA), quantified in years, was normally distributed (parametric data), the Shapiro-Wilk Test p-value is 0.021, and was therefore expressed in the form of the mean. N = number of cases and Std. Dev. = Standard deviation (SD). The mean age was 26.02 years, with a SD of 7.525 and a median of 26.5.

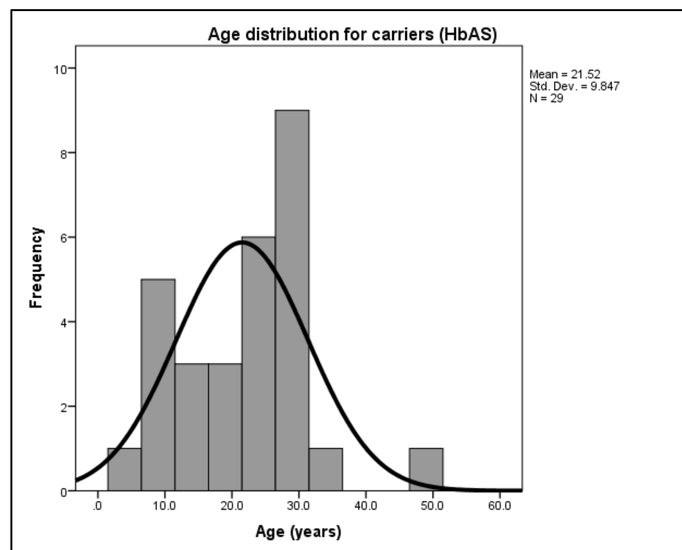


Figure 10: Distribution curve for the age variable for carriers (HbAS) in the study cohort

The age variable for the carrier cohort (HbAS), quantified in years, was normally distributed (parametric data), the Shapiro-Wilk Test p-value is 0.168, and was therefore expressed in the form of the mean. N = number of cases and Std. Dev. = Standard deviation (SD). The mean age was 21.52 years, with a SD of 9.847, and a median of 24.0.

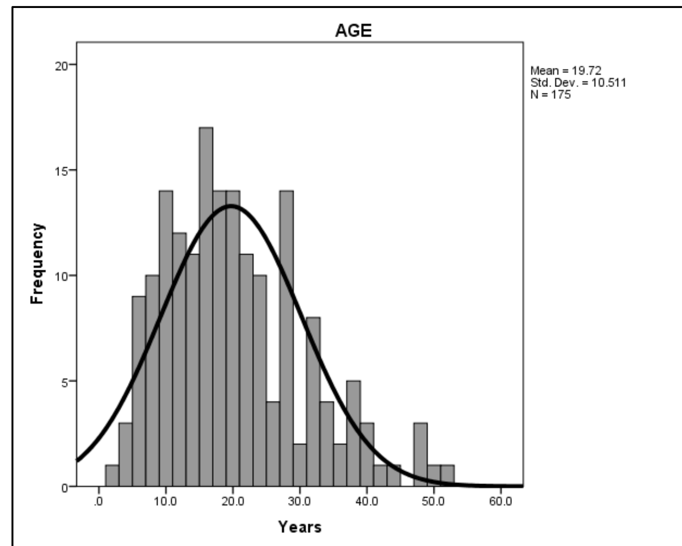


Figure 11: Distribution curve for the age variable for the patient cohort

The age variable for the patient cohort, quantified in years, was mildly skewed to the left illustrating a lower number of patients above the third decade. The Shapiro-Wilk Test, p-value was <0.0001, indicating that the data was abnormally distributed (p<0.05). N = number of cases and Std. Dev. =Standard deviation (SD). The mean age was 19.72 years, with a SD of 10.511, and a median of 17.5 years.

The patient cohort presents individuals who were not on any treatment for SCA. This untreated state may therefore present some biases. A detection/surveillance bias may be present where there was an inclination towards considering an outcome in one of the comparison groups. There may have been a length-time bias in which patients whose disease was discovered by screening for sickle cell anaemia, appeared to be asymptomatic, or they present with a milder phenotype when compared to individuals whose disease presents clinically with symptoms. Screening inclines towards identifying diseases that develop slowly and, therefore, present a 'good' prognosis.

4.2.2 Ethnicity

Individuals in the study cohort lived in the urban and peri-urban areas of the Yaoundé and Douala cities in Cameroon, with various ethnicities. These ethnicities refer to various regions within Cameroon. The 'mixed ancestry' group refers to individuals who have multiple ethnicities. For individuals in the HbAA group, one was of Bami descent and for individuals in the HbAS group there was one individual from each of the following groups: Bami, Beti, Ewondo, Haoussa, Maka and Mixed Ancestry (Table 7).

Table 7: Ethnicities of the patients in the study cohort

ETHNICITY	%	ETHNICITY	%	ETHNICITY	%
Abbo	0.8	Banen	1.6	Eton	6.5
Bafia	3.2	Bassa	8.8	Ewondo	12.9
Bafut	0.8	Batoufam	0.8	Foulbe	0.8
Bakaka	0.8	Bene	0.8	Haoussa	3.2
Bamenda	0.8	Beti	2.4	Maka	0.8
Bami	6.5	Bgnte	0.8	Mbamois	1.6
Bamileke	5.6	Bulu	1.6	Nkambe	0.8
Bamoun	3.2	Douala	1.6	Yambassa	3.2
Bandjoun	0.8	Dschang	6.5	Mixed ancestry	21.8

4.3 CLINICAL PROFILE

4.3.1 Haematological parameters

The red blood cell count (RBC) in a million cells/µl, haemoglobin level (HbA) in g/dl, mean corpuscular volume (MCV) in fL, mean corpuscular haemoglobin concentration (MCHC) in g/dl, white blood cell count (WBC) in X10^9/L, lymphocyte count in X10^9/L, monocyte count in X10^9/L, platelet level in X10^9/L, foetal haemoglobin (HbF) as a percentage and haemoglobin alpha-2 (HbA2) as a percentage, was presented as the haematological parameters examined. These abbreviations and their respective units apply to all tables with these haematological parameters.

Table 8: Reference values for the haematological parameters investigated

	HEALTHY RANGE	REFERENCE
RBC	4.2 - 6.1 million cells/µl	Bunn (2011)
HbA	12 - 17.5 g/dl	Mayo Foundation for Medical Education and Research (2014)
MCV	80 - 99 fL	Schoenborn and Snyder (2012)
MCHC	32 - 36 g/dl	Bunn (2011)
WBC	4.5 - 10 X10^9/L	Bagby (2007); Dinauer and Coates (2008)
LYMPHOCYTE COUNT	1 - 3.5 X10^9/L	Thomas (2007)
MONOCYTE COUNT	0.2 - 0.8 X10^9/L	Thomas (2007)
PLATELET LEVEL	150 - 450 X10^9/L	Schmaier (2008)
HbF	Up to 2%	Boyer et al. (1975); Wood et al. (1975); Rochette et al. (1994); Wojda et al. (2002)
HbA2	2 - 3%	Liebhaber et al. (1980)

Table 9: Haematological profile for the study cohort: a comparison between HbAA, HbAS, and HbSS

	HbAA					HbAS					HbSS				
	N	**Mean ± SD**	**Median**	**Min.**	**Max.**	**N**	**Mean ± SD**	**Median**	**Min.**	**Max.**	**N**	**Mean ± SD**	**Median**	**Min.**	**Max.**
RBC	47	4.5 ± 1	4.3	2	9	29	4.6 ± 1.3	4.6	2	9	160	3.5 ± 6.1	2.8	1	79
HbA	47	13.6 ± 2.3	13	7.9	18.7	29	12.4 ± 3	12.7	6.9	18.7	160	8.3 ± 2.2	7.8	3.2	17.9
MCV	47	81.6 ± 6.5	81	64	95	29	77.2 ± 7.1	79	56	85	160	82.5 ± 11.1	82	17.8	112
MCHC	47	36.7 ± 4.1	36.5	28.6	45.2	29	34.9 ± 4.9	33.8	29.1	45.2	159	34.7 ± 4.3	33.9	28.6	54.3
WBC	47	6.1 ± 3.4	5.3	2.8	24.4	29	6.9 ± 4.4	5.4	2	20.4	160	14 ± 7.1	12.6	2.9	42.4
LYMPHOCYTE COUNT	46	2.4 ± 1	2.2	0.4	5.6	29	2.8 ± 1.8	2.3	0.6	9	135	5.5 ± 3.2	4.9	0.6	21.6
MONOCYTE COUNT	46	2.8 ± 13.8	0.7	0	94	28	0.7 ± 0.5	0.5	0	2	135	1.4 ± 0.9	1.2	0	8
PLATELET LEVEL	47	238.9 ± 111.5	228	2.1	651	29	237.9 ± 109.1	214	108	586	159	374.5 ± 131.3	356	110	725
HbF	28	7.8 ± 4.3	8.6	1	22	15	8.3 ± 5.6	7.3	1	20	124	13.9 ± 9.6	13.5	0	86
HbA2	43	3.0 ± 1.3	3.3	0.1	5	26	3.3 ± 1.4	3.3	0.6	7.2	155	4.1 ± 2.1	3.8	0.9	18.2

N = Number of cases; SD = Standard deviation; Min. = Minimum; Max. = Maximum

The number (N) of individuals differed across the genotypes because the haematological indices for each individual were not adequately recorded. Hence, there were a few incomplete profiles. However, this did not affect the study, as the numbers were sufficient for meaningful comparisons. HbAA and HbAS were asymptomatic and had haematological parameters that fell within the normal range. HbSS had haematological parameters that fell out of the normal range, with the exception of the platelet level.

Table 10: Significance levels for the haematological profile for the study cohort: a comparison between HbAA, HbAS and HbSS

Haematological parameter	Genotype	p-value
RBC	HbAA vs. HbAS	0.999
	HbAA vs. HbSS	0.433
	HbAS vs. HbSS	0.548
HbA	HbAA vs. HbAS	0.346
	HbAA vs. HbSS	**<0.0001**
	HbAS vs. HbSS	**<0.0001**
MCV	HbAA vs. HbAS	0.192
	HbAA vs. HbSS	0.785
	HbAS vs. HbSS	**0.030**
MCHC	HbAA vs. HbAS	0.269
	HbAA vs. HbSS	**0.017**
	HbAS vs. HbSS	0.909
WBC	HbAA vs. HbAS	0.772
	HbAA vs. HbSS	**<0.0001**
	HbAS vs. HbSS	**<0.0001**
LYMPHOCYTE COUNT	HbAA vs. HbAS	0.767
	HbAA vs. HbSS	**<0.0001**
	HbAS vs. HbSS	**<0.0001**
MONOCYTE COUNT	HbAA vs. HbAS	0.425
	HbAA vs. HbSS	0.471
	HbAS vs. HbSS	0.876
HbA2	HbAA vs. HbAS	0.901
	HbAA vs. HbSS	**0.007**
	HbAS vs. HbSS	0.133

Pot-hoc analysis revealed that there were significant differences for: (1) the HbA levels between unaffected individuals and patients (**$p<0.0001$**) and between carriers and patients (**$p<0.0001$**), (2) the MCV between carriers and patients (**0.030**; $p<0.05$), (3) the WBC between

unaffected individuals and carriers (**$p<0.0001$**), and between carriers and patients (**$p<0.0001$**), and, (4) the HbA2 level between unaffected individuals and patients (**0.007**; $p<0.05$).

Table 11: Literature derived haematological profile for HbSS

	CAMEROON		TANZANIA (Cox et al. 2013)		TURKEY (Karazincir et al. 2013)		AMERICA (AFRICAN-AMERICANS) (Lettre et al. 2008)		BRAZIL (Lettre et al. 2008)		CARIBBEAN (AFRO-CARIBBEAN) (Gati et al. 2013)	
	N	**Mean ± SD**	**N**	**Mean ± SD**	**N**	**Mean ± SD**	**N**	**Mean ± SD**	**N**	**Mean ± SD**	**N**	**Mean ± SD**
RBC	160	3.5 ± 6.1	458	2.9 ± 0.7	-	-	1275	2.8 ± 0.6	350	2.5 ± 0.6	-	-
HbA	160	8.3 ± 2.2	458	7.4 ± 1.2	32	8.8 ± 0.8	1275	8.6 ± 1.3	350	7.5 ± 1.0	99	8.6 ± 1.2
MCV	160	82.5 ± 11.1	458	79.5 ± 15.3	32	90.1 ± 3.6	1275	89.4 ± 9.0	350	94.4 ± 9.7	-	-
MCHC	159	34.7 ± 4.3	458	31.9 ± 1.5	32	32.5 ± 1.2	-	-	-	-	-	-
WBC	160	14.0 ± 7.1	-	-	-	-	1275	11.9 ± 2.6	350	13.0 ± 4.3	-	-
LYMPHOCYTE COUNT	135	5.5 ± 3.2	-	-	-	-	-	-	-	-	-	-
MONOCYTE COUNT	135	1.4 ± 0.9	-	-	-	-	-	-	-	-	-	-
PLATELET LEVEL	159	374.5 ± 131.3	-	-	-	-	1275	442 ± 151	350	412 ± 134	-	-
HbF	124	13.9 ± 9.6	458	4.7	-	-	1275	6.4 ± 4.7	350	9.2 ± 6.0	-	-
HbA2	155	4.1 ± 2.1	-	-	-	-	-	-	-	-	-	-

N = Number of cases; SD = Standard deviation

Table 11 compares the haematological parameters of SCA patients from certain regions of the world. The Cameroonian patients in this study had a higher RBC, MCHC, WBC and HbF level, and a lower platelet level. The HbA and MCV values were variable across the regions.

4.3.2 Gender-based haematological indices

A non-parametric statistical test (Kruskal-Wallis Test for significance) was performed to investigate the presence of any differences between the haematological parameters based on gender. Analyses were performed separately for each genotype: HbAA- male vs. female, HbAS-male vs. female and HbSS-male vs. female. There were no significant differences ($p>0.05$) between the haematological parameters for HbAA based on gender. For HbAS, there was a significant difference (**$p=0.047$**; $p<0.05$) in the MCV between males and females. Females had a higher MCV (82fL) when compared to males (74fL). The MCV for males fell below the healthy range (80 – 99fL) (Table 9; Table 10), indicating smaller erythrocytes and therefore microcytic anaemia. For HbSS, there was a significant difference (**$p<0.0001$**) in the HbF level between males and females. Females had a higher HbF level (15.7%) when compared to males (10.8%). These values were higher than the normal range (Table 8; Table 9).

4.3.3 Clinical indices

After the review and validation of the clinical data provided via interviewing the parent (in the case of a minor under 18 years of age) or patient (in the case of an adult), and by consulting their medical records, only 121 patients had data that was suitable for analysis. The analyses have been tabulated below.

Table 12: Demographic data for the SCA patients in the study cohort

	N	Mean ± SD	Median	Minimum	Maximum
Age of Diagnosis (Years)	111	4.2 ± 4.5	3	0.5	21

N = Number of cases; SD = Standard deviation; Min. = Minimum; Max. = Maximum

Table 13: Clinical characterisation for the SCA patients in the study cohort

		N	Mean ± SD	Median	Min.	Max.
BMI (kg/m^2)		80.0	18.5 ± 3.5	18.2	12.0	27.0
SBP (mmHg)		82.0	108.7 ± 12.8	108.0	86.0	156.0
DBP (mmHg)		82.0	59.5 ± 9.1	58.0	43.0	93.0
No. of VOCs/year		118.0	3.0 ± 4.2	2.0	0.0	40.0
Overt stroke	YES	8.0	-	-	-	-
	NO	105.0	-	-	-	-
No. of consultations/year		108.0	2.2 ± 2.7	1.0	0.0	12.0
No. of hospitalisations/year		110.0	1.9 ± 2.5	1.0	0.0	12.0

N = Number of cases; SD = Standard deviation; Min. = Miminum; Max. = Maximum

The BMI (kg/m^2), systolic blood pressure (SPB) (mmHg), diastolic blood pressure (DBP) (mmHg), number of vaso-occlusive pain crises (VOCs) per year, incidence of stroke, the number of hospital consultations per year and the number of hospitalisations per year was established for the SCA patients (HbSS) in the study cohort.

Patients displayed a median BMI of 18.2 kg/m^2 (25th percentile = 15.7 kg/m^2; 75th percentile = 21.4 kg/m^2), SBP of 108 mmHg (25th percentile = 101mmHg; 75th percentile = 116mmHg), DBP of 58mmHg (25th percentile = 53mmHg; 75th percentile = 62.2mmHg), VOCs at 2/year (25th percentile = 1/year; 75th percentile = 4/year), and number of hospitalisations at 1/year (25th percentile = 0/year; 75th percentile = 2/year). A high number of VOCs (>3/year) was found among 48% of patients, with a few cases of overt stroke (7%), which is indicative of a more severe phenotype. Linear regression analysis, which incorporated the patient's age and gender, did not reveal any significant difference in the number of VOCs, and hospitalisations, per year. However, males tended to have a higher number of VOCs per year (Likelihood Ratio: p=0.09).

4.4 HAPLOTYPES OF THE *HBB* GENE CLUSTER

The molecular characterisation of the 5'Gγ, Gγ, Aγ, 3'ψβ and 5'β regions of the *HBB* gene was performed to determine which of the five (Arab-/Saudi-Indian, Bantu/Central African, Benin, Cameroon and Senegal) haplotypes occurred in the study population.

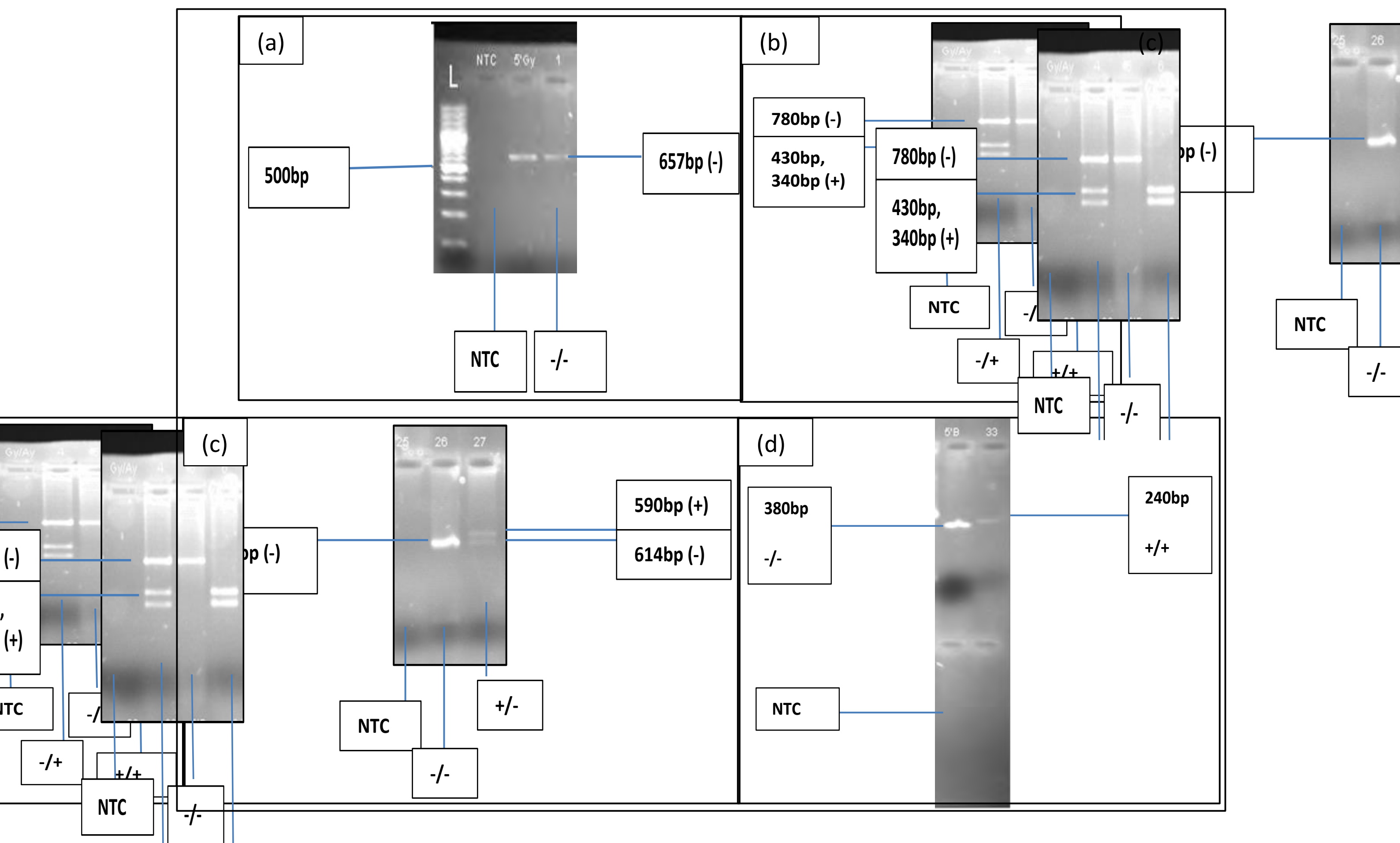


Figure 12: Gel electrophoresis images of the restriction enzyme profiles for *HBB* gene haplotyping

Each restriction endonuclease created different banding patterns, which led to the formulation of a pattern, consisting of pluses (+) and minuses (-) for the determination of a haplotype. L= GeneRuler 100bp plus DNA Ladder (Thermo Scientific, California, USA); NTC= No-template control: clear, thereby indicating that no contamination had occurred. (a) 5'Gy: 657bp fragment, cut with *Xmn*I (Promega, Madison, USA) produced -/- banding pattern. (b) Gy/Ay: 780bp fragment; cut with *Hind*III (Thermo Scientific, California, USA) produced the following banding patterns: -/+, -/-, and +/+. (c) 3'ψβ: 614bp fragment, cut with *Hinc*II (Thermo Scientific, California, USA) produced the following banding patterns: +/+ and -/+. (d) 5'β: 386bp fragment, cut with *Hinf*I (Thermo Scientific, California, USA) produced the following banding patterns: +/+, -/+, and -/-.

Table 14: Frequencies of the *HBB* gene haplotypes in SCA patients

	Frequency	
	N chromosome	%
BENIN	173	66.3
CAMEROON	55	21.0
ATYPICAL	29	11.1
BANTU	4	1.3
ARAB-/SAUDI-INDIAN	1	0.3
TOTAL	262	100

The Benin, Cameroon, Bantu and Arab-/Saudi-Indian haplotypes are the four known haplotypes. Atypical haplotypes refers to haplotypes that are not distinctive as a type, group, or class, and are classified as unusual haplotypes. In chromosomal frequencies, the Benin and Cameroon haplotypes were most prevalent in the patient cohort.

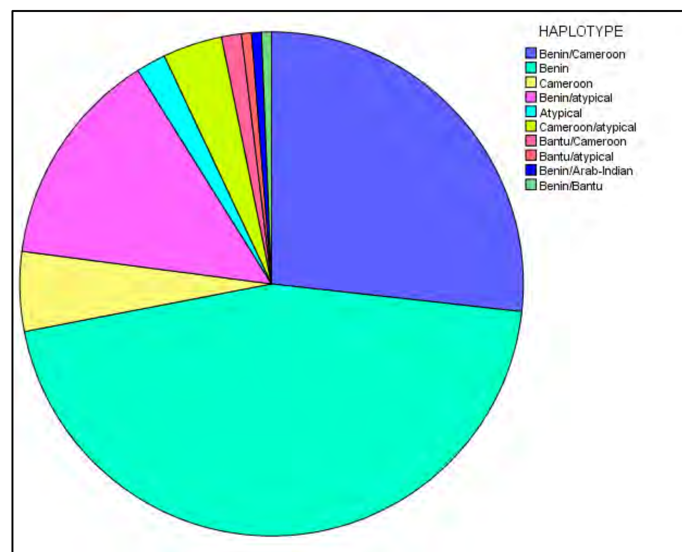


Figure 13: *HBB* gene haplotype distribution in SCA patients

The following haplotype combinations (N total = 157) occurred in the patient cohort: Benin/Benin (N = 71) 42.2%, Benin/Cameroon (N = 42) 26.8%, Benin/atypical (N = 22) 14%, Cameroon/Cameroon (N = 8) 5.1%, Cameroon/atypical (N = 6) 3.8%, atypical (N = 3) 1.3%, Bantu/Cameroon (N = 2) 1.3%, Bantu/atypical (N = 1) 0.6%, Benin/Arab-/Saudi-Indian (N = 1) 0.6% and Benin/Bantu (N = 1) 0.6%.

In combination, the Benin/Benin, Benin/Cameroon, Benin/atypical and Cameroon/Cameroon haplotypes were most prevalent in the patient cohort. Further analysis was performed on these four haplotype combinations.

Depicted below, is the classification of patients according to their *HBB* haplotype status, in relation to their ethnicities. N represents the number of patients with a specific haplotype. The 'mixed ancestry' group refers to patients who have multiple ethnicities.

