Evaluation of Spleen Size and Function: Relationship with Malaria and Bacterial Infections in Sickle Cell Disease Patients in North-Eastern Nigeria

Thesis submitted in accordance with the requirements of Liverpool School of Tropical Medicine for the degree of Doctor of Philosophy

By

Adama Isah Ladu

LSTM
LIVERPOOL SCHOOL
OF TROPICAL MEDICINE

31st July 2023

DECLARATION

I, Adama Isah Ladu, declare that this thesis: Evaluation of Spleen Size and Function: Relationship with Malaria and Bacterial Infections in Sickle Cell Disease Patients in North-Eastern Nigeria', is submitted for the degree of Doctor of Philosophy in Clinical Science of the Liverpool School of Tropical Medicine. This thesis has not been submitted for any other degree or award at this or any other institution. I confirm that this thesis is the result of my own work, and all materials used from other sources have been appropriately acknowledged and referenced in the thesis.

Supervisors

Prof Imelda Bates, Primary Supervisor (Liverpool School of Tropical Medicine)

Dr Caroline Jeffery, Second Supervisor (Liverpool School of Tropical Medicine)

Progress Review Advisors

Prof Stephen Allen (Liverpool School of Tropical Medicine)

Mr Russell Dacombe (Liverpool School of Tropical Medicine)

DEDICATION

I dedicate this piece of work to the memory of my late father, Alhaji Isah Ladu, who saw greatness in me at a very young age and always believed I would reach great heights. Your sacrifice to give us the best still remains fresh in my memory and will always be appreciated.

…… To all the Sickle cell disease patients, your pains and sufferingare felt through every piece of this work…better days lie ahead...

ACKNOWLEDGEMENT

All praise and thanks are due to the Almighty God who blessed me with this life changing opportunity, and to go through this PhD programme successfully. It still feels like a dream!

I am grateful to the Commonwealth Scholarship Commission for the full funding for my PhD programme. I also thank the management of University of Maiduguri and the University of Maiduguri Teaching Hospital, Borno state Nigeria, for granting me a study leave to pursue my PhD.

My sincerest appreciation goes to my supervisory team, Prof Imelda Bates (primary) and Dr Caroline Jeffery, and my Progress Assessment Panel members, Prof Stephen Allen and Mr Russell Dacombe for their tremendous support, timely feedback, advise and constructive criticism during my PhD research work. I have benefited immensely from their vast experience, expertise and knowledge which has contributed to the successful completion of this research work and thesis.

Especially to Imelda, words alone cannot express how grateful I am for your support, guidance, and advice at every stage of my PhD journey. You provided me so many opportunities for career growth, networking and always checked on my wellbeing in all aspect's throughput my PhD journey. Your unique and exceptional way of mentoring is unrivalled and on a different level. I have learnt so much from the time I have been with you. I thank you for allowing me to grow in research in my own ways and at my own pace. I will remain forever grateful to you. Lastly, I appreciate those coffee times on Wednesday, I will surely miss them!

I would like to express my gratitude to Prof Adekile Adekunle, although not part of my formal supervisory team, you were with me at the beginning of my PhD and

contributed at every stage of my progress. You never got tired of reviewing my works and giving me advice. I am indeed grateful for your continued mentorship!

I appreciate the support of my colleagues, Dr Aisha and Dr Fatima for your selfless support and friendship. I am really grateful. Also, many thanks to my other colleagues and resident doctors of the Haematology, Microbiology and Paediatric departments for assisting with the various aspects of patient's recruitment into the project.

I want to thank my ex- PhD colleagues – Mtisunge, Lilian and Wesam, your advice and support during those difficult times are much appreciated. You were all like sisters to me. Thanks to all those good memorable times shared; they really helped in making the PhD journey bearable. Also, to my office colleagues at the Liverpool of Tropical Medicine, Centre for Capacity Research Unit, especially Susie Crossman, for always offering your time and skills to help with my conference posters and your usual helpful tips, and Heather for the encouragement and diverse support during my studies. I will miss those little, little office talk! I also want to thank the PGR team – Charlotte Blakeburn and Richard Madden for their support, especially during the COVID 19 period.

I would like to say thank you to Greg and Dan of the Dagnall laboratory at LSTM for helping me during preparation for my fieldwork in Nigeria. To staff at the UMTH: Alhaji Bukar of Histopathology lab, Malam Haruna of Chemical pathology, Malam Ali Dogo of Immunology lab, Mustapha of Microbiology lab and Mr John of Haematology department, I thank you for your massive help during the laboratory processing and analysis of my project samples. My great appreciation also to Malam Habu of Aminu Kano Teaching hospital for organising the transport and analysis of samples for HPLC at your centre; your selfless help and dedication in this aspect of

the project left me dumbfounded on how to say thank you! I also want to thank the head of department of Radiology UMTH, Dr Sani Ahmadu, for allowing me the use of your facility for performing the ultrasound scans for my research participants.

My deepest gratitude goes to all the patients and their parents who made time to answer the questionnaires, agreed to be scanned and donated blood samples for the research. You all made the project a reality!

My special and heartfelt gratitude goes to my Mum and siblings for their support and encouragement during my studies.

Finally, to my husband, a lifelong friend and partner, thank you for being at the heart of all I do. Your exceptional understanding, support and sacrifice is what has brought me this far. I remain loyal and grateful to you!

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ASSC	Acute splenic sequestration crisis
AI	Agyrophylic inclusions
ASAT	Aspartate amino acid transferase
EDTA	Ethylene diamine tetra acetic
HbSS	Homozygous sickle cell anaemia
HbAS	Heterozygous sickle cell disease
Hb F	Foetal Haemoglobin
HJB	Howell-Jolly Bodies
HbS	Sickled haemoglobin
HPLC	High performance liquid chromatography
HU	Hydroxyurea
LMIC	Low-to-middle-income countries
RBC	Red Blood cells
SCD	Sickle cell disease
SSA	Sub-Saharan Africa
USS	Ultrasound scan
WBC	White blood cells

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Sickle cell disease (SCD) is a collective name for a group of conditions that cause clinical symptoms from the formation of sickled red cells. The spleen is one of the earliest organs affected in SCD. During the course of the disease, the spleen declines in both its structure and function; however, the course of events is variable amongst individuals with the disease and depends on clinical and biological factors. The loss of splenic function may be associated with increased susceptibility to infection; however, spleen function is rarely documented among SCD patients in Africa, due partly to the non-availability of sophisticated techniques such as scintigraphy and pitted red cell counts by contrast-enhancing microscopy, thus the role of splenic dysfunction and infections have not been well studied.

With the aim of better understanding the spleen size and function among our SCD patients, and the risk associated with infections, I investigated: 1) baseline spleen sizes amongst the SCD patients and healthy controls based on ultrasonography and the clinical and laboratory correlates associated with splenic size preservation. 2) splenic function by comparing the frequencies of red cells containing Howell-Jolly bodies (HJB) and argyrophilic inclusions (AI) in SCD patients with those of healthy controls, and explored factors associated with splenic dysfunction. 3) the prevalence of malaria infection in SCD patients and healthy controls, the prevalence of bacterial infection in acutely-ill SCD patients, and their association with spleen size and function.

On the evaluation of baseline spleen sizes, about half of the SCD patients had no visible spleens on ultrasonography (47%). The spleen was visualised in all the children aged five years and below. The spleen size in SCD patients during the first two years of life was three-fold higher than those of age-group matched, healthy controls, then followed by a progressive age-related decline in size. Among the laboratory factors

explored, high HbF and low mean corpuscular haemoglobin concentration (MCHC) were associated with preservation of the spleen.

In the spleen function assessment study, the frequency of HJB and AI- containing red cells were higher in the SCD patients than healthy controls. The HJB and AI counts were higher in patients without visible spleens than patients whose spleens were visualised on ultrasonography. The percentages of HJB- and AI- red cells rose significantly with increasing age, and both counts showed a significant positive association with MCH and a negative association with HbF level.

In the spleen and infection study, *P. Falciparum* prevalence and parasite densities were higher among the acutely-ill SCD patients than steady-state SCD controls. Severe malaria events were more frequent among the less than five years old SCD patients. There was no significant association between prevalence of parasitaemia or parasite density and the visualisation or non-visualisation of the spleen on ultrasonography, nor with the frequency of HJB red cells. The overall prevalence of bacteraemia among the SCD patients in the study was low (5.2%), and the majority of isolates cultured were Gram-negative organisms. There was no significant association between prevalence of bacteraemia and the visualisation or non-visualisation of the spleen on ultrasonography, nor with the frequency of AI and HJB red cell counts.

Overall, by exploring spleen size, spleen function and infections risk related to splenic parameters, my research has provided new insights into the spleen and SCD among patients in an African setting. It showed that the spleen can be visualised among SCD patients up to the age of five years before autosplenectomy occurs. It provides evidence that assessment of spleen function is feasible among SCD patients even in resource-limited settings. Importantly, my research provides evidence about the consistency of

HbF as predictor of both spleen size preservation and function among our SCD patients. Lastly, evidence regarding the role of the spleen in malaria and bacterial infection was obtained, thereby filling an important gap in knowledge regarding the role of the spleen and risk of infection.

CHAPTER 1. BACKGROUND TO MY RESEARCH

1.1 Chapter overview

My thesis evaluated spleen size and function among children and adults living with SCD in malaria-endemic region of North-Eastern Nigeria. This chapter provides a description of the research process, and a brief description of sickle cell disease (SCD), its epidemiology, clinical manifestations, and management. An overview of the spleen including the anatomy, function, and clinical presentation of splenic dysfunction is provided and various methods of assessing spleen function are described and its importance in SCD is discussed. The chapter ends with the aim and objectives of the thesis.

1.2 Sickle cell disease

1.2.1 Background

Sickle cell disease is a collective name for a group of inherited conditions that causes clinical symptoms from the formation of sickled red cells. The homozygous form of the condition (Hb SS or sickle cell anaemia) is the most common and arises from a single nucleotide change in the β globin gene (Hββ), whereby adenine is substituted by thymine (GAG = GTG) at the 6^{th} codon of the gene. This results in the substitution of valine for glutamic acid at the 6^{th} position of the β globin chain of the haemoglobin molecule (Fig.1) (Kato *et al.*, 2018). The resulting Haemoglobin S (Hb S) has poor solubility when deoxygenated and can polymerise within the red cells resulting in the classical 'sickled-shaped' red cells from which the disease derives its name (Fig.1). The sickled red cells also have a shortened life cycle as result of both intravascular and extra vascular haemolysis. Sickle cell haemoglobin C is a compound heterozygous state for Hb S and C (Hb SC); this compound state often results in a milder form of

SCD (Hoffbrand, Catovsky and Tuddenham, 2005). Sickle cell beta-thalassemia compound heterozygotes account for less than 10% of SCD; the majority of patients have the β^+ genotype which is characterised by HbA level between 5% to 30% and Hb A_2 level of up to 6% and is associated with a mild clinical course (Steinberg, 2008). The less common co-inheritance of the sickle cell gene and β zero genotype give rise to a severe form of SCD (Nagel and Fleming, 1992).

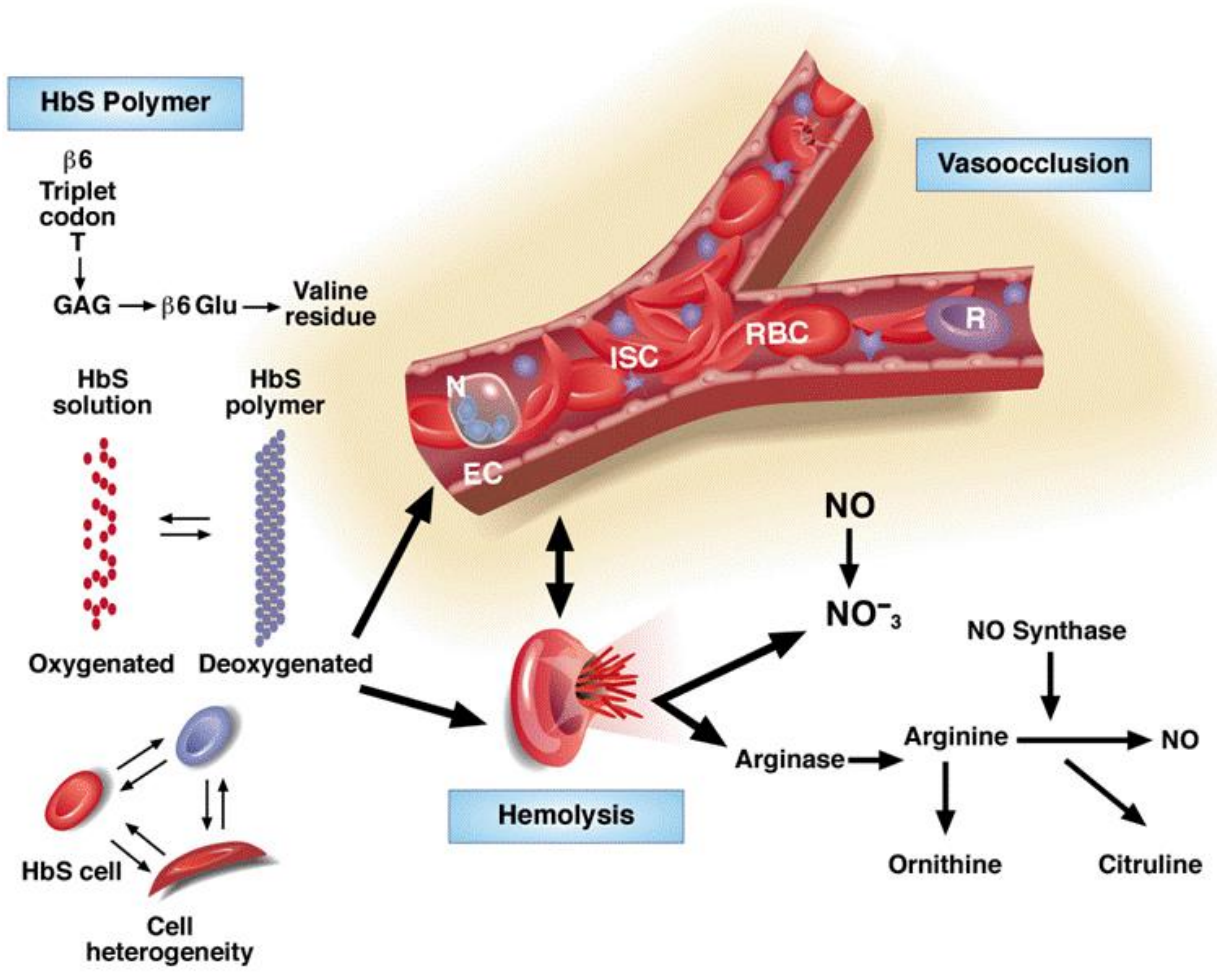


Figure 1: Pathophysiology of SCD

Legend: the HbS mutation arises from the substitution of adenine with thymine (GAG = GTG) at the 6th codon of the β globin gene (Hββ). This results in the substitution of valine for glutamic acid at the 6th position of the β globin chain of the haemoglobin molecule. The resulting Haemoglobin S (Hb S) has poor solubility when deoxygenated and can polymerise within the red cells. The HbS containing red cells have shortened

life span due to ongoing haemolysis; intravascular haemolysis results consumption of nitric oxide (adapted from Steinberg 2008)

.

1.2.2 Epidemiology of SCD

The sickle cell gene is common among populations of Sub-Saharan Africa (SSA), India, the Middle East, and some part of the Mediterranean (Piel *et al.*, 2013a). The heterozygous form of the disorder (Hb AS), otherwise referred to as sickle cell trait, confers a survival benefit against malaria infection (Luzzatto, 2012); the Hb S containing red cells inhibit the proliferation of *plasmodium falciparum* thereby increasing their clearance from the circulation. This explains the overlap between the geographical distribution of SCD and malaria endemic regions (Fig.2A) (Piel *et al.*, 2010). Movement of populations through slave trades, and in recent times, through trade routes and voluntary migration, have resulted in the dissemination of the sickle mutation to many parts of the world, including North America and Western Europe (Hoffbrand, Catovsky and Tuddenham, 2005; Piel *et al.*, 2013b).

The number of births with SCD in SSA was estimated to be about 230,000 in 2010, consisting of about 75% of the global births of newborn with SCD (Piel *et al.*, 2013a); this figure is expected to rise to 400,000 by 2050. The majority of affected babies are found in SSA countries of Nigeria, the Democratic Republic of Congo, and Tanzania (Fig.2B). However, in a recent global assessment, completed as part of the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2021, the results showed that between the year 2000 and 2021, the number of new-borns with SCD has risen from 453 000 to 515 000 - a 13.7 % increment in the global SCD birth rate to 382 per 100 000 live births (Thomson *et al.*, 2023). Within this period, the incidence in SSA showed a 27.1% increment in the births of SCD babies to 405 000 in 2021. Globally, all-age SCD prevalence increased from 5.46 million cases in 2000 to 7.74 million in

2021. The observed increment was partly due to population growth and changes in disease frequency associated with increased survival. In SSA, the all-age prevalence in 2021 was 5·68 million cases, a 67·4% increase since 2000 (Thomson *et al.*, 2023).

Estimates of survival from several large studies in the United States (US), Europe and the Caribbean show improvement in survival over the last 40 years, attributable to implementation of newborn screening and improvement in preventive care (Quinn *et al.*, 2010). However, survival in similar period has not improved in SSA where an estimated 50-90% of children born with sickle cell anaemia (SCA) die before the age of five (Makani *et al.*, 2013; Grosse *et al.*, 2011; Aygun and Odame, 2012). The majority of these deaths are due to anaemia and infections, such as malaria and pneumococcal sepsis (Albert *et al.*, 2009; McAuley *et al.*, 2010; Alima Yanda *et al.*, 2017).

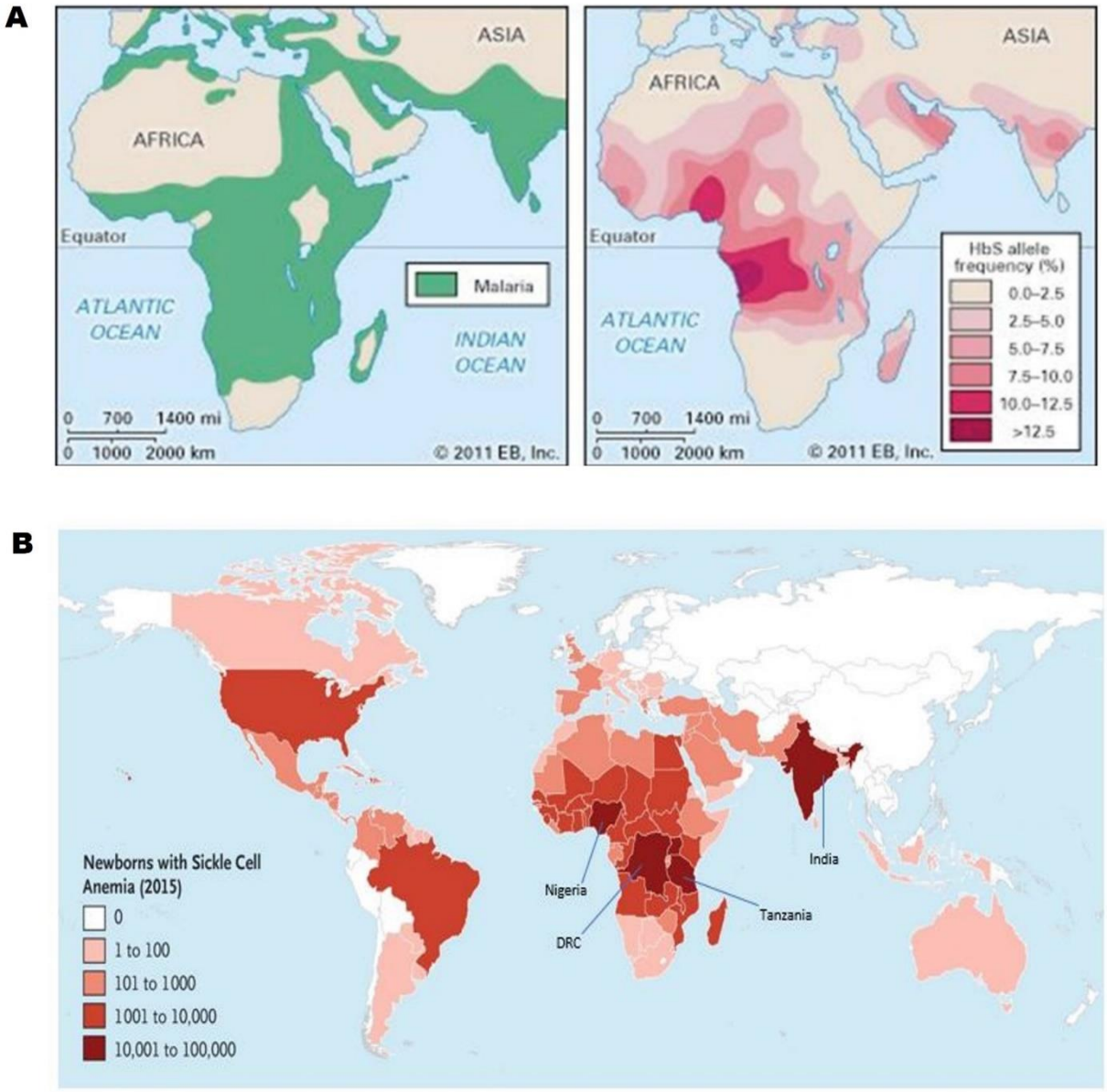


Figure 2: Maps showing the global burden of SCD.