Table 15: *HBB* gene haplotype distribution in SCA patients according to ethnicity

Ethnicity	N	Haplotype	Ethnicity	N	Haplotype	Ethnicity	N	Haplotype
Abbo	1	Benin/Cameroon	**Banen**	1	Benin/Benin	**Ewondo**	1	Bantu/Cameroon
Bafia	1	Atypical/Cameroon		1	Cameroon/Cameroon		1	Atypical/Cameroon
	1	Benin/Benin	**Bassa**	1	Cameroon/Cameroon		3	Benin/Atypical
	2	Benin/Atypical		3	Benin/Atypical		4	Benin/Benin
Bafut	1	Benin/Atypical		7	Benin/Benin		5	Benin/Cameroon
Bakaka	1	Benin/Atypical	**Batoufam**	1	Benin/Atypical	**Haoussa**	1	Atypical/Atypical
Bamenda	1	Benin/Benin	**Bene**	1	Benin/Cameroon		3	Benin/Benin
Bami	1	Benin/Cameroon	**Beti**	1	Benin/Atypical	**Mbamois**	1	Benin/Atypical
	1	Atypical/Atypical		1	Atypical/Atypical	**Yambassa**	1	Benin/Atypical
	2	Benin/Atypical	**Bgnte**	1	Benin/Benin		1	Bantu/Cameroon
	3	Benin/Benin	**Bulu**	2	Benin/Benin		1	Atypical/Atypical
Bamileke	1	Benin/Cameroon	**Douala**	1	Benin/Benin	**Mixed ancestry**	2	Atypical/Atypical
	1	Benin/Atypical		1	Benin/Atypical		3	Benin/Cameroon
	6	Benin/Benin	**Dschang**	1	Benin/Cameroon		10	Benin/Benin
Bamoun	1	Benin/Cameroon		1	Atypical/Atypical		11	Benin/Atypical
	1	Benin/Atypical		6	Benin/Benin			
	2	Benin/Benin	**Eton**	1	Benin/Benin			
Bandjoun	1	Benin/Benin		1	Atypical/Atypical			
				2	Benin/Atypical			
				2	Benin/Cameroon			

N = Number of cases

Table 16: Haematological profile for the frequently occurring haplotype combinations in the patient cohort

	BENIN/BENIN					BENIN/CAMEROON					BENIN/ATYPICAL					CAMEROON/CAMEROON				
	N	Mean ± SD	Median	Min.	Max.	N	Mean ± SD	Median	Min.	Max.	N	Mean ± SD	Median	Min.	Max.	N	Mean ± SD	Median	Min.	Max.
RBC	67	2.8 ± 0.8	2.7	2	5	38	2.8 ± 0.8	2.6	1	6	15	7.6 ± 19.7	2.5	1	79	8	2.9 ± 0.7	2.7	2	4
HEMOGLOBIN LEVEL	67	8 ± 1.6	7.8	5.4	14.3	38	7.9 ± 1.7	7.7	4.9	13.7	15	6.9 ± 1.5	6.7	3.4	9.4	8	8.5 ± 1.4	8.5	6.4	10.8
MCV	67	83.8 ± 10.8	84	59	112	38	83.5 ± 8.2	82.5	64.8	100	15	81 ± 21.1	82	17.8	103.3	8	80 ± 6.6	80.5	66.0	88.0
MCHC	67	34.9 ± 4.7	33.9	28.6	52.9	38	34.8 ± 4.1	34.1	28.8	47.2	14	34 ± 2.6	34	31	40	8	36.6 ± 7.4	35.0	31.6	54.3
WBC	67	14.1 ± 6.2	12.8	4.1	35.5	38	14.2 ± 6.2	13.4	4.9	33.9	15	18.8 ± 10.2	15.1	9.2	42.4	8	13.2 ± 5.0	12.2	6.2	21.2
Lymphocyte count	61	5.8 ± 3.2	4.8	1.6	17.5	32	5.9 ± 3.5	5.5	1.6	21.6	8	6 ± 2.6	5.9	1.9	11.3	8	6.6 ± 2.8	5.9	2.8	10.2
Monocyte count	61	1.5 ± 0.8	1.3	0	4	32	1.4 ± 0.8	1.1	0	4	8	1.2 ± 0.5	1.2	0	2	8	1.3 ± 0.3	1.3	1	2
Platelet level	67	402.9 ± 144.8	402	110	725	38	343.7 ± 90.5	342	147	482	14	339.6 ± 159	264	148	615	8	449.9 ± 80.6	448.0	350	584
HbF	56	16 ± 11.8	14.6	0	86	28	13.1 ± 7.2	14.8	0	27	14	15.1 ± 7	15.7	0	27	8	9.3 ± 4.1	9.9	2	16
HbA2	65	4.7 ± 2.9	4	1.6	18.2	38	4.1 ± 1	4	2.1	6.6	15	3.5 ± 0.8	3.4	2.4	5.1	8	3.7 ± 0.6	3.6	2.6	4.5

N = Number of cases; SD = Standard deviation; Min. = Minimum; Max. = Maximum

After performing Post-hoc analysis, for the haematological parameters vs. each haplotype (Benin/Benin vs. Benin/Cameroon; Benin/Benin vs. Benin/Atypical; Benin/Benin vs. Cameroon/Cameroon; Benin/Cameroon vs. Benin/Atypical; Benin/Cameroon vs. Cameroon/Cameroon; and Benin/Atypical vs. Cameroon/Cameroon), it was revealed that there were no significant differences between and within haplotype groups for all of the haematological parameters investigated.

The age of diagnosis, in years, was classified according to the four most prevalent haplotypes found in this study. For Benin/Benin patients, the age of diagnosis (Mean ± SD) was 4.3 ± 4.3 years, Benin/Cameroon 3.7 ± 3.2 years, Benin/Atypical 5.4 ± 7.5 years and Cameroon/Cameroon 6.8 ± 7.9 years. A one-way ANOVA Tukey’s Post-hoc Test was performed to determine if there were any significant differences between Benin/Benin vs. Benin/Cameroon, Benin/Benin vs. Benin/Atypical, Benin/Benin vs. Cameroon/Cameroon, Benin/Cameroon vs. Benin/Atypical, Benin/Cameroon vs. Cameroon/Cameroon, and Benin/Atypical vs. Cameroon/Cameroon. There was no significant difference between the ages of diagnosis across the haplotype groups.

Table 17: Clinical characterisation for the frequently occurring haplotypes in the patient cohort

		BENIN/BENIN					BENIN/CAMEROON					BENIN/ATYPICAL					CAMEROON/CAMEROON				
		N	Mean ± SD	Median	Min.	Max.	N	Mean ± SD	Median	Min.	Max.	N	Mean ± SD	Median	Min.	Max.	N	Mean ± SD	Median	Min.	Max.
BMI (kg/m^2)		34	19.5 ± 3.6	20.4	12	27	22	17.8 ± 3.5	17.5	13	27	7	17.7 ± 3.5	19.1	12	21	7	19.5 ± 3.5	18.5	14.7	23.5
SPB (mmHg)		35	110.2 ± 13.6	110	86	156	24	109.0 ± 12.1	109.5	88	135	7	103.7 ± 12.7	102	87	127	8	114 ± 12.8	116.5	89	133
DPB (mmHg)		35	61.6 ± 10.2	62	43	93	24	59.5 ± 7.7	58.5	47	79	7	58.1 ± 11.3	55	43	76	8	56.6 ± 7.9	54.5	47	71
No. of VOC/year		61	2.7 ± 2.6	2	0	15	31	3.3 ± 2.6	3	0	10	8	1.9 ± 1.2	2	0	4	8	1.4 ± 1.2	2	0	3
Stroke	YES	4	-	-	-	-	1	-	-	-	-	0	-	-	-	-	0	-	-	-	-
	NO	53	-	-	-	-	29	-	-	-	-	7	-	-	-	-	7	-	-	-	-
No. of consultations/year		55	2.2 ± 2.6	1	0	10	28	2.7 ± 3.2	1.5	0	12	7	0.6 ± 1.1	0	0	3	7	1.0 ± 1.4	0	0	3
No. of Hospitalisations/year		55	1.8 ± 2.2	1	0	12	30	1.8 ± 2.4	1	0	12	8	1.5 ± 2.1	0.5	0	6	7	1.9 ± 4.5	0	0	12

N = Number of cases; SD = Standard deviation; Min. = Minimum; Max. = Maximum

Post-hoc analysis revealed that there were no significant differences between Benin/Benin vs. Benin/Cameroon, Benin/Benin vs. Benin/Atypical, Benin/Benin vs. Cameroon/Cameroon, Benin/Cameroon vs. Benin/Atypical, Benin/Cameroon vs. Cameroon/Cameroon, and Benin/Atypical vs. Cameroon/Cameroon in any of the clinical parameters investigated.

4.5 CO-INHERITANCE: SCA-α-THALASSEMIA

The co-inheritance of α-thalassemia in SCA patients has been associated with a less severe phenotype. The molecular characterisation of the α-globin chain deletions associated with α-thalassemia was determined by multiplex gap PCR.

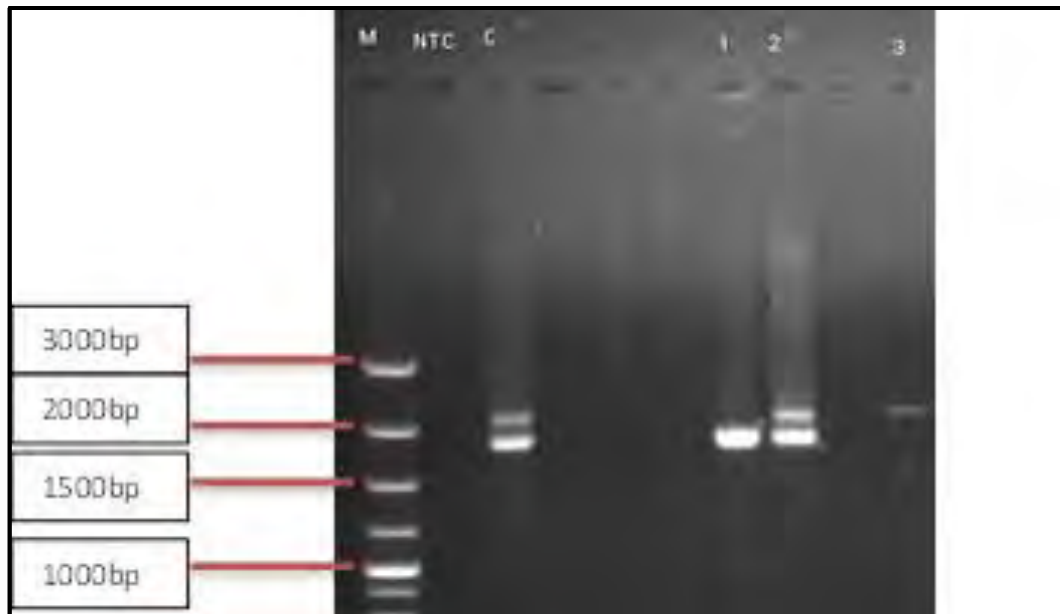
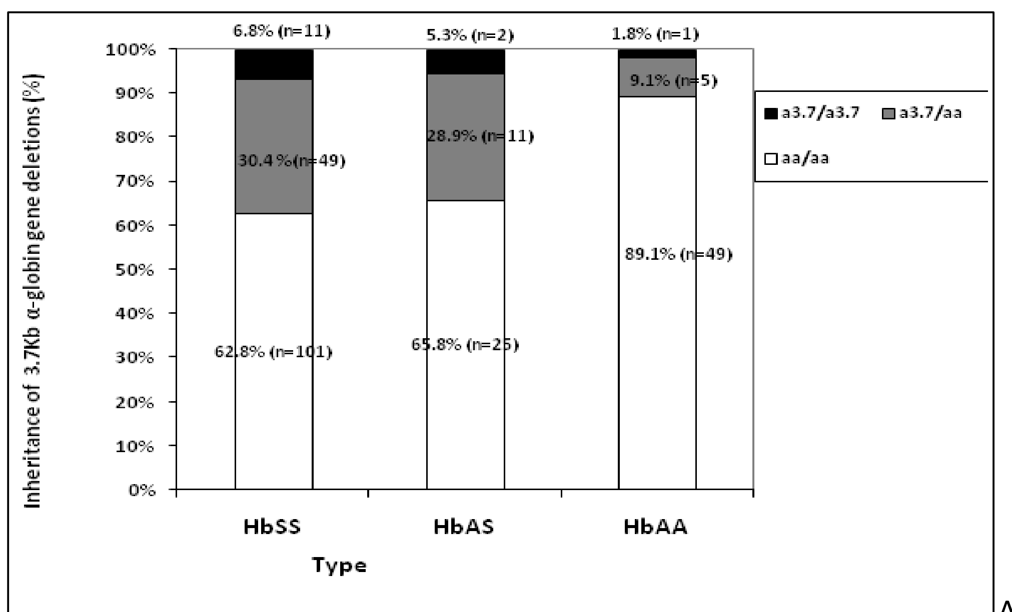


Figure 14: Gel image depicting the molecular analysis of the α-thalassemia deletion screening process, post electrophoresis

M = GeneRuler 100bp plus DNA Ladder (Thermo Scientific, USA); NTC = No-template control; C = 3.7kb heterozygote (αα/α3.7) control; 1, 2, 3 = Patient DNA. The NTC lane was clear, thereby indicating that there was no contamination. The 3.7kb heterozygote control represented two bands, one for the normal (αα/αα) allele, and one for the 3.7kb deletion (α3.7/α3.7) allele, with 1800bp and 2020bp bands, respectively. In lane 1, a 1800bp band was seen, indicating that this patient was "normal" (had all four α-globin chains). In lane 2, a 3.7kb heterozygote was seen. In lane 3, a 2020bp band was seen, indicating the 3.7kb homozygous deletion. Individuals with one or two α-globin deletions are carriers.

There were 55 unaffected individuals (HbAA), 38 carriers (HbAS), and 161 patients (HbSS) who were screened to determine their α-globin deletion status. For unaffected individuals, 89.1% were (αα/αα), 9.1% (αα/α3.7) and 1.8% (α3.7/α3.7). For carriers, 65.8% were (αα/αα), 28.9% (αα/α3.7) and 5.3% (α3.7/α3.7). For patients, 62.8% were (αα/αα), 30.4% (αα/α3.7) and 6.8% (α3.7/α3.7).



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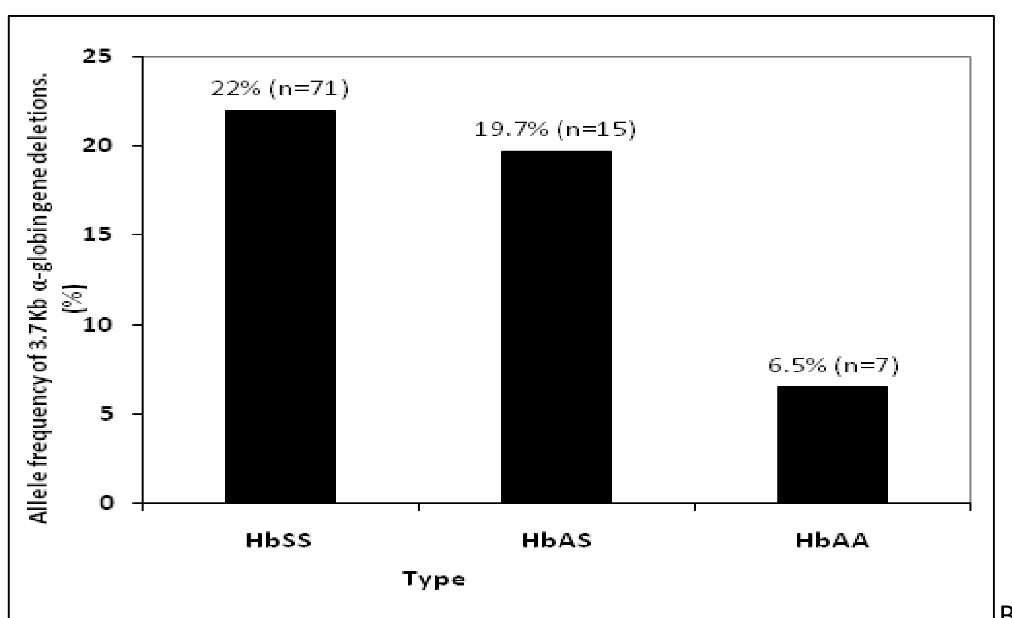


B

Figure 15: Prevalence and allele frequency of the 3.7kb deletion among patients and controls

A. The prevalence of the 3.7kb α-globin gene deletions, expressed as a percentage (%; y-axis) is depicted for unaffected individuals (HbAA), carriers (HbAS) and patients (HbSS) (x-axis) in the following proportions, respectively, where (αα/αα), (α3.7/αα), and (αα/αα) represents no, one and two, α-globin gene deletions: (1) HbAA-(αα/αα) 89.1% (n=49), HbAA-(α3.7/αα) 9.1% (n=5), HbAA-(α3.7/α3.7) 1.8% (n=1); (2) HbA-(αα/αα) 65.8% (n=25), HbAS-(α3.7/αα) 28.9% (n=11), HbAS-(α3.7/α3.7) 5.3%(n=2); (3) HbSS-(αα/αα) 62.8% (n=101), HbSS-(α3.7/αα) 30.4% (n=49), HbSS-(α3.7/α3.7) 6.8% (n=11). B. The allele frequency of the 3.7kb α-globin gene deletions in the HbAA, HbAS and HbSS cohorts was 6.5% (n=7), 19.7% (n=15) and 22% (n=71) respectively.

Allele frequencies of the 3.7kb α-globin gene deletions were 11.8% and 22% in controls (HbAA and HbAS) and patients, respectively (**p=0.006**). Individually, the allele frequencies for HbAA and HbAS were 6.5% and 19.7%, respectively. Multinomial logistic regression analysis was

performed, which incorporated SCA, α-thalassemia, age and gender, which indicated that differential frequency in the 3.7kb α-globin gene deletions among patients and controls was driven by unaffected individuals (HbAA). HbAA individuals were four times less likely to have a single 3.7kb α-globin gene deletion [exponentiation of the β coefficient =4.02 (95% CI) (1.45-11.13)] and five times less likely to have a double 3.7kb α-globin gene deletion than HbSS individuals [exponentiation of the β coefficient =5.42 (95% CI) (0.65-44.79)].

Considering HbSS, the 3.7kb α-globin gene deletions and gender, multinomial analysis indicated that, being male increased the likelihood of having the 3.7kb α-globin gene deletion (Likelihood Ratio **p=0.013**). HbAA individuals were four to five times more likely to have the α-thalassemia trait, when compared to their HbSS counterparts.

Table 18: Frequency of α-globin chain deletions in the world

	CAMEROON						AMERICAN (African-American) (Guasch et al. 1999)		URUGUAY (Afro-Uruguayan) (Da Luz et al. 2006)		INDIA (Pandey et al. 2011)		BRAZIL (Belisário et al. 2010)		OMAN (Wali et al. 2002)		GUADELOUPE (Nebor et al. 2010)		FRANCE (Bernaudin et al. 2008)		TANZANIA (Cox et al. 2013)	
	UNAFFECTED INIDVIDUALS (HbAA)		CARRIERS (HbAS)		PATIENTS (HbSS)		PATIENTS (HbSS)		PATIENTS (HbSS)		PATIENTS (HbSS)		PATIENTS (HbSS)		PATIENTS (HbSS)		PATIENTS (HbSS)		PATIENTS (HbSS)		PATIENTS (HbSS)	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
All 4 α-globin chains (αα/αα)	49	89.1	25	65.8	101	62.8	45	59	44	84	39	68	147	71	39	72	117	64	170	52	188	42
1 deletion (αα/α3.7)	5	9.1	11	28.9	49	30.4	16	21	8	16	9	16	58	28	8	14	60	33	125	39	192	41
2 deletions (α3.7/α3.7)	1	1.8	2	5.3	6.8	6.8	15	20	0	0	9	16	3	1	8	14	6	3	30	9	78	17
TOTAL	55	100	38	100	152	100	76	100	52	100	57	100	208	100	54	100	183	100	325	100	458	100

N = Number of cases

The frequency of α-globin deletions in SCA patients (HbSS) from the Cameroonian study cohort was determined and then compared to other reports in the literature. The results were tabulated from America (African-American; Guasch et al.1999), Uruguay (Afro-Uruguayan; Da Luz et al. 2006), India (Pandey et al. 2011), Brazil (Belisário et al. 2010), Oman (Nebor et al. 2010), France (Bernaudin et al. 2008), and Tanzania (Cox et al. 2013). N refers to the number of cases. The frequency of individuals with all four α-globin chains (αα/αα), individuals with one deletion (αα/α3.7) and individuals with two deletions (α3.7/α3.7) were compared. The incidence of the α-globin deletions differs based on the region of the world, with the deletion of one α-globin chain being most common.

4.5.3: Socio-demographic characterisation for the Control and Patient cohorts with (αα/αα), (αα/α3.7) and (α3.7/α3.7)

The mean ages (mean ± SD) for the unaffected (HbAA) subjects who were (αα/αα) was 25.7± 3.0 years, (αα/α3.7) was 27.0± 3.7 years and (α3.7/α3.7) was 35.0 ± 0.0 years. The mean ages (mean ± SD) for the carriers (HbAS) who were (αα/αα) was 21.4± 6.8 years, (αα/α3.7) was 19.3± 11.1 years and (α3.7/α3.7) was 30.5 ± 16.5 years. The mean ages (mean ± SD) for the patient (HbSS) subjects who were (αα/αα) was 19.7 ± 10.1 years, (αα/α3.7) was 20 ± 12 years and (α3.7/α3.7) was 20.1 ± 16 years.

The majority of the study cohort was female. A one-way ANOVA Tukey's Post-hoc Test, revealed that there were no significant differences between age and gender across the α-thalassemia genotypes, (αα/αα)/ (αα/α3.7)/ (α3.7/α3.7), where HbAA and HbAS was compared separately. The following combinations were compared for HbAA and HbAS cohorts, separately: (αα/αα) vs. (αα/α3.7), (αα/αα) vs. (α3.7/α3.7), (αα/α3.7) vs. (α3.7/α3.7), and then the combinations for HbAA vs. HbAS.

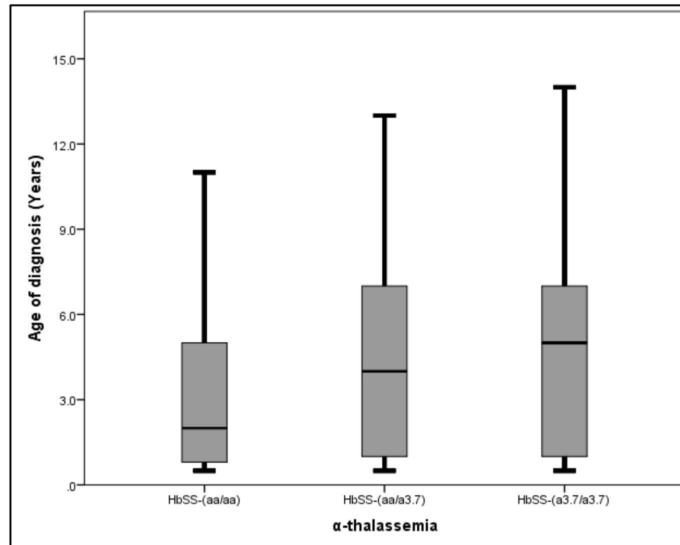


Figure 16: Age of diagnosis for the co-inheritance of SCA and α-thalassemia

The age of diagnosis, in years, (y-axis) was compared to SCA patients who were HbSS-(αα/αα) (patients with no α-globin gene deletions), HbSS-(αα/α3.7) (patients with one α-globin gene deletion), and HbSS-(α3.7/α3.7) (patients with two α-globin gene deletions) (x-axis). HbSS-(αα/αα) had a median age of diagnosis of 2 years, HbSS-(αα/α3.7) 4 years and HbSS-(α3.7/α3.7) 5 years. There was a significant difference between the age of diagnosis and the α-thalassemia genotypes in SCD patients ($p=0.026$; $p<0.05$).

The increasing number of 3.7kb α-globin gene deletions, with the age of diagnosis (**$p=0.026$**), is depicted by the box and whisker plot. Boxes have lines at the lower quartile, median and upper quartile. In the absence of neonatal screening, the age of diagnosis often corresponds to that of clinical manifestations that brings SCA to the clinician's attention.

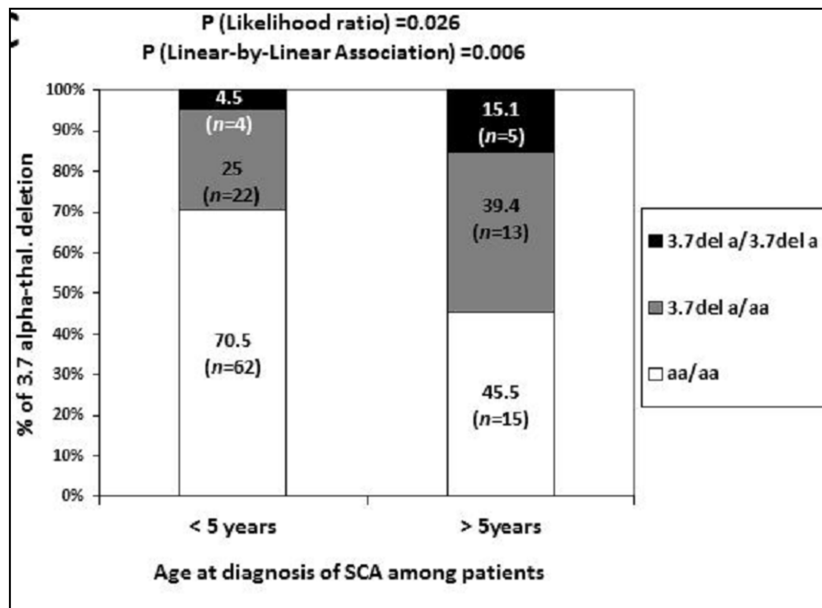


Figure 17: Age of diagnosis of HbSS patients with no, one and two α-globin gene deletions at an age less than and greater than five years.

The proportion of patients with 3.7kb α-globin gene deletions diagnosed before and after five years of age. The number of patients within each category that have no, one and two, α -globin gene deletions, (αα/αα), (αα/α3.7) and (α3.7/α3.7), respectively, is expressed as a percentage (y-axis), based on diagnosis before and after five years (x-axis). For diagnosis before five years, patients with no, one and two α-globin deletions were 70.5%, 25% and 4.5%, respectively. For diagnosis after five years of age, patients with no, one and two α-globin deletions were 45.5%, 39.4%, and 15.1%, respectively.