Legend: A) Correlation between malaria distribution and sickle cell anaemia in Africa and Asia (https://sqonline.ucsd.edu/wp-content/uploads/2018/07/). B) Global distribution of newborns with SCD in 2015 (adapted from Piel *et al*. 2013)

1.2.3 Clinical manifestation

The clinical symptoms of SCD vary significantly among patients with this disorder, being more severe in those with the homozygous form (i.e Hb SS) or Hb $S\beta^{-}$ thalassemia than in those with Hb SC or Hb $S\beta^{+}$ (Quinn, 2016; da Guarda *et al.*, 2020; Vincent *et al.*, 2016). The co-inheritance of one or two alpha globin gene deletions ameliorates the clinical manifestation (Benesch, Kwong and Benesch, 1982; Quinn, 2016). The presence of high F (HbF; the heterodimeric combination of two alpha and two gamma globin proteins) in individuals with hereditary persistence of foetal haemoglobin (HPFH) modifies the condition to a milder form (Alsultan *et al.*, 2011). Some of the clinical manifestations of SCD are described below:

1.2.3.1 Anaemia

Both intravascular and extra vascular haemolysis occur in SCD. Repeated episodes of polymerisation and sickling leads to the formation of irreversible sickled red cells which have a shortened life span because of their accelerated removal from circulation (Edelstein, Telford and Crepeau, 1973; Benesch, Kwong and Benesch, 1982). Patients with very low baseline haemoglobin are at increased risk of stroke and renal dysfunction; on the other hand, a higher haemoglobin is associated with more frequent painful episodes and acute chest syndrome (Rees, Williams and Gladwin, 2010). Further exacerbation of the pre-exiting anaemia can occur for several reasons including folate or iron deficiency, inadequate erythropoietin from renal dysfunction. Sudden exacerbation can also be observed in aplastic crisis or splenic sequestration crises (Hoffbrand, Catovsky and Tuddenham, 2005).

1.2.3.2 Acute painful episodes

This is the most common symptom for which patients with SCD seek medical attention. It is caused by repeated episodes of vascular occlusion by the sickled red

cells (Edelstein, Telford and Crepeau, 1973). Individuals with Hb SS, low HbF, alpha-thalassemia and a high baseline haemoglobin report more frequent painful episode. In the majority of cases, no identifiable cause for the painful episode can be identified, although some attacks may be precipitated by cold or hot weather, infections, stress, and menstruation (Ahmed and Ibrahim, 2017; Slovis, Talley and Pitts, 1986).

1.2.3.3 Infection

Early loss of splenic function in patients with SCD increases the risk of sepsis (Booth, Inusa and Obaro, 2010). There is variation in the relative incidence of bacterial organisms that cause septicaemia in patients with SCD in different parts of the globe. Pneumococcal infection is an important cause of sepsis especially in children with SCD less than five years of age (Lesprit and Lesprit, 2004). The incidence of *pneumococcal* sepsis and *H. infleunza* sepsis has declined due to penicillin prophylaxis and vaccination of infants in developed countries (Hirst and Owusu-Ofori, 2014). In adults, bacteraemia, and urinary tract infection due to *E.coli* and other Gram-negative organisms are frequent. Patients with SCD are also prone to osteomyelitis owing to bone infarction from repeated vaso-occlusion; the infection is typically caused by *Salmonella* or *Staphylococcus aureus* (Anand and Glatt, 1994). In Africa, *Salmonella spp, Klebsiella, E.coli* and *S. aureus* predominates (Kizito *et al.*, 2007; Magnus *et al.*, 1999). Malaria infection in SCD patients residing in endemic region is one of the major causes of crises (Aloni *et al.*, 2013; Akinyanju and Johnson, 1987; Ambe, Fatunde and Sodeinde, 2001).

1.2.4 Treatment

Although the first discovery of SCD was made several decades ago, treatment remains limited, especially in low to middle income countries (LMIC). Supportive management for infection, pain and anaemia remains important (Okpala *et al.*, 2002).

An elevated temperature in children with SCD needs prompt attention. Work-up involves performing a full blood count, blood and urine cultures and chest radiograph when indicated. Patients with recurrent episodes of pain are better managed in a familiar ambulatory setting. Evaluation for potential cause of the pain is essential. Management includes adequate hydration along with analgesics. Patient's education, penicillin prophylaxis, routine immunization for *pneumococcal, H influenza*, hepatitis B, and antimalarial prophylaxis in endemic region are highly recommended (Okpala *et al.*, 2002; Tluway and Makani, 2017). The use of hydroxyurea and hematopoietic stem-cell transplantation, and very recently gene therapy, are some of the options that can significantly enhance survival and quality of life in SCD (Hoban, Orkin and Bauer, 2016; Ware, 2015)

1.2.4.1 Hydroxyurea

The use of hydroxyurea (HU) to treat SCD started about four decades ago and long-term studies have shown benefits in both children and adults (Fig. 3) (Ware and Aygun, 2009). The ability of the drug to increase level of HbF is used for this purpose. The myelosuppressive effect of HU results in stress erythropoiesis and ultimately increased level of HbF (Hoffbrand, Catovsky and Tuddenham, 2005; Agrawal *et al.*, 2014). The rise in level of HbF in response to HU is variable among patients with SCD, while in some, no change from the baseline value may occur. Some of the clinical effects observed with HU treatment include decrease in frequency of pain episodes, acute chest syndrome and transfusion requirement (Gulbis *et al.*, 2005; Luzzatto and Makani, 2019; Tshilolo *et al.*, 2019). Patients on HU require frequent monitoring of their blood counts, renal and hepatic function. Myelosuppression is one of the side effects and dose reduction or temporary cessation of therapy may be required if it occurs.

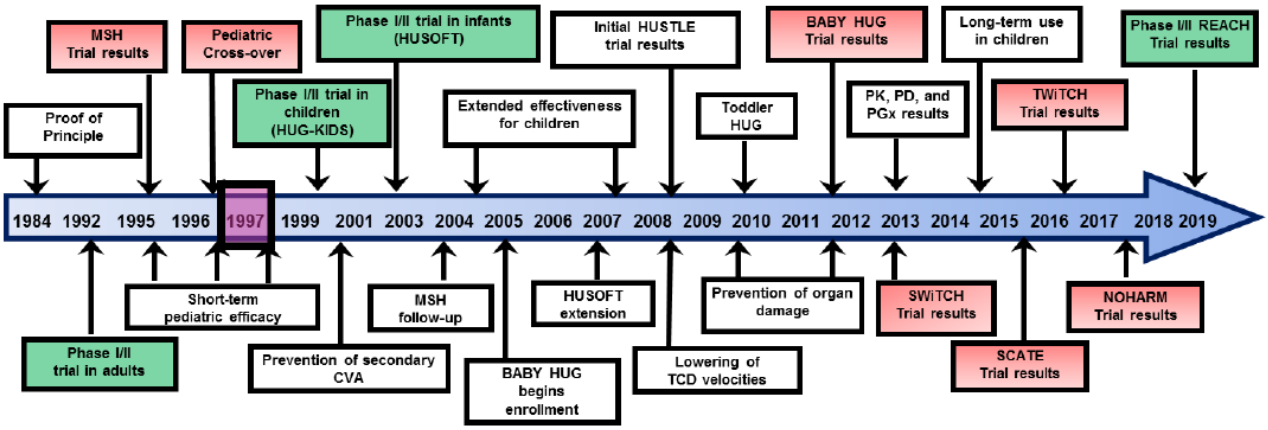


Figure 3: Timeline for clinical trials of hydroxyurea

Legend: several proof of principle studies was carried out in adults in the 80's. In the early 90s, a prospective phase 1/2 study in adults using HU at maximum tolerated dose was followed by a double-blinded placebo-controlled phase 3 MSH trial and showed the clinical efficacy of HU in preventing vaso-occlusive events. Towards the late 90s, studies describing the short-term efficacy and safety of HU among the paediatric population were reported. Subsequently, several multicentre randomized clinical trials involving infants, adolescent, and adults with SCD have been conducted.(Adapted from El Hoss et al 2019).

1.2.4.2 Stem cell transplantation

Haemopoietic stem cell transplantation (HSCT) offers the prospect of cure from SCD and should be considered in symptomatic patients with HLA matched family donor; globally, nearly 2000 SCD patients have undergone SCT, with survival rates of more than 90% from published reports (Kato *et al.*, 2018; Walters *et al.*, 2016). The excellent outcome in a cohort study involving 1000 children and adults with SCD transplanted over the course of three decades confirms the role of HLA-identical sibling transplantation in SCD (Gluckman *et al.*, 2017).

Although stem cell transplantation offers a high prospect of cure from SCD, this therapy is mainly available in high income countries. Also donor availability and associated SCD related morbidity with age and disease severity remains a challenge to

HSCT in SCD (Hulbert and Shenoy, 2018). The procedure is best indicated in symptomatic patients and should be considered in patients with SCD under 16 years of age with stroke, recurrent acute chest crisis or vaso-occlusive crisis (Kassim and Sharma, 2017).

1.3 The spleen

1.3.1 Overview of the spleen

The spleen serves as the major filter of blood to remove senescent red cells and micro-organisms. It contains the largest single aggregate of lymphoid tissue in the body and plays a role in both humoral and innate immunity (Gomez-Perez *et al.*, 2014; William and Corazza, 2007; Riva *et al.*, 2019).

1.3.2 Structure and functions of the spleen

The normal spleen weighs 150 to 250 g, but there is considerable variation within an individual with age. The spleen reaches its maximum weight of 200-300 g during puberty. After the age of 65 years, it declines to100 - 150 g or less (Hoffbrand, Catovsky and Tuddenham, 2005)). In adults, the spleen length is between 8-13 cm, the width between 4.5-7.0 cm, the surface area between 45-80 cm^2 and volume of about 275 cm^3 (Mebius and Kraal, 2005). The spleen receives 5% of the total cardiac output per minute; blood enters the spleen through the splenic artery, which then divides into trabeculae arteries that permeates the organ and give rise to central arteries within the white pulp (Fig. 4) (William and Corazza, 2007; Gomez-Perez *et al.*, 2014). The central arteries give rise to many arterioles and capillaries, some of which end up within the white pulp, while the remaining go on to enter the red pulp. The function of the spleen is subserved by its two anatomic compartment, the red pulp and the white pulp, interconnected by a perifollicular zone (Gomez-Perez *et al.*, 2014). These highly

organized microanatomical structures serve the spleen's two main functions, that is, blood filtration and immune defence.

1.3.2.1 Red pulp

This region of the spleen is made up of cords and vascular sinuses. The splenic cords consist of a meshwork of macrophages and fibroblast. The vascular sinuses are lined by discontinuous layer of endothelial cells. These small pores between the endothelial cells (referred to as inter-endothelia slits) allow for blood cells to traverse between the cords and sinuses (Brousse, Buffet and Rees, 2014). The red pulp is involved in the sequestration and phagocytosis function of the spleen. Sequestration is the temporary trapping of red cells within the splenic cords during their passage. Here the cells become conditioned or modified before going back into the circulation, for example, red cell inclusions including Howell-Jolly bodies, Heinz bodies and siderotic granules are removed during this process and the remainder of the cells returned back into the circulation (Brousse, Buffet and Rees, 2014; Safeukui *et al.*, 2008). Phagocytosis is the irreversible uptake by macrophages of non-viable cells, particulate matter or viable cells that have been damaged or coated with antibodies.

1.3.2.2 White pulp

This region of the spleen, which consists of T and B lymphocytes, is involved in the immunological function of the spleen. The T cells are located along the peri-arteriolar sheet lining the central arteries (Fig.4), while the B cells are located within the germinal follicles where they produce immunoglobulin (Gomez-Perez *et al.*, 2014). During the primary immune response to blood borne bacteria or damaged red cells, CD8 T cells act together with macrophages to eliminate such materials. Secondary stimulation with such antigens facilitates antibody production, usually IgG. This process is important for the protection from bacterial infections with encapsulated

organisms including *S. Pneumonia, H.influenza* and *N.Meningitides*. This mechanism also helps protect against infection with intra-erythrocytic parasites such as *plasmodium* and *Babesia* (Gomez-Perez *et al.*, 2014; Safeukui *et al.*, 2008).

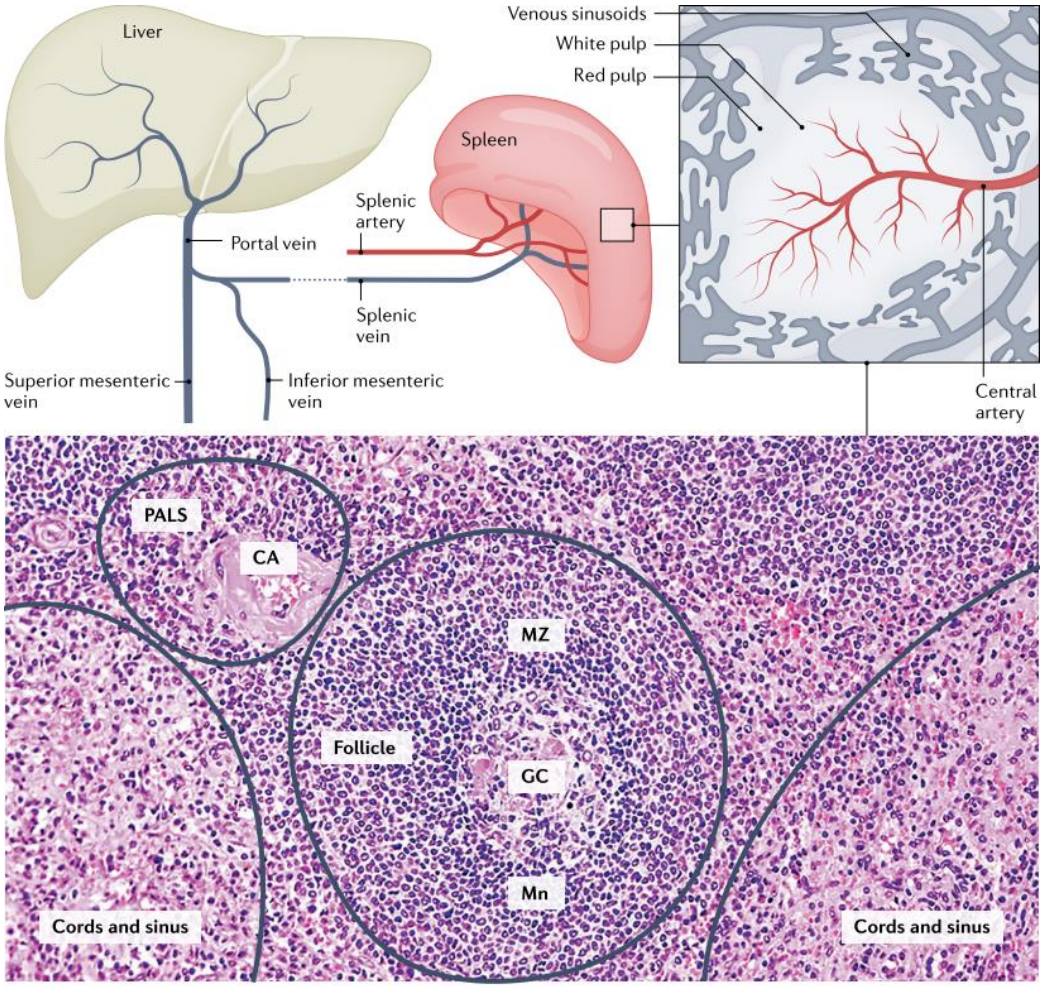


Figure 4: Structure of the spleen.

Legend:

Anatomy and histology of a healthy spleen: blood enters the spleen through the splenic artery, which then divides into trabeculae arteries that permeates the organ and give rise to central arteries within the white pulp. The splenic vein drain blood from the spleen, and together with the superior and inferior mesenteric veins converge into the portal vein that carries blood to the liver. The internal structure of the spleen consists of two functional unit – the white pulp and red pulp. Follicles in the white pulp are composed of the germinal centre (GC), the marginal zone (MZ) and the mantle zone (Mn; also termed the corona). The MZ contains memory B-cells and specialized macrophages. Here, pathogens are exposed to macrophages and dendritic cells, which

present them to the T and B lymphocytes of the white pulp. The red pulp is made up of the splenic cords and sinuses (adapted from Lenti et al 2022).

1.3.3 Clinical manifestation of splenic dysfunction

1.3.3.1 Splenomegaly

Enlargement of the spleen can occur in variety of conditions or from various pathological processes. The prevalence is also subject to geographical variation. In Western countries, haematological malignancies including leukaemia, lymphomas and myeloproliferative disorders predominate as causes of splenic enlargement (William and Corazza, 2007). In tropical countries, parasitic infections including malaria, leishmaniasis, and schistosomiasis are the predominant cause of splenic enlargement (Ehimwenma and Tagbo, 2011). In malaria infection, several pathogenic mechanisms are involved in splenic enlargement as demonstrated in hyper-reactive malarial splenomegaly (Bates *et al.*, 1997; Bedu-Addo and Bates, 2002). Inherited conditions are also important causes of splenic enlargement; in compound heterozygous forms of SCD, such as, Hb SC and Hb SBeta thalassemia, the spleen persists into adulthood more commonly than in Hb SS (Belhani *et al.*, 1984; Abjah and Aken'Ova, 2003).

1.3.3.2 Hypersplenism

Some cases of splenomegaly may be complicated by hypersplenism. The diagnostic features include enlargement of the spleen, cytopenias, normal or hypercellular marrow with normal maturation of the cell lines and normalisation of the blood count following splenectomy (El Hoss and Brousse, 2019). The cytopenias result from sequestration of blood cells within the enlarged spleen. About a third of the platelet pool resides within the spleen, thus, an enlarged spleen can sequester a large number of platelets causing thrombocytopenia. Similarly, a considerable number of red cells

and white cells can be trapped within the enlarged spleen causing anaemia and neutropenia (Diagne *et al.*, 2010).

1.3.3.3 Hyposplenism

This refers to derangement in the filtration and immune function of the spleen. Several disease conditions such as celiac disease, dermatitis herpetiformis, haematological malignancies and haemoglobinopathies have been associated with hyposplenism (William and Corazza, 2007; Lenti *et al.*, 2022). The clinical manifestation is characterised by increased risk to infection, particularly pneumococcal sepsis, and other encapsulated bacteria. The loss of filtration function results in red cell with abnormal inclusions such as Howell-Jolly bodies, acanthocytes (spur cells), Heinz bodies (denatured haemoglobin), target cells and pappenheimer bodies (iron inclusions) appearing in the peripheral circulation (William and Corazza, 2007). Leucocytosis and thrombocytosis may also be observed.

1.3.4 Assessment of spleen function

Several methods have been developed to assess the functions of the spleen. These methods are based on scintigraphy, haematological and immunological techniques (de Porto *et al.*, 2010; Lammers *et al.*, 2012; El Hoss *et al.*, 2018; Corazza *et al.*, 1990; Harrod *et al.*, 2007; Tham *et al.*, 1996). The scintigraphy method is the gold standard and assesses the ability of the spleen to filter the blood of abnormal cells and particles. However, this method is expensive, invasive and involves injecting radio-labelled substance into patients; moreover, it is not readily available in LMICs (de Porto *et al.*, 2010; Lammers *et al.*, 2012).

The haematological method assesses the inability of the spleen to phagocytose poorly deformable red cells because of their abnormal shape or presence of inclusion materials. One such method involves the detection of red cells containing Howell -

Jolly bodies (HJBs) (Casper *et al.*, 1976). Howell-Jolly bodies can be estimated from a peripheral blood smear by manual counting or by use of flow cytometry (Corazza *et al.*, 1990; Harrod *et al.*, 2007; El Hoss *et al.*, 2018). In patients with diminished splenic function, the membrane of the red cells contains 'pits' when viewed under an interference phase-contrast microscope. Pitted red cell counts of 0 - 4% is observed with normal splenic function, while a value above 4% is indicative of hyposplenism (Lammers *et al.*, 2012). The pitted red cell count method represents a sensitive technique for evaluating splenic function, however, it requires contrast-enhancing microscopy techniques and trained personnel. It is not readily available for routine use in LMICs.

Another haematological method of assessing splenic function is estimation of red cells containing argyrophilic inclusions (AIs). While studying the argyrophilic nucleolar organizer region, the authors of one study noticed most red cells from patients with history of splenectomy and SCD contained argyrophilic inclusions (Tham *et al.*, 1996). These inclusion bodies were thought to represent HJBs, and other inclusion bodies seen in patients with diminished or absent splenic function by electron microscopy. On that basis, the authors decided to assess this feature as marker of splenic function. Using the pitted red cells count as the gold standard for their study, the AIs count method had a sensitivity of 88.9% of and specificity of 97.1%. (Tham *et al.*, 1996).

1.4 The spleen in SCD

1.4.1 Early and progressive loss of splenic function

The spleen is one of the earliest organs to be affected in SCD (Pearson, Spencer and Cornelius, 1969; Pearson *et al.*, 1979). In Hb SS individuals in particular, loss of splenic function occurs as early as first 6 months of life. Serial pitted red cell counts

performed among 130 Hb SS children followed from birth rose with age, and were present in 23% at age 1 year, 42% at age 2 years and 52% at age 3 years (Rogers, 1982). In a study using a combination of HJB counts (by flow cytometry), pitted red cell counts and splenic scintigraphy to evaluate spleen function in 193 Hb SS children aged 8 to 18 months, loss of splenic function was observed in 86% before 12 months of age (Rogers *et al.*, 2011). The high number of damaged sickled red cells may clog up the filtration capacity of the spleen, causing initial enlargement of the organ and later a progressive splenic fibrosis. However, the function of the enlarged spleen has been shown to be defective as indicated by its inability to clear red cell inclusions like HJB or radiocolloids from the blood, hence the term 'functional hyposplenism/asplenia' (Pearson *et al.*, 1985; Pearson *et al.*, 1979). While the loss of splenic function is a common feature in SCD, the age at which irreversible loss of the organ (anatomical asplenia or autosplenectomy) occurs is variable.

1.4.2 Pathophysiology of splenic damage in SCD

Early observations presented a confusing picture of splenomegaly in some SCD patients and splenic atrophy in others (Serjeant, 2002), however, the precise mechanism of splenic damage in SCD is not completely understood. A detailed description of the sequence of splenic pathology in SCD was first described by Diggs (Diggs, 1935). The earliest demonstrable lesions in the spleen were congestion of the reticular space with sickled red cells and dilation of the capillaries in the white pulp. The spleen at this stage appears enlarged and soft. Perivascular haemorrhages around the terminal arteriole become organised producing thickening and hyperplasia of the vessels. Infarcts, usually small sized and multiple, surrounded by a congested pulp are common and are eventually replaced by fibrous tissue. These series of events result in the progressive destruction and contraction of the spleen which eventually becomes a

small, wrinkled mass of fibrous tissue. In general, large spleens were found in infants and young children and small spleens in older children and adults (Diggs, 1935).

1.4.3 Consequence of splenic dysfunction

In SCD, the loss of splenic function is the natural outcome of splenic dysfunction. Both functional hyposplenism and anatomical asplenia (autosplenectomy) may be associated with increased risk of early death from infections with encapsulated organisms or parasitised red cells (Rogers, Serjeant and Serjeant, 1982; Rogers, 1982). In a US study, SCD and splenectomised patients had lower levels of circulating non-switched memory B-cells which are located in the marginal zone of the spleen and this was associated with decreased response to pneumococcal vaccination (Lammers *et al.*, 2012). Similarly, SCA patients in SSA had up to 36-fold increased risk of infection with *S.Pneumonia* compared to those without SCA (Ramakrishnan *et al.*, 2010). A low incidence of bacterial and pneumococcal sepsis in a cohort of SCD patients with preserved spleen function compared to those with impaired function was observed in a study from Kuwait (Adekile *et al.*, 1996).

The majority of SCD patients reside in SSA, where malaria is also endemic. Both SCD and malaria can cause altered splenic function (Brousse, Buffet and Rees, 2014; Tubman and Makani, 2017; Gomez-Perez *et al.*, 2014). Malaria infection (acute or chronic) results in the excessive proliferation of the spleen reticuloendothelial macrophages (Adekile *et al.*, 1988; Franceschi *et al.*, 2005). The phagocytic action of these macrophages against parasitised and non-parasitised red blood cells results in splenic enlargement. *Pneumococcal* infections and bacteraemia from other organisms have also been reported to occur concurrently with malaria in SCD, further compounding the morbidity and mortality (Makani *et al.*, 2015; Brown *et al.*, 2017; Alima Yanda *et al.*, 2017).

In view of the important role the spleen plays in immunity against infections, my research sought to determine splenic function in SCD patients in Maiduguri, a malaria endemic region in North-Eastern Nigeria. Only a few studies have looked at the incidence and prevalence of bacterial infection in SCD patients in this area, where most febrile illnesses are generally treated as malaria. Both children and adults with SCD were included in my study, to provide important information regarding the course of spleen size changes over a spectrum of ages and the implications for risk of infection. The study was conducted in two parts: in the first part (spleen baseline study), the spleen sizes of SCD patients of various ages using ultrasonography were determined, and evaluation of their splenic function was performed. In the second part (spleen and infection study), the prevalence of acute malaria and bacterial infection in SCD patients, and their relationship with spleen size and function were determined.

1.5 Study aim and objectives.

1.5.1 Aim

The aim of my research was to evaluate spleen size and function among SCD patients in North-Eastern Nigeria, and to determine the association with malaria and bacterial infections. This was achieved by addressing the following specific objectives:

1.5.2 Specific objectives

1. Assessment of baseline spleen size amongst SCD patients and assessment of clinical and laboratory correlates associated with spleen size preservation (Spleen baseline study)
2. Evaluation of two laboratory methods for the assessment of spleen function and investigation of the relationship between clinical and laboratory variables with these markers of splenic dysfunction (Spleen baseline study).

3. Determination of the prevalence of malaria infection in SCD patients and healthy controls, and prevalence of bacterial infection in acutely-ill SCD patients, and their association with spleen size and function (Spleen and infection study).

1.6 Study significance

The anticipated outputs from this study were:

- Information about the spectrum of spleen size changes across children and adults with SCD will be obtained and the pattern compared with those in the literature.
- Reference ranges and percentiles curves for different splenic dimensions will be generated from normal controls in the same environment which can be used to monitor spleen size changes in the SCD patients.
- The role of HbF in splenic preservation in SCD patients will be obtained, which can provide new information that can be used to guide clinical management.
- Information about the feasibility of assessing splenic function in a resource-limited setting will be obtained.
- Evidence regarding the role of the spleen in malaria and bacterial infection will fill an important gap in knowledge regarding the role of the spleen and risk of infection.

1.7 Thesis structure

The thesis comprises ten chapters. The current chapter, **Chapter 1,** is a brief introduction providing background information into the research. It provides background knowledge on SCD, the spleen and the spleen in SCD.

Chapter 2 is a systematic review of published literature on splenic complications in patients with SCD in Africa. The main purpose of this chapter is to identify information on this subject area and identify existing gaps in knowledge.

Chapter 3 describes the study setting and research methodology.

Chapter 4 addresses objective one; it assesses baseline spleen size amongst steady state SCD patients and compares it with those of normal controls.

Chapter 5 also addresses objective one; it describes the clinical and laboratory factors associated with persistence of the spleen in SCD patients.

Chapter 6 addresses objective two; it describes the use of inclusions contained in red cells to evaluate spleen function.

Chapter 7 addresses the second aspect of objective two; it explores the association between the two markers of splenic dysfunction and clinical and laboratory factors.

Chapter 8 addresses objective 3 and compares the prevalence of malaria in SCD patients with normal controls; the association of splenic parameters and malaria infection is explored.

Chapter 9 addresses the second aspect of objective three; the prevalence and pattern of bacteraemia among SCD patients is explored. The association of splenic parameters and risk of bacteraemia is also evaluated.

Chapter 10 summaries the research findings, discusses the study strengths, challenges, limitations, future research, and recommendations.

1.8 My role and responsibilities in the research

My role and responsibilities during the course of the project were as follow:

1. I conceived and designed the study, developed the research proposal and data collection tools.

2. I secured approval from the Ethics Committees in LSTM, UK and UMTH, Nigeria

3. I conducted a systematic review (Chapter 2) with the support of a second reviewer from LSTM and with feedback from my supervisors, I wrote the first draft of the paper and published the results.

4. I collected all the data with the support of colleagues and research assistants in Nigeria.

5. I analysed the data and drafted all chapters of the thesis and with feedback from the supervisors, I revised and finalised the thesis.

6. I drafted seven manuscripts and with feedback from the supervisors, I submitted them for publication as the lead and corresponding author.

CHAPTER 2: LITERATURE REVIEW

2.1 Chapter overview

In this chapter, I review the existing evidence regarding the spleen in the context of its size, assessment of its function and relationship with infections. The aim of the systematic review was to describe and evaluate existing data on the spectrum of splenic complications and associated morbidities among SCD patients in Africa. The method section of this chapter describes how the systematic review was conducted. The result section summarizes key findings in narrative forms and tables. The discussion section describes the significance of the results, compares findings with those in the literatures and the implication of findings for clinical practice.

I led the study conceptualization and data extraction. Together with a second reviewer (AO), we performed the data screening and quality assessment of retrieved articles. I wrote the original draft, revised and final manuscript with the support of my supervisors, this chapter was published in Brit J Haem in 2021. The publication can be found at the link below:

https://doi.org/10.1111/bjh.17179

2.1 Abstract

Background

The majority of the global population of sickle cell disease (SCD) patients resides in Africa. Individuals with this condition are at great risk of serious infections and early mortality secondary to splenic dysfunction without preventative measures. This review investigated the spectrum of splenic complications encountered in SCD among populations in Africa.

Method

A systematic search of several databases for all articles published through March 3,2020 was performed. Fifty-five studies from 14 African countries were included.

Results

This review shows the difference in frequency of splenic complications in SCD in Africa when compared with their counterparts in the United State and Europe. While several studies (n=45) described splenomegaly with a prevalence of 12% to 73% among children, and 4% to 50% among adults with HbSS, the reported prevalence for acute splenic sequestration crisis (n=6 studies) and hypersplenism (n=4 studies) was <10% and <5% respectively. A total of 30 surgical splenectomies was reported across eight studies. Only two (3.7%) studies provided data on spleen function. A conflicting pattern was observed amongst studies that evaluated the relationship between splenomegaly and the presence of bacterial and malaria infections.

Conclusion

This review reveals the paucity of studies describing the role of SCD-induced splenic dysfunction in morbidity and infection related mortality in Africa.

2.2 Introduction

About two thirds of the global population of SCD patients reside in Africa (Makani, Williams and Marsh, 2007; Piel *et al.*, 2013a), and an estimated 50–90% of children born with SCD in Sub-Saharan Africa die before their fifth birthday (Grosse *et al.*, 2011). This high mortality has partly been attributed to infections secondary to splenic dysfunction (Booth, Inusa and Obaro, 2010; Williams *et al.*, 2009). The repeated cycle of vaso-occlusion and ischemia leads to progressive fibrosis, atrophy and autosplenectomy. Therefore, the spleen is rarely palpable beyond the age of five years in SCD patients in the US and Europe (Rogers *et al.*, 2011; Brousse, Buffet and Rees,

2014). However, enlargement of the spleen (splenomegaly) tends to persist into late childhood or even adulthood in SCD patients in Africa; earlier studies have linked this finding to the effect of malaria infection in a manner similar to what is observed in patients with the hyper-reactive malarial splenomegaly syndrome (Adekile *et al.*, 1988; Bedu-Addo and Bates, 2002). Also, persistently high level of haemoglobin F (HbF), co-inheritance of alpha thalassemia trait and presence of other compound heterozygous forms of SCD have associated with the persistence of splenomegaly (Al-Salem, 2011; Tubman and Makani, 2017; Adekile *et al.*, 1996). The enlarged spleen may be complicated by worsening anaemia due to trapping of blood within the spleen (Durosinmi *et al.*, 2005; Diagne *et al.*, 2010), and haemolytic crisis (Adeodu and Adekile, 1990). Other serious complications are acute splenic sequestration crisis (ASSC), massive splenic infarction, splenic abscess and hypersplenism, some of which may require splenectomy (Al-Salem, 2011; Al-Salem *et al.*, 1998b; Jama, Salem and Dabbous, 2002; Adekile *et al.*, 2002a).

The spleen serves as the major filter of blood from senescent red cells and micro-organisms and is involved in both humoral and innate immunity (Gomez-Perez *et al.*, 2014; William and Corazza, 2007; Riva *et al.*, 2019). The spleen parenchyma is divided into a white pulp and red pulp compartment and separating these two is the marginal zone. The white pulp corresponds to the T and B lymphocyte cells zone and is responsible for adaptive immunity. Within the marginal zone, cells interaction and cell to cell sorting take place; specialized marginal zone B lymphocyte cells respond to capsule polysaccharide antigens by differentiating into IgM-producing memory B cells. The red pulp is mainly responsible for the filtration function of the spleen; immature, damaged, or aging red cells adhere to the reticular meshwork in this zone

through specific signals or are captured by the macrophages and prevented from circulating again (Gomez-Perez *et al.*, 2014).

At birth, the spleen appears morphologically and functionally normal in SCD patients. The sequence of events that results in splenic injury begins following the haemoglobin switch that occurs at about 6 months of life (Brousse, Buffet and Rees, 2014). The blood flow within the red pulp is particularly slow; the resulting high haematocrit promotes red cell stagnation, leading to hypoxia, acidosis, and further sickling of the red cells. Splenic dysfunction develops from blockage of the small inter-endothelial slits within the sinuses by the stiff and sickled red cells. An earlier study revealed that the phagocytic function becomes impaired first, while the filtration function may persist longer before it becomes compromised (Adekile *et al.*, 2002b).

Given the critical role the spleen plays in defence against micro-organisms, the loss of splenic function contributes significantly to the increased predisposition to bacterial infections observed in patients with SCD (Obaro and Tam, 2016; Adekile *et al.*, 1996). However, studies describing the role of SCD-induced splenic dysfunction as a cause of morbidity and mortality in patients with SCD in Africa are limited. The objective of this systematic review was to describe and evaluate the existing data on the spectrum of splenic complications and associated morbidities amongst SCD patients in Africa. The findings are a synthesis of the scarce information available from Africa about the relationship between size and function of the spleen, and the outcomes of SCD, and can provide the basis for more rational strategies to manage this common, chronic condition which places a high burden on resource-limited health services across the continent.

2.2 Methodology

2.2.1 Inclusion Criteria

The review comprised studies conducted across African countries involving patients of all ages with SCD. For this review, SCD was defined as haemoglobin SS, haemoglobin SC, haemoglobin $S\beta^+$ thalassemia, or haemoglobin $S\beta^0$ thalassemia genotypes. Cross-sectional studies (prospective, retrospective), case-control studies, case series and case reports on splenic complications, in which the splenic complications were established through clinical assessment, abdominal ultrasound scan and other modalities were all considered. To be included in this review, articles needed to have original data on at least one splenic complication as a primary or secondary outcome of the study. Articles in all languages were included. Review articles and studies conducted outside of Africa were excluded.

2.2.2 Search strategy

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines was followed (Liberati *et al.*, 2009). Four databases were searched from the dates shown in parentheses up to March 3rd, 2020: Medline (1963), Global Health Database (1973), Web of Science (1970) and CINAHL (2002). Boolean logic with search terms including “sickle cell anaemia”, “spleen”, “splenic dysfunction”, and “Africa south of Sahara” was used. Controlled vocabularies (eg, Medical Subject Heading terms) to identify synonyms was used (Appendix 1). Additional searches in other electronic resources were performed including Google Scholar and African Journals Online and several grey literature resources including Google Advanced, EThOS library, BASE search, Networked digital library of thesis and dissertations, and open access theses and dissertations. The reference lists of all retrieved articles for

additional articles were screened. Citations were uploaded into an EndNote X9 library where duplicates were removed. No language restriction to the search criteria was applied and all non-English articles (i.e., ten in French) were translated.

2.2.3 Data Extraction

Titles and abstracts of studies retrieved from the databases and additional sources mentioned above were screened independently by two investigators (AIL and AOA) for their eligibility for full text review. Differences of opinion were settled through discussion with a third author (IB) until consensus was achieved. Excluded studies were documented with reasons. Data were extracted using a pre-defined proforma which contained sections for authors, date of publication, location, study population, sample size, study design, age, gender, and method of assessing spleen size and assessment of spleen function. We also extracted laboratory data on haemoglobin, counts for white blood cells, platelets, and reticulocytes, HbF, and the presence of malaria and bacterial infections.

2.2.4 Quality assessment of studies

The Joanna Briggs Institute Critical Appraisal tools for assessing the quality of observational studies was used (Appendix 2) (Moola *et al.*, 2017). Two authors (AIL, AOA) independently appraised each article for quality using the following assessment criteria: description of study selection criteria and population; ascertainment of spleen size using a standard approach; reliable and valid method for measuring exposure variables; identification of confounding factors and strategies to deal with them; definition of outcome and use of appropriate statistical analysis. The quality of each of the criteria was assessed as either “yes” or “no” or ‘’unclear’’ or ‘not applicable’. Points were assigned to each yes question for a total of seven points. Articles scoring

seven points were graded as A quality, those scoring five to six points as B quality, and those scoring less than five as C quality (Appendix 2).

2.2.5 Data synthesis and analysis

Variations in the study population (children, adults, mixed population), study design, methods of assessing spleen size (manual palpation using different cut-off values, ultrasound scan), and reported outcomes in the studies retrieved made direct comparisons inappropriate, so, a descriptive method for data synthesis was employed and a narrative approach used for data analysis.

2.3 Results

2.3.1 Search results

A total of 202 articles were identified in the initial search and 28 in the additional search (Fig.5). After removal of duplicates, 188 articles remained. Their abstracts and titles were screened and 68 relevant articles, which met the inclusion criteria were selected for full text review. Fifty-five studies were selected for inclusion into the final review. Twelve studies were excluded on the following grounds: duplication of data (n = 3), reviews (n = 3), lack of original data (n = 5), study outside Africa (n = 1). Another article was excluded because the authors inferred in the abstract that changes in haematological parameters (raised WBC and platelets) were related to spleen effect, however, on full text review, the study design did not include evaluation of the spleen. Of the 55 studies included in the systematic review 10 were in French and 45 in English.

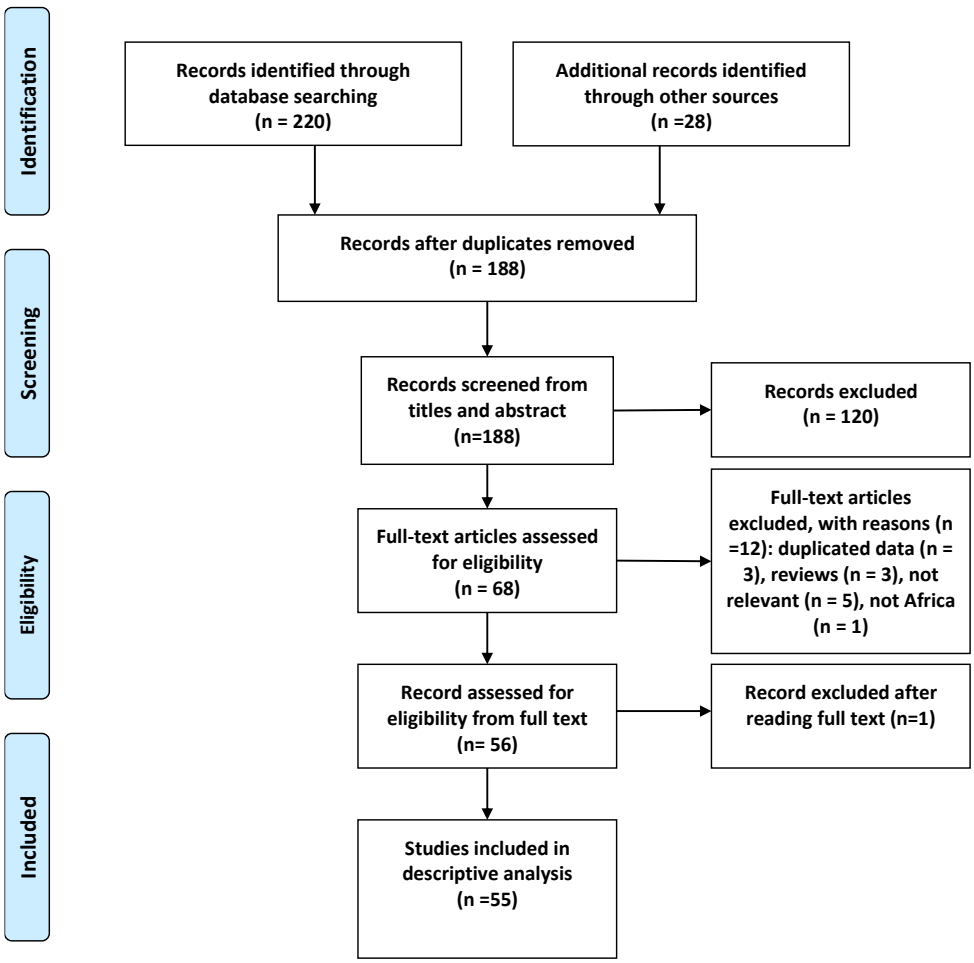


Figure 5: Summary of data extraction

2.3.2 General study description

The 55 studies were published between 1982 to 2019. Thirty-three (60.0%) studies had a cross-sectional design, 10 (18.2%) were retrospective descriptive studies, 11 (20.0%) were case control studies and one (1.8%) was a case study (Appendix 3). Following quality assessment of all studies, seven (12.7%) were classified as A grade, 36 (65.5%) as B grade, and the remaining 12 (21.8%) as C grade. The studies were conducted across 14 African countries. Seven of the countries were from the West African region, three from East Africa, and two each were from the Central and North African region.

However, most studies (33 [60.0%]) were conducted in Nigeria, four (7.2%) from the Democratic Republic of Congo (DRC) and two each (3.6%) from Congo, Senegal, and Ghana.

Seventeen (51.5%) of the studies from Nigeria were conducted in the South-Western part of the country across various institutions; four of these studies conducted between 1988 to 1993 were from a single author. The remaining 16 (48.5%) studies were conducted across various regions of the country.

2.3.3 Description of methodologies used in the studies

Apart from two large cross-sectional studies (n = 2305 and n = 4359), the sample size ranged between 40 to 591 for the prospective cross-sectional studies and 25 to 300 for the case control studies. Whereas 19 studies included children and adults with SCD, 23 studies consisted of children only and 13 adults only. Thirty-two studies included only individuals with Hb SS, 12 had a combination of SCD genotypes (i.e. Hb SS, Hb SC, Hb S-β-thalassemia (Hb S-β-thal), and the remaining 11 consisted of Hb SS patients and normal controls (Hb AA). Forty-five studies provided prevalence rates for splenomegaly amongst their study population; we found substantial variation in the criteria used in defining splenomegaly. Based on ultrasound scan (USS), splenomegaly was defined as the long axis of the spleen greater than 12 cm in three studies (Abdullahi, Hassan-Hanga and Ibrahim, 2014; OT Ojo, WA Shokunbi and Agunloye, 2014; Ezeike, 2019), and greater than 13 cm in four studies (Babadoko *et al.*, 2012; Luntsi *et al.*, 2018; G B Inah and Ekanem, 2018; F.A. Fasola and Adekanmi, 2019). In six studies involving children, using manual palpation, the authors defined massive splenomegaly as spleen size of at least 10 cm below the coastal margin during steady state condition, and persistent gross splenomegaly as spleen size of 10 cm or more during steady state (Adekile *et al.*, 1988; Adeodu and Adekile, 1990; Adekile *et al.*,

1991; Adekile *et al.*, 1993; Adegoke, Adeodu and Adekile, 2015; Akinlosotu *et al.*, 2018). In seven other studies, the Hackett classification was used in grading spleen size (Abjah and Aken'Ova, 2003; Tshilolo, Mukendi and Girot, 1996; Doumbo *et al.*, 1992; Gnassingbe *et al.*, 2007; Kazadi *et al.*, 2019; Shongo *et al.*, 2014; Tolo-Diebkile *et al.*, 2010). Twenty-five studies did not report on the parameters used to define splenomegaly. Only two studies (3.6%) reported an assessment of splenic function. The method employed in both studies was counting of pitted red cells using direct interference, phase-contrast microscopy (Adekile *et al.*, 1991; Adekile *et al.*, 1993)

2.3.4 Spectrum of splenic complications

2.3.4.1 Splenomegaly

Of the 45 (81.8%) studies that provided data on the prevalence of splenomegaly in Hb SS patients, 19 (42.2%) consisted of children only, 11 (24.4%) adults only, and the remaining 15 (33.3%) consisted of children and adult participants (Appendix 3). In studies involving only children, the estimate ranged from 12% in Nigeria and Mali to 73.2% in the DRC. Four of these studies determined spleen size using USS and the remaining 15 by manual palpation. Notably, all 11studies that provided data on the prevalence of splenomegaly in the adult SCD population were conducted in Nigeria (n = 10) and Ghana (n=1). The estimate ranged from 4% to 50%. Seven of these studies determined spleen size by manual palpation and the remaining four by USS. Seven studies, all from the West African countries of Nigeria (n=5), Senegal (n=1) and Ghana (n=1), reported on splenomegaly in Hb SC individuals. The prevalence varied from 4% to 42% in children and adolescent and 15% to 67% in adults with HbSC respectively (Olatunji and Olatunji, 2001; Bedu-Addo and Bates, 2002; Abjah and Aken'Ova, 2003; Akinola, Bolarinwa and Faponle, 2009; Diagne *et al.*, 2010; B. J. Brown; Adegoke, Adeodu and Adekile, 2015). Three other studies, two from Northern

Africa (Bayoumi *et al.*, 1988; Belhani *et al.*, 1984) and one from West Africa (Diagne *et al.*, 2010) reported data from individuals with Hb S-β-thal^{0} and Hb S-β-thal $^{+}$. In these studies, the prevalence of splenomegaly ranged from 54% to 88% (Belhani *et al.*, 1984; Diagne *et al.*, 2010).