The proportion of patients with the 3.7kb α-globin gene deletion, diagnosed after five years of age was significantly different to those diagnosed before five (Likelihood ratio, **p=0.026**; Linear-by-Linear Association, **p=0.006**).

4.5.4: Haematological characterisation for the Control and Patient cohorts with (αα/αα), (αα/α3.7) and (α3.7/α3.7)

The haematological indices were tested for significance across the α-thalassemia genotypes, (αα/αα)/ (αα/α3.7)/ (α3.7/α3.7), for HbAA and HbAS. The following combinations were compared separately for HbAA and HbAS cohorts: (αα/αα) vs. (αα/α3.7), (αα/αα) vs. (α3.7/α3.7), (αα/α3.7) vs. (α3.7/α3.7), and then the combinations for HbAA vs. HbAS. A ONE-WAY ANOVA Tukey's Post-hoc Test, revealed that there were no significant differences between the haematological indices across the α-thalassemia genotypes and HbAA or HbAS. The lack of significance is due to the small sample size of 46 for HbAA-(αα/αα), 5 for HbAA-

(αα/α3.7), 1 for HbAA-(α3.7/α3.7), 23 for HbAS-(αα/αα), 7 for HbAS-(αα/α3.7) and 2 for HbAS-(α3.7/α3.7).

Table 19: Haematological characterisation for the Patient cohort (HbSS): HbSS-(αα/αα), HbSS-(αα/α3.7) and HbSS-(α3.7/α3.7)

	HbSS - (αα/αα)					HbSS- (αα/α3.7)					HbSS-(α3.7/α3.7)					
	N	**Mean ± SD**	**Median**	**Min.**	**Max.**	**N**	**Mean ± SD**	**Median**	**Min.**	**Max.**	**N**	**Mean ± SD**	**Median**	**Min.**	**Max.**	**SD**
RBC	100	3.6 ± 7.7	2.6	1	79	49	3.3 ± 1	3.1	2	6	9	3.5 ± 1.1	3.7	1.9	5.5	1.1
HAEMOGLOBIN LEVEL	100	8.1 ± 2.2	7.7	3.2	17.9	49	8.8 ± 2.4	8.1	4.9	14.5	9	8.4 ± 2.5	8.5	5.4	14.3	2.5
MCV	100	85.4 ± 11.1	86	17.8	112	49	79.6 ± 7.8	79.6	66	100	9	70.8 ± 12.6	69	59	101	12.6
MCHC	99	35.2 ± 3.9	34.4	28.8	52.9	49	34 ± 4.9	32.7	28.6	54.3	9	33.2 ± 4.8	31.4	28.8	44.1	4.8
WBC	100	15.7 ± 7.6	13.7	3	42.4	49	11.1 ± 4.4	10.5	2.9	24	9	9.3 ± 3.9	9.1	4.1	17.4	3.9
Lymphocyte count	84	6.3 ± 3.6	5.5	1.4	21.6	42	4.3 ± 1.9	4.2	0.6	8.3	9	3.9 ± 2.2	2.9	1.6	8.2	2.2
Monocyte count	84	1.6 ± 1.1	1.4	0.2	7.8	42	1.1 ± 0.6	1.1	0.2	3	9	1 ± 0.6	0.8	0.4	2.3	0.6
Platelet level	99	386.7 ± 131.5	372	110	725	49	371.5 ± 127.9	375	147	615	9	281.6 ± 117.8	247	163	559	117.8
HbF	81	14 ± 10.8	12.6	0	86.2	33	12.9 ± 6.4	13.5	0	26	8	13.8 ± 7.7	14.9	3	27.3	7.7
HbA2	96	4 ± 2.4	3.7	0.9	18.2	48	4.1 ± 1.8	3.9	1.1	13.2	9	4.2 ± 0.9	4.4	2.5	5.3	0.9

N = Number of cases; SD = Standard deviation; Min. = Minimum; Max. = Maximum

Table 20: Significance values for the haematological characterisation for the patient cohort (HbSS): HbSS-(αα/αα), HbSS-(αα/α3.7), and HbSS-(α3.7/α3.7)

Haematological parameter	α-thalassemia genotype	p-value
RBC	HbSS-(aa/aa) vs. HbSS-(aa/a3.7)	0.971
	HbSS-(aa/aa) vs. HbSS-(a3.7/a3.7)	1.000
	HbSS-(aa/a3.7) vs. HbSS-(a3.7/a3.7)	0.989
Hb	HbSS-(aa/aa) vs. HbSS-(aa/a3.7)	0.221
	HbSS-(aa/aa) vs. HbSS-(a3.7/a3.7)	0.949
	HbSS-(aa/a3.7) vs. HbSS-(a3.7/a3.7)	0.833
MCV	HbSS-(aa/aa) vs. HbSS-(aa/a3.7)	**0.004**
	HbSS-(aa/aa) vs. HbSS-(a3.7/a3.7)	**<0.0001**
	HbSS-(aa/a3.7) vs. HbSS-(a3.7/a3.7)	**0.015**
MCHC	HbSS-(aa/aa) vs. HbSS-(aa/a3.7)	0.259
	HbSS-(aa/aa) vs. HbSS-(a3.7/a3.7)	0.211
	HbSS-(aa/a3.7) vs. HbSS-(a3.7/a3.7)	0.711
WBC	HbSS-(aa/aa) vs. HbSS-(aa/a3.7)	**<0.0001**
	HbSS-(aa/aa) vs. HbSS-(a3.7/a3.7)	0.153
	HbSS-(aa/a3.7) vs. HbSS-(a3.7/a3.7)	0.965
LYMPHOCYTE COUNT	HbSS-(aa/aa) vs. HbSS-(aa/a3.7)	**0.002**
	HbSS-(aa/aa) vs. HbSS-(a3.7/a3.7)	0.083
	HbSS-(aa/a3.7) vs. HbSS-(a3.7/a3.7)	0.956
MONOCYTE COUNT	HbSS-(aa/aa) vs. HbSS-(aa/a3.7)	**0.024**
	HbSS-(aa/aa) vs. HbSS-(a3.7/a3.7)	0.129
	HbSS-(aa/a3.7) vs. HbSS-(a3.7/a3.7)	0.873
HbA2	HbSS-(aa/aa) vs. HbSS-(aa/a3.7)	0.946
	HbSS-(aa/aa) vs. HbSS-(a3.7/a3.7)	0.980
	HbSS-(aa/a3.7) vs. HbSS-(a3.7/a3.7)	1.000

Significance is set at the 0.05 level ($p<0.05$).

To correct for multiple testing, a one-way ANOVA Tukey's Post-hoc Test was performed. There were significant differences between patient groups for the following haematological parameters: (1) MCV – (αα/αα) and (αα/α3.7) (**0.004**; $p<0.05$), (αα/αα) and (α3.7/α3.7) (**$p<0.0001$**), and (αα/α3.7) and (α3.7/α3.7) (**0.015**; $p<0.05$); (2) WBC - (αα/αα) and (αα/α3.7) (**$p<0.0001$**); (3) lymphocyte count - (αα/αα) and (αα/α3.7) (**0.002**; $p<0.05$); and (4) monocyte count - (αα/αα) and (αα/α3.7) (**0.024**; $p<0.05$). The co-inheritance of the α-globin gene deletions suggested an improved haematological phenotype for SCA patients in the study cohort as there was a significant decrease in the MCV, WBC, lymphocyte, and monocyte counts, with an increase in the number of α-globin gene deletions.

Table 21: Literature-derived haematological parameters for SCA patients with α-thalassemia

	CAMEROON			AFRICAN AMERICAN (Embury et al. 1982a)			BRAZILIAN (Belisário et al. 2010)			AFRICAN AMERICAN (Guasch et al. 1999)			AFRICAN AMERICAN (Steinberg and Embury 1986)		
	HbSS-(αα/αα) (64%)	HbSS-(αα/α3.7) (31%)	HbSS-(α3.7/α3.7) (5%)	HbSS-(αα/αα) (53%)	HbSS-(αα/α3.7) (38%)	HbSS-(α3.7/α3.7) (9%)	HbSS-(αα/αα) (71%)	HbSS-(αα/α3.7) (28%)	HbSS-(α3.7/α3.7) (1%)	HbSS-(αα/αα) (59%)	HbSS-(αα/α3.7) (21%)	HbSS-(α3.7/α3.7) (20%)	HbSS-(αα/αα) (53%)	HbSS-(αα/α3.7) (29%)	HbSS-(α3.7/α3.7) (18%)
	Mean ±SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Age	19.7 ± 10.1	20 ± 12.0	20.1±11.6	-	-	-	6.5 ± 2.3			30	31		-	-	-
RBC	3.6 ± 7.7	3.3 ± 1	3.5 ± 1.1	-	-	-	-	-	-	-	-	-	-	-	-
HAEMOGLOBIN LEVEL	8.1 ± 2.2	8.8 ± 2.4	8.4 ± 2.5	7.9 ± 0.9	9.8 ± 1.6	9.2 ± 1.0	7.85 ± 0.95	8.12 ± 1.15	8.30 ± 0.75	8.1 ± 0.2	8.3 ± 0.2		8.0 ± 1.1	8.6 ± 1.1	9.2 ± 1.3
MCV	85.4 ± 11.1	79.6 ± 7.8	70.8 ± 12.6	-	-	-	93.52±6.34	85.70±5.51	71.93±3.20	99 ± 3	86 ± 2		92±7	83 ± 7	72 ± 4
MCHC	35.2 ± 3.9	34 ± 4.9	33.2 ± 4.8	-	-	-	-	-	-	34.9 ± 0.2	33.9 ± 0.2		-	-	-
WBC	15.7 ± 7.6	11.1 ± 4.4	9.3 ± 3.9	-	-	-	17.28±4.07	14.95±3.60	11.10±1.57	13 ± 1	13 ± 1		-	-	-
Lymphocyte count	6.3 ± 3.6	4.3 ± 1.9	3.9 ± 2.2	-	-	-	-	-	-	-	-	-	-	-	-
Monocyte count	1.6 ± 1.1	1.1 ± 0.6	1 ± 0.6	-	-	-	-	-	-	-	-	-	-	-	-
Platelet level	386.7 ± 131.5	371.5 ± 127.9	281.6 ± 117.8	-	-	-	396.52±83.58	395.78±79.70	386.0±95.38	390 ± 14	398 ± 21		-	-	-
HbF	14 ± 10.8	12.9 ± 6.4	13.8 ± 7.7	-	-	-	18.63±8.02	17.95±9.08	13.33±5.51	-	-	-	-	-	-
HbA2	4 ± 2.4	4.1 ± 1.8	4.2 ± 0.9	-	-	-	-	-	-	-	-	-	2.8 ±0.4	3.3 ± 0.6	3.8 ± 0.4

N = Number of cases; SD = Standard deviation

Other studies in the literature reported on a decrease in the HbA level, MCV, MCHC, WBC, platelet level and HbF, and a decrease in the HbA2 level, with an increase in the number of α-globin gene deletions. The Cameroonian study cohort revealed a decrease in the HbA level, MCV, MCHC, WBC, platelet, and HbF levels.

The mean ± SD for the age of diagnosis for HbSS-(αα/αα), HbSS-(αα/α3.7), and HbSS-(α3.7/α3.7) is 3.5 ± 3.9, 5.1 ± 5.1 and 5.7 ± 5.3 years respectively. The following groups were compared: HbSS-(aa/aa) vs. HbSS-(aa/a3.7), HbSS-(aa/aa) vs. HbSS-(a3.7/a3.7), and HbSS-(aa/a3.7) vs. HbSS-(a3.7/a3.7). A ONE WAY ANOVA Tukey's Post-hoc test, revealed that there were no significant differences between and within groups when comparing the age of diagnosis across the groups.

Table 22: Clinical characterisation for the patient cohort with (αα/αα), (αα/α3.7) and (α3.7/α3.7)

		HbSS - (αα/αα)					HbSS- (αα/α3.7)					HbSS-(α3.7/α3.7)					
		N	**Mean ± SD**	**Median**	**Min.**	**Max.**	**N**	**Mean ± SD**	**Median**	**Min.**	**Max.**	**N**	**Mean ± SD**	**Median**	**Min.**	**Max.**	**SD**
BMI (kg/m^2)		48	18.5 ± 3.5	18	12	27	25	18.3 ± 4	17.7	13	27	7	19.6 ± 2.4	18.3	17	23	2.4
SBP (mmHg)		49	108.6 ± 13.6	108	86	156	27	109.5 ± 12.7	108	89	135	6	106.2 ± 6.2	105.5	99	116	6.2
DBP (mmHg)		49	58.5 ± 9.3	56	43	93	27	61.7 ± 8.6	60	47	79	6	58.2 ± 9.2	60.5	45	70	9.2
No. of VOC/year		75	2.9 ± 2.4	2	0	15	34	3.7 ± 6.9	2	0	40	9	1.7 ± 1.1	1	1	4	1.1
Stroke	**YES**	6	-	-	-	-	1	-	-	-	-	1	-	-	-	-	-
	NO	65	-	-	-		33	-	-	-		7	-	-	-		-
No. of consultations/year		70	2.3 ± 2.7	1	0	12	31	2.1 ± 2.9	1	0	12	7	1.3 ± 1.6	1	0	4	1.6
No. of hospitalisations/year		70	1.9 ± 2.4	1	0	12	33	1.9 ± 3	1	0	12	7	1.9 ± 1.6	2	0	4	1.6

N = Number of cases; SD = Standard deviation; Min. = Minimum; Max. = Maximum

The following groups were compared: HbSS-(aa/aa) vs. HbSS-(aa/a3.7), HbSS-(aa/aa) vs. HbSS-(a3.7/a3.7) and HbSS-(aa/a3.7) vs. HbSS-(a3.7/a3.7). Post-hoc analysis, one-way ANOVA Tukey's Post-hoc Test, revealed that there were no significant differences between and within groups of patients with the various genotypes relating to α-thalassemia for the clinical characteristics investigated.

4.6 SNP GENOTYPING IN THE *HMIP, BCL11A, HGB XmnI-158&OR51B5/6* LOCI

The effects of α-thalassemia on key clinical and haematological indices in the generalised linear regression model, adjusted for age, sex and the five SNPs that influence HbF levels, have been tabulated.

Table 23:Effects of α-thalassemia on key clinical and haematological indices, in generalised linear regression models, adjusted for age, gender and five SNPs* that influence HbF level.

Outcomes	Unit of measurement	Single deletion vs. No deletion			Double deletion vs. No deletion		Number of Observations
		Estimates (SE)	p-values		Estimates (SE)	p-values	
Consultations	Day/year	-1.32 (0.63)	**0.038**		-1.31 (1.06)	0.221	110
Hospitalisation	Day/year	-0.17 (0.34)	0.608		-0.37 (0.57)	0.514	104
Vaso-occlusive crisis	Number/year	-0.57 (0.50)	0.253		-1.49 (0.84)	0.079	121
RBC count	million cells/µl	0.37 (0.16)	**0.021**		1.03 (0.27)	**0.0002**	149
Hemoglobin	g/dl	0.58 (0.37)	0.120		0.72 (0.63)	0.25	150
HbA2	(%)	0.23 (0.52)	0.656		0.04 (0.87)	0.956	143
HbF	(%)	-1.55 (1.57)	0.327		0.97 (2.64)	0.715	147
MCV	fl	-5.72 (1.89)	**0.003**		-18.18 (3.19)	**<0.0001**	150
WBC count	X10^9/L	-3.43 (1.04)	**0.001**		-4.31 (1.75)	**0.015**	150
Lymphocytes count	X10^9/L	-2.15 (0.655)	**0.001**		-2.41 (1.102)	**0.030**	130
Monocytes count	X10^9/L	-0.51 (0.20)	**0.01**		-0.67 (0.34)	**0.05**	130
Platelets count	X 10^9/L	-4.80 (27.49)	0.86		-45.42 (46.23)	0.32	149

SE = Standard Error
***HbF related SNPs are: BCL11A rs4671393, HBS1L-MYB rs28384513, HBS1L-MYB rs9399137, HBS1L-MYB rs9402686, HBS1L-MYB rs9494142 and OR51B5/6 rs5006884.**

The co-inheritance of α-thalassemia was associated with a lower consultation rate (**p=0.038**). The effects of the co-inheritance of α-thalassemia on the RBC count, MCV and lymphocyte

count were observed. Two SNPs were significantly associated with specific haematological indices: *BCL11A rs4671393* with the HbF level (**p=0.005**) and *HMIP rs9399137* with a lower lymphocyte count (estimate=-2.09816; SD =1.02912; **p=0.044**). In addition, females had a significantly higher MCV (estimate=3.88, SD =1.73; **p=0.02**), HbA2 (estimate=1.02; SD =0.48; **p=0.035**) and HbF level (estimate=3.82; SD =1.44; **p=0.009**).

CHAPTER 5: DISCUSSION

5.1 PRIMARY FINDINGS

Currently, there is little data available on the prevalence of α-thalassemia occurring with SCA (HbSS) within the Cameroonian context. This study showed that Cameroonian SCA patients were more likely to co-inherited α-thalassemia than the controls and exhibited predominantly 3.7kb α-globin single gene deletion. Haplotype analysis illustrated a high prevalence of the Benin haplotype and a lower prevalence of the Cameroon haplotype. The co-inheritance of α-thalassemia was associated with an improved clinical phenotype in SCA patients. The improved clinical phenotype explains the age of diagnosis, which increased with the number of α-globin gene deletions. The results from this study have implications for clinical follow-ups, anticipatory guidance, or counselling of SCA patients and their families, and future research in Cameroon, and on the African continent.

A high prevalence of the 3.7kb α-globin gene deletion has been reported among SCA patients in Brazil (29 %) (Belisário et al. 2010), India (32%) (Pandey et al. 2011), the UK among African Britons (34%) (Day et al. 2012), Guadeloupe (36%) (Tarer et al. 2006), Saudi Arabia (40%) (Alsultan et al. 2012), the USA among African Americans (41%) (Guasch et al. 1999), Oman (43%) (Wali et al. 2002), France among Africans (48%) (Bernaudin et al. 2008), and Tanzania (58%) (Cox et al. 2013). However, none of these studies compared the prevalence of the 3.7kb α-globin gene deletion to HbAA and HbAS individuals from the same setting. The present study has provided a unique contribution toward consolidating the hypothesis of a possible positive effect of the 3.7kb α-globin gene deletion on the survival of SCA patients. A decade ago, in Congo, researchers reported a significant difference in coinheritance, with 67.2% of SCA patients who had co-inherited the 3.7kb α-globin gene deletion, as compared to 54.8% of HbAA adults (Mouélé et al. 2000). In Yemen, a similar trend was reported with 34.6% of SCA patients carrying the 3.7kb α-globin gene deletion, compared to 26.3 % in the HbAA group (El-Hazmi and Warsy 1999). The present study shows that the prevalence of the 3.7kb α-globin gene deletions in the control cohort (HbAS and HbAA) was comparable to that reported previously in many other settings across Africa, such as, (1) 15.8% in Kenya (Foote et al. 2013), (2) 15.1 % in Rwanda (Gahutu et al. 2012), (3) 20.8% in Guinea (Millimono et al. 2012), and, (4) 10%-25% in high-altitude villages in Northern Tanzania (Enevold et al. 2007). The

differential frequency among various populations was attributed deleterious environmental forces, like the prevalence of malaria or bacteraemia in patients (Steinberg 2005), although this needs to be confirmed by future research.

This study is the first of its kind to study SCA in a Cameroonian population (according to a PubMed search, using the key words 'Cameroon sickle cell disease/anaemia'). The clinical components examined in this study such as the haematological parameters, BMI, SBP, DBP, VOCs, incidence of stroke, number of hospital consultations and the number of hospitalisations has not been previously examined in a single study, to the best of the investigator's knowledge. These findings have contributed to the pool of knowledge regarding the molecular characterisation of SCA in Cameroon. Furthermore, these findings could have implications on the selection of the site and population for future studies in the field of SCA and α-thalassemia. This study also had a capacity-building dimension in genomics research relating to SCA. This study, from design and sample collection to the performing of the molecular experiments, data analysis and reporting, was performed, in full, on the African continent. These findings add to the global pool of data that will become available to researchers, clinicians, and genetic counsellors across the world, with the added aim of improving disease prevention and management within Cameroon and eventually extending to the rest of Africa.

5.2 HAEMATOLOGICAL INDICES: PATIENTS vs. CONTROLS

The haematological indices in Cameroonian SCA patients were similar to what was reported in patients in the literature, with moderate to severe anaemia and elevated levels of WBCs (Table 11). In this study, the haematological profile for the unaffected individuals was compared to the haematological profile of the SCA patients (Table 9). The haematological parameters from this study were compared to a reference list compiled and represented by Table 8. The unaffected and carrier cohorts had a haematological profile that fell within the healthy range (Tables 8 and 9).

There was a significant difference in the HbA level between HbAA and HbSS individuals, and HbAS and HbSS individuals (Tables 9 and 10). Patients had a lower HbA level, which is an indicator of anaemia (Rochester and Minn 2011). The literature supports this finding (Herrick 1910; Nicoll et al. 2008; Hussein and Hadad 2010).

A significantly high WBC count was found in the Cameroonian SCA cohort under study, between unaffected individuals (HbAA) and HbSS patients, and between carriers (HbAS) and HbSS patients, where patients displayed marginally higher levels (Tables 8, 9 and 10). Similar results were found in other studies (Kasschau et al. 1996; Hebbel and Vercellotti 1997; Lard et al. 1999; Bagby 2007; Dinauer and Coates 2008). Olatunji and Davies (2000) reported that an increased WBC leads to a rise in the number of hospitalisations, and, a decrease in the HbA and HbF levels, in SCA patients.

Lymphocytes and monocytes are classified as white blood cells (Thomas 2007; Kaushansky et al. 2010) and had an elevated count similar to WBC (Tables 9 and 10). Since high WBCs are associated with an increase in VOCs, the patients in the study cohort should have had high VOC incidences; however, this was not the case (Kasschau et al. 1996; Hebbel and Vercellotti 1997). Patients in the study cohort had low incidences of VOCs (Median = 2/year) (Table 13), which indicated a milder form of SCA.

HbA2 is found in adults in minute amounts (2-3%) (Liebhaber et al. 1980). High levels of HbA2 were reported in HbSS patients (Craver et al. 1997; Huisman, 1997). There was a significant difference between the HbA2 level of HbAA and HbSS individuals in the study cohort, which resulted in patients having a higher level than the normal range (Tables 8, 9 and 10). This finding is similar to other studies (Craver et al. 1997; Huisman, 1997). Deviations from normal HbA levels, WBC, lymphocyte count and HbA2 levels for patients in the study cohort was also similar to other studies (Tables 8, 9, 10 and 11; Kasschau et al. 1996; Hebbel and Vercellotti 1997; Lard et al. 1999).

The haematological indices were classified in the literature based on gender, specifically RBC, HbA level, MCV and MCHC (Bunn 2011; Rochester and Minn 2011; Schoenborn and Snyder 2012). Since the individuals in the study cohort were not gender-matched and there were no major differences between the haematological parameters across gender, the values specific to male or female were negligible. Furthermore, the other studies in the literature did not consider the haematological parameters based on gender, which allowed this study to be comparable to the other studies in the literature (Table 11).

5.3 CLINICAL SEVERITY IN SCA PATIENTS

The BMI, SBP, DBP were in the normal ranges for patients in the study cohort (Table 13). However, comparative data for the unaffected and carrier individuals was not gathered, hence a phenotypic profile of the BMI, SBP, and DBP was only compiled for the patient cohort.

Body mass index (BMI) was of importance because SCA has been associated with hindering the growth of pre-adolescents, irrespective of gender (Stevens et al. 1986; Akodu et al. 2014). The BMI scale classified a number less than 18.5kg/m^2 as underweight, 18.5kg/m^2-24.9kg/m^2 as normal/healthy, 25kg/m^2-29.9kg/m^2 as overweight and 30kg/m^2 or more as obese (WHO 1995). Patients in the study had a normal BMI (Table 13).

Blood pressure (BP) is an important factor as it is linked to the occurrence of stroke (Akingbola et al. 2014; Gosmanova et al. 2014). For adults, a healthy SBP reading is between 110mmHg - 140mmHg, with the optimal reading at 120mmHg, and a healthy DBP reading is between 60mmHg - 90mmHg, with the optimal reading at 80mmHg. HbSS patients generally have a lower than normal SBP and DBP (De Jong et al. 1982; Pegelow et al. 1997; Ernst et al. 2000; Ekure et al. 2012). SCA patients in this study (all were adults) had a slightly lower SBP and DBP (Table 13). This is due to (1) vasodilatation and (2) an elevation in the renal tubular water and sodium secretion thereby decreasing the arterial pressures and in turn lowering the SBP (Grell et al. 1981; Homi et al. 1993; Rodgers et al. 1993; Pegelow et al. 1997; Aderibigbe et al. 1999).

Pain crises are a measure of disease severity and are used by clinicians to classify SCA as mild (less than three pain crises/year) or severe (more than three pain crises/year) (Nebor et al. 2011). The median number of VOCs per year was two, which indicated a less severe level of pain experienced by patients in this study cohort (Table 13). However, this was not an accurate measure of severity, as each individual's pain threshold differed and pain episodes were not often reported.

Stroke is a severe, frequent complication associated with SCA (Verduzco and Nathan 2009; Gueguen et al. 2014) with debilitating effects on the patient’s life (Tengs et al. 2001). Stroke is used as a proxy of severity to identify patients with SCA (Platt et al. 1994). Seven percent of the patients in the study cohort were affected by stroke (Table 13). This was a relatively high proportion, which is confirmed by previously published data on Cameroonian SCA patients (Njamnshi et al. 2006).