The relationship between spleen size and age was described in 12 studies (Appendix 4). In studies involving children only, persistence of the spleen beyond five years was noted in most of the SCD patients (Thuilliez *et al.*, 1996; Sadarangani *et al.*, 2009; Diagne *et al.*, 2010; Abdullahi, Hassan-Hanga and Ibrahim, 2014). In studies with a mixed population of adults and children, while some demonstrated decreasing spleen size with increasing age (Kaine, 1982; Adekile *et al.*, 1993; Akpan, 2015; Ugwu *et al.*, 2018), a few showed a steady increase in the spleen size up to the second (Eze *et al.*, 2015) and third decade (Olatunji and Olatunji, 2001) before the size begins to decline. In one study involving adults with SCD above 30 years, a third of the patients still had palpable spleen (Yetunde and Anyaegbu, 2001). Three studies used USS to compare the spleen sizes in SCD patients with those of normal controls (Eze *et al.*, 2015; Ezeike, 2019; Olatunji and Olatunji, 2001). In one of these studies involving children (age range, 2 - 17 years), the mean spleen length in SCD patents was 97.67 ± 39.61mm, while that of the controls was 80.84 ± 16.89 mm (*P <0.05*) (Eze *et al.*, 2015). In another report, the mean longitudinal length of the spleen in patients with SCD (age range, 3 - 47 years) was 101.7 ± 18 mm, while in the controls was 95.6 ± 13 mm (*P <0.02*) (Olatunji and Olatunji, 2001). In the third study, the mean splenic volume and anterior posterior diameter in patients with SCD (age range, 0 - 30 years) was 267.3 mm^3 and 4.63mm and differed significantly from the values in the controls of 161.3 mm^3 (*P=0.001*) and 4.12 mm (p=0.048), respectively. However, there was no

significant difference between the spleen length (*P=0.659*) and transverse diameter (p=0.433) (Ezeike, 2019).

2.3.4.2 Hypersplenism

Only four studies provided information on hypersplenism. This was defined in one of the studies as splenomegaly of at least 5 cm in association with haemoglobin level of less than 1g/dl from baseline value, low platelets (<200 $x10^9$/l) and increased reticulocytes (>150 $x10^9$/l) observed on at least two occasions in the absence of any other cause of hyperhaemolysis (Diagne *et al.*, 2010). The prevalence of hypersplenism was generally low across all studies. Two studies involving children in Nigeria and Senegal reported rates of 0.1% (Okoro, Kaine and Okeahialam, 1989) and 5% (Diagne *et al.*, 2010) respectively. Similar figures of 1% (Banza *et al.*, 2019) and 4.2% (Durosinmi *et al.*, 2005) were also reported in the adult studies in Nigeria and the DRC respectively. Improvement in the haematological indices following splenectomy was observed in three studies (Durosinmi *et al.*, 2005; Diagne *et al.*, 2010; Okoro, Kaine and Okeahialam, 1989).

2.3.4.3 Acute splenic sequestration crisis (ASSC)

Only six studies from four countries reported on the occurrence of acute splenic sequestration crisis (ASSC). This was defined in two of the studies as sudden enlargement of the spleen accompanied by worsening anaemia, requiring immediate blood transfusion (Diagne *et al.*, 2010; Goussanou *et al.*, 2003).The highest prevalence of 27.3% was reported from Nigeria (Ambe, Fatunde and Sodeinde, 2001), whereas, two studies, from Senegal (Thiam *et al.*, 2017) and Congo (Kazadi *et al.*, 2019) reported a prevalence rate of 7%. The prevalence was low at 3% in a study from the Republic of Benin (Goussanou *et al.*, 2003), and 2% in two studies from Senegal (Diagne *et al.*, 2010) and DRC (Banza *et al.*, 2019). Two studies provided information

on ASSC- related mortality in their patient population. In both studies, all 11 deaths recorded were related to non-availability of blood for transfusion (Ambe, Fatunde and Sodeinde, 2001; Goussanou *et al.*, 2003).

2.3.4.4 Surgical splenectomy

A total of eight studies reported data on splenectomy across six countries including Nigeria (n=3), Algeria (n=1), Senegal (n=1), Ivory Coast (n=1), Togo (n=1) and the DRC (n=1). The indications for splenectomy included hypersplenism in three studies (Diagne *et al.*, 2010; Durosinmi *et al.*, 2005; Okoro, Kaine and Okeahialam, 1989), therapeutic for symptomatic splenomegaly in two studies (Alufohai and Odusanya, 2006; Belhani *et al.*, 1984), and drainage of splenic abscess in one study (Gassaye *et al.*, 2000). In one other study, splenectomy was performed following traumatic rupture of the spleen in five patients and as prophylaxis in three other patients (Gnassingbe *et al.*, 2007). The indication for splenectomy was not indicated in one study (Tolo-Diebkile *et al.*, 2010). In the small series from Togo, the authors reported on eight children with SCD (5 Hb SS and 3 Hb SC; age range 6-13 years), who underwent splenectomy between 1987 to 2004. All were on prophylaxis with penicillin V, and received pre- and post-operative vaccination against *pneumococcus*, *meningococcus*, and *Haemophilus influenzae b*. There was a reduction in their transfusion requirement when followed up for three years. In another small series from Eastern Nigeria, the authors reported on 17 patients who underwent splenectomy over a 12-year period for various conditions including SCD patients with splenomegaly (n= 5; 29%) (Alufohai and Odusanya, 2006). The outcome of surgery was uneventful and none of the SCD patients required further blood transfusion when compared to the pre-operative period.

2.3.4.5 Splenic rupture

Traumatic rupture of enlarged spleen was reported in five out of eight SCD patients who underwent splenectomy as described in a retrospective review conducted over a 17-year-period from Togo (Gnassingbe *et al.*, 2007). The complication occurred in three patients with Hb SS and two with Hb SC. Clinical examination on presentation revealed evidence of peritoneal irritation and ascites; abdominal ultrasound scan confirmed rupture of the enlarged spleen. All the patients had a total splenectomy performed.

2.3.4.6 Splenic abscess

Two studies from Nigeria and the DRC reported on splenic abscesses. In one of these studies, a case report from Nigeria, the abscess was treated by laparotomy and surgical drainage and the spleen preserved (Jebbin and Adotey, 2011). Whereas splenectomy was performed in all three SCD patients with splenic abscess from the DRC study (Gassaye *et al.*, 2000).

2.3.4.7 Splenic infarction and calcification

Only one study each, reported on splenic infarction (Eze *et al.*, 2015) and splenic calcification (Luntsi *et al.*, 2018). In both studies, these complications were asymptomatic and picked up during ultrasonography in steady state HbSS patients.

2.3.4.8 Autosplenectomy

Sixteen studies provided information on autosplenectomy. This was defined as the non-visualisation of the spleen on ultrasonography in the absence of surgical splenectomy by most of the studies. The reported prevalence varied from 4% to 20% among children, and 20% to 54% among adults' patients with SCD.

2.3.5 Other associations with spleen size in patients with SCD

2.3.5.1 Splenic function

Although only two of the 55 studies reported on spleen function, both studies showed increased spleen size was associated with retained function (Adekile *et al.*, 1991; Adekile *et al.*, 1993). One of the studies was a multi-centre study, involving seven centres across Nigeria, and a comparative arm in the USA (Adekile *et al.*, 1993). The mean pitted erythrocyte count was 4.3% in Hb SS patients with splenomegaly and 12.3% in those without splenomegaly (*P=0.001*) (Adekile *et al.*, 1991), indicating that spleen function was better in those with splenomegaly compared to those without. The mean pitted erythrocyte count was 11.1% in Hb SS subjects when compared to 1.8% in the Hb AA population *(P = 0.001)* (Adekile *et al.*, 1991).

2.3.5.2 HbF level

While twelve studies provided results for HbF level in their SCD patients, only five of these studies evaluated the relationship with spleen size. Three of these studies from Nigeria (Akinlosotu *et al.*, 2018), Uganda (Mpalampa *et al.*, 2012) and Senegal (Diagne *et al.*, 2010) found no significant relationship between spleen size and HbF level in children with Hb SS. In contrast, two other studies conducted in Nigeria amongst adults with Hb SS, reported a parallel increase in HbF level with increased spleen size (Durosinmi *et al.*, 2005; Kotila, Fawole and Shokunbi, 2000). In two studies, one involving children (Adegoke, Adeodu and Adekile, 2015) and the other a mixed population of children and adults (Banza *et al.*, 2019), the authors indicated that 5.4% and 29.2% of their study population were on hydroxyurea respectively. However, no relationship between spleen size and hydroxyurea therapy was mentioned in either study.

2.3.5.3 Alpha thalassemia trait

Two out of the 55 studies evaluated the association between alpha-thalassemia trait and spleen size. One of the reports from the DRC indicated that patients with the alpha-thal-2 deletion were more likely to have splenomegaly (Mouélé *et al.*, 1999). However, the second study from Nigeria showed that 33.3% of Hb SS patients with splenomegaly were heterozygous for alpha-thal-2 deletion compared to 39.0% for those without splenomegaly; suggesting alpha-thal-2 deletion was not related to splenomegaly (Adekile *et al.*, 1993).

2.3.5.4 Haematological parameters

There was no consistent association between spleen size and haematological parameters among the studies. For example, while some studies found a negative correlation between haematocrit and spleen size (Diagne *et al.*, 2010; Brown, Fatunde and Sodeinde, 2012), one found a positive correlation (F.A. Fasola and Adekanmi, 2019), and others found no correlation between the two variables (Durosinmi *et al.*, 2005; Olatunji and Olatunji, 2001). Similarly, splenomegaly was associated with cytopenia in some studies (Abdullahi, Hassan-Hanga and Ibrahim, 2014; Adegoke, Adeodu and Adekile, 2015; Adeodu and Adekile, 1990), but not others (Ugwu *et al.*, 2018). Likewise, the reticulocyte count increased with increasing spleen size in one study (Abdullahi, Hassan-Hanga and Ibrahim, 2014), while another report found no relationship between the spleen size and reticulocyte count (Durosinmi *et al.*, 2005).

2.3.6 Association between spleen size and infections

Splenomegaly was noted in fifteen studies from six countries that reported on the frequency of bacterial and malaria infections in SCD patients (Table 1). Most of these studies (n=11) involved children. Malaria diagnosis was based on peripheral blood smear in most of the reports. The prevalence of symptomatic malaria was highest in

Nigeria and the DRC at greater than 50%, compared to prevalence rates from Mali (4%), Kenya (6%), Tanzania (3%) and Uganda (9.7%). Eight studies reported on bacterial infections; three of these studies reported using blood culture for diagnosis. The reported prevalence rates were 23.% (Adegoke, Adeodu and Adekile, 2015) and 25.6% (Ambe, Fatunde and Sodeinde, 2001) from Nigeria, and 28% from Uganda (Kizito *et al.*, 2007). The remaining five studies provided no clear description of how infection was diagnosed.

Among the 15 studies reporting on both malaria and bacterial infections, 10 evaluated the relationship between spleen size and infections (Table 1). Three of these studies indicated no relationship between the frequency of malaria parasitaemia or parasite density, clinical malaria and spleen size (Durosinmi *et al.*, 2005; Sadarangani *et al.*, 2009; Makani *et al.*, 2010a), while one study reported a higher parasite density in patients with normal sized or enlarged spleens compared to those with autosplenectomy (Awotua-Efebo, Alikor and Nkanginieme, 2004). Three other studies mentioned a direct association between increasing spleen size and levels of serum IgM and antimalaria IgG (Adekile *et al.*, 1988; Adekile *et al.*, 1993; Abjah and Aken'Ova, 2003). The spleen size was also reported to have decreased following treatment with antimalaria therapy (Adekile *et al.*, 1988).

One study evaluated the relationship between splenomegaly and bacterial infections (Kizito *et al.*, 2007), while two studies evaluated the association between autosplenectomy and risk of infections (Awotua-Efebo, Alikor and Nkanginieme, 2004; Okongwu *et al.*, 2018). Notably, the relationship between risk of infections and spleen function was not evaluated in two of the studies that assessed function.

Table 1: Summary of studies reporting on the relationship between spleen size and infections in SCD patients.

Country	Study year	Mean age (SD, range)	Study size	Bacterial infection	Malaria infection	Spleen size	Evaluation of spleen size and infection (s)	Reference
Nigeria	1988	7.1 (4.2, 0.5-15)	139	NA	Serum IgM level and response to antimalaria treatment assessed in patient with persistent gross splenomegaly (PGS)	**Splenomegaly** 33.8% **PGS** 10.8%	Significant reduction in spleen size followed treatment with antimalaria (proguanil) over a six-month period (P=0.01). Serum IgM levels were significantly higher in patients with PGS compared to HbSS without splenomegaly and HbAA controls (P=0.01)	Adekile et al
Sudan	1988	6.4 (NR, 0.5-38)	50	**Diagnosis-** Not described **Prevalence** 8.0%	NA	**Splenomegaly** 42.0%	NA	Bayoumi et al
Mali	1992	NR (NR, 0- 12)	236	NA	**Diagnosis** Rectal temp - $>38^0$ C Thick and thin blood smears for parasites **Prevalence**	**Splenomegaly** 12.0%	NA	Doumbo et al

					4.0%			
Nigeria	1993	9.7 (0.3, 1 - 25)	310	**Diagnosis** Not explicitly defined. Clinical syndrome of pneumonia, osteomyelitis and septicaemia stated. **Prevalence** 7.4%	**Diagnosis** Thick blood smears for parasites **Prevalence** 8.1%	**Splenomegaly** 23.3%	Immunoglobulin IgA, IgG, IgM levels increased with increasing spleen size. IgG antimalaria antibody titre also increased with increasing spleen size.	Adekile et al
DRC	1996	NR (NR,0.5 – 15)	591	**Diagnosis** Not described **Prevalence** 2.4%	NA	**Splenomegaly** 44.4%	NA	Tshilolo et al
Nigeria	2001	NR (NR, 0.5 -15)	104	**Diagnosis** Positive blood culture **Prevalence** 25.6%	**Diagnosis** Blood smears for parasites **Prevalence** 66.0%	**NR**	NA	Ambe et al
Nigeria	2003	NR (NR,15 - 54)	50	NA	Antimalaria IgG levels assessed in	**Splenomegaly** HbSS - 50.0% HbSC - 67.0%	The mean plasmodium falciparum IgG correlated directly with the spleen size.	Abjah et al

					asymptomatic patients			
Nigeria	2004	NR (NR, 0.5 -15)	100	NA	**Diagnosis** Thick blood smears for parasites **Prevalence** 30.0% (asymptomatic)	**Splenomegaly** 27.0% **Autosplenectomy** 20.0%	Parasite density was higher in SCA patients with splenomegaly and normal sized spleen when compared with those with autosplenectomy.	Awotua-Efebo
Nigeria	2005	Median 21 (16 - 48)	72	NA	**Diagnosis** Thick blood smears for parasites **Prevalence** 46.3% (asymptomatic)	**Splenomegaly** 26.8%	There was no difference in malaria parasite density between patients with PGS and those without. There was no significant correlation between malaria parasitemia and splenomegaly (r=0.06)	Durosinmi et al
Uganda	2007	**Median** 4.4 (0.3 - 14.8)	165	**Diagnosis:** Positive blood & urine cultures; +/- CXR; Fever >38^0 C **Prevalence** Bacteraemia= 28.0%	**Diagnosis:** Blood smears: parasite density graded from +1 to +3 **Prevalence** 9.7% (symptomatic)	**Splenomegaly** 36.0%	There was no relationship between splenomegaly and positive blood cultures, or type of organism isolated.	Kizito et al

				UTI = 11.0%				
Kenya	2009	**Median** 6.3 (0.8 - 13.7)	124	NA	**Diagnosis** Thick and thin blood smears for parasites; parasite density computed against WBCs. **Prevalence** 6.0%	**Splenomegaly** 33.0%	There was no relationship between spleen size and the number of episodes of malaria, malaria parasitaemia or use of proguanil.	Sandaragani et al
Tanzania	2010	Median 11 (0.3 -47)	1808 - OPD 697 - Inpatients	NA	**Diagnosis** RDT; Thick blood smears; parasite density computed against WBCs. **Prevalence** 0.7%: OPD visits 3.0%: Inpatients	**Splenomegaly** OP visits 10% Inpatients 22.0%	The prevalence of splenomegaly and malaria parasitemia in patients with SCA was higher during hospitalization, however splenomegaly was not a predictor of malaria parasitemia.	Makani et al
DRC	2013	Median 5.4 (0.5 - 13)	90	NA	**Diagnosis** Fever >38^{0}C; clinical malaria, positive blood smears **Prevalence**	**Splenomegaly** 37.8%	Acute malaria and splenomegaly were more common in those under 5 years of age during acute crisis.	Aloni et al

					63.3% (symptomatic)			
Nigeria	2015	5.9 (3.7, 0.5 -15)	240	**Diagnosis** Positive blood cultures. **Prevalence** 23.8%	**Diagnosis** Thick blood smears for parasites **Prevalence** 53.0%	**Splenomegaly** HbSS - 12.5%, HbSC- 4.6%	NA	Adegoke et al
Nigeria	2018	29.3 (8,17 - 51)	46	**Diagnosis** Not explicitly described. Annual frequency of hospitalization and fever assessed.	NA	**Splenomegaly** 12.5% **Autosplenectomy** 20.0%	Infection rate of more than once a year was reported in 87.5% of those with autosplenectomy compared to 50% in those without. No correlation between interferon gamma (IFN-γ) level and spleen size	Okongwu et al
DRC	2019	8.4 (4.9, 0.5 - 24)	256	**Diagnosis** Not described **Prevalence** 3.9%	**NA**	**Splenomegaly** 41.7%	NA	Kazadi et al

DRC: Democratic republic of Congo; IgG: Immunoglobulin G; IgM: Immunoglobulin M; NA: not assessed; OPD: Outpatient department; PGR: Persistent gross splenomegaly; RDT: Rapid diagnostic tests; UTI: Urinary tract infection; WBCs: White blood cells

2.4 Discussion

In this review we identified 55 studies with data on various forms of splenic complications among patients with SCD in Africa. Several studies (n=45 studies [81.8%]) described splenomegaly either by palpation or imaging in SCD patients with a prevalence of 12% to 73% among children, and 4% to 50% among adults with Hb SS. Amongst studies involving patients with Hb SC, the prevalence of splenomegaly varied from 4.3% to 33% among children, and 19% to 67% among adults. The reported rates in the Hb SS population from this review appears high, compared to the figures of 5% to 19% reported from the United States in children and young adults with Hb SS (Fatunde and Scott, 1986; Gale *et al.*, 2016). Splenomegaly in SCD patients in Africa appears to be less common than in the Middle East, where the occurrence of splenomegaly in SCD patients ranges from 69% to 82% across all age groups (Al-Jam'a *et al.*, 2000; Al-Salem *et al.*, 1998a; Alsultan *et al.*, 2014). This may be attributed to the inclusion of individuals with Hb S-β-thal^{+} in studies from this region.

In patients with SCD, the spleen commonly enlarges during childhood, but then undergoes autosplenectomy by about 5 years of age due to repeated attacks of vaso-occlusion and infarction (Madani *et al.*, 2007). A palpable spleen is unusual beyond this age in Hb SS patients in the United States and Europe (Powars, 1975; El Hoss and Brousse, 2019; Rogers *et al.*, 2011), and only few reports from the West have documented persistence of the spleen beyond childhood (Gale *et al.*, 2016). This, however, was not what we observed in African SCD patients in this review. We noted persistence of the spleen into late childhood and adulthood across several studies. In one of the adult studies, comprising SCD patients from age 30 years and above, more than a third still had an enlarged spleen (Yetunde and Anyaegbu, 2001). Indeed, haemoglobinopathies accounted for 3% of the causes of massive splenomegaly in

adults patients in Ghana (Bedu-Addo and Bates, 2002). This finding indicates that the late persistence of spleen in SCD patients in Africa is comparable to what is observed in the Middle East, where a large number of adult patients still have their spleen palpable (Al-Salem *et al.*, 1998a; Chopra, Al-Mulhim and Al-Baharani, 2005). In their report, Al-Salem (1998a) observed the spleen size increased with age in their SCD patients until about 40 years of age before slowly decreasing.