The number of consultations per year was assessed to determine how often patients frequented the clinic. This allowed for the establishment of the degree of patient care required for each patient. The median number of consultations per annum was one (Table 13). This indicated that there was a lower level of care required, which can be attributed to the majority of the patients in the study cohort having had a milder form of SCA (median of two per year – less than three; Table 13; Clarke and Higgins 2000). However, this low number could also be attributed to the inability of patients to afford regular consultations, in terms of the associated medical and transportation costs (Wonkam et al. 2014a). The majority of care is provided by family members, which may indicate a greater level of care required by patients (Wonkam et al. 2014a).

SCA is characterised by severe pain crises due to the obstruction of the blood vessels by the sickled erythrocytes thereby making hospitalisation for pain or other complications a common occurrence (Wilson et al. 1979; Brousseau et al. 2010). A study by Allen-Liles et al. (2014) reported that the hospital-led management of VOCs in SCA patients decreased their length of stay, thus emphasising the need to examine the number of hospitalisations to assess the degree of disease severity and to establish the type of patient care required. The median number of hospitalisations for the patient cohort was one (Table 13), which suggested a relatively mild phenotype. In a first world context, VOCs will result in a hospitalisation. However, within the Cameroonian context, this is not the case (Wonkam et al. 2014a). Cameroonians, on average, are unable to afford medical aid and transportation costs to the hospital, therefore relying on care from their family members (Wonkam et al. 2014a). Thus, the low number of hospitalisations could be due to a lack in affordability and not entirely due to a milder phenotype.

5.4 HAPLOTYPES OF THE *HBB* GENE

The allele frequencies of the haplotypes that occurred in this patient cohort, illustrated that the Benin haplotype was the most common (Table 14). In addition, the combination of haplotypes in patient cohort illustrated that the Benin/Benin (42.2%), Benin/Cameroon (26.8%), Benin/atypical (14%) and Cameroon (5.1%) types were most prevalent (Figure 14).

The Benin haplotype was initially found in people from Central West Africa (Curtin 1969). High (95%) frequencies of the Benin haplotype have been reported in Tunisian populations

(Fattoum 2006), with lower frequencies in the Venezuelan (29.2%) and Brazilian populations (20 - 28.8%; Moreno et al. 2002; Da Luz et al. 2006; Adorno et al. 2008). The Benin/Cameroon haplotype occurs in 1.6% of the Brazilian population (Adorno et al. 2008).

This study found a relatively low proportion of the Cameroon haplotype (Table 14; Figure 14). The Cameroon haplotype occurs more frequently in SCA patients in Sudan (Mohammed et al. 2006; Elderdery et al. 2012). This raises the question of the geographical origin of this haplotype. It is possible that the origin of the HbSS mutation was associated with the Cameroon haplotype in Sudan and not Cameroon and that the first description in Cameroon was due to population migration, which introduced this haplotype into Cameroon (Mohammed et al. 2006; Elderdery et al. 2012). A comparative study of the Cameroon haplotype in both Cameroonian and Sudanese patients could unravel this mystery.

The Bantu and Arab-/Saudi-Indian haplotypes occurred in low percentages (less than 1%) in the patient cohort under study. Atypical haplotypes were previously shown to be common in the Cameroonian population (Sarnaik and Ballas 2001) and deserves further investigation by way of deep sequencing. This method allows for the establishment of the pattern of particular nucleotides in a sequence of DNA, where the depth of the method is several times greater than the length of the sequence being analysed (Meyerson et al. 2010; Ajay et al. 2011; Hampton et al. 2011). This makes the estimation of other quantities possible including the detection of SNPs (Meyerson et al. 2010; Ajay et al. 2011; Hampton et al. 2011). Deep sequencing offers high coverage, which overcomes inaccuracies in base calling and assembly, as well as high-level accuracy (Meyerson et al. 2010; Ajay et al. 2011; Hampton et al. 2011). The accuracy of sequencing is increased by sequencing individual genomes several times (Meyerson et al. 2010; Ajay et al. 2011; Hampton et al. 2011). This aids the determination of additional SNPs in the *HBB* gene region, as well as gives further insight into the classification of the atypical haplotypes discovered in the Cameroonian cohort under study.

The patients in the study cohort were of various Cameroonian ethnicities (Table 15). The majority of patients with the Benin/Benin haplotype were of mixed ancestry origin, for Benin/Cameroon patients were Ewondo, for Benin/Atypical patients were mixed ancestry, and for Cameroon/Cameroon patients were Banen and Bassa (Table 15).

There were no significant associations between the haematological parameters and the haplotypes that occurred in the Cameroonian study cohort (Table 16). This was in agreement with the study by Mohammed et al. (2006). The comparison of the clinical parameters across the four most frequently occurring haplotype combinations did not reveal any major differences (Table 17). These findings were in agreement with the study by Steinberg et al. (1998).

5.5 CO-INHERITANCE: SCA and α-THALASSEMIA

The current study examined the effect of two α-globin gene deletions, the 3.7kb, and 4.2kb, deletion. The 4.2kb deletion was not detected in this study cohort (Figure 15). This implies that the 3.7kb deletion may only apply to a Cameroonian setting (sample size of 262 individuals).

This study revealed a high frequency of the co-inheritance of α-globin gene deletions (36%) in SCA patients, which was higher than the prevalence of these deletions in the Cameroonian unaffected (HbAA) and carrier individuals (HbAS) (Table 18). The high frequency in HbSS individuals was comparable to previous reports in the literature (Table 18; Guash et al. 1999; Bernaudin et al. 2008; Belisário et al. 2010; Nebor et al. 2010; Cox et al. 2013).

HbAA-(αα/αα) individuals had a healthy haematological profile. These were healthy/normal/unaffected individuals. HbAA-(αα/α3.7) were unaffected individuals with one α-globin deletion and HbAA-(α3.7/α3.7) were unaffected individuals with two α-globin deletions. HbAS-(αα/αα) individuals had a healthy haematological profile. These were carriers with no α-globin gene deletions. HbAS-(αα/α3.7) were carriers with one α-globin deletion and HbAS-(α3.7/α3.7) were carriers with two α-globin deletions. The only abnormality displayed, for HbAA-(αα/α3.7), HbAA-(α3.7/α3.7), HbAS-(αα/α3.7), and HbAS-(α3.7/α3.7) was a MCV that was below the normal range, thereby indicating smaller erythrocytes (Murphy 1999; Culligan and Watson 2009).

The co-inheritance of α-thalassemia and SCA was significantly associated with a lower MCV between and within groups, WBC between (αα/αα) and (αα/α3.7) patients, lymphocyte count between (αα/αα) and (αα/α3.7) patients, and monocyte count between (αα/αα) and (αα/α3.7) patients, all of which were reported to be associated with an improved clinical phenotype (Table 19; Table 20; Table 21). Other studies reported an increase in HbA (Table

21) and HbA2 (Steinberg and Embury 1986), and a decrease in MCV (Table 21; Steinberg et al. 1995), and WBC (Belisário et al. 2010). These results are similar to results from this study (Table 19). A reduction in the MCV is due to the loss of the α-globin genes (Belisário et al. 2010), which in turn affords a protective effect against the SCA phenotype by decreasing the amount of intravascular sickling, thereby alleviating pain (Condon et al. 1983). The decrease in the WBC is due to a decrease in the haemolytic rate, the amount of sickled erythrocytes and the inflammatory process (Belisário et al. 2010).

The literature has reported an improved haematological phenotype for SCA patients with an increase in α-globin gene deletions when compared to patients with no deletions (Belisário et al. 2010; Pandey et al. 2011). Even so, α-thalassemia's protective effect against the severity of SCA is contested (Joly et al. 2011), although the majority of the literature suggests that α-thalassemia is associated with an improved phenotype in SCA patients (Condon et al. 1983; Belisário et al. 2010; Dabari et al. 2012; Pandey et al. 2011).

The relative delay of diagnosis was explained by the non-existence of any SCA screening policies in Cameroon (Wonkam et al. 2014a). The median age of diagnosis for the patients in the study cohort was three years (Table 12). Other studies reported the age of diagnosis to be approximately ten years in Senegal (Diop et al. 1999), two years in Nigeria (Brown et al. 2010) and three years in Uganda (Mpalampa et al. 2012). The data represented indicates that the co-inheritance of α-thalassemia could delay the clinical manifestations of SCA, thereby positively affecting the survival of Cameroonian patients. This explains the elevated allele frequency of the 3.7kb α-globin gene deletions among patients vs. controls (Figure 17; Figure 18).

Fabry et al. (1984) reported that in the first ten years of life among African American HbSS individuals, the prevalence of the 3.7kb α-globin gene deletion was comparable to that in the general African American population (17%), while after 20 years of age, the prevalence increased to 49%. Similarly, in Cuban patients, the prevalence of the 3.7kb α-globin gene deletions increased with age (Martinez et al. 1996). Alpha-thalassemia may be linked to improved survival in patients (Martinez et al. 1996). The age of diagnosis associated with the co-inheritance of the α-thalassemia trait is useful in describing a context where neonatal

screening for SCA is non-existent. This is especially true for Cameroon, where SCA diagnosis is only made once patients have manifested symptoms.

Multivariate analysis revealed that the co-inheritance of α-thalassemia trait was associated with a lower consultation rate in this cohort (Table 23). There were no significant differences between the co-inheritance of the 3.7kb α-globin gene deletions and the clinical parameters (Table 22; Table 23). This finding may be due to the small sample size and the challenge to define a SCA severity scoring system that could be applied universally (Coelho et al. 2012). Furthermore, the potential deleterious effect of α-thalassemia on the number of painful episodes reported previously (Tarer et al. 2006; Dabari et al. 2012) could be difficult to validate within the Cameroonian context, where free medical services to patients are unavailable. Pain tolerance and socio-economic factors could have influenced the number of hospital visits and thereby biased our evaluation of clinical events.

It has been shown that individuals who have SCA and the 3.7kb α-thalassemia deletion, displayed a low RBC and serum iron level, indicative of an afforded protection against severe anaemia (Stuart and Nagel 2004; Morris 2008; Mariani et al. 2009; Pandey et al. 2012). The relationship between α-thalassemia and environmental factors like bacteraemia and malaria should be studied further in terms of the survival of patients.

The association with α-thalassemia, number of VOCs, hospital consultations, and hospitalisations should be interpreted with caution due to the self-reported nature of these clinical measures and the variable degree of pain tolerance. There were no significant differences between the α-thalassemia genotypes, BMI and blood pressure in SCA patients. A study by Joly et al. (2011) highlighted the difficulties of using clinical outcomes to assess the effect of α-thalassemia. However, the most established effect of α-thalassemia was its influence on the haematological parameters.

The improved haematological indices could be the major factor that contributes to the general well-being and life expectancy of SCA patients. Ultimately it may explain the higher prevalence of the 3.7kb α-globin gene deletion among patients than unaffected individuals. In the present study, the results of the generalised linear regression models seem to indicate

that other genomic and demographic factors could also affect the haematological indices (Table 23).

5.6. LIMITATIONS AND RESEARCH RECOMMENDATIONS

One shortcoming of this project is that the study cohort was not age and gender matched. In terms of gender, there were no major differences between the haematological parameters when comparing them across genders. The majority of the literature has not taken gender into account, although when clinically reporting on the haematological indices, gender is taken into account. This study had a gender bias as the majority of the study cohort was composed of females.

The majority of subjects in the study cohort were adults. We recommend that the subjects be carefully age and gender matched for any future studies, as well as addressing the comparison of the BMI and blood pressure of controls. Medical records were not adequately completed and although the statistical programme utilised was able to deal with missing data, a complete record is always advisable. In addition, the age distribution of patients may have biased the *HBB* haplotypes and the distribution of α-thalassemia. Further investigation is required to investigate *HBB* gene haplotypes and the α-thalassemia genotypes in different age groups.

The quality of DNA for certain individuals was poor as DNA was extracted from frozen blood by the salting out method, which is most effective on fresh blood samples. This resulted in additional repetition of experiments several times (up to five times) and having to further purify the DNA samples. The latter did not always render positive results. With every purification, the amount of DNA is drastically reduced, even though the quality of the sample is enhanced. A commercial purification kit should be utilised as the DNA yield and the quality of the sample is better. Of the 300 samples analysed, 38 samples was discarded due to poor quality.

HbF quantification was limited by age and methodology. The median ages of individuals in the study cohort were 26, 23 and 18 years for HbAA, HbAS and HbSS respectively (Table 6). This is essentially an adult cohort. An HbF level of 2% is the maximum found in a healthy individual (Table 8), with up to 20% as the maximum in SCA individuals (Kar et al. 1986; Akinsheye et al. 2011; Jain et al. 2012). The median HbF levels were 8.6%, 7.3% and 13.5%

respectively, for HbAA, HbAS and HbSS in the study cohort (Table 9). Other studies in the literature reported lower mean HbF levels for patients (6.4% for African American and 9.2% for Brazilian SCA patients) (Table 10), compared to those found in this study (13.9%; Table 9). The HbF level for HbSS-males and HbSS-females is 10.8% and 15.7% respectively. For the haplotype groups the HbF levels were 14.6%, 14.8%, 15.7% and 9.9% respectively, for Benin/Benin, Benin/Cameroon, Benin/Atypical and Cameroon/Cameroon (Table 16). For the co-inheritance section of the study, high levels of HbF was seen with an increase in the amount of α-globin gene deletions (Table 19), whereas Belisário et al. (2010), reported a decrease in HbF with an increase in the number of α-globin gene deletions. Although the HbF mean-values for patients fell within the acceptable range (<20%), these values were widely discrepant with maximums of 86% (Table 9; Table16; Table 19). These discrepancies indicate that there was a limitation in the precision of HbF quantification. The majority of the quantification was performed by ADT. This method is cost-efficient, easily reproducible and allows for the detection of HbA, HbS, HbF, and HbC (Ondei et al. 2007). It does not allow for the detection of HbD, HbG, HbO, and HbE (Ondei et al. 2007). In addition, it does not provide precise identification of the variant due to the co-migration of resembling forms (Ondei et al. 2007). In contrast, the HPLC method is a fast, precise, and more accurate method of HbF quantification (Ondei et al. 2007). We recommend using HPLC whenever possible in future studies, which will allow for more accurate measurements.

Multiple SNPs within the *Hind*III restriction site, located in the gamma-globin intervening sequence II, were observed which produced the same RFLP pattern. This suggests the incapacity of RFLP analysis to decode the complexity of sequence variations that effects genomic structure in this gene region. The data generated by this study suggests that high-density SNP mapping may be necessary to accurately explain the association between the *HBB* gene haplotypes and the varying clinical phenotypes relating to SCA.

There were no positive control samples available for the HbSS diagnostic testing section and for the *HBB* gene haplotyping section of the project. Guidance was obtained from the available literature and the positive control samples will be established in future research.

For future research iniatives, the role of malaria in relation to the data obtained in this study could be analysed. Clinical data on whether or not patients had malaria was not recorded.

Specifically for Cameroon, which is a high malaria endemic area affecting 90% of the population (WHO, 2014), as a future study, the role of co-inheritance of malaria in SCA and α-thalassemia can be explored, specifically for the Cameroonian population. This will add data to the pool of information that becomes available for epidemiological purposes. Furthermore, future studies can look at identifying certain domain proteins or amino acid motifs which are selectively expressed in the malaria parasite, that infect HbAS individuals (Beaudry et al. 2014). These research approaches could provide deeper insight into the mechanism of protection by HbAS in malaria co-infection cases. In general, there is lack of well-designed epidemiological and molecular studies to confirm the malaria hypothesis for the high prevalence of these genetic disorders. Moreover, accurate estimation of these hemoglobin disorders would be desirable for proper utilization of health resources in developing countries.

5.7. POLICY AND PRACTICAL IMPLICATIONS

SCA patients had an elevated possibility of many complications that compromised their state of health and wellbeing. The extent of the phenotypic diversity seen in these patients re-iterated the significance of alternative genetic loci that may contribute to the ever-changing disease severity. The confirmation of the knowledge that α-thalassemia has a positive effect on, and possibly a strong selection for the survival of SCA patients, along with the capacity to test for this disease within an African setting, could offer the possibility of profiling patients from birth.

The molecular exploration of SCA and α-thalassemia made it possible to gain insight into the development of valuable local molecular diagnostic laboratories, diagnostic tools, and additional tools for use in a clinical setting to improve the quality of medical care provided to patients. This study identified the genotype to phenotype connection of SCA in Cameroon and it further helped towards the anticipatory guidance in the care of patients, by identifying those who are susceptible to SCA.

CHAPTER 6: CONCLUSION AND PERSPECTIVES

The co-inheritance of α-thalassemia with SCA was associated with an improved haematological profile, with an increase in the number of α-globin gene deletions. The possible positive effect of the co-inheritance of α-thalassemia on SCA patients' survival could explain the high proportion of α-thalassemia among SCA patients when compared to the unaffected controls. These results have implications for disease management in Cameroon in terms of genetic counselling and the detection of SCA.

Despite the fact that more than 70% of SCA sufferers live in Africa (about 13 million people), expenditure on care and research on the African continent, remains negligible. Most advances in the understanding and management of this condition have been based on research conducted in the in other parts of the world. The present study illustrated the capacity of an African team to conduct molecular research on SCA in Africa and thereby provide a clearer perspective for major collaborations and research opportunities with researchers in the rest of the world. Regional collaborations should be built to fast track SCA molecular research on the continent. The possibility of novel haplotypes could shed insight on other aspects of the disease.

If designed appropriately, future studies in Africa could explore the potential beneficial effect of the co-inheritance of the α-thalassemia trait and SCA, on specific phenotypes such as (1) haemolysis (Nouraie et al. 2013), (2) the frequency of gallstones (Vasavda et al. 2007), (3) albuminuria (Guasch et al. 1999; Nebor et al. 2010; Day et al. 2012) or (4) the risk of stroke (Hsu et al. 2003; Belisário et al. 2010; Flanagan et al. 2011). The comparison of the frequency of the 3.7kb α-globin gene deletion in SCA patients, across populations from different ethnicities and geographical locations, should be made with caution, as the advantageous effect of α-thalassemia has been associated with age and the occurrence of malaria (Enevold et al. 2007).

Our confirmation of the knowledge that α-thalassemia has a positive effect on and possibly a strong selection for the survival of SCA patients, along with the capacity to test for this disease within an African setting, could offer the possibility of profiling patients from birth. The data presented by this dissertation is encouraging. In spite of gaps in medical records and

incomplete phenotyping data in the control, the statistical programmes used were able to adequately account and work with missing data to produce accurate statistical outcomes. If patients are profiled, a closer follow-up protocol could be implemented. Adult and neonatal screening of SCA in Cameroon could improve the ability of practitioners to provide appropriate genetic counselling and care.

As much as there is a gap in research there is also a gap in care. Even with the information provided by additional research, there is still a lot of work that must be done on the ground. Cameroonians are often unable to afford medical aid and transportation costs to the hospital, therefore relying on care from their family members (Wonkam et al. 2014a). To lift this burden and aleviate this disease will require work from all sectors.

APPENDIX

A1: INTRODUCTORY EXPLAINATION

Dear patients, parents, and tutors

A research study titled "Sickle Cell Anaemia in Cameroon: Co-Inheritance of α-Thalassemia, *HBB* Gene Haplotypes, Clinical & Haematological Characterisation" is to be carried out by the Faculty of Biomedical Science, Faculty of Medicine at the University of Yaoundé and the University of Cape Town's Division of Human Genetics. The project is under the supervision and the direction of biologists, clinicians, geneticists, and haematologists at these institutions. This study relates to a Cameroonian sickle cell anaemia (SCA) context, without distinction of age, gender, or ethnicity. Unaffected individuals, carriers, and sickle anaemia patients will be required for this study.

SCA is a chronic disease, which is characterised by anemia. It is marked by sickle-shaped red blood cells, and manifests in the form of episodic pain in the joints, fever, leg ulcers and jaundice. The disease occurs in individuals who are homozygous (HbSS) for the mutant haemoglobin (HbS) gene. There is no cure for SCA. It is frequent in Cameroon, affecting approximately 20% of the general population. It was declared a Public Health Priority in Cameroon.

Alpha-thalassemia (α-thalassemia) is a blood disorder, which leads to the decreased production and increased destruction of red blood cells. Hemoglobin in the red blood cells carries oxygen for all organs in the body. The loss of red blood cells results in low haemoglobin. This is as the result of loss of α-globin genes (1, 2, 3, or 4).

The aim of this study is to investigate the prevalence of α-thalassemia in a sample of Cameroonian SCA patients, and to study the correlation between the α-thalassemia genotypes and phenotypes in SCA patients.

The study will consist of interviewing individuals, patients, and/or parents of the patients, to collect the following information:

1. Socio-demographic information such as: age, gender, ethnicity, and level of education for all participants.

2. Clinical information such as: BMI, blood pressure, incidence of stroke, number of vaso-occlusive pain crises per year, the number of consultations per year, and the number of hospitalisations for the patients.

3. The haematological indices such as: the red blood cell count (RBC), haemoglobin level (HbA), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white blood cell count (WBC), lymphocyte count, monocyte count, platelet level, foetal haemoglobin level (HbF), and haemoglobin alpha-2 (HbA2) will be determined from blood using the haematological testing service at Haematology Laboratory of the Centre Pasteur, Yaoundé for all participants.

Interviews will be conducted at the recruitment sites (Yaoundé Central Hospital, Laquitine Douala Hospital and the surrounding sites). All information is and will remain confidential. The data generated will only be used for research purposes.

If you decide to partake in this study, you will be required to provide the above mentioned information (1, 2 and 3) and 10ml of blood (taken from the arm). The drawing of blood will be performed by professional nurses and the haematological report generated by haematologists. The process will take 45 minutes.

The risks relating to participation in this study are minimal. The drawing of blood is painful in terms of the prick and the few seconds of extraction, which may result in blue mark forming. However, the process is not fatal, and therefore no other problems will be encountered due to the drawing of blood . The utmost care will be taken to ensure a sterile environment.

This study will not give the participant any direct benefit, monetary or otherwise. Participation is purely voluntary, free, and confidential.

The participant's results will be individually communicated to them. If the participant does not wish to be informed of their results, their decision will be respected and the results will not be disclosed.

The information from the questionnaire and the haematological analyses will be preserved in form of a computerized database at the Faculty Biomedical Science and the Faculty of Medicine at the University of Yaoundé, and the Division of Human Genetics at the University of Cape Town. This information will be accessible only to the persons involved in this study. The participant will be able to withdraw their consent at any time. No disadvantage for the subsequent medical monitoring will follow from this decision. The team guarantees the protection of the confidentiality of the participants in study by respecting the following principles:

- To interpret and transmit the results of all analyses to the participants who shall request for it.

- To assure respect for good clinical and biological practices.

- To respect the self-government and respectability of every subject.

- To guarantee the confidentiality of participant data.

- To share research conclusions with thestudy participants.

- To assure the appropriate broadcasting of the research results.

- To inform participants of the research-related evolution of knowledge.

For more information, contact:

1. Prof. J. Ngogang supervisor of the study (Laboratory of Biochemistry of CHUY, such: 99922353)

2. Prof. A. Wonkam, responsible for the study (Faculty of medicine of Yaoundé, Such: 96401593; University of Cape Town)

3. Dr Mr Monny Lobe, joint manager of the study, (doctor responsible for the Centre of reference to the Central Hospital of Yaoundé Tel: 77517004)

4. Ngo Bitoungui Valentina Josiane (PhD student, Tel: 99722383, email: vngobitoungui@yahoo.fr).

A2: TEMPLATE OF THE INFORMED CONSENT DOCUMENT

ASSENT _____________ OR CONSENT ___________

Relative : Mother Father Tutor

Name: ....

First name: ....

Date of birth : ....

Patient Name ....

First name ....

Date of birth ....

I hereby confirm that I have understood, with my satisfaction, and that I have had enough time to reflect on my decision and ask the necessary questions, the study.

I accept that the research of the biological, and genetic characteristics, mentioned below, are carried out on my sample of blood /sample of blood of my above-mentioned child.

- Hémogramme (Numeration Formulates Blood).
- Haemoglobin electrophoresis for the confirmation of the diagnosis, and evaluation of the rate of foetal haemoglobin.
- Genetic analysis: (1) genetic confirmation of the diagnosis of SCA, (2) investigation of the genetic factors that influence the severity of SCA (identification of the *HBB* gene haplotypes and the α-globin deletions).

I accept that the data collected will be preserved in a computerized form, with the aim of research for the improvement of SCA. The data will be treated in a strictly confidential manner.
I voluntarily partake in this study. I may withdraw my agreement of participation at any time without having to give reasons.

I approve the conservation of my/child's sample of blood or DNA sample:

- For further and future research, while maintaining the strictest confidentiality.

Signature Date and place ....

Investigator

I hereby confirm that I have informed the patient above, of every aspect of the study and I have answered all his/her questions.

Name .... First name ....

Place and date ....Signature ....

A3: TEMPLATE OF THE QUESTIONAIRE

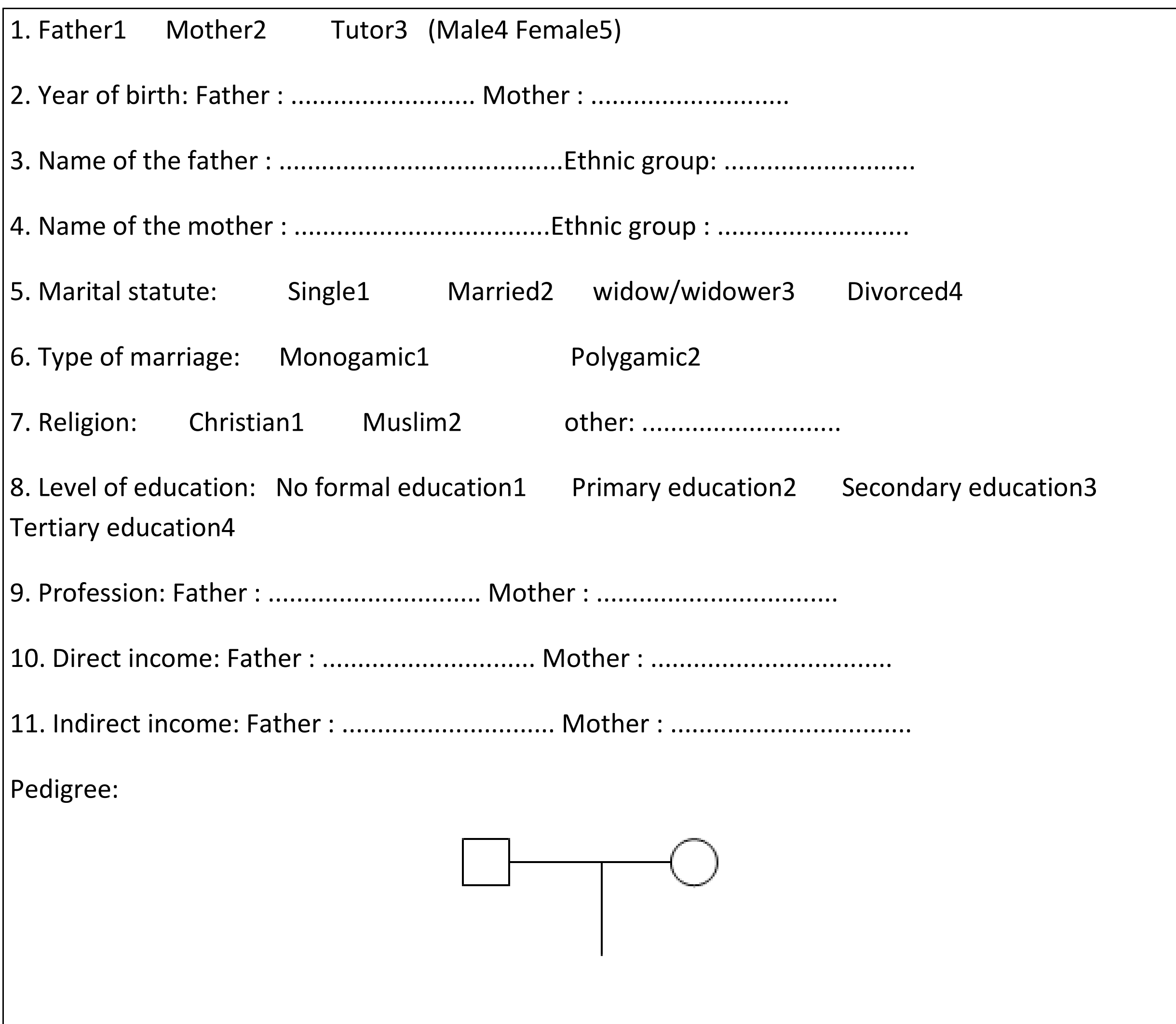
NRO……………………

Name of the patient : .... Address:
.... Such:......................./........................

Date of birth: ....

Level of education: No formal education1 Primary education2 Secondary education3 Tertiary education4

SOCIO-DEMOGRAPHICS DATA OF THE PARENTS



1. Father1 Mother2 Tutor3 (Male4 Female5)

2. Year of birth: Father : .... Mother : ....

3. Name of the father : ....Ethnic group: ....

4. Name of the mother : ....Ethnic group : ....

5. Marital statute: Single1 Married2 widow/widower3 Divorced4

6. Type of marriage: Monogamic1 Polygamic2

7. Religion: Christian1 Muslim2 other: ....

8. Level of education: No formal education1 Primary education2 Secondary education3 Tertiary education4

9. Profession: Father : .... Mother : ....

10. Direct income: Father : .... Mother : ....

11. Indirect income: Father : .... Mother : ....

Pedigree:

CLINICAL PHENOTYPE OF SCA PATIENTS

Frequency of VOCs/annum during last 5 years

Age of the diagnosis ....

Painful crises ....

Episode of cerebral vascular accident: yes1 no 2 unknown 3

Acute thoracic syndrome ....

Painful crisis - swelling of the back of the hands and feet often associated with a swelling with the fingers (Dactylitise)………

Acute splenic sequestration ....

Number of consultations/year ....

Number hospitalisations/year ....

Date from the last transfusion: less than 6 months1 more than 6 months2

Use of hydroxyurea: Yes1 No2

HAEMATOLOGICAL PARAMETERS OF SCA PATIENTS

1- Hémogramme

GR. ....

Hb ....

MCV ....

MHCC ....

GB ....

Distribution: Lymphocytes ....Monocytes ....

Platelets ....

2 - Electrophoresis of haemoglobin: (HbSS)

Hb F ....

Hb A ....

Hb A2 ....

Hb C ....

GENOTYPAGE AND SEQUENCING

1-Molecular confirmation of SCA by ADT (S)

YES1

NO2

2-Haplotype of the *HBB* gene:

- Cameroon1 - Senegal2 - Benin3

- Bantu4 - Atypical5 - Arab-Indian6

3-Specific Variations of the genome, which affects the rate of HbF – α GLOBIN GENE ANALYSIS:

(αα/αα) No deletion1

(–α/αα) Type 2-heterozygote2

(–α/–α) Type 2-homozygote3

(ααα/-) Alpha triplication4

A4: CLINICAL AND EXPERIMENTAL DATA OF INDIVIDUALS IN THE STUDY COHORT

Table 24: Characterisation of patient information, clinical and experimental data

The characterisation of subject information used in the study is presented in this table. Colours characterise the study cohort according to unaffected controls (HbAA; green), carriers (HbAS; blue) and patients (HbSS; red). The following abbreviations and their units are as follows: male (M), female (F), age in years, red blood cell count (RBC) in a million cells/µl, haemoglobin level (HbA) in g/dl, mean corpuscular volume (MCV) in fL, mean corpuscular haemoglobin concentration (MCHC) in g/dl, white blood cell count (WBC) in10^9X/L, lymphocyte count (L) in 10^9X/L, monocyte count (M) in 10^9X/L, platelet level (P) in 10^9X/L, foetal haemoglobin (HbF) as a percentage and haemoglobin alpha-2 (HbA2) as a percentage, the age of diagnosis (AOD) in years and the number of vaso-occlusive crises (VOCs) per year. Phase I, II and III refers to the data collected from the three experimental parts performed.

SUBJECTS	SEX	AGE	ETHNICITY	EDUCATION	RBC	HbA	MCV	MCHC	WBC	L	M	P	HbA2	HbF	AOD	VOCs	STROKE	CONSULTATIONS	HOSPITALISATIONS	PHASE I	PHASE II	PHASE III
RC42																				HbAA		(aa/aa)
RC43																				HbSS	BENIN/CAMEROON	(aa/aa)
RC44																				HbSS	BENIN/CAMEROON	(aa/aa)
RC45	F	17			2.6	7.1	86.8	31.5	17.5			433.0	3.2	0.0						HbSS	BENIN/CAMEROON	(aa/aa)
RC46																				HbSS	BENIN/BENIN	(aa/aa)
RC47	F	43			2.9	9.2	92.6	34.5	13.7			315.0	3.0	20.0						HbSS	BENIN/BENIN	(aa/aa)
RC49	M	9			2.5	6.6	81.3	32.9	24.0			373.0	3.2	18.1						HbSS	BENIN/CAMEROON	(aa/a3.7)
RC50	M	14			3.1	7.6	74.2	32.7	15.8			396.0	3.5	0.0						HbSS	BENIN/BENIN	(aa/a3.7)
RC51	M	8			3.5	8.5	79.3	30.6	24.4			414.0	2.3	21.6						HbAA		(aa/aa)
RC52																				HbAS		(aa/aa)
RC53	F	11			2.5	6.4	82.0	31.9	23.2			246.0	4.4	26.5						HbSS		(aa/aa)
RC54	M	7			2.2	6.2	80.0	34.5	21.9			148.0	3.4	26.2						HbSS	BENIN/ATYPICAL	(aa/a3.7)
RC55																				HbSS	ATYPICAL/CAMEROON	(aa/aa)
RC59	F	9			2.8	7.5	86.3	31.0	22.5			233.0	3.4	17.8						HbSS	BENIN/ATYPICAL	(aa/aa)
RC60																				HbSS	BENIN/BENIN	(aa/a3.7)
RC61	F	28			78.8	3.4	17.8		19.4				5.1	13.3						HbSS	BENIN/ATYPICAL	(aa/aa)
RC62	M	17			2.5	7.1	86.2	33.3	24.5			404.0	3.3	22.4						HbSS	BENIN/BENIN	(aa/aa)
RC63	M	10			3.0	6.9	68.3	34.2	16.9			515.0	4.2	0.0						HbSS	BENIN/BENIN	(aa/aa)
RC69	M	16			3.9	8.4	66.6	32.2	14.3			378.0	6.9	0.0						HbSS	BENIN/ATYPICAL	(aa/aa)
RC70	F	18			4.7	9.6	68.0	30.5	29.9			354.0	3.3	19.0						HbSS		(a3.7/a3.7)
RC71	F	11			2.5	6.4	82.0	31.9	23.2			246.0	4.4	26.5						HbSS	BENIN/ATYPICAL	(aa/aa)
RC72																				HbSS	BENIN/ATYPICAL	(aa/a3.7)
RC73	F	10			2.6	7.4	79.0	36.2	26.4			345.0	3.0	0.0						HbSS	BENIN/BANTU	(aa/aa)

RC74	F	40			3.2	8.4	77.1	34.5	33.9			399.0	3.1	14.6						HbSS	BENIN/CAMEROON	(aa/aa)
RC76	F	27			3.9	8.1	68.8	30.2	12.7			339.0	4.3	20.1						HbSS	BENIN/CAMEROON	(aa/a3.7)
RC77																				HbSS	BENIN/CAMEROON	(aa/aa)
RC78																				HbSS	ATYPICAL/CAMEROON	(aa/aa)
RC79	F	9			3.4	8.4	79.6	30.7	20.7			408.0	3.1	14.6						HbSS	ATYPICAL/CAMEROON	(aa/a3.7)
RC81	M	13			1.8	6.0	103.3	32.3	39.3			266.0	2.5	13.6						HbSS	BENIN/ATYPICAL	(aa/aa)
RC83	M	15			3.2	6.8	64.8	32.7	14.7			177.0	4.5	27.3						HbSS	BENIN/CAMEROON	(a3.7/a3.7)
RC84	F	37			3.2	8.9	79.9	34.8	9.2			225.0	2.7	17.9						HbSS	BENIN/ATYPICAL	(aa/aa)
RC85	F	13			2.4	6.8	86.2	33.5	35.5			442.0	6.8	86.2						HbSS	BENIN/CAMEROON	(aa/aa)
RC87	F	33											3.6	12.2						HbSS	BENIN/ATYPICAL	(aa/aa)
RC88	M	18			2.8	7.6	79.9	33.4	23.6			294.0	3.5	0.0						HbSS	BENIN/BENIN	(aa/aa)
RC89	F	16			4.2	8.6	71.9	28.6	15.0			474.0	3.7	0.0						HbSS	BENIN/ATYPICAL	(aa/a3.7)
RC90	M	11																		HbSS	BENIN/CAMEROON	(aa/a3.7)
RC91																				HbSS	BENIN/BENIN	(aa/aa)
RC92	F	24			3.7	8.6	71.2	32.3	16.9			457.0	3.3	15.4						HbSS		(aa/a3.7)
RC93	F	9			2.9	8.0	81.7	33.5	42.4			524.0	2.8	18.3						HbSS	BENIN/ATYPICAL	(aa/aa)
RC95																				HbSS	BENIN/ATYPICAL	(aa/a3.7)
RC98																				HbSS	BENIN/BENIN	(aa/aa)
RC103																				HbSS	BENIN/ATYPICAL	(a3.7/a3.7)
RC105																				HbSS		(aa/aa)
RC106																				HbSS		(aa/aa)
RC107																				HbAA		(aa/aa)
RC108																				HbAA		(aa/aa)
C1	F	26			4.8	16.0	76.0	44.3	5.7	2.6	0.6	296.0	2.4	9.4						HbAA		(aa/aa)
C2	F	24			9.3	13.3	81.0	35.3	4.1	0.4	94.0	73.0	3.6	10.6						HbAA		(aa/aa)
C3	F	42			4.0	8.1	66.0	30.5	4.8	0.6	1.4	300.0	1.9	12.1						HbSS	BENIN/ATYPICAL	(aa/a3.7)
C4	F	16			4.7	13.2	79.0	35.2	7.8	0.5	1.2	354.0	4.0							HbAA		(aa/aa)
C5	F	20			4.7	12.0	78.0	33.0	4.1	1.5	0.4	203.0	1.1							HbSS	ATYPICAL/SENEGAL	(aa/a3.7)
C6	M	48			5.2	13.6	82.0	31.0	2.9	1.1	0.2	157.0								HbSS		(aa/a3.7)
C7																				HbAA		(aa/aa)
C8																				HbSS	BENIN/ATYPICAL	(aa/aa)
C9	F	19			4.5	11.6	75.0	34.7	4.6	2.6	0.4	189.0	2.8							HbAA		(aa/aa)
C10	M	21			5.1	13.3	74.0	35.5	8.9	4.4	1.3	249.0	3.6							HbSS		(aa/a3.7)
C11	F	32			8.2	14.3	83.0	34.2	3.0	1.4	0.2	196.0	1.7							HbSS		(aa/aa)
C12	F	24			4.2	12.8	81.0	37.4	5.9	2.5	0.7	242.0	5.0							HbAA		(aa/aa)
C13	F	24			3.9	11.5	84.0	35.8	8.3	2.7	2.3	284.0	3.9							HbAS		(aa/aa)
C14	M	23			4.9	14.2	85.0	34.0	4.8	1.6	0.8	174.0	2.2							HbAS		(aa/a3.7)
C15	F	17			4.6	12.9	88.0	31.4	4.5	2.1	0.5	377.0	2.2							HbSS		(aa/aa)
C16	F	22			4.0	11.8	81.0	36.6	11.2	4.0	1.3	224.0	3.3	9.7						HbAA		(aa/aa)
C17	F	29			5.4	16.1	85.0	35.0	5.8	2.8	0.8	228.0	3.7	9.9						HbAA		(aa/aa)

C18	M	24			4.7	14.7	90.0	34.7	6.8	3.1	0.3	193.0	1.8							HbAA		(aa/aa)
C19	M	12			6.2	15.9	74.0	34.6	5.8	1.4	0.6	252.0	2.8							HbAS		(aa/a3.7)
C20	M	21			4.3	13.0	78.0	38.7	4.4	1.8	0.4	2.1	0.9							HbAA		(aa/aa)
C23	M	42			4.3	13.2	94.0	33.1	6.9	3.3	0.4	183.0	4.3							HbAA		(aa/aa)
C26	F	27			4.8	13.0	87.0	31.3	5.5	2.2	0.4	221.0	2.6							HbSS	BENIN/BENIN	(aa/a3.7)
C27	F	47			4.3	10.0	72.0	31.9	4.9	2.5	0.3	137.0	2.9	17.1						HbAS		(a3.7/a3.7)
C29	F	35			3.9	11.8	82.0	30.6	5.2	0.6	1.1	181.0	0.6	7.3						HbAS		(aa/aa)
C30	M	4			5.1	11.7	73.0	31.3	2.0	1.2	0.3	278.0	4.8							HbAS		(aa/a3.7)
C31	F				4.5	13.2	87.0	33.9	4.9	2.0	1.1	256.0								HbSS		(aa/aa)
C32	F	24			4.3	10.4	64.0	37.6	4.8	1.6	0.7	334.0	1.2	5.4						HbAA		(aa/a3.7)
C34																				HbAS		(aa/aa)
C36	F	30			4.4	14.0	83.0	38.6	8.4	4.4	1.3	99.0	4.9	9.9						HbAA		(aa/aa)
C39	F	4			2.7	8.5	95.0	31.4	9.2	5.6	0.8	379.0	2.8							HbAA		(aa/aa)
C40	F	23			4.2	14.2	85.0	40.5	6.1	3.4	0.5	201.0	1.3	3.8						HbAA		(aa/aa)
C41	F	23			5.4	18.7	79.0	43.5	5.7	2.2	1.1	152.0	3.4							HbAS		(aa/aa)
C42	F	28			4.8	13.5	85.0	33.2	5.1	2.1	0.5	262.0	4.1							HbAA		(aa/aa)
C43	M	21			5.2	16.2	81.0	38.3	4.7	2.1	0.8	163.0	0.4	7.8						HbAA		(aa/aa)
C44	M	16			5.1	12.9	81.0	31.2	4.3	2.9	0.9	340.0	3.3							HbAA		(aa/aa)
C46	F	36			4.7	12.9	88.0	30.8	3.6	1.8	1.1	250.0	3.2	0.8						HbAA		(aa/aa)
C47	F	24			3.8	15.0	91.0	43.8	4.8	2.1	1.3	228.0	4.2	10.4						HbAA		(aa/aa)
C48	F	23			4.1	13.0	82.0	38.4	5.3	1.9	0.4	210.0	3.3	10.7						HbAA		(aa/aa)
C49	M	31			5.6	13.1	77.0	30.5	5.6	2.5	0.4	138.0	2.3							HbAS		(aa/a3.7)
C50	F	28			4.9	13.5	84.0	32.6	4.7	2.0	0.4	160.0	4.4							HbAS		(aa/aa)
C51	F	22			4.7	12.5	74.0	36.5	7.0	1.7	0.8	290.0	1.2	7.0						HbAA		(aa/aa)
C52	M	34			5.3	18.7	80.0	44.3	5.1	2.5	0.9	181.0	3.0	10.0						HbAA		(aa/a3.7)
C53	M	42			5.4	16.5	82.0	37.2	4.0	1.7	0.7	258.0	4.2	11.0						HbAA		(aa/aa)
C54	F	21			4.1	11.5	77.0	36.1	4.6	2.3	0.4	278.0	4.6	10.8						HbAA		(aa/aa)
C55	F	22			4.6	9.8	63.0	33.8	6.5	2.3	0.9	254.0	0.9	7.3						HbSS	ATYPICAL/ATYPICAL	(aa/aa)
C58	F	18			4.5	13.2	87.0	33.9	4.9	2.0	1.1	256.0	2.3							HbSS		(aa/aa)
C63	F	27			4.0	10.9	69.0	38.9	5.0	0.8	1.1	111.0		11.0						HbAA		(aa/a3.7)
C64	F	27			4.1	13.1	88.0	36.3	5.7	3.0	0.7	212.0	4.0							HbAA		(aa/aa)
C70	F	29			4.3	10.7	56.0	44.1	5.7	1.4	0.4	260.0	2.3							HbAS		(aa/aa)
C73	M	35			4.1	12.3	82.0	35.9	4.6	2.1	0.6	247.0	0.5							HbAA		(a3.7/a3.7)
C75	F	28			5.5	11.3	68.0	29.9	5.8	1.8	1.3	454.0	4.2	<0,8						HbAA		(aa/aa)
C78	F	29			4.0	10.3	83.0	31.3	6.9	3.3	0.4	235.0	3.3							HbAS		(aa/aa)
C79	M	38			5.0	14.5	91.0	32.3	4.9	1.4	0.7	230.0	2.2	AS						HbSS		(aa/a3.7)
C82	F	8			5.1	11.6	74.0	30.6	6.9	4.3	0.6	340.0	4.3	1.3						HbAS		(aa/aa)
C83	M	8			5.2	12.8	80.0	30.6	9.3	3.7	0.9	153.0	4.0							HbAS		(aa/aa)
C85	F	26			5.2	16.4	76.0	41.7	5.2	2.5	0.8	178.0	3.6							HbAA		(aa/aa)
C86	F	19			9.3	16.0	78.0	33.8	3.2	1.1	0;3	147.0	2.5	4.3						HbAS		(aa/aa)

C96	F	26			4.3	12.0	87.0	32.4	5.0	2.2	0.8	272.0	3.1	0.9						HbAA		(aa/a3.7)
C97	F	43			3.7	10.5	77.0	36.5	3.1	1.5	0.2	99.0	3.9	4.9						HbAA		(aa/aa)
C99	F	24			4.6	13.4	84.0	33.5	7.7	4.5	0.8	214.0	3.7	10.0						HbAS		(aa/aa)
C103	M	36			4.9	17.9	83.0	44.6	6.7	1.9	0.6	153.0	4.2	9.5						HbSS		(aa/aa)
C106	F	22			3.8	13.5	79.0	45.1	5.6	2.1	0.8	341.0		11.1						HbAA		(aa/aa)
C108	F	26			4.2	12.4	78.0	37.6	2.8	2.0	0.3	193.0	4.5	4.6						HbAA		(aa/aa)
C113	F	27			4.2	13.0	86.0	36.5	4.7	2.3	0.8	246.0	3.9	10.8						HbAA		(aa/aa)
C114	M	28			5.2	15.8	83.0	36.7	4.4	2.1	0.6	306.0	1.6	3.5						HbSS		(aa/aa)
C121	F	24			4.4	11.2	72.0	35.2	8.4	2.1	1.6	226.0	1.6	4.5						HbAA		(aa/a3.7)
C124	F	20			4.2	13.0	81.0	38.1	5.5	3.3	0.6	123.0	3.4	4.5						HbAA		(aa/aa)
C125	F	30			4.5	13.6	81.0	37.7	4.0	1.7	0.6	235.0	0.1							HbAA		(aa/aa)
C127	F	14			2.1	12.8	82.0	32.0	4.8	2.4	0.5	291.0	4.0							HbAS		(a3.7/a3.7)
C133	F	23			4.3	16.1	82.0	45.2	3.3	1.8	0.4	249.0		5.4						HbAA		(aa/aa)
C134	M	28			4.8	13.3	79.0	34.7	5.7	3.2	0.7	190.0	1.6							HbAA		(aa/aa)
C137	M	27			5.5	15.7	80.0	35.6	5.0	2.7	0.9	133.0	3.8	3.9						HbAA		(aa/aa)
C139	F	38			4.1	12.6	92.0	32.9	5.9	2.1	0.5	249.0	3.3	<0,8						HbAA		(aa/aa)
C140	M	33			4.2	12.8	81.0	37.4	5.9	2.5	0.7	242.0	3.0							HbAA		(aa/aa)
C141																				HbAA		(aa/aa)
C145	F	24			5.0	15.6	90.0	34.8	8.7	2.8	0.5	651.0	4.8							HbAA		(aa/aa)
57	M	27	EWONDO	SECONDARY	3.80	8.3	68.0	32.0	20.4	4.7	0.8	586.0	3.2	3.7	0.5	2	NO	9	3	HbAS		(aa/aa)
75	M	28			5.7	16.7	78.0	37.3	3.6	1.4	0.3	364.0	1.2	0.7						HbAS		(aa/aa)
78																				HbAS		(aa/aa)
80	M	27			4.8	14.8	81.0	38.3	4.1	0.8	0.4	145.0	3.2	12.6						HbAS		(aa/aa)
83	M	27			4.8	15.8	90.0	36.7	3.3	1.1	0.3	164.0	3.3	3.6						HbAA		(aa/aa)
84	F	19	MIXED ANCESTRY	SECONDARY	2.4	6.9	83.0	34.2	12.2	6.3	1.6	190.0	2.9	12.6	0.5	0	NO	0	1	HbAS		(aa/aa)
87	F	16			4.6	12.7	78.0	35.6	4.5	2.7	0.6	220.0		9.1						HbAS		(aa/aa)
99	F	10	BAMI	SECONDARY	3.9	8.5	70.0	31.0	5.4	2.2	0.3	318.0	2.7	19.9	6	0	NO	5	5	HbAS		(aa/aa)
108	M	27	BAMI	SECONDARY	2.0	7.9	88.0	28.6	13.5	5.6	1.3	433.0								HbAA		(aa/aa)
120	F	15	MIXED ANCESTRY	SECONDARY	3.0	6.3	71.0	29.7	12.1	6.0	1.1	432.0	5.5	15.3	9	2	NO	0	0	HbSS	BENIN/CAMEROON	(aa/a3.7)
123	M	11	ETON	SECONDARY	3.6	9.7	83.0	32.6	15.9	8.3	0.8	340.0	3.8	9.4	2	2	NO	2	3	HbSS	BENIN/ATYPICAL	(aa/aa)
126	M	12	DSCHANG	PRIMARY	2.6	7.7	87.0	34.6	10.6	4.3	1.2	316.0	2.3	15.2	5	1	NO	2	4	HbSS	BENIN/CAMEROON	(aa/aa)
127	M	10	BAMI	PRIMARY	2.8	7.0	79.0	31.9	16.8	4.7	2.4	164.0	3.8	7.2	4	2	NO	0	0	HbSS	BENIN/ATYPICAL	(aa/a3.7)
128	M	37	MIXED ANCESTRY	TERTIARY	2.1	6.0	80.0	36.3	8.0	3.4	2.1	583.0	3.8	10.9	2	1	NO	1	4	HbSS	BENIN/BENIN	(aa/aa)
129	M	13	BAMI	SECONDARY	2.40	7.7	88.0	36.3	16.4	9.2	1.8	408.0	4.5	10.7						HbSS	BENIN/ATYPICAL	(aa/aa)
130	M	19	MIXED ANCESTRY	SECONDARY	1.4	5.5	95.0	40.0	19.5	11.3	1.6	350.0	4.8	0.0	0.8	2	NO	1	0	HbSS	BENIN/ATYPICAL	(aa/aa)
131	M	10	EWONDO	PRIMARY	3.1	6.4	66.0	31.9	12.0	6.6	1.2	472.0	3.8	7.8	3	2	NO	0	0	HbSS	ATYPICAL/CAMEROON	(aa/a3.7)
134	F	49	YAMBASSA	SECONDARY	3.8	8.5	72.0	30.8	7.2	2.9	0.7	283.0	4.5	5.2	6	2	NO	3	4	HbSS	ATYPICAL/ATYPICAL	(a3.7/a3.7)
135	F	32	BASSA	PRIMARY	2.4	6.9	82.0	35.7	10.6	6.2	0.9	540.0	3.8	10.7	2	1	NO	0	0	HbSS	BENIN/ATYPICAL	(aa/aa)
136	F	11	MIXED ANCESTRY	SECONDARY	2.1	7.2	91.0	37.6	13.0	5.0	0.8	484.0	2.4	9.3	0.5	0	NO	0	0	HbSS	BENIN/ATYPICAL	(aa/aa)
137	F	11	HAOUSSA	PRIMARY	3.7	9.4	81.0	31.2	6.7	2.7	0.6	355.0	2.7	9.5	0.6	2	NO	10	12	HbSS	ATYPICAL/ATYPICAL	(aa/a3.7)