The persistence of splenomegaly in patients with SCD in Africa has been attributed to exposure to bacterial and parasitic infections, in particular malaria infection. It is suggested that the parasite causes hyperplasia of the reticuloendothelial tissues in the host spleen that counteract the natural progression to autosplenectomy, and hence the persistence of the spleen (Franceschi *et al.*, 2005). This view is supported by reports from this review that documented increasing IgG antimalaria antibody titre with increasing spleen size (Adekile *et al.*, 1993; Abjah and Aken'Ova, 2003), high malaria parasite density in children with splenomegaly compared to those without (Awotua-Efebo, Alikor and Nkanginieme, 2004), and significant reduction in the spleen size following treatment with the antimalaria drug proguanil (Adekile *et al.*, 1988). Additionally, significantly high lgM values for Hb SS children with persistent splenomegaly when compared with those without splenomegaly was observed, and the splenic reticuloendothelial function was intact in some Hb SS patients with splenomegaly (Adekile *et al.*, 1991; Adekile *et al.*, 1993). Taken together, the late persistence of the spleen beyond childhood in SCD patients in Africa may represent highly active immune response mechanisms against malaria and other infective organisms in the region. We also observed that, all the studies that provided data about the persistence of splenomegaly in adults with SCD were from the West African countries of Nigeria and Ghana. This raises the question as to whether the persistence

of splenomegaly in SCD in Africa could also be due to geographical variation. Nevertheless, few studies in this review found no association between splenomegaly and malaria parasitaemia or episodes of clinical malaria in both children and adults (Sadarangani *et al.*, 2009; Durosinmi *et al.*, 2005; Makani *et al.*, 2010a). Given the number of studies that provided data on the prevalence of splenomegaly in SCD (n=35), and the limited number of studies exploring the association between spleen size and infections (n=10), along with the conflicting findings from these reports, further studies are needed to ascertain the relationship between splenomegaly and the risk of infections in SCD in Africa.

Reports from other parts of the world suggest that the persistence of splenomegaly is linked to modifiers of disease severity, such as, co-inheritance of alpha thalassemia trait and high foetal haemoglobin (HbF) (Padmos *et al.*, 1991; Alsultan *et al.*, 2011). The high HbF level, by its ameliorating influence on the sickling process, may play a role in the persistence of splenomegaly. However, the effects of these factors remain unclear in the African setting as evidenced by this review, as only two of the nine studies that evaluated level of HbF showed an association with presence of splenomegaly (Durosinmi *et al.*, 2005; F.A. Fasola and Adekanmi, 2019). The two studies that provided information about the co-inheritance of the alpha-thalassemia trait and splenomegaly showed conflicting findings (Mouélé *et al.*, 1999; Adekile *et al.*, 1993).

2.4.1 Splenomegaly - related complications

The review highlighted that the presence of an enlarged spleen may predispose SCD patients to further morbidities as described below:

2.4.1.1 Acute splenic sequestration crisis (ASSC)

Splenomegaly was complicated by acute splenic sequestration crisis (ASSC) in six studies and in most of these studies (n=5), the reported prevalence was less than 10%. This is lower than reported in children from other parts of the world (Brousse *et al.*, 2012; Powell *et al.*, 1992) and in adults with SCD (Al-Jam'a *et al.*, 2000). In a cohort of 216 children with HbSS followed since birth, fifty-two (24%) suffered from at least one episode of ASSC over the study period of six years (Topley *et al.*, 1981). Also, a total of 437 episodes (0.06 / patient-year) of ASSC was reported over a nine-year period in the French cohort (Brousse *et al.*, 2012). ASSC also seems to be less frequent in SCD patients in Africa compared to the Middle East, a region with comparable rates of enlarged spleens persisting into adulthood (Alsultan *et al.*, 2014; Padmos *et al.*, 1991; Al-Jam'a *et al.*, 2000). The low report of ASSC in Africa may be related to the late age of patients recruited in most of the studies. It could also be linked to the expression of different haplotypes across the geographical regions.

Regular clinical examination and parental education to identify acute enlargement of the spleen in febrile patients can facilitate prompt diagnosis of suspected cases. Urgent treatment with top-up transfusion or red blood cells exchange is mandatory as delays can lead to circulatory collapse and death from acute anaemia as evidenced in studies from Nigeria (Ambe, Fatunde and Sodeinde, 2001) and Republic of Benin (Goussanou *et al.*, 2003) that provided data on ASSC related mortality. All the ASSC related deaths (n=4) occurred during the first year of the Benin study, which was designed to determine the effect of a comprehensive clinical care program on disease course in SCD patients. With sustained and intensive parental education on spleen palpation, no other death was recorded in the remaining four years of the study (Goussanou *et al.*, 2003)

2.4.1.2 Hypersplenism

Only few studies provided information on hypersplenism, and within these studies, the reported prevalence rates were low (<5%). The chronic sequestration of blood within the enlarged spleen and the accompanying red cell haemolysis results in cytopenia. The increased transfusion need linked to hypersplenism was the main indication for splenectomy in most of the studies in this review (Diagne *et al.*, 2010; Durosinmi *et al.*, 2005; Okoro, Kaine and Okeahialam, 1989). Also, the resulting expansion in erythropoietic drive to compensate for the excessive haemolysis imposes a high metabolic requirement in SCD patients and may interfere with normal growth in children (Singhal *et al.*, 1995). This is of particular concern in SCD patients in most low-income countries of Africa where malnutrition is high, because any additional malnutrition from increased demand can exacerbate the susceptibility to infection (Hyacinth, Adekeye and Yilgwan, 2013).

2.4.1.3 Surgical splenectomy

Thirty patients had splenectomy performed across 10 studies over a four-decade period in this review. This is low compared to reports from the Middle East, where splenectomy was performed in 44 (20%) patients (age range, 4-52 years) with SCD over a four-year period (Chopra, Al-Mulhim and Al-Baharani, 2005), and 134 children (mean, 7.6 years) over a 14-year period for various splenic complications (Al-Salem, 2011; Al-Salem, 2006). The splenectomy rate in a US study (9 children [8%]) over 12 years was higher than that in any study in our review (Gale *et al.*, 2016). Due to the better availability of specialist surgical expertise, as well as conjugate vaccine (Adamkiewicz *et al.*, 2013), and prophylactic antibiotics (Hirst and Owusu-Ofori, 2014) in developed countries, splenectomy is more likely to be considered feasible across all age groups of patients with SCD (Lesher *et al.*, 2009). However, in Africa,

where these resources are limited, coupled with the high burden of infection, surgeons may be reluctant to perform splenectomy in SCD patients. The operative risk of removing an enlarged spleen is also high when blood and components for transfusion may not be reliably available. General anaesthesia may induce vaso-occlusive crisis, therefore, maintenance of optimal oxygenation and hydration intraoperatively, and minimising anaesthetic duration is critical (Alufohai and Odusanya, 2006; Gnassingbe *et al.*, 2007).

Despite the limited data on splenectomies performed in SCD patients in Africa, most of the studies (n=5 [83.3%]) reported improved clinical symptoms and haematological parameters following the procedure. Two studies reported absence of infection-related complications in the post splenectomy period after 18 months (Okoro, Kaine and Okeahialam, 1989) and 3 years (Gnassingbe *et al.*, 2007) of follow-up in their patients; while the remaining eight studies did not provide information about the risk of infection post-splenectomy. Careful patient selection and adequate preventive measures before and after splenectomy to control for infections is crucial to improve the outcome.

2.4.1.4 Assessing spleen function in SCD in Africa and implication for future research

The two studies that provided information on spleen function, noted that most of the patients with palpable spleen had lower red cell pit counts, indicating that the splenic reticuloendothelial function was still preserved (Adekile *et al.*, 1991; Adekile *et al.*, 1993). One of the studies compared splenic function between Hb SS patients from a malaria zone (Nigeria) and those living in a malaria-free zone (US). Patients with palpable spleens in both groups had significantly lower pit counts when compared to those without splenic enlargement (Adekile *et al.*, 1993). This would imply that,

although functional asplenia has been described in SCD patients with splenomegaly, reticuloendothelial function is not always compromised. This finding is corroborated by reports from the Middle East which also showed preservation of spleen function into adulthood among SCD patients with splenomegaly (Al-Awamy, Wilson and Pearson, 1984; Al-Jam'a *et al.*, 2000). In contrast however, reports from the West suggest loss of splenic function in most patients by two years of age (El Hoss *et al.*, 2019; Rogers *et al.*, 2011). In view of these conflicting data across regions, further studies are needed to fill this gap in knowledge regarding the age and prevalence of splenic dysfunction in SCD patients in Africa. Additionally, several studies have evaluated the role of hydroxyurea in improving spleen function in SCD patients (Hankins *et al.*, 2008; Nottage *et al.*, 2014). The splenic filtration function was preserved after three years on treatment in a third of the patients following hydroxyurea; starting treatment at a younger age and baseline spleen function were both associated with a favourable outcome. Recently, the safety and efficacy of hydroxyurea in young children with SCD residing in malaria endemic regions of Africa has been demonstrated (Opoka *et al.*, 2017; Tshilolo *et al.*, 2019). Therefore, the development of new tools to assess splenic function or the optimization of existing ones in low-income countries can play a role in identifying those patients who can benefit from such therapy, especially in Africa, where there is a high prevalence of diseases, such as, SCD and malaria that can affect the spleen concurrently.

Splenic function can be measured using several methods including liver spleen scintigraphy, enumeration of Howell-Jolly bodies in red blood cells either manually or by flow cytometry, and counting of pitted red cells using direct interference, phase-contrast microscopy (de Porto *et al.*, 2010). Unfortunately, these tests are not readily available in most low-income settings of Africa as evidenced by the scarcity of data in

this review. A simple method has been described which is based on counting red cells containing argyrophilic inclusions using a light microscope. This method has a good correlation with the pitted red cells count method and a good inter- and intra-observer reliability (Tham *et al.*, 1996). This method may be suitable to assess splenic function in SCD patients in Africa, where resources for spleen scintigraphy and interference phase-contrast microscopy are absent.

2.4.1.5 Strengths and limitations of this review

The major strength of our study is the comprehensive inclusion of many studies reporting on various splenic complications across Africa. It included articles from non-indexed journals to avoid publication bias, and there were no age, language, or time inclusion restrictions. However, the findings of the review should be interpreted with caution. The studies measured spleen size in SCD patients using different approaches, which made it difficult to compare reported spleen sizes among the studies. Criteria used to define some of the splenic complications and infections were not stated or were inconsistent. Only a few studies reported on spleen-related complications, so we were unable to combine the data to provide pooled estimates for these complications.

2.5 Conclusion

SCD is a prevalent, chronic condition in Africa which places a high burden on the health services and on patients and their families. Evidence from this review indicates that splenomegaly is prevalent among SCD patients; however, splenomegaly-related complications are under reported and may contribute to significant but unrecognised complications. The spleen seems to persist longer in SCD patients in Africa when compared to their counterparts in the West; although this maybe similar to what is observed in the Middle East, however, factors explaining this occurrence may be

different. In SCD patients in Africa there was less association between splenomegaly and factors such as HbF level and the presence of alpha thalassemia traits compared to similar patients in the Middle East; instead, a link between malaria and splenomegaly seems to be indicated by data from this review. There was evidence of an association between splenomegaly and retention of the spleen function in patients with splenomegaly, although the data are limited. Furthermore, there was no information in any of the studies in the review regarding the link between spleen function and the risk of infection. In Africa, where majority of children with SCD may die because of infections and with splenic dysfunction contributing a significant part, the importance of detecting those at high risk cannot be over emphasized. Thus, further studies are needed to fill this gap in knowledge regarding the age and prevalence of splenic dysfunction in SCD patients in Africa. Such knowledge can be employed in the management of infection risk in SCD patients and ensure a more effective use of the limited resources available.

CHAPTER 3: METHODOLOGY

3.1 Chapter overview

Having described the study aim and objectives and gaps in the literature, this chapter describes the location where this project was conducted. It describes the research methodology used in this study to achieve the objectives outlined in Chapter 1. The project was divided into two parts: spleen baseline study and spleen and infection study. The baseline study involved steady state SCD patients, whereas the spleen and infection study involved acutely-ill SCD patients. The method used for assessing spleen size and the two methods used to assess spleen function are described. Details for the methods used to analyse malaria and bacterial infection are provided too. The selection criteria and sample size calculation for the two parts of the study are also highlighted.

Further details of the statistical analyses used in the research are provided in the respective results chapters as appropriate to the objective of the particular chapter.

3.2 Study setting

The study was conducted at the University of Maiduguri Teaching Hospital (UMTH), located in Maiduguri the capital of Borno State. Borno state is located in the North-Eastern part of Nigeria. It spans an area of about 69,455sq. km and has a population 5,860,200 million people as of 2016 (Statistics, 2016). It occupies the greatest part of the Chad basin, and shares borders with Republic of Niger to the North, Republic of Chad to the North-East, and Cameroon to the East. On the South and West, it borders the Nigerian states of Adamawa, Gombe and Yobe. It has diverse ethnicity with Kanuri being the dominant ethnic group. Maiduguri is the largest city in North-East Nigeria, with an estimated population of about 800 thousand people (Bell and Card, 2021).

The University of Maiduguri Teaching Hospital (UMTH) is a 500 bed - capacity tertiary health facility, but functions both as a general and referral hospital (Fig.6). An adult haematology day-care unit runs daily and provides supportive care for an average of 10 to 12 SCD patients/day including blood transfusion service, intravenous rehydration, and treatment of painful crisis. Patients requiring in - hospital management are admitted onto the medical wards. The out - patient clinic runs every Tuesday for adults and Wednesday for paediatric patients, and an average of 40 - 50 SCD patients are seen per clinic. The patients attend clinic at 8 - 12 weeks intervals depending on the severity of symptoms; or as short as a 1- or 2-weeks interval if there is need for closer monitoring or review of investigation results. The average travel time to the hospital is 20 to 40 minutes using public transport from most parts of the city.

3.2.1 Demographic and socio-economic context of study participants

The majority of the study participants were from Maiduguri and its surroundings. Most of the adult participants were students at secondary and tertiary levels of education; only a few of were employed, hence most of them rely on their parents/guardians to provide for their health care. The majority of study participants have to pay for all aspects of their health care. The state has a national health service scheme but less than 10% of the patients are enrolled in the scheme. All eligible patients who met the study criteria were included in the study as they did not have to pay for any investigations. Therefore, the socio-economic status of an individual patients did not affect their eligibility to be enrolled in the research.

3.2.2 Routine care for SCD

The current clinic policy in paediatric and haematology at the UMTH is to advise SCD patients to take prophylactic antimalarial (proguanil) and folate supplementation.

These treatments are available and affordable for most SCD patients as evidenced by a high compliance among the study participants. The current national immunisation policy requires all children to be vaccinated against the routine childhood vaccines and immunisation is free of charge to all eligible age groups. From 2017 the new routine vaccination scheme has incorporated pneumococcal vaccination. Penicillin is no longer prescribed routinely for paediatric SCD patients as all children born after 2017 are likely to have received the conjugate pneumococcal vaccine. For those not immunised, oral penicillin may be given up to the age of five years.

Also, the policy in the paediatric unit is to provide hydroxyurea (HU) to patients who meet the criteria and can afford the drug. In paediatrics, the criteria for prescribing HU to SCD patients is based on the frequency of crises, blood transfusion and stroke. In adult SCD patients, HU is given for patients with increased transfusion requirements and for those with end organ damage. However, the use of HU remains low among the SCD patients; during enrollment, less than a quarter of the study participants were on hydroxyurea, and the majority have never heard of it. I used the opportunity of my research to provide education on the importance and benefits of using HU. All parents were also provided with information about the spleen, how to identify an enlarged spleen in their child and what steps should be taken if one is identified.

Figure 6: University of Maiduguri Teaching Hospital

3.3 Study Methodology

3.3.1 Study population and sampling

The study population consisted of children and adults with SCD (both inpatient and outpatient) receiving medical treatment at the paediatric and adult haematology units of UMTH. The study also included non-SCD participants; the non-SCD population served as controls for comparison of spleen size and function, and malaria results with those of the SCD participants. Given the variation in the literature regarding the age at which anatomical asplenia (autosplenectomy) and functional hyposplenism occurs, the patients were divided into four age groups; 1: Less than 5 years; 2: 5 - 9 years; 3: 10 - 14 years and group 4: 15 years and above. This will also provide us a snapshot of changes in spleen size across a broad spectrum of age.

3.3.2 Study period

The study data were collected over a 15-month period from September 2020 to Nov 2021.

3.3.3 Study design

The project was performed as a prospective cross-sectional study and consisted of two parts: a spleen baseline study and a spleen and infection study. In the first part, baseline data about spleen sizes and function and their relationship with clinical and laboratory parameters were obtained. In the second part of the project, the prevalence of acute malaria infection and bacteraemia amongst the study participants were determined and their relationship with spleen size and function analysed. All enrolled study participants underwent history taking; self-reported medical history from the patients (or their carers) including frequency of hospitalisation, febrile episodes, and painful crises over the preceding year including acute splenic sequestration crisis were obtained. The history of use of folate supplementation and antimalaria prophylaxis and the current use of hydroxyurea therapy was also obtained. Clinical examination including a general physical examination, weight, height, pulse rate, blood pressure and temperature was performed. The spleen size was assessed by palpating the anterior axillary and mid clavicular line by a single examiner. Palpable splenomegaly was reported as the distance in cm that it extended under the left coastal margin in the mid clavicular line. The information obtained was entered into a pre-designed case-report form (Appendix 5-7)

3.3.4 Study Participants

3.3.4.1 Inclusion criteria

- Spleen baseline study: SCD patients in steady state (free of painful crises in the last 4 weeks, no history of intercurrent illness such as infection, inflammation in the previous 4 weeks and blood transfusion in the last 4 months) (Ballas, 2012).
- Spleen and infection study: acutely ill SCD patients (fever, symptomatic anaemia, bone pain, jaundice).

- Provided informed consent to participate in the study.

3.3.4.2 Exclusion criteria

Spleen baseline study

- Splenectomy in the past: surgical removal of the spleen eliminates the primary filter for circulating red cells with inclusions such as HJB.
- Blood transfusion in the past 3 months: this will interfere with the peripheral blood film finding of the spleen function assessment and HPLC results.
- Acute, severe illness: this may interfere with splenic size and function assessment.
- Refusal to provide consent: this was to respect patient's wish.

Spleen and infection study

- Splenectomy in the past: Surgical removal of the spleen eliminates the primary filter for circulating HJB.
- Refusal to provide consent: to respect patient's wish.

Controls

- Individuals with ongoing febrile illness such as malaria, sepsis, and diarrhoea; or with features suggestive of haematological conditions including passage of dark urine, bleeding tendencies and bone pains were excluded.

3.3.5 Sample size calculation

3.3.5.1 Spleen baseline study

The sample size was based on one of the primary objectives of the study - to determine the prevalence of enlarged spleen , and was based on the formula for a cross sectional study with finite population correction (Daniel, 1999) as follow:

$n = N * X / (X + N - 1)$

where, $X = Z_{\alpha/2}^2 * p (1-p) / MOE^2$ and $Z_{\alpha/2}$ is the critical value of the normal distribution at $\alpha/2$ (for a confidence level of 95%, α is 0.05 and the critical value is 1.96), MOE is the margin of error set at 5%, p is the sample proportion, and N is the population size. A prevalence rate of 24.9% for enlarged spleens among SCD patients from a previous study in the same hospital (Luntsi *et al.*, 2018) and N = 700 as the population size (the number of registered patients with SCD) were used.

$n = N * X / (X + N - 1)$, where $X = Z_{\alpha/2}^2 * p (1-p) / MOE^2$

$X = (1.96)^2 * 0.249 (1-0.249) / (0.05)^2$

$X = 3.8416 * (0.249 \times 0.751) / 0.0025$

$X = 3.8416 * 0.186 / 0.0025 = 287.35$

Therefore,

$n = N * X / (X + N - 1)$

$n = 700 * 287.35 / (287.35 + 700 - 1)$

$n = 201{,}061 / 986.23$

$n = 203.9286$

Hence, 1 aimed to enrol 204 SCD patients for this study

3.3.5.2 Spleen and infection study

The sample size was based on one of the primary objectives of the spleen and infection study - to determine the prevalence of bacteraemia. The sample size was calculated using the same formula as for the spleen baseline study. A prevalence rate of 13.8% for bacteraemia from a previous study among SCD was used (Brown *et al.*, 2017). Calculating the sample size using p = 13.8% (prevalence of bacteraemia)

$n = N * X / (X + N - 1)$ where, $X = Z_{\alpha/2}^2 * p (1-p) / MOE^2$

$X= 1.96^2 * 0.138 (1- 0.138) / 0.05^2 = 3.84 * 0.1189 / 0.0025 = 182.7925$

X= 182.7925

Therefore,

n = N * X / (X + N – 1)

n= 700 * 182.7925 / (182.7925 + 700 – 1) = 128,100 / 882 = 145.1076

Hence, 1 aimed to enrol 146 patients for this study.

3.3.5.3 Controls

Due to financial and time constraints, I aimed to enrol one control subject for every two SCD patients enrolled in the spleen baseline study. Hence, I aimed to enrol 100 controls.

3.3.6 Recruitment strategy

Spleen baseline study

All Patients with SCD or the parents or guardians of patients with SCD between September 2020 to November 2021 were invited to participate in the study by their managing physicians. They were provided with information leaflets (Appendices 8-9); SCD patients who met the inclusion criteria were enrolled after signing the consent forms (Appendices 12-13). For those who were unable to read or didn't speak English, the information sheets and consent forms were read out and explained to them. Patients for this part of the project were recruited from the out-patient clinics which ran every Tuesdays for the adult patients and Wednesdays for the paediatric patients.

Spleen and infection study

All patients with SCD or the parents or guardians of patients with SCD during the period of October 2020 to May 2021 were invited to participate in the study by their managing physicians. They were provided with information leaflets (Appendices 10-11); SCD patients who met the inclusion criteria were enrolled after signing the

consent forms (Appendices 14-15). For those who are unable to read or didn't speak English, the information sheets and consent forms were read out and explained to them. Patients for this part of the study were recruited from the haematology day-care unit, accident and emergency unit and the paediatric emergency unit. Patients presenting in acute crisis to either the adult or paediatric clinics were also recruited.

Controls

Non-SCD controls consisted of children of hospital personnel and paediatric patients on post-op follow-up in the surgical clinic. For the adult population, medical students were invited to participate (Appendices 16-18). Participants who met the inclusion criteria were enrolled after signing the consent forms (Appendices 19-21)

3.3.7 Blood sample collection

Spleen baseline study

Adults: 8 ml of venous blood was collected from each patient into two K_2EDTA containing tubes (3 ml each) and one plain tube (2 ml) for full blood count, High performance liquid chromatography (HPLC) and chemistry.

Children: 6 ml of venous blood was collected from each patient into two K_2EDTA containing tubes (2 ml each) and one plain tube (2 ml) for full blood count, HPLC and chemistry.

Spleen and infection study

Adults: 4 ml of venous blood was collected from each patient into a K_2 EDTA containing tube and plain tube for full blood count, HPLC and chemistry, and 8 ml into blood culture bottles

Children: 4 ml of venous blood each was collected from each patient into a K_2 EDTA containing tube and plain tube for full blood count, HPLC and chemistry, and 1-3ml into blood culture bottles

3.4 Laboratory analysis

3.4.1 Laboratory testing for haematological parameters, serum biochemistry and HbF level

Aliquots from the K_2EDTA sample was used for haematologic analyses including full blood count (FBC), using an autoanalyzer (Sysmex XN 550 model). Reticulocytes count was performed manually using standard protocol (Bain, Bates and Laffan, 2016). The plain bottle samples were used for analysing aspartate amino transferase (ASAT), total and conjugated bilirubin; the analysis was performed using Roche/Hitachi autoanalyzer C311 model. The blood in the second K_2EDTA tube was stored at 2 - 8°C degree and transported to Aminu Kano Teaching hospital (AKTH) laboratory for estimation of HbS, HbF and HbA2 level using HPLC (Bio-Rad D-10 dual program 220-0201). The remaining tests, including FBC, reticulocytes and haemolytic markers were performed at the haematology and chemical pathology departments of the UMTH.

The remainder of the blood samples collected were centrifuged. The resulting plasma from the K_2EDTA tubes and serum from the plain tubes were harvested and aliquoted into duplicate Eppendorf tubes and stored at minus 80ºC at UMTH. The red cells were disposed of in accordance with the local policy. All peripheral blood smears were fixed using appropriate fixatives and stored within sealed slide boxes with ample desiccant.

3.4.2 Estimation of argyrophilic-inclusion containing red cells (AI)

The percentage of AI red cells was obtained from a blood smear as previously described with some modification (Tham *et al.*, 1996) and observing strict universal precaution (Appendix 22). A blood smear from the EDTA sample was fixed in ethanol-formalin solution for 3 minutes before washing with distilled water. The smear was

then covered with three drops of silver staining solution and allowed to stain in complete darkness for 20 minutes. The slides were rinsed with distilled water, counterstained with 1% eosin and dehydrated through increasing concentrations of ethanol. The presence of AI red cells was determined by the number of red cells with one or more distinct black inclusions per 500 red cells. I prepared and read all the slides myself

3.4.3 Estimation of Howell-Jolly body (HJB) red cells

Howell-Jolly bodies (HJB) containing red cells were estimated from peripheral blood smear using basophilic staining (combination of May-Grunewald and Giemsa- MGG) (Appendix 23). To obtain a uniform quality of staining, the slides were stained simultaneously in batches using a staining tank containing freshly made May-Gruenwald (Sigma-Aldrich) working solution for 5 minutes and washed in 2 to 3 changes of distilled water. The slides were next immersed in Giemsa stain (Lab Tech Chemicals) for 10 minutes before washing in 2 to 3 changes of distilled water. The slides were allowed to dry upright as this prevented residue from forming on the slides. Any residual water on the slides was wiped off with dry gauze, which also improved the clarity of the film. The HJB slides were not mounted as this did not affect the clarity of the slide. The presence of HJBs was determined by the ratio of HJB-containing red cells per 400 red cells. I prepared and read all the slides myself.

3.4.4 Blood culture analysis

Blood samples for culture were collected aseptically by venepuncture using appropriately sized BD Bactec safety lock blood collection set (21 G for adults and 23 G for paediatric patients) directly into a BACTEC Peds plus bottle for children or BACTEC Aerobic plus bottle for adults' patients. This avoided the need to change the needle before transferring blood sample into the culture bottles. Samples were

immediately transferred to an incubator at 37^0 C and agitated daily. Samples were incubated for a total of 7 days before being declared negative. Samples were routinely sub-cultured blindly on days 3, 5 onto a Blood agar (BA), MacConkey agar (MAC) and Chocolate agar (CA), and onto CA only on day 7. All bacterial isolates were subjected initially to a gram stain to identify the class of bacteria. Also, the gram stain served as a guide for choice of media for secondary sub-subculture (Appendix 24). Quality control in terms of adherence to standard operating procedure (SOP) was built into each step in the isolation and identification of bacteria from culture samples. Control organisms for Gram positive cocci (GPC), Staphylococcus aureus (ATCC 25923) and Gram-negative bacteria (GNB), Escherichia coli (ATCC 25922) were used to test new batches of media. Sterility test was also performed on newly prepared media. All media were stored in the refrigerator and any media with evidence of contamination was discarded immediately. Results of blood culture was given to the physicians to aid the management of patients.

3.4.5 Malaria parasite identification and quantification

Thick film are the standards for malaria quantification from smears, however due to quality assurance issues when I assessed the use of thick films in practice, I chose to report the thin film result; hence testing for malaria parasitaemia and density were performed using a Giemsa-stained thin blood film following standard method (Appendix 25).

3.4.6 Spleen size assessment

A real-time ultrasound machine General Electric - Lp5 Pro- Europe, model number: 5415172, was used to perform spleen size assessment for the study. Curvilinear probe with frequencies ranging from 1–4 MHz and 4–9 MHz was used to evaluate the spleen size and echotexture. All scans were obtained by a single Radiologist with more than

15 years' experience in paediatric and adult sonography. The patients were asked to lie down in a supine position or a lateral position when optimal scans were not achievable in the supine position. Uncooperative infants were scanned on their mother's lap. A water-based medium was applied to both the probe and the body area being scanned to ensure good transmission of the ultrasound beam. Optimal images for a complete spleen evaluation were obtained through sagittal, transverse, and occasionally through the intercostal space if necessary. From the obtained images (Appendix 26), the following splenic parameters were obtained spleen length (SL), spleen width (SW), spleen depth (SD) and spleen volume (SV) as described previously (Gale *et al.,* 2016). Two sequential measurements of each splenic parameters were obtained. The spleen was also assessed for parenchymal homogeneity and focal abnormalities. Given the variation in spleen size with age, the mean value for each splenic parameter was calculated and compared against the age specific values of the controls. We classified spleen sizes using both the spleen length and spleen volume. The spleen size was considered normal if the measured value fell within the 2.5^{th} and 97.5^{th} centile of the expected value for the age specific population. Spleen sizes below and above the cut off values were considered small-sized spleens and enlarged spleens (splenomegaly) respectively. Autosplenectomy was defined as the non-visualisation of the spleen in the splenic bed (Mustapha *et al.,* 2010).

3.5 Statistical analysis

The specific statistical analysis used are highlighted in the respective chapters (2, 4-9) as appropriate. Baseline characteristics including age, sex was reported as mean (SD), median (IQR) and frequencies following assessment of distribution using histogram and test of normality. Descriptive statistic was carried out to describe spleen sizes and reported as mean and standard deviation (±) if normally distributed, or median and

interquartile range if the data was skewed. One way ANOVA test or Kruskal-Wallis tests were used to compare the means of continuous variables across more than two groups of the population. To determine the relationship between spleen size and function with clinical and laboratory parameters, baseline comparison using Student T test for continuous variables that were normally distributed, Mann–Whitney U test for those that were not normally distributed and chi-squared test for categorical variables were performed as necessary. Multivariate regression analysis was performed to determine the predictors of spleen persistence and splenic dysfunction. The independent variables selected including clinical (age, rate of vaso-occlusive crisis, hospitalisation) and laboratory parameters were based on association from previous observational studies.

3.6 Ethical consideration and confidentiality

3.6.1 Confidentiality

Each study participant was given a unique identifier which was used in the case report form (CRF), on any diagnostic tests and in data analyses. Consent forms and other paper documentation were kept separately in locked drawers in UMTH, Nigeria under my care for the duration of the study. The CRF and laboratory results were recorded in the participant's register and stored electronically in a computer which was encrypted with a password. Electronic data files were stored on the LSTM server, United Kingdom. All published data were completely anonymised.

3.6.2 Informed consent

At the start of the research, I briefed the local collaborators and study team members on the study and its protocols. Potential participants were approached by their managing physician about the study and were provided with information leaflets about

the spleen, its function, the importance of assessing splenic function, the procedures involved and how patients could participate in the study (appendices 8-11, 16-18). Participants were not coerced, rushed or forced to take part in the study at any point and were assured of their right to opt out at any point; no participant was interviewed, or requested to submit samples without a signed consent form (appendices 12-15,19-21).

3.6.3 Compensation for participation

Participants did not receive financial or other assistance for participating in the study. However, a token amount was given as reimbursement for their time and to cover their transportation to attend for the initial and follow up visit for ultrasonography. Children were compensated with educational items (e.g. pen, crayon, school bags).

3.6.4 Ethical approval

The study was funded by the Commonwealth Scholarship Commission. The study sponsor was Liverpool School of Tropical Medicine. Patients were recruited from the University of Maiduguri teaching Hospital (UMTH), the study partner in Nigeria. Permission for my research was sought from the hospital director and head of departments of paediatric, haematology and radiology of UMTH prior to study commencement and data collection. I applied for and secured approvals from the following ethics committees:

1. Liverpool: Ethics Committee, Liverpool School of Tropical Medicine (Reference number 20-010; dated 1st June 2020 (Appendix 27).

2. Nigeria: University of Maiduguri Teaching Hospital Research & Ethics Committee (UMTH/REC/20/606; dated 10th February 2020) (Appendix 28).

CHAPTER 4: ULTRASONOGRAPHIC ASSESSMENT OF SPLEEN SIZE AND PATTERN OF CHANGE AMONG SICKLE CELL DISEASE PATIENTS AND HEALTHY CONTROLS IN NORTH-EASTERN NIGERIA

4.1 Chapter overview

This chapter focused on identifying baseline spleen sizes among the SCD participants. Given the variation in parameters used to define various spleen size in the literature, we initially obtained spleen size measurements among normal individuals in the same environment and determined the reference ranges for spleen length and volume for various age groups which was then used to compare spleen sizes among the SCD group.

Chapter 4 was submitted to Ultrasound on the 17th of July 2023 and the reviewers have recommended for publication (21/1023) upon completion of minor revisions.

4.2 Abstract

Background

Ultrasonography is an established and reliable method for assessing the spleen. Because of variation due to genetic and other environmental factors including malaria endemicity, interpretation of splenic sizes requires a knowledge of the normal reference range for a given population. The aim of this study was to determine spleen size in different age groups among healthy people in North-Eastern Nigeria and use this as a reference to determine spleen size amongst sickle cell disease (SCD) patients.

Methods

Using a cross-sectional study design, spleen size was measured in healthy people of different age groups, and steady-state SCD patients (children and adults) using abdominal ultrasonography. Using the age-group specific reference values obtained

from the controls, spleens were classified into small, normal size, or enlarged among the SCD patients.

Results

The study consisted of 109 (34.8%) healthy controls and 204 (65.2%) steady-state SCD patients. The spleen was visualized in all the controls (n=109). However, 97(47.6%) of the SCD patients had no visible spleen; among the remaining 107 SCD patients with visible spleens, small, normal, and enlarged spleens were observed in 8.8%, 33.3% and 10.3%, respectively. Compared to the control group, splenic length was three-fold higher in the first two years of life in SCD patients, followed by a progressive age-related decline in size.

Conclusion

Model-based age-specific reference ranges and percentile curves for splenic dimensions based on ultrasonography among normal controls in North-Eastern Nigeria were established and may be of value in assessing spleen sizes among SCD patients living in malaria-endemic regions of Africa.

4.3 Introduction

Sickle cell disease (SCD) is an inherited condition of red blood cells widely prevalent across Sub-Saharan Africa, affecting up to 3% of births in some parts of the continent (Grosse *et al.*, 2011). The spleen is the largest organ in the reticulo-endothelial system with an active role in immune defence against infection and is one of the earliest organs to be affected in patients with SCD. In SCD the spleen initially enlarges, followed by progressive atrophy due to repeated episodes of vaso-occlusion and infarctions (Serjeant, 2002). Acute splenic sequestration crisis (ASSC), characterized by a sudden enlargement of the spleen can cause an abrupt drop in haemoglobin by at least 20% from baseline value, resulting in death in severe cases (Topley *et al.*, 1981). Part of the

routine clinical evaluation of patients with SCD involves examining for the spleen (Emond *et al.*, 1985). However, clinical examination by palpation has a low sensitivity for the detection of splenic enlargement because the organ would have to enlarge two to three times its normal size before becoming palpable on abdominal examination (Zhang and Lewis, 1989); consequently, subclinical splenic sequestration crisis could go undetected during abdominal examination.

Ultrasonography provides a non-invasive method of assessing the spleen size; it is the test of choice in most climes because of its accuracy, low cost, flexibility, and safety profile (Mustapha *et al.*, 2010). Other diagnostic modalities used in the radiological assessment of the spleen include CT and MRI scan (Catalano *et al.*, 2005; Luna *et al.*, 2006), however, routine use of CT or MRI imaging is not feasible in most developing countries because of high cost and limited availability. Despite the widespread use of ultrasonography in the clinical evaluation of splenic enlargement, there is no consensus on how to define splenomegaly among SCD patients, as various cut-off values have been used in the literature. Some studies have considered a spleen length of ≥13 cm to indicate enlarged spleens (Babadoko *et al.*, 2012; Luntsi *et al.*, 2018; Kaushal *et al.*, 2014), while others have defined splenic enlargement as a spleen length of > 11cm (Okongwu *et al.*, 2018) or > 12 cm (OT Ojo, WA Shokunbi and Agunloye, 2014). Some authors have used spleen length to define splenomegaly (Ugwu *et al.*, 2018; Akinlosotu *et al.*, 2018), while others have used spleen volume or index (McCarville *et al.*, 2011; Yakubu CI, 2017).

Furthermore, prior knowledge of the normal size of the spleen in a healthy population is required to interpret changes in spleen size because geographical variations in spleen size due to environmental factors like infections (e.g., malaria) and genetic variation can occur. Thus, establishing a reference range for populations living in the same area

as the patients' population is needed to be able to interpret ultrasonography assessments. However, few studies are available on normal spleen dimensions in children and adults in Africa, particularly those living in malaria-endemic regions (Tsehay *et al.*, 2021; Ezeofor *et al.*, 2014; Eze *et al.*, 2013).

Therefore, the aim of this study was to determine various splenic dimensions for different age groups in apparently healthy children and adults in North-Eastern Nigeria to generate a reference range for normal spleen size. This served as baseline for assessing changes in spleen size in patients with SCD in a tertiary hospital. We have also compared the spleen length and volume to assess spleen size, in order to identify which parameter is preferable and practical to reliably diagnose and follow up patients with splenomegaly in a resource-limited setting.

4.4 Materials and methods

4.4.1 Study design and participants

See section 3.3.1

4.4.2 Data collection

See section 3.3.3

4.4.3 Sonographic evaluation

See section 3.4.6

4.4.4 Statistical analysis

The data was analysed using Statistical Package for the Social Sciences (SPSS) (version 25; SPSS, Chicago, IL, USA). Categorical data were summarised using frequency and proportions while continuous data were summarised using descriptive statistics. Model-based age-specific reference ranges for the various spleen dimensions

were computed with age modelled as fractional polynomials using MedCalc® v.20.114 (MedCalc Statistical Software Ltd, Ostend, Belgium) (Wayne, 2008). Log-transformation of variables was applied before model fitting as needed. The model-based 2.5th, 10th, 50th, 90^{th,} and 97.5th percentiles of the (log-transformed) variables were then plotted against age. Comparison of splenic dimensions between controls and SCD patients was performed using non-parametric analysis. The level of significance was set at two-tailed P-value <0.05.

4.4.5 Ethical considerations

See section 3.6.4

4.5 Results

4.5.1 General characteristics of the study population

A total of 214 patients with SCD (median age 13.0 years; range 1- 45 years) and 111 apparently normal controls (median age 14.5 years; range 1- 34 years) were enrolled into the study. The median weight (29.0 kg vs 36.0 kg; P=0.006) and BMI (15.5 kg/m^2 vs 17.9 kg/m^2 ; P=0.001) were significantly lower in the SCD patients compared to the controls (table 2). Ultrasonography data were available for 109 of the controls and 204 of the SCD patients. The remaining 12 participants (2 controls and 10 SCD patients) failed to turn up for the scan and were excluded from further analysis. The haemoglobin (Hb) phenotypes of the SCD patients consisted of homozygous sickle cell disease (Hb SS) (n = 196), sickle-haemoglobin C disease (Hb SC) (n = 5), and sickle cell β-thalassaemia (Hb Sβ) (n=3). The Hb phenotypes of the controls consisted of Hb AA (n=98), Hb AS (n=8) and Hb AC (n=3). The spleen was clinically palpable in five (2.4%) of the SCD patients (all HbSS) (ranges from 2 to 10 cm below the left

coastal margin) but was not palpable in any of the controls. Three of the HbSS patients (aged 1, 6 and 24 years) had past history of acute splenic sequestration.

Table 2: Baseline characteristics of SCD patients and healthy controls

Parameter	SCD patients	Controls	P value
No of Subjects	214	111	
Age (years, range)	13.0 (1.1 – 45)	14.5 (1.5 – 34)	0.900
No (%) of subjects per group			
1 – 4 years	44 (67.7)	21 (32.3)	0.962
5 - 9 years	38 (63.3)	22 (36.7)	
10 – 14years	42 (66.7)	21 (33.3)	
≥15 years	90 (65.7)	62 (34.3)	
Gender, n , (%)			
Male	107 (50.0)	69 (62.1)	0.046^^
Female	107 (50.0)	42 (37.9)	
Weight (kg, median, range)	29.0 (8 - 94)	36.0 (9 -100)	0.006*
Height (cm, median, range)	141.5 (73 – 188)	145 (74 -190)	0.152
BMI ((kg/m^2, median, range)	15.5 (8 -30.4)	17.9 (11 -30.8)	0.001*

P value ^^ Chi square, *Mann Whitney U test. BMI body mass index

4.5.2 Sonographic evaluation of the spleen among study participants

The spleen was visualised on ultrasonography in all the control participants; their spleen dimensions by age groups and non-parametric reference limits are shown in Table 3. Four (4.1%) of the Hb AA had enlarged spleen (i.e above the 97.5 centile) and were all children in the less than five years age group. All the Hb AS and Hb AC controls had spleen size within the normal range. Specific (log-transformed) reference limits for spleen dimensions in relation to age are shown as curves in Figs. 7A-D.

Table 3: Splenic dimensions according to age group and non-parametric reference ranges among the controls

Parameter	**Number (n)**	**Mean (SD)**	**Median**	**2.5th centile**	**97.5th centile**
Spleen length (cm)					
< 5 years	21	6.3 (0.7)	6.1	5.2	7.0
5 – 9 years	22	6.8 (0.9)	6.9	5.3	8.3
10 – 14 years	21	8.0 (1.2)	7.7	6.0	11.1
≥15 years	45	9.0 (1.4)	8.8	7.2	12.5
Spleen depth (cm)					
< 5 years	21	6.3 (0.7)	6.2	5.1	8.1
5 – 9 years	22	7.1 (0.9)	7.1	5.4	8.5
10 – 14 years	21	8.1(1.3)	7.7	6.2	10.7
≥15 years	45	9.2 (1.5)	8.9	6.9	12.4
Spleen width (cm)					
< 5 years	21	2.6(0.3)	2.6	2.1	3.1
5 – 9 years	22	3.0 (0.5)	3.0	2.2	3.8
10 – 14 years	21	3.3 (0.4)	3.2	2.6	4.0
≥15 years	45	4.0 (0.8)	3.9	2.9	5.8
Spleen vol (cm^3)					
< 5 years	21	54.0(14.9)	53.5	32.0	86.4
5 – 9 years	22	76.9(27.3)	78.6	33.0	133
10 – 14 years	21	117(48.7)	102.8	56.2	226
≥15 years	45	180.8(87)	152.7	80.0	414.7

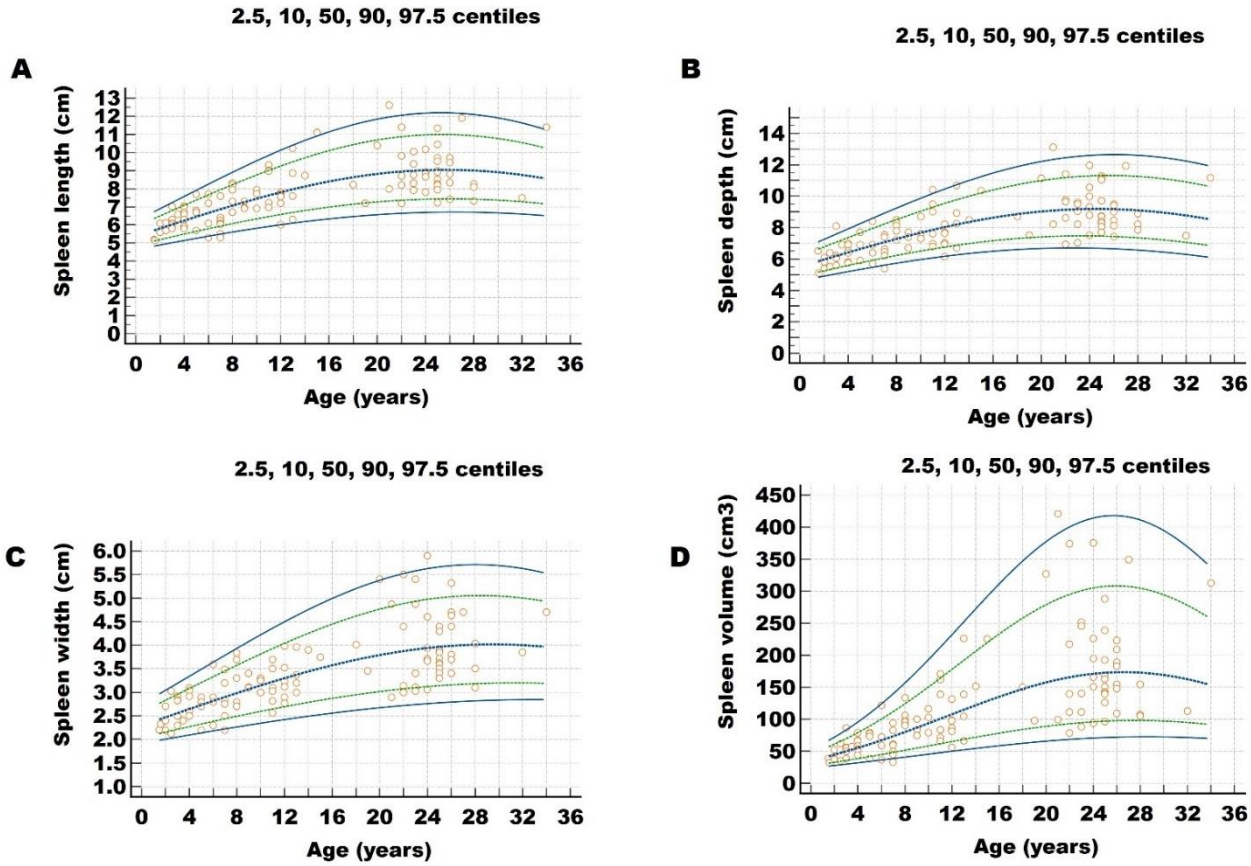


Figure 7: Percentile curves of the age-based reference limits

Legend: Specific reference limits for age 1 to 36 years are shown for splenic length (A), depth (B), width (C) and volume (D)

4.5.3 Spleen sizes among the SCD patients

Of the 204 SCD patients, the spleen was visualised in 107 (52.3%) and not visualised in 97 (47.5%) by ultrasonography. Based on the reference ranges generated from the control participants, spleen sizes were classified as small in 18 (n=18/204; 8.87%), normal in 68 (n=68/204; 33.3%) and enlarged in 21 (n=21/204; 10.3%) (Figs. 8-9). Further classification of spleen size across age groups and genotype among the SCD patients is shown in Table 3. Enlarged spleens were more frequently found in children less than five years old by both spleen length (31%) and volume (35.7%), and among patients 15 years above (25% and 20% by length and volume respectively). Small-

sized spleens were more frequently encountered in the age group 10-14 years using either the spleen length (21.1%) or volume (31.6%) respectively (Table 4).