138	M	18	ETON	SECONDARY	2.7	7.8	80.0	35.7	6.2	2.8	1.0	350.0	2.6	15.6	1	0	NO	0	12	HbSS	ATYPICAL/ATYPICAL	(aa/aa)
140	M	7	MIXED ANCESTRY	PRIMARY	2.6	7.6	86.0	34.3	21.2	10.2	1.3	514.0	3.4	9.1	2	2	NO	3	0	HbSS	BENIN/ATYPICAL	(aa/aa)
141	M	23	BATOUFAM	SECONDARY	3.8	9.3	78.0	31.6	12.3	5.0	0.9	356.0	4.4	6.7	16	1	NO	0	0	HbSS	BENIN/ATYPICAL	(aa/aa)
142	M	17	BAKAKA	SECONDARY	2.6	9.5	81.0	36.3	18.0	9.8	1.4	424.0	3.4	12.1	0.5	1	NO	1	0	HbSS	BENIN/ATYPICAL	(aa/aa)
143	F	29			4.6	11.7	83.0	31.0	4.4	2.3	0.2	204.0	6.0							HbAS		(aa/a3.7)
144	M	20	EWONDO	SECONDARY	2.8	8.9	88.0	36.7	10.8	3.9	2.1	465.0			5	1	NO	0	2	HbSS	BENIN/ATYPICAL	(aa/aa)
145	M	6	MIXED ANCESTRY	PRIMARY	3.1	6.6	62.0	34.3	19.3	11.1	2.1	483.0	3.5	0.8	0.5	0	NO	0	0	HbSS	ATYPICAL/ATYPICAL	(aa/aa)
146	M	21	EWONDO	SECONDARY	2.4	7.0	84.0	35.8	9.0	2.3	1.6	334.0			2	2	NO	4	0	HbSS	BENIN/BENIN	(aa/aa)
177	M	7	BETI	PRIMARY	2.8	6.9	73.0	33.9	19.0	9.0	1.9	287.0	4.2	6.8	3	1	NO	0	0	HbAS		(aa/aa)
181	F	24	MIXED ANCESTRY	SECONDARY	3.3	8.9	87.0	31.2	7.2	3.3	0.6	529.0	3.2	22.3	4	0	NO	4	3	HbSS	BENIN/BENIN	(aa/aa)
183	F	18	BETI	TERTIARY	2.10	7.8	94.0	39.3	18.9	4.5	1.6	501.0	4.4	8.0	0.6	2	NO	0	0	HbSS		(aa/aa)
184	F	37	EWONDO	SECONDARY	1.4	5.8	98.0	41.8	31.9	21.6	3.8	477.0	3.5		0.5	0	NO	0	3	HbSS	BENIN/CAMEROON	(aa/aa)
185	F	6		PRIMARY	2.7	8.4	85.0	36.6	11.2	4.7	1.2	602.0	5.7	11.2						HbSS	BENIN/BENIN	(aa/aa)
189	M	7	EWONDO	PRIMARY	3.0	7.2	76.0	31.9	10.2	4.4	0.9	376.0	3.4	7.8	2	2	NO	1	4	HbSS		(aa/a3.7)
190	M	4	BAMOUN	PRIMARY	2.9	6.8	67.0	35.4	20.3	8.3	1.6	362.0	2.9	4.6	1	2	NO	0	12	HbSS	BENIN/BENIN	(aa/aa)
193	F	27	BASSA	TERTIARY	4.3	9.6	73.0	30.3	10.3	5.9	0.9	375.0	6.1	23.4	7	0	NO	3	0	HbSS	BENIN/ATYPICAL	(aa/a3.7)
194	F	18	MIXED ANCESTRY	SECONDARY	3.3	8.0	73.0	33.5	25.4	4.4	3.0	406.0	4.1	6.8	0.5	2	NO	2	1	HbSS	BENIN/BENIN	(aa/aa)
195	M	16	BASSA	SECONDARY	2.3	6.2	80.0	33.9	16.2	10.1	1.8	273.0	3.2	12.3	2	2	NO	4	4	HbSS	BENIN/BENIN	(aa/aa)
197					1.6	3.2	90.0	34.0	4.0	2.3	0.4	290.0								HbSS		(aa/aa)
198	F	9	YAMBASSA	PRIMARY	3.00	7.6	77.0	33.1	11.9	3.8	0.6	526.0	2.6	6.8	6	1	NO	0	4	HbSS	BANTU/CAMEROON	(aa/a3.7)
200	M	6	BAMI	PRIMARY	1.9	5.5	89.0	32.9	8.5	3.1	0.9	471.0	3.3			2	NO	3	4	HbSS	BENIN/CAMEROON	(aa/a3.7)
201	M	8	BAFUT	PRIMARY	3.0	7.7	84.0	30.5	23.2	9.6	2.8	650.0	3.5	11.0	2	2	NO	0	1	HbSS	BENIN/ATYPICAL	(aa/aa)
202	F	10	BETI	PRIMARY	3.2	8.2	75.0	34.0	23.7	8.4	2.2	535.0	4.0		2	1	NO	2	0	HbSS	ATYPICAL/ATYPICAL	(aa/aa)
210	F	14	EWONDO	SECONDARY	2.5	7.8	89.0	37.7	13.5	5.6	1.3	579.0	4.8		3	1	NO	1	1	HbSS	BANTU/CAMEROON	(aa/aa)
211	M	8	MIXED ANCESTRY	PRIMARY	2.9	8.2	81.0	34.4	17.4	6.4	1.3	208.0	4.3	8.2	3	0	NO	0	4	HbSS	BENIN/ATYPICAL	(aa/aa)
214	M	19	BAMI	SECONDARY	2.2	6.6	99.0	32.1	13.1	6.1	1.7	262.0	3.1	18.1	3	1	NO	3	6	HbSS		(aa/aa)
216	M	13	HAOUSSA	PRIMARY	2.9	7.7	85.0	31.6	18.0	4.9	1.4	233.0	2.6	15.6	5	2	NO	3	0	HbSS	BENIN/BENIN	(aa/aa)
217	M	10	BAMI	PRIMARY	3.8	8.3	71.0	30.4	14.9	6.6	1.0	341.0	4.1	15.9	3	2	NO	0	0	HbSS	BENIN/BENIN	(aa/a3.7)
218	M	5	EWONDO	PRIMARY	2.9	8.0	83.0	33.0	11.2	5.5	1.1	460.0	4.3							HbSS	BENIN/CAMEROON	(aa/a3.7)
219	F	35	EWONDO	SECONDARY	2.8	8.0	93.0	31.4	9.5	4.0	0.9	486.0	4.0		3	1	NO	0	0	HbSS	BENIN/BENIN	(aa/aa)
223	F	15	YAMBASSA	SECONDARY	1.8	4.9	92.0	29.7	10.6	4.8	3.0	228.0	3.1	9.2	3	1	NO	1	0	HbSS	BENIN/ATYPICAL	(aa/a3.7)
224	M	51	BASSA	SECONDARY	2.1	6.7	90.0	35.3	9.7	1.9	0.4	155.0	3.6	8.8	13	1	NO	0	0	HbSS	BENIN/ATYPICAL	(aa/a3.7)
225	F	21	BAMILEKE	TERTIARY	2.60	8.2	86.0	36.6	10.4	4.4	0.8	449.0	3.9	16.4	0.5	2	NO	5	5	HbSS	BENIN/BENIN	(aa/aa)
227	F	31	EWONDO	TERTIARY	2.3	7.7	92.0	36.0	10.0	5.5	0.7	351.0	3.6	3.0	0.6	1	NO	0	0	HbSS	BENIN/CAMEROON	(aa/aa)
229	F	10	EWONDO	PRIMARY	2.6	7.7	89.0	33.6	10.5	4.9	0.6	463.0	4.7	16.4	7	2	NO	1	1	HbSS	BENIN/CAMEROON	(aa/a3.7)
230	F	39	BENE	SECONDARY	2.5	9.1	94.0	38.2	13.9	8.2	1.8	374.0	3.2	18.4	0.8	1	NO	4	4	HbSS	BENIN/CAMEROON	(aa/aa)
231	F	27	BAFIA	NONE	1.5	6.0	100.0	40.1	14.4	5.7	2.0	444.0	3.9		4	1	NO	1	3	HbSS	BENIN/ATYPICAL	(aa/aa)
233	F	23			4.4	15.9	80.0	45.2	5.2	2.2	0.8	275.0	3.3	10.0						HbAS		(aa/aa)
234	M	18	MAKA	SECONDARY	4.9	9.2	64.0	29.1	4.4	2.2	0.2	169.0	7.2	4.2	5	0	NO	0	0	HbAS		(aa/aa)
236	F	26			4.1	14.9	83.0	44.1	3.5	2.2	0.3	138.0								HbAS		(aa/aa)

321	F	10	MIXED ANCESTRY	PRIMARY	3.2	6.1	60.0	31.4	11.9	6.7	1.2	209.0	4.0	18.4	0.7	1	NO	0	3	HbSS	BENIN/ATYPICAL	(a3.7/a3.7)
322	M	34	MIXED ANCESTRY	SECONDARY	2.8	8.2	93.0	31.8	9.9	3.1	1.2	517.0	7.2		0.5	1	NO	0	5	HbSS	BENIN/ATYPICAL	(aa/a3.7)
323	F	28			4.3	16.4	84.0	45.1	5.7	2.9	0.5	108.0	1.0	4.8						HbAS		(aa/a3.7)
324	F	32			4.3	16.4	84.0	45.1	5.7	2.9	0.5	108.0	1.0	4.8						HbAA		(aa/aa)
325	F	28	DOUALA	TERTIARY	2.7	8.4	100.0	31.4	10.5	5.7	1.2	615.0	3.4		21	1	NO	0	2	HbSS	BENIN/ATYPICAL	(aa/a3.7)
326	F	4	FOULBE	NONE	4.3	9.9	77.0	28.8	9.6	4.5	0.8	323.0								HbSS		(aa/aa)
328	F	25	MIXED ANCESTRY	SECONDARY	3.9	14.0	78.0	46.3	4.9	2.6	0.8	265.0	3.1	19.8	7	1	NO	0	0	HbSS		(aa/a3.7)
329	M	27	ETON	TERTIARY	3.3	10.0	90.0	33.3	7.7	3.4	0.4	281.0	3.9	25.9	6	1	NO	3	0	HbSS		(aa/aa)
330	F	20	BAMI	NONE	1.7	5.7	112.0	30.5	28.7	17.5	2.9	270.0	2.3	17.6	1	2	NO	3	3	HbSS	BENIN/BENIN	(aa/aa)
331	M	22	MBAMOIS	SECONDARY	2.6	8.1	90.0	34.9	10.0	5.1	1.5	262.0	2.1	1.1	6	1	NO	1	2	HbSS		(aa/aa)
333	M	18	EWONDO	SECONDARY	2.3	5.4	67.0	35.2	17.4	8.2	2.3	163.0	3.1	3.0	1	1	YES	0	2	HbSS	BENIN/ATYPICAL	(a3.7/a3.7)
334	M	16	EWONDO	SECONDARY	3.6	9.8	90.0	30.6	11.2	5.2	1.0	589.0	4.0	11.1		2	NO	3	3	HbSS	BENIN/BENIN	(aa/aa)
335	M	48	BASSA	SECONDARY	4.1	10.8	81.0	32.5	11.6	4.3	1.5	584.0	3.3	1.6	20	0	NO	0	0	HbSS	CAMEROON/CAMEROON	(aa/a3.7)
336	F	19	MIXED ANCESTRY	SECONDARY	2.2	7.7	104.0	33.6	12.5	6.1	0.5	598.0	4.2		5	1	NO	10	6	HbSS	BENIN/ATYPICAL	(aa/aa)
337	F	31	BAMILEKE	SECONDARY	1.7	5.8	97.0	35.4	12.2	3.2	0.8	572.0	5.5	13.6	7	1	NO	2	2	HbSS	BENIN/BENIN	(aa/aa)
339	M	8	HAOUSSA	PRIMARY	4.1	9.4	67.0	34.5	15.1	5.0	1.8	498.0			2	1	NO		1	HbAS		(aa/a3.7)
340	F	8	DSCHANG	PRIMARY	3.3	8.4	81.0	32.0	10.4	4.3	1.3	394.0	3.3	27.3	0.5	2	NO	6	1	HbSS	BENIN/BENIN	(aa/aa)
341	F	34	HAOUSSA	PRIMARY	2.4	7.6	91.0	34.4	14.0	4.1	1.6	325.0	4.2	15.9	15	1	NO	0	0	HbSS	BENIN/BENIN	(aa/aa)
342	F	31	YAMBASSA	TERTIARY	3.3	7.9	72.0	33.0	9.6	2.3	0.9	442.0	4.0	17.0	0.5	1	NO	0	0	HbSS		(aa/a3.7)
343	F	24	BANEN	SECONDARY	2.1	9.2	80.0	54.3	8.1	5.2	1.3	491.0	3.9	10.8	5	1	NO	3	1	HbSS	CAMEROON/CAMEROON	(aa/a3.7)
344	F	20	MIXED ANCESTRY	TERTIARY	2.1	6.9	93.0	36.0	13.1	5.1	1.3	358.0	3.4	23.2	1	2	NO	6	1	HbSS	BENIN/BENIN	(aa/aa)
346	F	6	BAMILEKE	PRIMARY	3.4	8.5	75.0	32.6	8.8	4.8	1.7	438.0	4.9	21.9	5	1	NO	1	1	HbSS	BENIN/BENIN	(aa/a3.7)
347	M	23	DSCHANG	SECONDARY	2.8	7.7	80.0	34.8	10.5	5.4	0.9	556.0	4.0		2	2	NO	8	6	HbSS	ATYPICAL/ATYPICAL	(aa/aa)
348	M	29	ETON	SECONDARY	5.5	12.1	76.0	28.8	9.4	3.7	0.7	392.0	4.9	4.8	0.6	1	NO	1	1	HbSS	BENIN/ATYPICAL	(aa/a3.7)
349	M	24	MIXED ANCESTRY	SECONDARY	4.2	7.7	64.0	28.8	10.9	2.8	0.6	559.0	5.3	15.4	7	1	NO	4	1	HbSS	BENIN/BENIN	(a3.7/a3.7)
350	M	32	MIXED ANCESTRY	TERTIARY	1.8	6.2	88.0	38.9	12.3	6.2	1.8	412.0	3.6	10.4	0.5	1	NO	3	1	HbSS	BENIN/BENIN	(aa/aa)
351	F	47	DSCHANG	PRIMARY	2.6	6.8	86.0	30.1	9.4	3.9	2.0	200.0	8.0	14.8	17	1	NO	4	4	HbSS	BENIN/BENIN	(aa/aa)
352	F	14	BASSA	SECONDARY	5.5	14.3	59.0	44.1	7.3	2.0	1.2	200.0	4.0	14.3	0.5	1	NO	0	0	HbSS	BENIN/BENIN	(a3.7/a3.7)
353	M	21		SECONDARY	1.9	8.9	101.0	32.1	10.4	3.9	0.8	354.0	4.4	10.5	3					HbSS	BENIN/ATYPICAL	(a3.7/a3.7)
354	M	5			5.0	11.3	70.0	32.7	7.9	3.7	1.7	440.0	1.9							HbSS	BENIN/BENIN	(aa/a3.7)
355	M	24	MIXED ANCESTRY	SECONDARY	2.9	8.8	89.0	34.4	16.9	6.7	7.8	192.0	4.8	11.0	6	1	YES	2	3	HbSS	ATYPICAL/ATYPICAL	(aa/aa)
356	F	12	BAMILEKE	PRIMARY	2.6	9.4	93.0	38.4	14.2	6.8	2.3	243.0	2.2	26.3	0.5	2	NO	0	0	HbSS	BENIN/BENIN	(aa/aa)
357	M	8	MIXED ANCESTRY	PRIMARY	2.5	7.5	82.0	36.3	13.5	5.4	1.1	320.0	5.5	8.9	2	2	NO	12	1	HbSS	BENIN/CAMEROON	(aa/a3.7)
358	M	15	DSCHANG	NONE	2.7	6.8	83.0	29.9	12.8	5.5	1.5	661.0	3.5	13.5	12	1	NO	1	1	HbSS	BENIN/BENIN	(aa/aa)
359	F	14	ETON	SECONDARY	4.3	8.7	69.0	29.6	5.8	2.8	0.4	278.0	4.7	16.3	14	1	NO	1	0	HbSS	BENIN/CAMEROON	(a3.7/a3.7)
360	F	17	BAMOUN	SECONDARY	2.3	7.7	83.0	39.7	8.6	3.1	1.2	410.0	4.4		2	1	NO		1	HbSS	BENIN/ATYPICAL	(aa/a3.7)
361	F	28	MIXED ANCESTRY	TERTIARY	2.2	7.4	92.0	36.3	12.5	4.4	0.9	259.0	4.2	14.1	6	1	NO	2	1	HbSS	BENIN/CAMEROON	(aa/aa)
362	F	11	BAFIA	PRIMARY	2.0	5.8	88.0	33.7	18.2	8.8	1.0	333.0	3.9							HbSS	BENIN/CAMEROON	(aa/aa)
363	F	19	BAMILEKE	SECONDARY	2.2	8.5	98.0	39.8	12.9	6.0	1.6	402.0	3.7	18.5	11	2	NO	4	1	HbSS	BENIN/BENIN	(aa/aa)
364	M	4	BANDJOUN	PRIMARY	2.0	9.8	93.0	52.9	14.7	7.8	2.9	296.0	2.7	14.1	2	1	NO	1	1	HbSS	BENIN/BENIN	(aa/aa)

365	F	34	BANEN	NONE	3.6	9.6	77.0	34.4	15.2	7.2	1.1	351.0	5.9	14.0	12	1	NO	1	2	HbSS	BENIN/BENIN	(aa/a3.7)
366	M	24	ETON	TERTIARY	3.5	13.7	82.0	47.2	4.9	1.6	0.7	147.0	3.8	13.3	7	1	NO	3	12	HbSS		(aa/a3.7)
367	F	16	DSCHANG	SECONDARY	3.7	8.5	76.0	30.6	4.1	1.6	0.5	241.0	2.5	27.3	14	1	NO	1	3	HbSS	BENIN/BENIN	(a3.7/a3.7)
368	F	5	ETON	PRIMARY	2.60	6.4	75.0	32.8	13.5	5.2	0.8	598.0	13.2	18.4	4	1	NO	3	3	HbSS	BENIN/BENIN	(aa/a3.7)
369	F	21	BAMILEKE	SECONDARY	2.0	7.8	94.0	42.4	22.5	1.9	2.5	482.0	3.6	14.9	0.5	2	NO	5	0	HbSS	BENIN/CAMEROON	(aa/aa)
370	F	9		PRIMARY	2.0	6.0	95.0	31.9	16.0	7.3	1.3	453.0	3.1	29.3						HbSS	BENIN/BENIN	(aa/aa)
371	M	16	BAMENDA	SECONDARY	2.1	6.9	81.0	40.7	17.3	3.3	3.5	328.0	4.9		2	1	NO	5	1	HbSS	BENIN/BENIN	(aa/aa)
372	M	19	BAMOUN	SECONDARY	2.70	11.1	80.0	51.1	18.5	9.7	3.3	110.0	4.0	8.9	0.5	1	NO	0	0	HbSS	BENIN/BENIN	(aa/aa)
373	M	15	BASSA	SECONDARY	2.6	6.6	76.0	33.6	16.8	7.2	1.8	625.0	3.9	9.4		1	NO	0	2	HbSS	BENIN/BENIN	(aa/aa)
374	M	11	DSCHANG	PRIMARY	2.1	7.8	94.0	40.3	14.9	8.3	2.1	243.0	3.9	13.5	7	1	NO	2	2	HbSS	BENIN/BENIN	(aa/a3.7)
375	M	11	ABBO	PRIMARY	2.6	7.4	91.0	31.4	13.4	7.1	2.2	283.0	3.3	15.3	5	2	NO	12	1	HbSS	BENIN/CAMEROON	(aa/aa)
376	M	40	MIXED ANCESTRY	TERTIARY	3.3	8.3	75.0	33.5	12.6	7.0	1.0	489.0	3.7		1	1	NO	0	0	HbSS	BENIN/BENIN	(aa/aa)
377	F	20	MIXED ANCESTRY	PRIMARY	2.5	7.1	90.0	30.9	8.9	3.8	1.2	596.0	4.5	17.2	8	2	NO			HbSS	BENIN/BENIN	(aa/a3.7)
378	M	24	BASSA	SECONDARY	2.2	7.3	97.0	33.6	12.3	4.7	1.3	615.0	3.2	12.6	1	1	NO	0	2	HbSS	BENIN/BENIN	(aa/aa)
379	F	5	MIXED ANCESTRY	PRIMARY	2.6	8.3	86.0	37.3	25.4	10.4	3.0	267.0	4.0		2	1	NO	1	2	HbSS	BENIN/ATYPICAL	(aa/aa)
381	M	21	BAFIA	SECONDARY	2.4	7.9	94.0	35.5	12.4	6.5	1.5	393.0	2.2	10.3	0.5	1	YES	5	1	HbSS	ATYPICAL/CAMEROON	(aa/aa)
382																				HbSS	BENIN/ATYPICAL	(aa/a3.7)
383	M	26	DOUALA	PRIMARY	2.4	7.1	77.0	37.8	8.7	4.0	1.1	378.0	4.1	12.2	3	1	NO	1	1	HbSS	BENIN/CAMEROON	(aa/a3.7)
384	M	14	ETON	SECONDARY	2.9	7.3	81.0	31.6	13.8	7.6	1.4	304.0	4.5	16.7	7	2	NO	6	0	HbSS	BENIN/CAMEROON	(aa/a3.7)
385	M	38	EWONDO	TERTIARY	3.2	9.0	78.0	35.6	13.7	6.6	0.9	472.0	3.8		3	1	NO	6	1	HbSS	BENIN/CAMEROON	(aa/aa)
386	F	18	MIXED ANCESTRY	SECONDARY	3.1	8.6	82.0	33.9	9.8	4.2	1.4	329.0	4.8		0.5	2	NO	4	1	HbSS	BENIN/BENIN	(aa/a3.7)
387	F	17	EWONDO	SECONDARY	1.6	7.1	111.0	41.2	29.3	15.2	3.9	248.0	18.2	22.9	1	2	YES	6	4	HbSS	BENIN/BENIN	(aa/aa)
388	M	21	BAFIA	SECONDARY	2.1	6.9	82.0	40.2	13.4	7.8	1.3	344.0	6.6		1	2	NO	6	4	HbSS	BENIN/ATYPICAL	(aa/a3.7)
389	F	22	DSCHANG	TERTIARY	2.2	6.5	83.0	36.1	12.8	6.8	1.5	290.0	3.2	16.0	10	0	NO	0	0	HbSS	BENIN/BENIN	(aa/aa)
390	F	28	EWONDO	TERTIARY	2.6	7.3	78.0	35.5	10.6	5.7	0.9	339.0	4.3		0.8	2	YES		1	HbSS		(aa/a3.7)
391	F	15	DULU	SECONDARY	2.1	7.7	95.0	39.4	17.4	4.4	2.6	190.0	4.9	23.6	2	1	NO	0	0	HbSS	BENIN/BENIN	(aa/aa)
393	F	31	MIXED ANCESTRY	TERTIARY	1.9	6.8	96.0	36.5	12.3	2.9	1.2	493.0	4.6	12.0	2	0	NO			HbSS	BENIN/ATYPICAL	(aa/aa)
394	M	19	BAMOUN	SECONDARY	3.2	10.1	85.0	37.5	14.0	4.1	2.5	296.0	4.8		1	1	NO	2	1	HbSS	BENIN/CAMEROON	(aa/a3.7)
395	M	27	BGNTE	SECONDARY	4.3	9.8	66.0	34.6	10.9	4.0	1.6	593.0	4.7		0.5	1	NO	0	0	HbSS	BENIN/ATYPICAL	(aa/a3.7)
397	F	15	BASSA	SECONDARY	3.0	7.4	69.0	36.1	9.1	4.6	1.1	247.0	5.3		5	1	NO			HbSS	BENIN/BENIN	(a3.7/a3.7)
398	M	12	BAMILEKE	SECONDARY	2.5	7.7	85.0	36.6	21.3	11.3	1.7	593.0	3.8	5.4	4	2	YES	1	1	HbSS	BENIN/ARAB-INDIAN	(aa/aa)
399	M	2	MIXED ANCESTRY	NONE	3.9	10.6	82.0	32.7	15.4	9.7	2.4	272.0	6.2	20.6	1	1	NO	0	0	HbSS	BENIN/ATYPICAL	(aa/aa)
400	F	14	BAMILEKE	SECONDARY	3.4	9.7	82.0	35.1	28.5	16.0	2.5	725.0	6.8	12.5	3	1	NO	2	0	HbSS	BENIN/BENIN	(aa/aa)
401	F	29	BASSA	SECONDARY	2.6	7.2	80.0	35.3	13.0	6.4	1.1	292.0	2.7	10.3	14	1	YES	0	0	HbSS	BENIN/BENIN	(aa/aa)
402	F	28	MIXED ANCESTRY	TERTIARY	3.5	8.7	78.0	32.3	10.2	3.3	0.8	456.0	18.1	11.2	7	1	YES	3	1	HbSS	BENIN/BENIN	(aa/aa)
403	M	22	MBAMOIS	PRIMARY	2.6	9.5	93.0	38.5	10.6	6.2	0.8	372.0	4.5	23.8	0.6	1	NO	1	1	HbSS	BENIN/ATYPICAL	(aa/aa)
404	F	15	BASSA	SECONDARY	2.9	9.2	84.0	37.2	10.9	5.2	2.6	403.0	3.7	16.1	8	2	NO	1	4	HbSS	BENIN/BENIN	(aa/aa)
405	F	8	DULU	PRIMARY	3.2	8.8	82.0	33.8	20.9	9.9	0.8	560.0	1.6	15.1	3	1	NO	10	2	HbSS	BENIN/BENIN	(aa/aa)
406	M	20	NKAMBE	SECONDARY	4.3	9.9	77.0	28.8	9.6	4.5	0.8	323.0	4.6	10.9	0.5	1	NO	1	1	HbSS		(aa/aa)

A5: PREPARATION OF 1XTE BUFFER

The following components were required: (1) 10mM Tris (Sigma-Aldrich, Munich, Germany), (2) HCl acid (Sigma-Aldrich, Munich, Germany) and (3) 1mM EDTA (Sigma-Aldrich, Munich, Germany). A 100ml solution was prepared by adding 1ml of 1M Tris-HCl (pH 8.0) and 0.2ml EDTA (0.5 M). These components were mixed and the remaining volume was topped up with distilled water until the 100ml mark. This buffer was stored at room temperature.