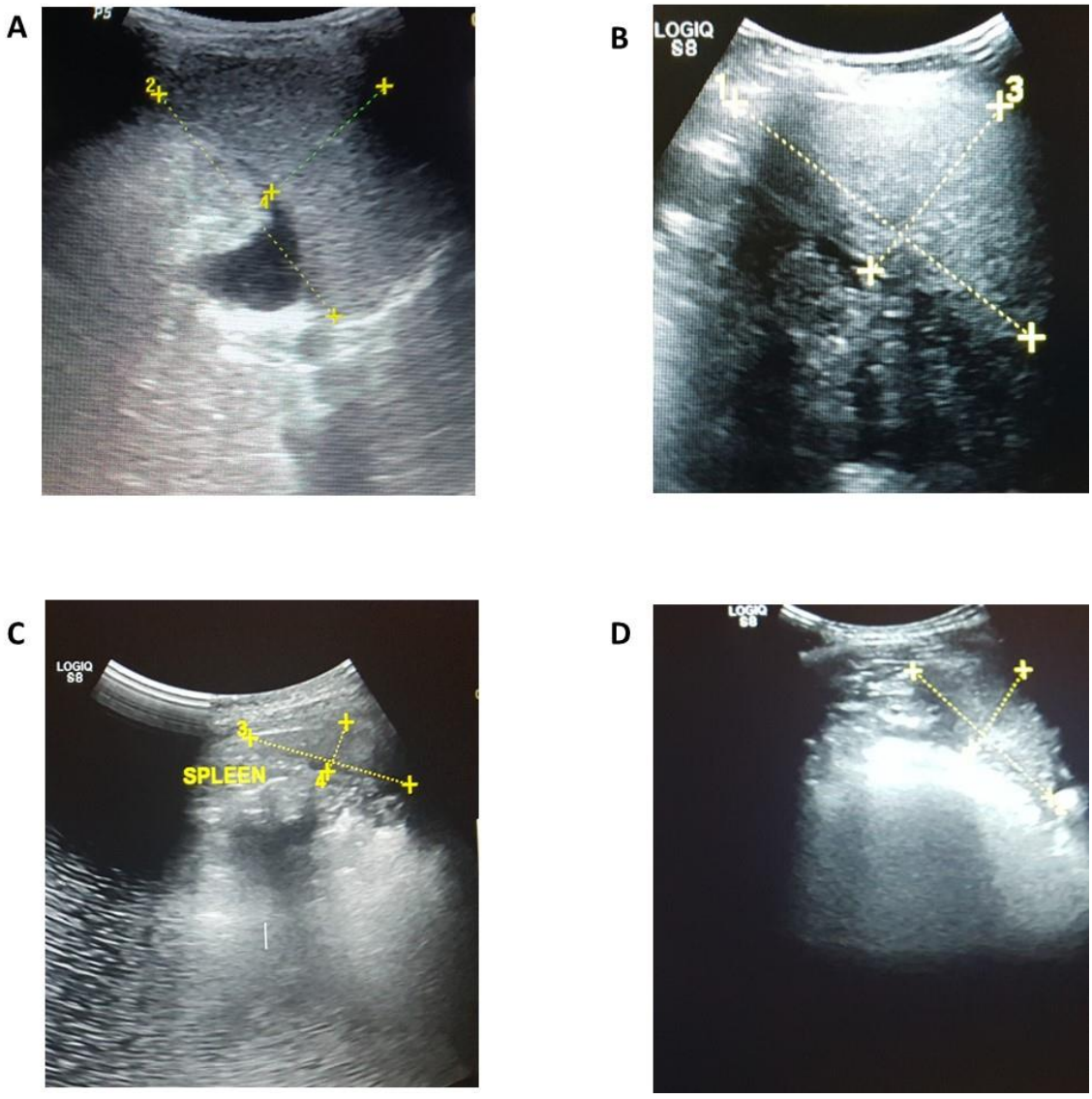


Figure 8: Abdominal ultrasonography showing normal and small-size spleens among SCD patients

Legend: Transverse view of the spleen in a: (A) Three-year-old Hb SS female child showing a normal sized spleen (spleen length 7.6 cm; spleen width 6.7cm) and normal echotexture. (B) 27-year-old Hb SS female with a normal sized spleen (spleen length 10.6 cm; spleen width 9.2 cm) and increased echotexture. (C) Seven-year-old Hb SS female with a small-sized spleen (spleen length 4.3 cm; spleen width 4.3 cm) and increased echotexture. (D) 20-year-old Hb SS male with a small sized spleen (spleen length 6.2 cm; spleen width 5.9 cm) and increased echotexture (spleen demarcated by callipers). (Spleen sizes are compared to controls values as shown in Table 2)

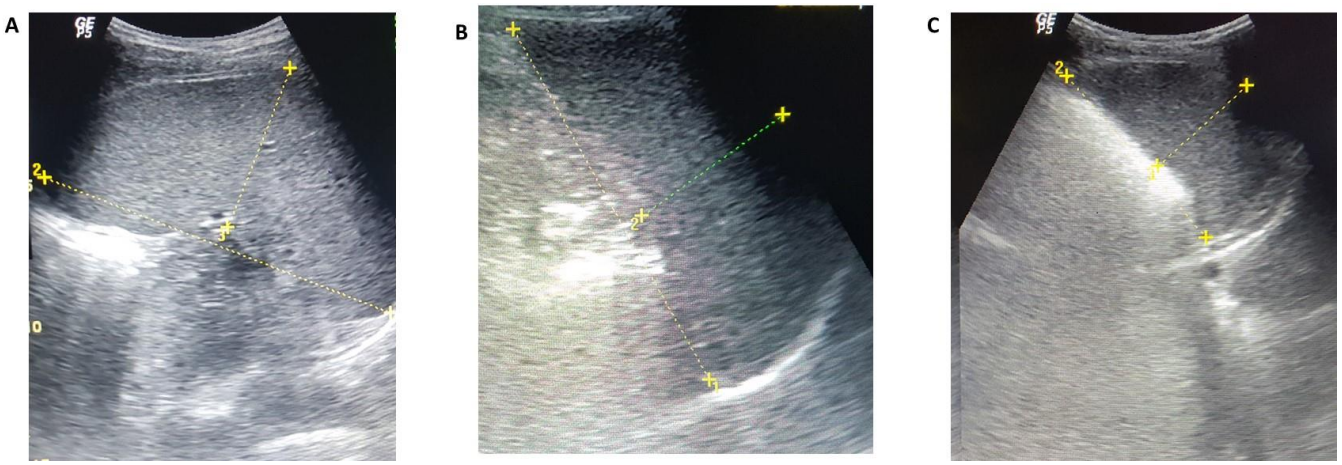


Figure 9: Abdominal ultrasonography showing enlarged spleens among SCD patients

Legend: Transverse view of an enlarged spleen in a: A) Thirty-year-old Hb Sβ thalassemia male (spleen length 13 cm; normal 7.2 cm – 12.5 cm). B) 16-year-old Hb SC female (spleen length 15 cm; normal 7.2 cm – 12.5 cm). C) 27-month-old Hb SS male child (spleen length 9.1cm; normal 5.2 cm – 7.0 cm). The spleen echotexture appeared normal in all three patients (spleen demarcated callipers).

Table 4: Frequency of different spleen sizes among the SCD patients by age group and genotype

Parameter	**Category of spleen size, n, (%)**			**Total**
	Small-sized	Normal	Enlarged	
Spleen length (cm)				
< 5 years	6 (14.3)	23 (54.8)	13 (31.0)	42
5 - 9 years	4 (15.4)	20 (76.9)	2 (7.7)	26
10 - 14 years	4 (21.1)	14 (73.7)	1 (5.3)	19
≥15 years	4 (20.0)	11 (55.0)	5 (25.0)	20
Spleen volume (cm^3)				
< 5 years	4 (9.5)	23 (54.8)	15 (35.7)	42
5 - 9 years	2 (7.7)	17 (65.4)	7 (26.9)	26
10 - 14 years	6 (31.6)	12 (63.1)	1 (5.3)	19
≥15 years	5 (25)	11 (55.0)	4 (20.0)	20
Genotype				
HbSS	17	67	19	103
HbSC	1	1	1	3
HbSβ thal	0	0	1	1

Age-group based reference ranges were generated from the corresponding spleen length and volume of the controls. Spleen size below the 2.5th centile were considered small size; normal-sized spleen are dimensions between the 2.5th and 97.5th centiles; enlarged spleens are values above the 97.5th centiles.

4.5.4 Comparison of spleen size and relationship with age between the SCD patients and the controls

Spleen length among the SCD patients with visible spleens was compared with those of normal controls. The mean spleen length (P = 0.0001) and volume (P = 0.002) were significantly higher in the control group compared to SCD patients. This difference was particularly striking in those aged 10 - 14 years (Table 5). Of note however, the spleen length was almost three-fold higher among the SCD patients compared to the controls in the first two years of life, followed by a rapid decline in spleen size around the third year (Fig.10A). Also, the fitted line between spleen size and age had a positive slope among different age groups of the control group (Fig.10B), while the slope was negative across all age groups among the SCD patients (Fig.10C).

Table 5: Comparison of spleen sizes between SCD patients and controls

Parameters	**Sickle cell disease patients (n=107)**			**Normal controls (n=109)**			**P value**
	N	**Mean(SD)**	**Median**	**N**	**Mean (SD)**	**Median**	
Spleen length (cm)							
< 5 years	42	6.4(1.2)	6.5	21	6.2(0.6)	6.1	0.423
5 – 9 years	26	6.6(1.3)	6.5	22	6.8(0.9)	6.9	0.096
10 -14 years	19	7.0(1.8)	7.0	21	8.0(1.2)	7.7	0.004*
>15 years	20	9.9(3.6)	8.8	45	9.0(1.3)	8.8	0.921
Total	107	7.2 (2.3)	6.7	109	7.8 (1.6)	7.7	0.000*
Spleen vol (cm^3)							0.020*
< 5 years	42	73.8(33.7)	66.4	21	53.9 (14.6)	53.5	0.979
5 – 9 years	26	85.7(55.0)	66.5	22	76.9 (28.0)	78.6	0.013*
10 -14 years	19	90.0(60.0)	70.4	21	117.0(48.3)	102.0	0.599
>15 years	20	258.0(270)	145.7	45	183.6(84.7)	154.4	0.002*
Total	107	114 (140)	70.4	109	124 (80.8)	99.5	

* P value: Mann Whitney U test for within age group analysis

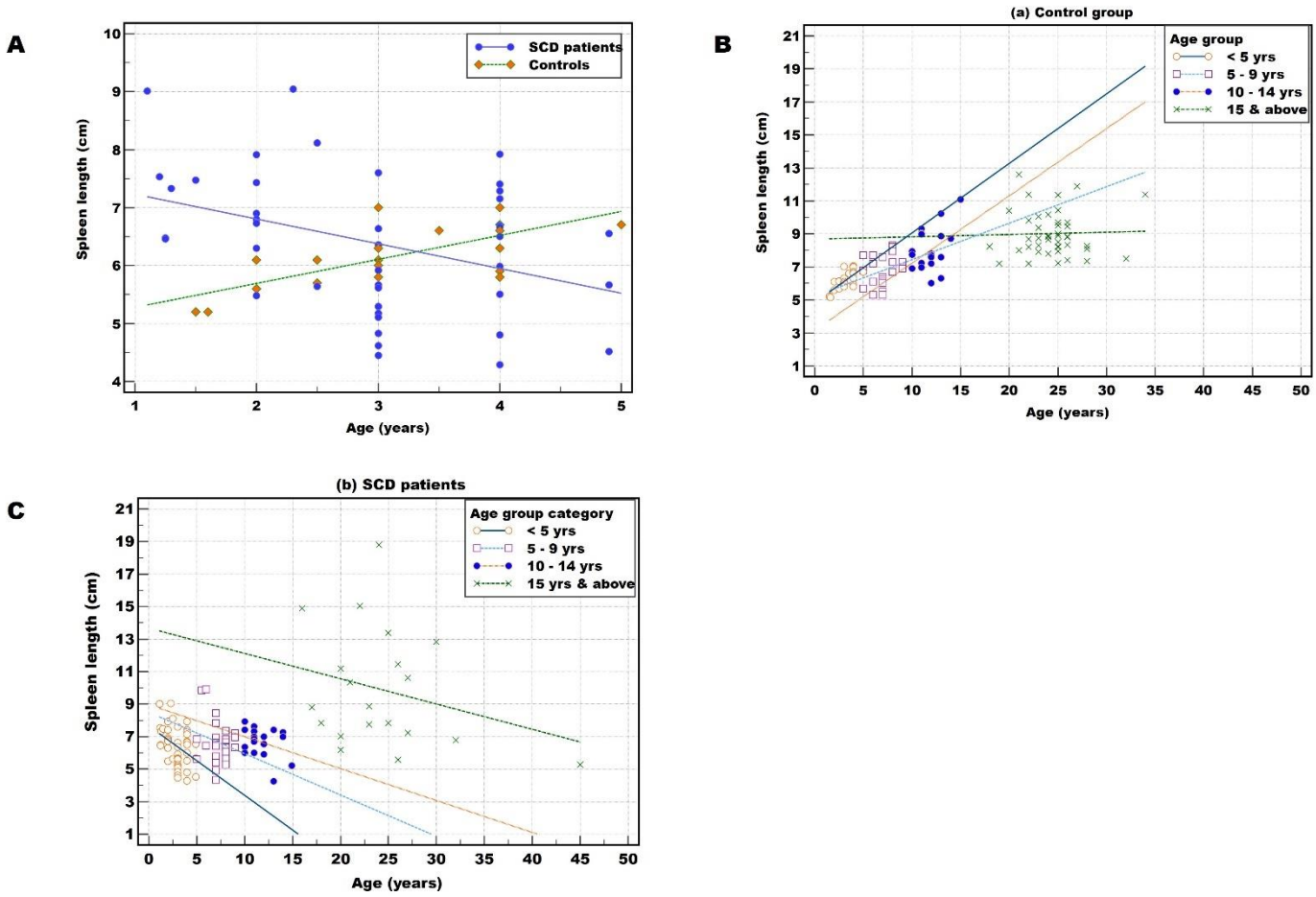


Figure 10: Scatter plot showing the relationship between spleen length and age among the SCD and controls

Legend: (A) Scatter plot showing the relationship between spleen length (Y-axis) and age (X-axis) among the less than five years SCD patients (n=42) and normal controls (n=22). The spleen size was almost three-fold larger in the SCD population in the first two years of life compared to the controls, before decreasing abruptly around the third year; thereafter, the spleen size becomes variable as SCD with both small and large spleens could be observed. Regression lines are superimposed for both populations. (B) Scatter plot showing the relationship between spleen length (Y-axis) and age (X-axis) among the control group (n=109). The spleen length shows a progressive rise with increasing age up to the third decade before levelling off. Regression lines are superimposed for all age groups. (C) Scatter plot showing the relationship between spleen length (Y-axis) and age (X-axis) among the SCD patients (n=107). The spleen length in the those less than five years (n=42) and those aged 5 to 9 years (n=26) were similar and both showed a downward trend. The spleen length becomes static in those aged 10 to 14 years (n=19) ; after 15 years the spleen length becomes variable with normal and enlarged spleens present (NB this represents the remaining 20 out of the 85 patients among this age group that still have their spleens visible). Regression lines superimposed for all age groups.

4.6 Discussion

4.6.1 Overview of findings

Ultrasonography is routinely used to evaluate the size of internal organs such as the spleen, because it is non-invasive, is not associated with radiation exposure, it provides real-time information and it is widely available in resource-limited settings (Pelizzo *et al.*, 2018); however, there are no standards for defining spleen size among SCD patients. Apart from the influence of age and geographical differences, variations in spleen sizes recorded in different studies can be attributed to differences in ultrasonography techniques and the parameters used to classify spleen size as normal or abnormal (Lamb *et al.*, 2002; Rosenberg *et al.*, 1991). We documented splenic size and volume among apparently healthy participants from the same geographical area and used this as a reference for assessing spleen size among our SCD patients. We generated non-parametric (2.5^{th} - 97.5^{th} percentiles) reference ranges according to four age groups and percentile curves to provide specific reference values for spleen sizes for individuals aged 1-36 years. The same radiologist obtained splenic measurements from all the study participants to ensure consistency and reduce inter-operator bias.

Differences in spleen sizes across race and geographic locations has been attributed to genetic and environmental factors including endemicity of infections associated with splenic enlargement. Our data on splenic length among the controls were similar to previous findings among paediatric (Tsehay *et al.*, 2021; Ezeofor *et al.*, 2014; Eze *et al.*, 2013) and adult population in Africa (Agwu and Okoye, 2005; Mustapha *et al.*, 2010; Ehimwenma and Tagbo, 2011). In contrast, the upper limit of spleen length in our controls aged 1-4 years (median, 6.1 cm), 5-9 years (median 6.9 cm) and 10-14 years (median 7.7 cm) were smaller when compared among a normal population in the USA aged 2-4 years (median 7.4cm), 6-8 years (median 8.2 cm) and 10-12 years

(median 9.9 cm) (Rosenberg *et al.*, 1991). Our data for the upper limit of spleen length were also smaller than those recorded among comparable age groups in Europe (Pelizzo *et al.*, 2018). In a study involving 631 American athletes, the spleen was larger in white Americans compared to the African-American athletes (Hosey *et al.*, 2006). These findings suggests that the spleen may be inherently smaller in the African population compared to the White population, contrary to the general notion of the spleen being bigger in areas where infections like malaria are endemic. Other studies from Nigeria have made similar observations of smaller spleen sizes among their population compared to published data among the White population (Agwu and Okoye, 2005; Mustapha *et al.*, 2010; Ehimwenma and Tagbo, 2011). This underscores the importance of using population-specific reference values in classifying spleen sizes among individuals with SCD and other disease conditions affecting the spleen.

4.6.2 Spleen size using spleen length or volume

Whereas most studies used spleen length to define splenomegaly in SCD (Ugwu *et al.*, 2018; Okongwu *et al.*, 2018; Luntsi *et al.*, 2018; Babadoko *et al.*, 2012; Akinlosotu *et al.*, 2018; Akpan, 2015; Kaushal *et al.*, 2014), others used spleen volume (McCarville *et al.*, 2011; Yakubu CI, 2017; Abdullahi, Hassan-Hanga and Ibrahim, 2014; Al-Salem *et al.*, 1998a) or both length and volume (Eze *et al.*, 2015; Olatunji and Olatunji, 2001). Given the strong positive correlation between spleen length and volume in our normal controls (rho = 86.4; P = 0.0001), consistent with earlier reports (Lamb *et al.*, 2002; Bezerra *et al.*, 2005), both parameters were used to classify spleen sizes. A drawback of using spleen volume lies with the use of the prolate ellipsoid volume method in its determination formula. However, the spleen can become irregularly shaped in SCD patients due to the recurrent vaso-occlusion and infarctions, therefore, estimating volume using this formula may not be accurate (Hosey *et al.*, 2006). Despite

similarities in the range of values obtained for the different categories of spleen sizes using spleen length and volume, we noted a tendency for higher frequencies of enlargement when using spleen volume. Furthermore, the ease of acquiring spleen length when compared to the cumbersome nature of assessing volume favours the use of spleen length to determine spleen size in routine clinical practice.