A6: PREPARATION OF AN AGAROSE GEL

The agarose gels used in this study were prepared according to the weight/volume (w/v) ratio. Various percentages were required:

1. A 1.5% agarose gel, was prepared using 1.5g of agarose (Seakem Lonza, Basel, Switzerland) mixed in 100ml of 1XTBE buffer (Appendix A8). The mixture was swirled by hand, and then heated, using a microwave, at medium power for 1min or until the agarose has completely dissolved. Upon cooling, EtBr (0.5ug/ml) (Sigma-Aldrich, Munich, Germany; Appendix A7) was added and mixed gently by swirling. The mixture was poured into a casting tray and allowed to solidify. After 30 minutes, the gel was ready for use.
2. A 2% agarose gel, was prepared using 2g of agarose (Seakem Lonza, Basel, Switzerland) mixed in 100ml of 1XTBE buffer (Appendix A8). The mixture was swirled by hand, and then heated, using a microwave, at medium power for 1 minute or until the agarose has completely dissolved. Upon cooling, EtBr (0.5ug/ml; Sigma-Aldrich, Munich, Germany; Appendix A7) was added and mixed gently by swirling. The mixture was poured into a casting tray and allowed to solidify. After 30 minutes, the gel was ready for use.

A7: PREPARATION OF EtBr (0.5µg/ml)

EtBr (0.5µl; Sigma-Aldrich, Munich, Germany) was added to 10ml of distilled de-ionised water and mixed gently by swirling in a container with a lid.

A8: PREPARATION OF 1XTBE BUFFER

A 5X stock solution of TBE was prepared dissolving 0.45M Tris (Sigma-Aldrich, Munich, Germany) and 0.44M Boric acid (Sigma-Aldrich, Munich, Germany) in 900ml deionized water. EDTA (0.5M; pH 8.0) was added and the solution was adjusted to a final volume of 1L. This buffer was stored at room temperature (~22°C). Thereafter, a working solution was prepared for agarose gel electrophoresis. The stock solution was diluted with deionized water in the 1:10 ratio. The final solute concentrations were 45mM Tris-borate and 1mM EDTA.

A9: PREPARATION OF LOADING DYE

The following components were required to prepare the loading dye: (1) 0.25% Bromophenol Blue (Merck; Darmstadt, Germany), (2) 40% Sucrose (Merck, Darmstadt, Germany) and (3) 59.75% sterile water. Components (1), (2) and (3) were mixed together, and half of a NaOH (Sigma-Aldrich, Munich, Germany) pellet was added to create a slightly basic mixture. The mixture was stored at -20°C.

A10: PREPARATION OF RBC LYSIS BUFFER

The following components were used to prepare the RBC lysis buffer: (1) 155mM NH_4Cl (Sigma-Aldrich, Munich, Germany), (2) 12mM $NaHCO_3$ (Sigma-Aldrich, Munich, Germany), (3) 0.1mM EDTA (Sigma-Aldrich, Munich, Germany), and (4) distilled water. A 1L solution was prepared by dissolving the NH_4Cl, $NaHCO_3$, and EDTA in distilled water. The mixture was then filter sterilised using a 0.2µm filter and stored at 4°C.

A11: PUBLICATIONS

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The Co-Inheritance of Alpha-Thalassemia and Sickle Cell Anemia Is Associated with Better Hematological Indices and Lower Consultations Rate in Cameroonian Patients and Could Improve Their Survival

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Abstract

Background: Co-inheritance of α-thalassemia was reported to be associated with a delayed age of disease onset among Cameroonian Sickle Cell Anemia (SCA) patients. The present study aimed to explore the correlation between α-thalassemia, hematological indices, and clinical events in these patients.

Methods and Findings: We studied 161 Cameroonian SCA patients and 103 controls (59.1% HbAA) with median ages of 17.5 and 23 years. RFLP-PCR was used to confirm SCA genotype and to describe haplotypes in the *HBB-like* genes cluster. Multiplex Gap-PCR was performed to investigate the 3.7 kb α-globin gene deletions. SNaPshot PCR, capillary electrophoresis and cycle sequencing were used for the genotyping of 10 SNPs in *BCL11A, HMIP1/2, OR51B5/6* and *HBG* loci, known to influence HbF levels. Generalised linear regression models adjusted for age, sex and SNPs genotypes was used to investigate effects of α-thalassemia on clinical and hematological indices. The median rate of vaso-occlusive painful crisis and hospitalisations was two and one per year, respectively. Stroke was reported in eight cases (7.4%). Benin haplotype was the most prevalent (66.3%; *n* = 208 chromosomes). Among patients, 37.3% (*n* = 60) had at least one 3.7 kb deletion, compared to 10.9% (*n* = 6) among HbAA controls (p<0.001). Among patients, the median RBC count increased with the number of 3.7 kb deletions [2.6, 3.0 and 3.4 million/dl, with no, one and two deletions (p = 0.01)]. The median MCV decreased with the number of 3.7 kb deletion [86, 80, and 68fl, with no, one and two deletions (p<0.0001)], as well as median WBC counts [13.2, 10.5 and 9.8×10^9/L (p<0.0001. The co-inheritance of α-thalassemia was associated with lower consultations rate (p = 0.038).

Conclusion: The co-inheritance of α-thalassemia and SCA is associated with improved hematological indices, and lower consultations rate in this group of patients. This could possibly improve their survival and explain the higher proportion of α-thalassemia among patients than controls.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. Data are from the study site whose authors may be contacted at: Associate Professor Ambroise Wonkam, Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, Anzio Road, Observatory, 7925, Cape Town, Republic of South Africa. Tel: 0027 21 406 63 07; Fax; 002721 406 6826; email:ambroise.wonkam@uct.ac.za

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Introduction

Sickle cell disease is a life-long genetic disease that begins in childhood, affecting the structure of erythrocytes. Typically, a single DNA mutation within the beta globin gene leads to a glutamic acid to valine substitution, changing normal hemoglobin (HbA) into abnormal sickle hemoglobin (HbS). In deoxygenating or dehydrating conditions, HbS polymerizes within the erythrocytes, leading to intracellular tactoids that deform the red blood cells into the characteristic sickled shape, inducing microvascular obstruction, abnormal adhesion of leukocytes and platelets,

inflammation and hypercoagulation. Individuals with SCA suffer a wide range of complications: increased susceptibility to infections, chronic hemolytic anemia; recurrent periodic acute vaso-occlusive events, and chronic damage affecting almost every organ system [1].

The allele frequency of the HbAS matches the regions of highest malaria endemicity, supporting the hypothesis that HbAS confers protection against severe malaria [2]. An estimated 305 800 neonates are affected annually with nearly two-thirds of these occurring in Africa. Sickle Cell Anemia (SCA; HbSS form) is by far the most prevalent and severe form of the disease [3]. The lack of effective early detection and treatment initiatives has resulted in a high SCA-related death rate in some African countries [4]. Cameroon is a country of about 20 million inhabitants and has a population growth of 3% per annum. The country has a high carrier frequency of SCA, ranging from 8 to 34% [5]. Although Cameroon has developed a national control programme for SCA, there is not yet provision of neonatal screening, nor is there a specialized center for lifelong medical care and surveillance for this major cause of morbidity and mortality in this country [6]. There is no universal medical insurance coverage in Cameroon, and care of SCA patients is heavily dependent on families. Poverty in Cameroon affects more than 50% of the rural population and up to 30% of the urban population [7], contributing to a high burden of SCA on parents [6]. Cameroonian SCA patients can present with exceptionally severe phenotypes, as illustrated by a high rate of stroke [8] and severely impaired neurocognitive functions [9].

Although SCA is genetically characterised by a single point mutation, there are various genetic modulators that affect the phenotype of this disease, and patients can manifest with varying degrees of clinical severity [10]. Increased levels of fetal hemoglobin (HbF), and genetic loci associated with this trait, have been shown to influence the clinical severity of SCA [11]; research has recently replicated these findings in Cameroonian SCA patients [12]. In addition, co-inheritance of α-thalassemia has been associated with a milder phenotype in SCA patients, e.g. lower stoke rate [13], but could also result in the increase of vaso-occusive painful episodes [14].

There is a scarcity of data on the co-inheritance of α-thalassemia and SCA in Africa [15;16,17], and surprisingly, there have been no reports on the impact of α-thalassemia on the clinical phenotype of SCA patients in Africa. SCA patients that live on the African continent, unlike e.g. African American patients, are exposed to malaria that can potentially alter the frequency of α-thalassemia [2], therefore the pattern of co-inheritance with SCA. Recently, we reported that co-inheritance of 3.7 kb α-globin gene deletion was associated with a delayed age at diagnosis and possibly improved survival of Cameroonian patients [15]. The present study aimed to explore the correlation between 3.7 kb α-globin gene deletion and hematological indices among patients and controls, and its relation to clinical severity among patients.

Materials and Methods

Ethical approval

The study was performed in accordance with guidelines of the Helsinki Declaration (Brazil, 2013). Ethical approval was given by the National Ethical Committee Ministry of Public Health, Republic of Cameroon (No 033/CNE/DNM/07); and the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee (HREC REF. 132/2010). Written and signed informed consent was obtained from participants who were 18 years or older, and for the children, consent was obtained from parents/guardians with an assent from the children participants older than seven years.

Patients and clinical events

The study was conducted at the Yaoundé Central Hospital, and Douala Laquintinie Hospital in Cameroon. Socio-demographic and clinical data were collected by means of a structured questionnaire. Parents/guardians as well as adult SCA patients were interviewed; patients' medical records were reviewed, to delineate their clinical features over the past three years. In the seminal Cooperative Study in the USA, three adverse events served as proxies for severe sickle cell disease: 1) the rate of vaso-occlusive painful crisis (VOC), 2) the occurrence of stroke and 3) the rate of acute chest syndrome [18]. In Cameroon, it was virtually impossible to assess the rates of acute chest syndrome episodes retrospectively, because of the inherent difficulties in diagnosing acute chest syndromes in the settings and poor medical records. Consequently, acute chest syndromes were not considered in our evaluation. Nevertheless, we included the number of consultations and hospitalisations, as additional proxies of clinical severity. In addition, blood transfusions and any administration of hydroxyurea were recorded. VOC events were defined as episodes that could not be attributed to causes other than SCA and required hospital visits and treatment with pain killers. Anthropometric variables [Body Mass Index (BMI), and Blood Pressures (BP)] were measured upon arrival at the hospital.

The sampling was not restricted to hospital-based patients to avoid overrepresentation of the most severe phenotypes. To fulfil this goal, two SCA patient associations in Cameroon were engaged for collaboration, and additional patients were recruited during their monthly meetings. No incentive was provided for participation in the study. Only patients who had not received a blood transfusion or hospitalisation in the past 6 weeks were included, with no patient receiving hydroxyurea treatment.

The control group were randomly selected individuals (HbAS and HbAA) who were apparently healthy blood donors, and who volunteered their participation in the study. The following information was collected from control participants: a complete hematological profile (full hematological indices and Hb electrophoresis results), and minimal socio-demographic data (age and gender).

Hematological phenotypes

Hemoglobin electrophoresis and complete routine blood count of the SCA-affected patients were conducted upon arrival at the hospital. Two methods of HbF detection were employed in this study: 1) the Alkali Denaturation Test (ADT) initially, and 2) High Performance Liquid Chromatography (HPLC), when it became available (BIORAD D-10, USA). HbF detection was performed at the hematological laboratory of the Centre Pasteur in Yaoundé. Measurements done in patients <5 years old were excluded from the analysis because HbF levels are not yet stable at this early age. ADT was used to measure HbF levels in 28% ($n=39$) of controls and 72% ($n=100$) of SCD patients. These two techniques displayed differences in median values ($p=0.001$) with ADT yielding a median value of 11.2% compared to 6.6% for the HPLC method.

Genotypes

DNA was extracted from peripheral blood of both patients and controls, following instructions accompanying the commercial DNA isolation kit [Puregene blood kit (Qiagen, USA)], in the molecular diagnostic laboratory, Gyneco-Obstetric and Paediatric Hospital, Yaounde, Cameroon. Genotypic analyses were per-

formed in the Division of Human Genetics, Faculty of Health Sciences, University of Cape Town.

Molecular diagnostic testing for SCA (HbSS). PCR primers were designed to optimally amplify a 770 bp segment of the β-globin gene: PCR was carried out in a thermocycler (BIORAD, USA) and analysis for the sickle-cell mutation involved restriction enzyme analysis of the PCR product, using the restriction endonuclease Dde I (GIBCO-BRL, USA). Only patients identified to be HbSS type were included in the analysis, according to a reported method [19].

Haplotyping of the β-globin gene cluster. Five restriction fragment length polymorphism (RFLP) regions in the β-globin genes cluster were amplified using published primers and methods to analyse the XmnI (5'Gγ), HindIII (Gγ), HindIII (Aγ), HincII (3"Ψβ), and HinfI (5'β) restriction fragments. RFLP sites and the fragments were visualised by agarose gel electrophoresis and β-globin gene haplotypes were defined by the study of the combination of the restriction sites [20].

Detection of 3.7 kb α-globin gene deletions. Alpha-thalassemia is caused most frequently by deletions involving one or both α-globin genes [21]. The 3.7 kb α-globin gene deletions is the most prevalent in sub-Saharan Africans, among whom point mutations has been seldom reported in α-globin gene [22]. Alpha-globin gene deletions were screened by multiplex gap-PCR following the method reported previously [23], with a few modifications: only five primers were required for the detection of 3.7 kb and 4.2 kb deletions. The Expand Long Template PCR system was utilised with buffer 3 (Roche, Mannheim, Germany), and DMSO (1.5%) (Thermo Scientific, California, USA) was added.

SNPs genotyping in the HMIP, BCL11A, HBG XmnI-158 and OR51B5/6 loci. In a separate study, we reported that sequence variants at *BCL11A* and *HBS1L-MYB* loci influenced HbF levels. In addition to *BCL11A* rs4671393 SNPs that was associated with wider range of hematological indices, independently of HbF levels and two SNPs in *HBS1L-MYB* that were associated with the number of hospitalisation [12]. In this paper we also investigated the effects of α-thalassemia on clinical and hematological indices, in relation to these variants. For this purpose, 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 *HBG* loci, SNP rs7482144. PCR was performed to determine genotypes using SNaPshot multiplex ready reaction mix (Applied Biosystems, California, USA); followed by capillary electrophoresis (Applied Biosystems California, USA) and cycle sequencing (Gene Amp PCR system 9700) were used for the genotyping of the 10 selected SNPs, as previously reported. We previously reported the details of these experiments [12].

Statistical analysis

A Hardy-Weinberg Equilibrium (HWE) test was performed on the genotype results of 3.7 kb α-globin gene deletions and the 10 selected SNPs. Observed 3.7 kb α-globin gene deletion allele frequencies in controls were consistent with HWE ($\chi^2 = 2.37$; p = 0.12), equally to SCA patients ($\chi^2 = 1.69$; p = 0.19). Two SNPs were dropped because of significant violation of HWE (rs1188686 in *BCL11A*; HWE p-value = 0.00030; and rs9389269 in the *HBS1L-MYB* locus; HWE P-value: 0.002876). And two others SNPs were monomorphic (rs9376090 in the *HBS1L-MYB* locus, all the patients were T/T homozygous; and rs7482144 in *HBG* loci, all the patients were G/G homozygous).

Descriptive statistics was performed for all quantitative data using SPSS (IBM, USA version 21.0). The distribution of variables of interest was assessed by the Shapiro-Wilk test and informed the use of non-parametric tests to compare groups of participants (Mann-Whitney U test, median test or the Kruskal-Wallis). Additive model per copy of the α-globin gene deletions were performed, as well as multinomial, or linear logistic regression analysis incorporating SCA genotype, α-thalassemia genotype, age, gender, or clinical events. In addition, to correct for the skewness of the HbF distribution, we log10-transformed and normalized the data to obtain the quantitative trait used in the association analysis (after correcting for age, gender, and electrophoresis technique). The effects of α-thalassemia on key clinical and hematological indices were investigated in generalised linear regression models, adjusted for age, sex and six SNPs genotypes (always assuming log-additive genetic effects) using the R statistical package version 3.0.3 [06.03.2014], The R Foundation for statistical computing, Vienna, Austria). Significance was set at the 0.05 level.

Results

Socio-demographic data

All 161 SCA patients and 103 controls (59.1% HbAA; $n = 55$) lived in the urban and peri-urban area of Yaoundé and Douala, the two biggest cities in Cameroon; 51% ($n = 76$) of patients and 67.8% ($n = 59$) of controls were female, with a significantly higher proportion of females among controls (p = 0.043).

Patients were relatively younger than controls ($p<0.001$) and the median age of SCA patients was 17.5 years (25th percentile = 11 years; 75th percentile = 24 years). The median age of HbAS controls was 24 years (25th percentile = 17.5 years; 75th percentile = 26 years) and that of HbAA controls was 26.5 years (25th percentile = 23.2 years; 75th percentile = 30 years).

Anthropometric variables and clinical events in SCA patients

The patients displayed median systolic and diastolic blood pressure of 108 mmHg (25th percentile = 101 mmHg; 75th percentile = 116 mmHg) and 58 mmHg (25th percentile = 53; 75th percentile = 62.2). The median BMI was 18.2 kg/m^2 (25th percentile = 15.7 kg/m^2; 75th percentile = 21.4 kg/m^2).

After the review and validation of clinical data/events from parents/patients' interviews and medical records, a maximum of 121 patients had data that were suitable for the analysis (Table 1). The median rate of VOC was 2 per year (25th percentile = 1 per year; 75th percentile = 4 per year) and the median rate of hospitalisations was 1 per year (25th percentile = 0 per year; 75th percentile = 2 per year) (Table 1). High rate of VOC (>3 per year among 43% patients) and relatively high rates of overt strokes (8 cases; 7.4%) were indicative of severe phenotypes among patients. Linear regression analysis incorporating the age of patient and gender does not revealed any differences in rate of VOC and hospitalisations. Though males tended to have a higher rate of VOC (Likelihood Ratio p = 0.09).

Hematological indices among SCA patients and controls

Hematological indices among patients and controls are summarised in Table 2, reporting in patients, a normocytic normochromic anemia, with higher lymphocyte and platelet counts, and also a higher HbF and HbA2 levels than those of controls.

Table 1. Co-inheritance of SCA-alpha thalassemia and of clinical events.

		HbSS-(αα/αα)		HbSS-(αα/α3.7)		HbSS-(α3.7/α3.7)		
		N	Median (Minimum-Maximum)	N	Median (Minimum-Maximum)	N	Median (Minimum-Maximum)	P-values
BMI (kg/m2)		49	17.5 (12.1–26.6)	27	17.7 (12.5–26.7)	7	18.3 (17.0–23.1)	0.91
Systolic blood pressure (mmHg)		49	108 (86–156)	29	108 (89–135)	6	105.5 (99–116)	0.71
Diastolic blood pressure (mmHg)		49	56 (41–93)	29	60 (47–60)	6	60.5 (45–70)	0.62
No. of vaso-occlusive pain crises/year		77	2 (0–15)	35	2 (0–40)	9	1 (1–4)	0.31
Overt Stroke	**YES**	6	-	1	-	1	-	0.48
	NO	65	-	33	-	7	-	
No. of consultations/year		70	2 (0–12)	31	1 (0–12)	9	1 (0–4)	0.52
No. of hospitalisations/year		70	1 (0–10)	33	1 (0–10)	9	1 (0–9)	0.28

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Haplotypes in the β-globin-like genes cluster among SCA patients

HBB gene haplotype data revealed the following frequencies, per number of chromosomes: Benin (66.3%; $n = 208$), Cameroon (21%; $n = 66$), atypical (11.1%; $n = 35$), Bantu (1.3%; $n = 4$), and Arab/Saudi-Indian (0.3%; $n = 1$). No Senegal haplotype was found. In combination, the Benin/Benin (42.2%; $n = 71$), Benin/Cameroon (26.8%; $n = 42$), Benin/atypical (14%; $n = 22$) and Cameroon/Cameroon (5.1%; $n = 8$) haplotypes were the most prevalent. There were no significant differences, when studying the association among the main groups of haplotype combinations and the hematological indices, clinical events or HbF levels (data not shown).

Prevalence and allele frequency of 3.7 kb α-globin gene deletion among patients and controls

Among controls (HbAS and HbAA), 20.4% ($n = 19$) had at least one 3.7 kb α-globin gene deletion, compared to 37.3% ($n = 60$) among patients ($p = 0.007$). HbAS controls had more 3.7 kb α-globin gene deletions than HbAA controls ($p = 0.02$) and the proportion of HbAS controls with one or two 3.7 kb α-globin gene deletions were 34.2% ($n = 13$); the proportion of HbAA controls with at least one 3.7 kb α-globin gene deletion was 10.9% ($n = 6$) (Fig 1A). Similarly, allele frequencies of the 3.7 kb α-globin gene deletions were 11.8% and 22% in controls (HbAA and HbAS) and patients ($p = 0.006$), respectively. Allele frequency HbAS controls of was 19.7% ($n = 15$) and that of Hb AA controls was 6.4% ($n = 7$) (Fig. 1B).

Multinomial logistic regressions analysis incorporating SCA genotype, α-thalassemia genotype, age and gender indicated that, the differential frequency of 3.7 kb α-globin gene deletions among patients and controls was mostly driven by the genotype of HbAA individuals. HbAA individuals were about 4 times less likely to have a single 3.7 kb α-globin gene deletion [exponentiation of the β coefficient (95% Confidence Interval) = 4.02 (1.45–11.13)] and 5 less likely to have a double 3.7 kb α-globin gene deletion than HbSS patients [exponentiation of the β coefficient (95% Confidence Interval) = 5.42 (0.65–44.79)].

In addition, multinomial logistic regression analysis showed that the allele frequencies among patients as compared to controls tended, non-significantly, to be influenced by gender and to a lesser extent by age. Considering sickle genotype, 3.7 kb α-globin gene genotype and gender, multinomial analysis indicated that, being male increased the likelihood of having the 3.7 kb α-globin gene deletion (Likelihood Ratio $p = 0.013$).

Co-inheritance of SCA and α-thalassemia: hematological indices and clinical events

After univariate analysis, the co-inheritance of 3.7 kb α-globin gene deletion and SCA was significantly associated with a lower MCV and higher RBC, WBC, monocyte and lymphocyte counts (Table 3) and no significant differences were observed across α-thalassemia genotypes, when comparing anthropometric variables and clinical events (Table 1).

Effects of α-thalassemia on key clinical and hematological indices in generalised linear regression models, adjusted for age, sex and five SNPs that influence HbF levels are summarised on Table 4. The co-inheritance of alpha-thalassemia was associated with lower consultation rate ($p = 0.038$). The effects of the co-inheritance of α-thalassemia on RBC count, MCV and lymphocytes count were still observed (Table 4). Two SNPs were associated with specific hematological indices: *BCL11A* rs4671393 was significantly associated with HbF level ($p = 0.005$;

Table 2. Hematological indices of SCA patients and controls (HbAA and HbAS).

	HbAA		HbAS		HbSS		
Haematological indices	N	Median (Minimum-Maximum)	N	Median (Minimum-Maximum)	N	Median (Minimum-Maximum)	P-values
RBC (million cells/μl)	64	4.4	36	4.6	149	2.7	**0.01**
		(2–9.3)		(2.1–9.3)		(1.7–5.5)	
Hb (g/dl)	65	13.2	38	12.7	150	7.7	**<0.0001**
		(7.9–19.5)		(6.9–18.7)		(3.3–14.5)	
MCV (fL)	65	81	38	79	150	82	**0.021**
		(64–95)		(56–102)		(66–112)	
CCMH (g/dl)	64	36.3	36	33.8	148	33.9	**0.021**
		(28.6–45.7)		(29.1–45.2)		(26.6–54.3)	
WBC ($X10^9$/L)	65	5.1	38	4.8	150	12.4	**<0.0001**
		(4.4–6.0)		(2–19)		(2.9–42.4)	
Lymphocyte count ($X10^9$/L)	64	2.1	38	2.2	130	5.1	**<0.0001**
		(2.8–24.4)		(0.2–9)		(1.2–21.6)	
Monocyte count ($X10^9$/L)	64	0.6	38	0.5	130	1.2	**0.001**
		(0.4–0.8)		(0.3–2.3)		(0.4–7.8)	
Platelet level (X 10^9/L)	65	224	29	219	149	354	**0.001**
		(174–651)		(137–371)		(110–802)	
HbF (%)	39	5.9	17	7.3	143	11.1	**0.002**
		(0–21.6)		(0–17.1)		(0–29.3)	
HbA2 (%)	58	3.2	32	2.8	147	3.8	**0.003**
		(0.1–5)		(0–7.2)		(1.6–18.2)	

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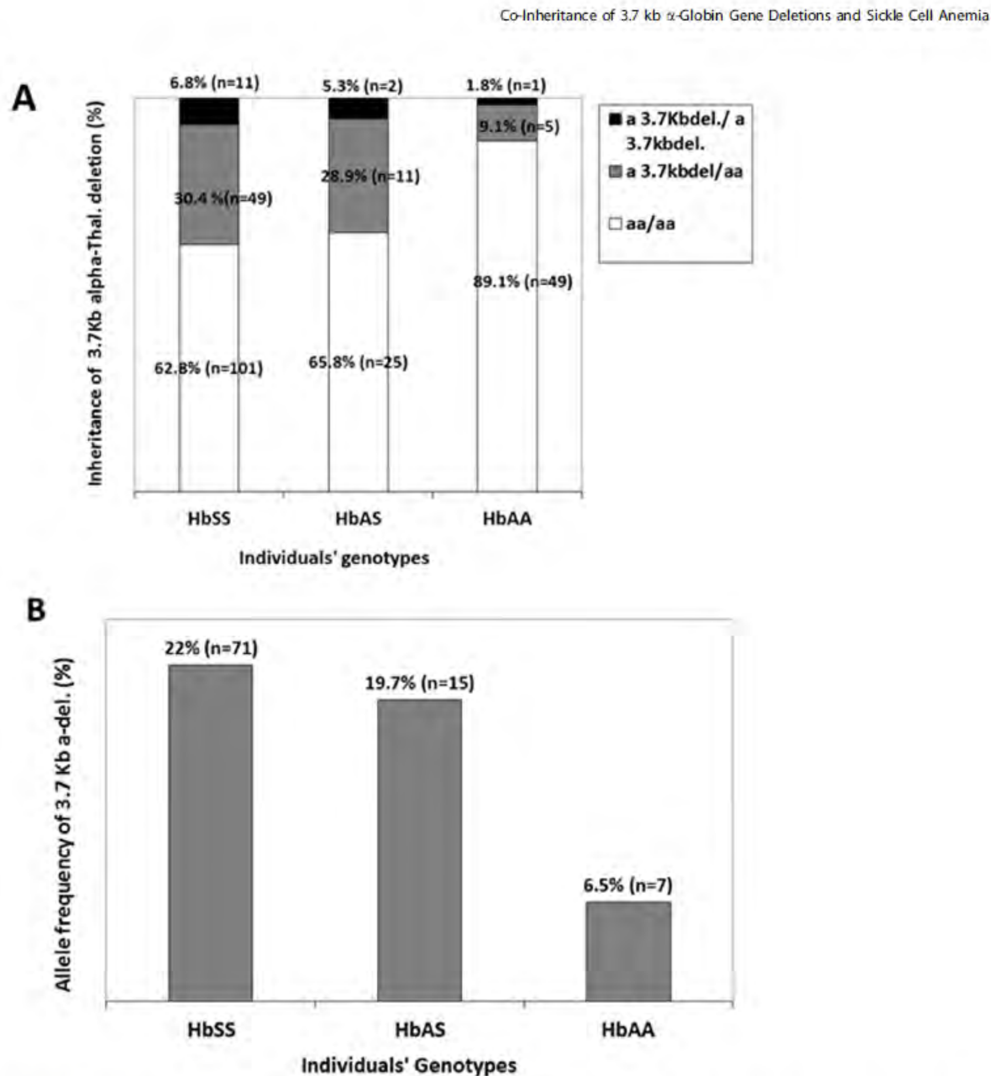

Figure 1. Co-inheritance of α-thalassemia among patients and controls. Panel A displays a much higher prevalence of 3.7 kb α-globin gene deletions among patients compared to unaffected controls (HbAA and HbSS combined (p = 0.003)). This difference was mostly driven by a much lower proportion of 3.7 kb α-globin gene deletions among HbAA controls. **Panel B** displays the allele frequencies of the 3.7 kb α-globin gene deletions among patient and control. The frequencies were 22% among patients and 11.8% among controls (HbAS and HbAA combined) (p = 0.006). HbAS controls had more 3.7 kb α-globin gene deletions than HbAA controls (p = 0.02).
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Table 4); *HMIP* rs9399137 was significantly associated with lower lymphocyte count (estimate = −2.09816; standard deviation = 1.02912; p = 0.044) and borderline associated with lower platelets count (estimate = −76.72; standard deviation = 43.16; p = 0.078). In addition, being female was associates with higher MCV (estimate = 3.88, standard deviation = 1.73; p = 0.02), higher HbA2 (estimate = 1.02; standard deviation = 0.48; p = 0.035) and higher HbF level (estimate = 3.82; standard deviation = 1.44; p = 0.009).

Discussion

A high prevalence of the 3.7 kb α-globin gene deletion has also been reported among SCA patients in Brazil (29%) [24], in India (32%) [25], in the UK among African Britons (34%) [26], in

Table 3. Co-inheritance of SCA and α-thalassemia and hematological indices.

	HbSS-(αα/αα)		HbSS-(αα/α3.7)		HbSS-(α3.7/α3.7)		
Haematological indices	N	Median (Minimum-Maximum)	N	Median (Minimum-Maximum)	N	Median (Minimum-Maximum)	P-values
RBC (million cells/μl),	94	2.6 (1.7–4.7)	46	3 (1.8–5.5)	10	3.4 (1.9–5.5)	**0.01**
Hb (g/dl)	93	7.7 (3.4–13.2)	45	8 (4.9–14.5)	10	8.1 (5.4–14.3)	0.55
MCV (fL)	93	86 (66–112)	45	80 (66–100)	10	68 (59–101)	**<0.0001**
CCMH (g/dl)	99	34.5 (28–54.3)	44	32.9 (28.6–54.3)	10	31.7 (28.8–44.1)	**0.01**
WBC ($X10^9$/L)	100	13.4 (4–42.4)	45	10.5 (2.9–24)	10	9.7 (4.1–17.4)	**0.001**
Lymphocytes count ($X10^9$/L)	80	6 (1.9–21.6)	39	4.2 (1.8–8.4)	9	2.9 (1.6–8.2)	**0.007**
Monocytes count ($X10^9$/L)	80	1.5 (0.4–7.8)	39	1.1 (0.4–3)	10	0.8 (0.4–2.3)	**0.002**
Platelets count ($X10^9$/L)	92	354.5 (110–650)	45	373 (148–802)	10	252 (177–559)	0.73
HbF (%)	89	12.1 (0–29.3)	42	8.8 (0–26.2)	10	14.8 (0.8–27.3)	0.11
HbA2 (%)	91	3.7 (1.6–18.2)	44	3.9 (1.9–13.2)	10	4.7 (2.5–5.3)	0.07

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Table 4. Effects of α-thalassemia on key clinical and hematological indices, in generalised linear regression models, adjusted for age, sex and five SNPs* that influence HbF level.

Outcomes	Unit of measurement	Single deletion vs. No deletion		Double deletion vs. No deletion		Number of Observations
		Estimates (Standard error)	p-values	Estimates (Standard error)	p-values	
Consultations	Day/year	−1.32 (0.63)	**0.038**	−1.31 (1.06)	0.221	110
Hospitalisation	Day/year	−0.17 (0.34)	0.608	−0.37 (0.57)	0.514	104
Vaso-occlusive crisis	Number/year	−0.57 (0.50)	0.253	−1.49 (0.84)	0.079	121
RBC count	million cells/μl	0.37 (0.16)	**0.021**	1.03 (0.27)	**0.0002**	149
Hemoglobin	g/dl	0.58 (0.37)	0.120	0.72 (0.63)	0.25	150
HbA2	(%)	0.23 (0.52)	0.656	0.04 (0.87)	0.956	143
HbF	(%)	−1.55 (1.57)	0.327	0.97 (2.64)	0.715	147
MCV	fl	−5.72 (1.89)	**0.003**	−18.18 (3.19)	**<0.0001**	150
WBC count	$X10^9/L$	−3.43 (1.04)	**0.001**	−4.31 (1.75)	**0.015**	150
Lymphocytes count	$X10^9/L$	−2.15 (0.655)	**0.001**	−2.41 (1.102)	**0.030**	130
Monocytes count	$X10^9/L$	−0.51 (0.20)	**0.01**	−0.67 (0.34)	**0.05**	130
Platelets count	$X 10^9/L$	−4.80 (27.49)	0.86	−45.42 (46.23)	0.32	149

*HbF related SNPs are: *BCL11A* rs4671393, *HBS1L-MYB* rs28384513, *HBS1L-MYB* rs9399137, *HBS1L-MYB* rs9402686, *HBS1L-MYB* rs9494142 and *OR51B5/6* rs5006884.
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Guadeloupe (36%) [27], in Saudi Arabia (40%) [28], in the USA among African Americans (41%) [29], in Oman (43%) [30], in France among Africans (48%) [31], and in Tanzania (58%) [16]. However, none of these studies compared the prevalence of 3.7 kb α-globin gene deletion to unaffected controls from the same setting. By doing this, the present study has provided a unique contribution toward consolidating the hypothesis of a possible positive effect of the 3.7 kb α-globin gene deletion on survival of SCA patients [15]. A decade ago in Congo, researchers reported a less stringent difference with 67.2% SCA patients who had co-inherited the 3.7 kb α-globin gene deletion, as compared to 54.8% of HbAA adults [17]. In Yemen, a similar trend was reported with 34.6% of SCA patients carrying the 3.7 kb α-globin gene deletion, compared to 26.3% in the HbAA group [32]. To support our findings, the prevalence of controls individual (HbAS and HbAA) who have at least one 3.7 kb α-globin gene deletion in the present study (20.4%) is comparable to that reported previously in many other settings across Africa. The prevalence of 3.7 kb α-globin gene deletion was: 15.8% in Kenya [33], 15.1% in Rwanda [34], 20.8% in Guinea (West Africa) [35], and 10%–25% in high-altitude villages in Northern Tanzania [36]. Nevertheless, comparison across populations from different ethnicities and geographical location needs some caution, as the advantageous effect of α-thalassemia is clearly associated with altitude, age of individuals and endemicity of malaria [36]. Individually, HbS and α-thalassemia, are protective against severe *Plasmodium falciparum* malaria, but, there is a possible negative epistasis between α-thalassaemia and sickle cell trait which can modulate the inter population variation [2]. Thus, the significance of a much higher prevalence of 3.7 kb α-globin gene deletion among HbAS than HbAA controls reported will require further investigations in relation to malaria protection.

Noticeably, in a seminal work in the USA, authors reported three decade ago that, in the first ten years of life among HbSS individuals, the prevalence of 3.7 kb α-globin gene deletions was comparable to that in the general African American population (17%), while after 20 years of age, the prevalence increased to 49% [37]. Equally, the prevalence of the 3.7 kb α-globin gene deletion increased with age in Cuban SCA patients [38]. In addition to the higher proportion of 3.7 kb α-globin gene deletions among patients, we reported that the co-inheritance of 3.7 kb α-globin gene deletions delayed the onset of clinical manifestations [15]. These data, in addition to previous report, are implying that co-inheritance of α-thalassemia could be associated with longer survival of SCA patients. Nevertheless, the delayed age of first symptoms may not necessarily be related to lifetime milder clinical expression. Interestingly, after multivariate analysis in the present study, the co-inheritance of α-thalassemia was shown to be associated with lower consultation rate. But, in the present first attempt in both Cameroon and Africa, we did not find any significant influence of the co-inheritance of the 3.7 kb α-globin gene deletion and other clinical events in SCA (Table 1). This is not necessarily unexpected, due to the small sample size and the challenge to define a SCA severity scoring that could be universally used [39]. Specifically, the potential deleterious effect of α-thalassemia on the number of painful episodes reported previously [14,27], could be difficult to validate in a context, such as Cameroon, where free medical services for patient is unavailable. Pain tolerance and socio-economic factors could have influenced the number of hospital visits and biased our evaluation of clinical events. If designed appropriately, future studies in Africa could explore the potential beneficial effect of the co-inheritance of α-thalassemia and SCA, on specific phenotype such as lower hemolysis [40], lower frequency of gallstones [41], lower albuminuria [26,29,42] or lower risk of the occurrence of a stroke [13,24,43]. Noticeably, the present study confirms that the presence of 3.7 kb α-globin gene deletion improved hematological indices and mitigates the degree of anemia in SCA patients (Table 2).

Indeed, α-thalassemia has been shown to diminish the severity of disease by reducing the amount of sickled RBC, increasing the HbF level and HbA2, and decreasing the intracellular HbS level, which results in a reduction in HbS prompted cellular destruction, thereby improving hemolysis [44,45]. The decrease in WBC was attributed to a drop in the hemolytic rate, the amount of sickled red blood cells and a reduction in the inflammatory process. The

extent of the protective effect was also shown to be in accordance with the amount of α-globin genes deleted [24]. The improved hematological indices could be the major factor that contributes to ameliorate the general well-being and possibly survival of SCA patients, and ultimately explain a much higher prevalence of 3.7 kb α-globin gene deletion among patients, than HbAA controls. Nevertheless, in the present study, the results of the generalised linear regression models seem to indicate that others genomic factors as well as demographic factors could also affect the hematological indices (Table 4).

Limitations

The first limitation of the study is the relatively small sample sizes of patients and controls that were not age gender or ethnically matched. Another methodological limitation is the limited use of the gold standard, i.e. HPLC method to measure HbF, because the ADT method is less precise [46], and this could have affected the association of haplotypes, to HbF levels and clinical events. Nevertheless, in a different study, we disaggregated SCA patients sample, based on the HbF assessment technique (ADT vs HPLC), and found that the significant associations with HbF levels, examined independently, were present in both sub-groups studied using the different assay methods, in rs4671393 (*BCL11A*), rs28384513 (*HMIP 1*) and rs9494142 (*HMIP 2*) [12]. The self-reported nature of clinical variables such as VOC episodes limits the interpretation of the results, as pain tolerance and the financial status could have been modifying factors for hospital attendances.

Practical implications and perspectives

The possibility that α-thalassemia could have a strong effect on survival of SCA patients, offers the prospect of profiling patients from birth and addressing a closer follow up. To define a global severity scale for the purpose of genomic studies of SCA is challenging; however, some preliminary data are encouraging. Indeed, in a recent study in Cameroon, two SNPs in *HBS1L-MYB* that influence HbF level were also associated with the number of hospitalisations and in the present study the co-inheritance of α-thalassemia was associated with lower consultation rate. If confirmed, these data could add to the evidence of the clinical effects that are associated with these variants, and a clue on how to measure them. It will be interesting in future on a much larger sample size, to study the concurrent effects of the various genomic loci that influence HbF level, α-thalassemia, socio-demographic and environmental factors such as bacteraemia and malaria, on the survival of patients, in both urban and rural settings.

Despite the fact that more than 70% of SCA sufferers live in Africa, most advances in the molecular understanding and management of SCA have been based on research conducted in the USA or Europe. In the context of Africa, SCA could be consider a neglected tropical disease [47]; thus, the capacity-building dimension that this study provides is worth to be underlined, as it was completely performed on the African continent and could create further research opportunities.

Conclusion

The study confirmed the co-inheritance of α-thalassemia with improved hematological indices and lower consultations rate, which could contribute ameliorate the general well-being and possibly the survival of patients; and ultimately explain the higher proportion of 3.7 kb α-globin gene deletion among SCA patients than controls, specifically HbAA individuals.

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Author Contributions

Conceived and designed the experiments: AW RR AAV. Performed the experiments: MBR VJN JN. Analyzed the data: AW MBR VJN APK. Contributed reagents/materials/analysis tools: RR JN AW. Contributed to the writing of the manuscript: MBR AW RR VJN JN AAV APK. Performed the molecular experiments: MBR. Drafted the manuscript: MBR AW. Acquired clinical data: VJN. Performed DNA extraction and molecular confirmation of SCA: VJN. Performed and supervised the molecular analysis: RR AAV. Model, perform and report on the statistical analysis: APK. Performed all the hematological indices: JN. Supervised the samples collection: JN. Designed the study: AW. Raised funding: AW. Provided general supervision of the research group: AW. Compiled the revisions: AW. Revised and approved the final version of the manuscript: AW MBR VJN AAV RR APK JN.

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CORRESPONDENCE

Coinheritance of sickle cell anemia and α-thalassemia delays disease onset and could improve survival in Cameroonian's patients (Sub-Saharan Africa)

To the editor: Although Sickle Cell Anemia (SCA) is genetically characterized by a single point mutation, the coinheritance of α-thalassemia has been associated with lower stroke rate and higher vaso-occlusive painful episodes [1]. There is a scarcity of data on the coinheritance of α-thalassemia and SCA in Africa; this study aimed to explore the prevalence of α-thalassemia among a group of Cameroonian SCA patients and unaffected controls and the correlation with the age of diagnosis among patients.

Ethical approval was given by the National Ethical Committee Ministry of Public Health, Republic of Cameroon (No 033/CNE/DNM/07); and the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee (HREC REF. 132/2010). The study was conducted at the Yaoundé Central Hospital and Douala Laquintinie Hospital in Cameroon. Socio-demographic data were collected by means of a structured questionnaire and the review of patients' medical records. The control group consisted of randomly selected individuals (HbAS and HbAA) who were apparently healthy blood donors. Hemoglobin electrophoresis and complete routine blood count were conducted on arrival at the hospital. Only patients with SCA (HbSS type) who had not received a blood transfusion or hospitalization in the past 6 weeks were included, with no patient receiving hydroxyurea treatment. Using multiplex gap-PCR following a reported method [2] with a few modifications (Supporting Information 1), 3.7 and 4.2 kb α-globin gene deletions were screened. Using SPSS (IBM, version 21.0), nonparametric tests compared groups of participants; additive model per copy of the α-globin gene deletions were performed, as well as multinomial logistic regression analysis incorporating SCA genotype, α-thalassemia genotype, age, and gender. Significance was set at the 0.05 level.

All 161 SCA patients and 93 controls (59.1% HbAA, $n = 55$) lived in urban areas; 51% ($n = 76$) of patients and 67.8% ($n = 59$) of controls were female ($P = 0.043$). Patients were relatively younger than controls ($P < 0.001$); the median ages of SCA patients, HbAS, and HbAA controls were 17.5, 24, and 26.5 years. Only 3.7 kb α-globin gene deletion was identified. Among patients, the median RBC count increased with the number of 3.7 kb deletions [2.6, 3.0, and 3.4 million/dl, with no, one, and two deletions ($P = 0.01$)] and the median WBC counts decreased with the number of 3.7 kb deletion [13.2, 10.5, and 9.8×10^9/L ($P < 0.0001$)]. Among controls, 20.4 % ($n = 19$) had at least one 3.7 kb α-globin gene deletion, compared to 37.3% ($n = 60$) among patients ($P = 0.007$; Fig. 1A). Multinomial logistic regressions analysis indicated that, HbAA individuals were about 4–5 times less likely to have a single or a double 3.7 kb α-globin gene deletion than patients (Supporting Information Table S1). In addition, being male increased the likelihood of having the 3.7 kb α-globin gene deletion (Likelihood Ratio $P = 0.013$). The median age of SCA diagnosis increased with the number of α-globin gene deletions ($P = 0.026$; Fig. 1B,C).

In a seminal work in the USA, authors reported that, in the first 10 years of life among HbSS individuals, the prevalence of 3.7 kb α-globin gene deletions was 17%, which was comparable to that in the general Black population, whereas in the group over 20 years of age, the prevalence increased to 49% [3]. Equally, the prevalence of the 3.7 kb α-globin gene deletion has also been shown to increase with age in Cuban SCA patients [4], implying that α-thalassemia could be associated with longer survival. To support this hypothesis, the high prevalence of 3.7 kb α-globin gene deletions was also reported in Tanzanian SCA patients (58%) [5]. Interestingly, the increasing age of diagnosis of SCA with number of 3.7 kb α-globin gene deletion, reported in the present study, is novel finding that was only possible to be described, in a context where neonatal screening for SCA is nonexistent. Thus, in Cameroon, the SCA diagnosis is often made when the onset of clinical manifestations brings the patient to medical attention [6]. Taken together with the higher proportion of 3.7 kb α-globin gene deletions among patients, this finding consolidates the hypothesis that the coinheritance α-thalassemia and SCA could ultimately improve survival of SCA patients. In future, data from a larger ethnically, age and sex matched patients and controls sample or ideally, data from a prospective SCA birth cohort, could definitively confirm or affirm the contribution of α-thalassemia in survival of SCA patients.

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AW designed the study, raised funding, provided general supervision of the research group, drafted the manuscript and compiled the revisions. MBR performed the molecular experiments and drafted the manuscript. VJN acquired clinical data and performed DNA

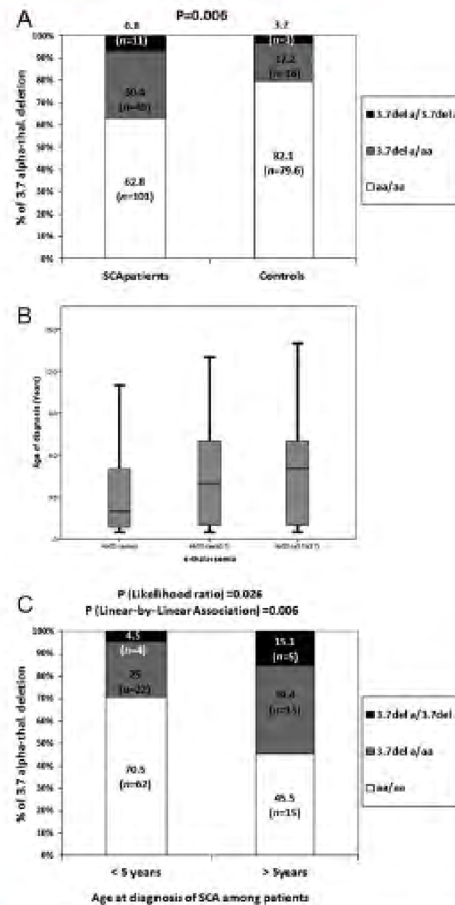

Figure 1. Coinheritance of α-thalassemia could improve SCA patient survival. Panel A displays a much higher frequency of 3.7 kb α-globin gene deletions among patients as compared to unaffected controls [HbAA and HbSS ($P = 0.003$)]. Similarly, allele frequencies of the 3.7 kb α-globin gene deletions were 11.8 and 22% in controls and patients ($P = 0.006$), respectively. HbAS controls had more 3.7 kb α-globin gene deletions than HbAA controls ($P = 0.02$) and the proportion of HbAS controls with one or two 3.7 kb α-globin gene deletions were 34.2% ($n = 13$) with an allele frequency of 19.7% ($n = 15$), which is almost similar to that of HbSS patients. The proportion of HbAA controls with the 3.7 kb α-globin gene deletion was 10.9% ($n = 6$), with an allele frequency of 6.4% ($n = 7$). Observed 3.7 kb α-globin gene deletion allele frequencies in controls were consistent with Hardy–Weinberg equilibrium ($\chi^2 = 2.37$; $P = 0.12$). Panel B displays the increasing number of 3.7 kb α-thalassemia gene deletion with the age at diagnosis ($P = 0.026$). Boxes have lines at the lower quartile, median, and upper quartile. In the absence of neonatal screening, the age at diagnosis often corresponds to that of clinical manifestations that bring the SCA patient to medical attention. Panel C illustrates that the proportion of patients with 3.7 kb α-globin gene deletions diagnosed after 5 years of age was significantly higher than diagnosed before 5 years of age (Likelihood ratio $P = 0.026$; Linear-by-Linear Association $P = 0.006$). Among patients who did not inherit the α-thalassemia gene deletion, 80.5% ($n = 62$) was diagnosed before 5 years of age, as compared to 62.8% ($n = 22$) and 44.4% ($n = 4$) of patients who has coinherited one or two deletions. Taken together, these data seem to indicate that the coinheritance of α-thalassemia could delay clinical manifestations and positively affect the survival of this group of Cameroonian SCA patients, which could explain the much higher allele frequency of the 3.7 kb α-globin gene deletions among SCA patients compared to control.

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extraction and molecular confirmation of SCA. RR and AAV performed and supervised the molecular analysis. JG performed all the hematological indices and supervised the samples collection. All the authors revised and approved the final version of the manuscript.

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