4.6.3 Comparison of spleen size between the SCD patients and controls

More than half of our SCD patients had their spleens visible on ultrasonography; we used the reference ranges generated from our controls to classify these spleens as normal-sized, small-sized or enlarged. About a third of patients (33.3%) had spleen length within the normal range. Small-sized and enlarged spleens were observed in 8.8% and 110.3% of the SCD patients respectively. The majority of the controls had normal-sized spleens; only 3.4% had enlarged spleens and they were mostly among those less than five years of age. We noted a difference in the pattern of age-related increase in spleen size between the controls and SCD patients. Among the controls, the splenic length showed a progressive increase in children less than five years to the adult mean size of 9.0 cm before levelling off, consistent with reports from the USA (Rosenberg *et al.*, 1991), Europe (Pelizzo *et al.*, 2018), and India (Arora *et al.*, 2010). The SCD patients in our cohort had larger spleens early in life, but the rate of increase in length was slow thereafter. Compared to the progressive increase in length observed in the control group, the splenic length remained steady in SCD patients aged 5 - 9 years group, with a slight increase in the 10-14 years group, before increasing to the adult (>15 years) mean length of 9.9 cm. The slow increase in spleen size observed during childhood and adolescence among the SCD patients is indicative of progressive

splenic injury, thereby counteracting the normal age-related physiological increase in spleen size observed in the control group.

4.6.4 Clinical implications

Although the spleen is usually relatively larger in infants and toddlers than in adults (Pelizzo *et al.*, 2018), we observed a more than 3-fold increase in size of the spleen among the SCD patients compared to the normal controls in the first two years of life. This is not unexpected as obstruction of the inter-endothelial slits in the basement membrane by the relatively rigid sickled red blood cells could result in passive splenic enlargement (Bowdler, 2001). Extra medullary haemopoiesis and infections may also account for enlarged spleens in early childhood among SCD patients (Topley *et al.*, 1981; Rogers, Vaidya and Serjeant, 1978). Although the majority of our older SCD patients had no visible spleen on ultrasonography, the spleens among a quarter (n=5/20) of those with visible spleens were found to be markedly enlarged. It is not clear if the presence of compound heterozygosity for the Hb S gene may be associated with this finding (they consisted of 3 HbSS, 1 HbSC and 1 HbS thal). Patients with enlarged spleens may be prone to complications related to splenomegaly including sub-clinical sequestration and hypersplenism resulting in worsening anaemia, splenic infarction (Diagne *et al.*, 2010) and may need close monitoring. Furthermore, the finding of enlarged spleens in 31% (n = 13/42) of our SCD patients less than five years of age using ultrasonography compared to 4.8% (n = 2/42) by clinical examination aptly demonstrates the low sensitivity of the latter technique and the fact that the spleen may be enlarged 2-3-fold before it becomes palpably enlarged. All the three patients with past history of acute splenic sequestration crisis, also had evidence of enlarged spleens on ultrasonography. It is possible that red cell sequestration and hypersplenism occurs within a spleen that is not palpable and so splenic

ultrasonography may be a useful adjunct to clinical management. The strong correlation among the splenic dimensions indicates that reliable measurements of the spleen can aid diagnosis and follow-up of this group of patients.

4.6.5 Limitation

Our study has some limitations. Being a hospital-based and single centre study with small sample size used to generate the reference ranges may limit generalizability of the findings. Having a single board-certified radiologist to obtain all images in healthy participants and SCD group brought about consistency and improved accuracy of comparison between the groups, but this may not be possible in routine practice.

4.7 Conclusion

This study has provided reference ranges and percentile curves for splenic dimensions for different ages based on ultrasonography among normal controls in North-Eastern Nigeria and may be of value in assessing spleen sizes among SCD patients living in malaria-endemic regions. The spleen length was three-fold higher in the first two years of life among the patients compared to controls, but this was followed by a progressive age-related decline in size suggesting progressive splenic injury. Regular spleen scans can help identify patients with enlarged spleens, who may require close monitoring for development of complications such as subclinical acute sequestration (especially in vulnerable age groups) and hypersplenism.

CHAPTER 5: DETERMINANTS OF SPLENIC PRESERVATION AMONG PATIENTS WITH SICKLE CELL DISEASE IN NORTH-EASTERN NIGERIA

5.1 Chapter overview

Having shown that the spleen in some SCD patients could not be identified on ultrasonography in the preceding chapter (Chapter 4), the focus of this chapter was on identifying both clinical and laboratory factors associated with splenic preservation among SCD patients. The role of some of the factors associated with splenic preservation mentioned in the literature review (Chapter two) are explored in this chapter.

Chapter 5 was published by the journal of 'Tropical Medicine and International Health' and can be accessed using the link: http://doi.org/10.1111/tmi.13910

5.2 Abstract

Background

In patients with sickle cell disease (SCD), the spleen commonly enlarges during early childhood, but undergoes reduction in size and fibrosis from repeated episodes of vaso-occlusion and infarction. The rate of progression of this process varies markedly among these patients. The aim of current study was to explore clinical and laboratory factors associated with the preservation of the spleen among these patients.

Methods

Two hundred and four SCD patients (103 females; age 1- 45 years) underwent abdominal ultrasonography at the University of Maiduguri Teaching Hospital, Nigeria between October 2020 to November 2021 to assess for splenic visualisation and echotexture. Steady-state clinical parameters and blood samples for full blood count,

serum chemistry, high-performance liquid chromatography, and malaria parasitaemia were obtained from all the patients.

Results

The spleen was visualised in 107 (52.4%; 95% CI, 46% - 59%) SCD patients on ultrasonography. While, the spleen was visualised in all children less than five years of age, it was visualized in only 23.5% of those aged 15 years and above. Visualisation of the spleen was significantly associated with low mean corpuscular haemoglobin concentration and high HbF in those less than 10 years. The odds of visualisation of the spleen on ultrasonography increased by a factor of 1.17% for every 1% increase in HbF level. Only 32 (15%) patients were on regular hydroxyurea therapy. The HbF level was significantly higher among patients on hydroxyurea (median 12.7 vs 7.4; $P < 0.0001$).

Conclusion

In patients with SCD, failure to visualise the spleen was not found in children less than 5 years old. Patients with visualised spleens had a higher level of HbF compared to those with non-visualised spleens. HbF was significantly associated with visualisation of the spleen before 10 years of age. Since early administration of hydroxyurea will increase Hb F level, we expect that it would help to preserve the spleen.

5.3 Introduction

Sickle cell disease (SCD) is an inherited condition characterized by recurrent haemolytic and vaso-occlusive episodes leading to acute and chronic tissue ischaemia, infarction, and chronic organ damage (Rosse *et al.*, 2000). The spleen is one of the organs affected by SCD early in life with evidence of hyposplenism present in the first 12 months of life (Rogers, Vaidya and Serjeant, 1978; O'Brien *et al.*, 1976; Pearson, Spencer and Cornelius, 1969). The spleen acts as a filter in the bloodstream by

removing old or damaged red cells, foreign bodies and microorganisms that have gained access to the blood. In SCD, the high number of damaged red cells may clog up this filter, causing an initial enlargement and later progressive splenic fibrosis. A detailed description of this process was first published by Diggs in 1935 (Diggs, 1935). During early childhood, the spleen is enlarged because of congestion of the reticular space with large numbers of sickled red cells and dilation of the capillaries in the white pulp. By late childhood, repeated episodes of vascular occlusion and infarction result in the progressive destruction and contraction of the spleen, which eventually becomes a small, wrinkled mass of fibrous tissue (Diggs, 1935). Furthermore, splenic function is usually lost early in life even in the presence of an enlarged spleen (functional asplenia) (Pearson, Spencer and Cornelius, 1969); the loss of splenic function renders the patients at increased risk of infection with encapsulated bacteria, which can be prevented by penicillin prophylaxis and immunization (Booth, Inusa and Obaro, 2010; Obaro and Tam, 2016).

In the majority of patients, vaso-occlusive events in the spleen are clinically silent and the rate of progression of this process varies markedly among individuals with the disease (Pearson *et al.*, 1985). Also, the sequence of events may be modified in different geographic areas by the frequency of other genetic factors, *HBB* haplotypes, and environmental factors (Serjeant, 2002). In a large Jamaican cohort followed from birth, 64% of children aged 9-17 years had visualized spleens on ultrasonography (Walker and Serjeant, 1993). The higher level of HbF, greater frequency of alpha thalassemia and Asian haplotype in some Arab and Indian populations with SCD results in persistence of the spleen into adulthood (Serjeant, Hambleton and Serjeant, 2021; Al-Salem *et al.*, 1998a; Adekile *et al.*, 1996). Malaria endemicity may promote the persistence of the spleen; the spleen was palpably enlarged up to adulthood among

15-35% of SCD patients in studies from Southern Nigeria (OT Ojo, WA Shokunbi and Agunloye, 2014; Yetunde and Anyaegbu, 2001). Also, within a particular region, the spectrum of events may differ from one locality to another, for instance in Nigeria, the spleen size varies among SCD patients in the North compared to those in the Southern part (Adekile *et al.*, 1993).

In the current study, we determined the frequency of SCD patients with spleens present or absent based on ultrasonography across various age groups. We assessed factors that could potentially influence persistence of the spleen and may be useful to improve their management including haematological parameters, HbF, hydroxyurea therapy, and malaria parasitaemia. We also investigated whether clinical makers associated with disease severity were associated with persistence of the spleen.

5.4 Material and methods

5.4.1 Study design and participants

See section 3.3.1 and 3.3.2

5.4.2 Clinical data and laboratory analysis

See section 3.3.3 and section 3.4.

5.4 3 Sonographic evaluation

See section 3.4.6

5.4.4 Statistical analysis

The data were analysed using Statistical Package for the Social Sciences (SPSS) (version 25; SPSS, Chicago, IL, USA). Categorical data were summarized using frequency and proportions, and continuous data using descriptive statistics. Comparison between gender group was performed using Mann-Whitney test, and

between age and Hb phenotype groups using the Kruskal-Wallis tests. Factors associated with visualisation of the spleen were determined using logistic regression. The goal of the analysis was to identify independent clinical and laboratory factors associated with visualisation or non-visualisation of the spleen on ultrasonography. Univariate regression analysis was performed on all variables of interest to obtain the candidate variables and their significance, which were included in the full model. The model effect was determined using the Robust estimator which allows for reliable estimates under a wider range of conditions. The Beta coefficient (logarithmic odds ratios) generated were transformed to normal odds ratios (ORs), so that reported ORs and 95% CIs were more clinically meaningful. The level of significance was set at the two-tailed P-value <0.05.

5.4.5 Ethical considerations

See section 3.6.4

5.5 Results

5.5.1 Demography and clinical characteristics of the study population

A total of 214 patients with SCD were enrolled in the study, however, only 204 of these participants presented for the ultrasonography (95.3%), while 10 patients failed to turn up. The general characteristics and baseline laboratory data of the patients are summarized in Table 6. The median age was 12.5 years (25^{th} to 75^{th} percentile: 7 – 22 years). The Hb phenotypes consisted of homozygous sickle cell disease (Hb SS) (n = 196/204; 96.1%), sickle-haemoglobin C disease (Hb SC) (n = 5/204; 2.4%), and sickle cell β-thalassaemia (Hb Sβ) (n=3/204; 1.5%). In the year preceding the start of the study, the majority of the patients had more than one episode of fever (90.2%) or painful crises (58%), and 32.7% of patients had been hospitalised. Thirty-two (15%)

patients had been taking hydroxyurea regularly over the preceding 12 months, whereas 10 patients previously on treatment with hydroxyurea stopped using it for various reasons including side effects and financial constraints; the remaining patients have never heard of the drug (n=162). The majority of the study participants showed a high compliance for the use of bed nets (84.8%) and were on regular antimalaria chemoprophylaxis (78.9%) with proguanil. History of completion of routine immunisation obtained from parents or guardians of the younger patients showed a high compliance, whereas most of the older patients were unsure of their immunisation history during childhood.

Table 6: Characteristics of the studied population

Variable	Median (25^{th} -75^{th} percentile) or %	Min-Max	Observation (n)
Gender (Male/Female), n	101/103		204
Age (years)	12.5 (7 - 22)	1.1- 45	204
Hb phenotype HbSS, n (%) HbSC, n (%) HbSB thal, n (%)	 196 (96.1) 5 (2.4) 3 (1.5)		204
Age group Less than 5 years, n (%) 5 to 9 years, n (%) 10 to 14 years, n (%) 15 years and above, n (%)	 42 (20.6) 35 (17.1) 42 (20.6) 85 (41.7)		204
Clinical parameters			
Hospitalisation over last 12 months	0 (0 -1)	0 - 5	204
Febrile episodes over last 12 months	2.0 (1 - 3)	0 - 12	204
Painful crisis over last 12 months	2.0 (1 - 3)	0 - 12	204
Proportion of patients on regular hydroxyurea, n (%)	32 (15.7)		204
Proportion of patients on regular antimalaria prophylaxis	161 (78.9)		204
Proportion of patients using bed net	173 (84.8)		204
Routine immunisation completed. Yes No Not sure	 94 (46.1) 21(10.3) 89 (43.6)		204
Proportion of patients on penicillin prophylaxis*	2 (4.8)		42
Laboratory parameters			
WBC count ($\times 10^3$ /µL)	13.0 (10.8 -16.3)	3.2 – 34.0	202
Haemoglobin (g/dl)	7.3 (6.4 – 8.3)	3.2 -14.2	203

Platelets ($\times 10^6/\mu L$)	377 (274 – 514)	53.0 – 854.0	203
Reticulocytes (%)	15.0 (8 - 25)	0.0 – 40.0	179
MCV (fl)	83.2 (77 – 89)	55.0 – 118.0	202
MCH (pg)	28.9 (27 – 31)	18.3 – 39.0	203
MCHC (g/d)	35 (33 – 36)	30.0 – 39.0	203
Bilirubin (total) (µmol/l)	30 (19 – 46)	7.0 – 151.0	176
ASAT (µmol/l)	15 (10 – 28)	3.0 – 89.0	176
HbF (%)	8.7 (4.8 – 13.7)	0.8 – 33.3	182
HbA_2 (%)	3.3 (2.7 -3.8)	2.7 – 3.8	143
Malaria parasite positive, n (%)	49 (31)		158

Continuous variables are presented as median, interquartile range, while categorical variables are presented as absolute (n) and relative (%) frequency. ASAT: aspartate amino transferase; WBC white blood cell; Hb haemoglobin; MCV mean corpuscular haemoglobin; MCH mean corpuscular haemoglobin concentration; MCHC mean corpuscular haemoglobin concentration. * Data obtained among the less than five years only.

5.5.2 Frequency of splenic visualisation on ultrasonography based on age, gender, and genotype.

The spleen was visualised in 107 (52.4%; 95% CI, 46% - 59%) SCD patients. The frequency of non-visualised spleens increased with age from 0% in all the children less than five years to 76.5% in those aged ≥15 (Fig.11). There was no difference in visualisation of the spleen by gender (P= 0.091) or Hb phenotype (P=0.691).

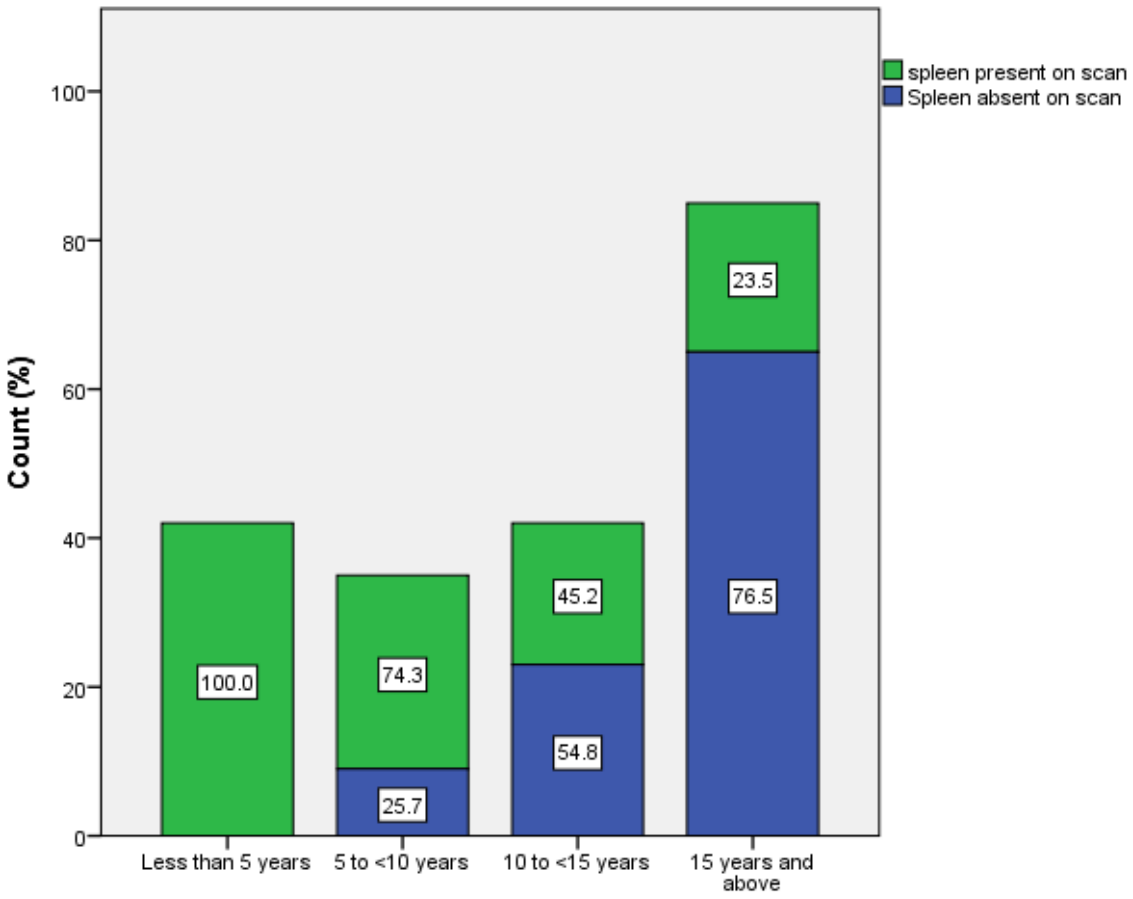


Figure 11: Prevalence of visualised or non-visualised spleens on ultrasonography

Legend: Chart showing frequency of visualised or non-visualised spleens across age groups among SCD patients (n=204). The frequency of non-visualised spleen increased with increasing age.

5.5.3 Spleen parenchymal echotexture on ultrasonography

The spleen echotexture was normal in 93 (86.9%) patients. It was increased in eight patients (7.5%), while six patients (5.6%) had heterogeneous and coarse-appearing spleens with nodules of varying sizes and numbers (Figs. 12A&B). Individuals from the age groups 5-9 years and 10-14 years had more heterogeneous-appearing spleens and a higher frequency of splenic nodules than SCD patients below 5 years or above 15 years.

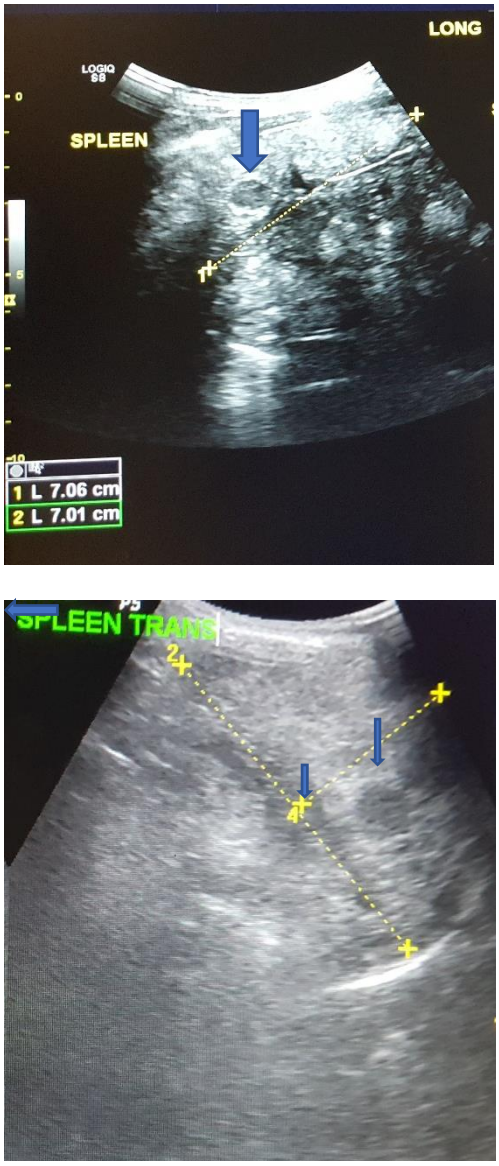


Figure 12: Abdominal ultrasonography showing splenic nodules among SCD patients.

Legend: A. Upper figure: solitary intrasplenic nodule in a 14-year-old male with HbSS. Longitudinal view of the spleen on ultrasonography shows a round hypoechoic nodule close to the hilum (blue arrow) and measures up to 2 cm. B. Lower figure Transverse view of the spleen showing multiple hypoechoic nodules (blue arrows) in a 24-year-old female with HbSS.

5.5.4 Association of clinical and laboratory factors with splenic visualisation on ultrasonography

Factors that were significantly associated with visualisation of the spleen on univariate logistic analysis included increased HbF and haemoglobin, decreased levels of platelets, decreased HbA_2, decreased red cell indices (MCH, MCHC), positive malaria parasitaemia and history of hydroxyurea therapy (Table 7). On multivariate logistic regression analysis, only HbF (OR 1.17, 95% CI, 1.05 - 1.29; $P = 0.003$) and MCHC (OR 0.69, 95% CI, 0.47 - 0.99; $P = 0.049$) remained significantly associated with spleen visualisation (Table 8). The odds of visualisation of the spleen on ultrasonography increased by a factor of 1.17% for every 1% increase in HbF level. A subgroup analysis was performed to determine the influence of HbF across the various age groups; as the spleen was visualised in all children less than five years, the analysis was confined to the remaining age groups. HbF was significantly associated with visualisation of the spleen among the 5-9 years group (OR 1.29, 95% CI, 1.03 - 1.62; $P = 0.028$), but not among the 10-14 years age group (OR 1.29, 95% CI, 0.93 - 1.14; $P = 0.591$) or those above 15 years (OR 1.03, 95% CI, 0.94 - 1.14; $P = 0.488$). Gender and clinical parameters including frequencies of hospitalisation, painful crises, and febrile episodes, had no association with splenic visualisation (Table 8). Only 32 (15%) patients were on regular hydroxyurea; HbF% level was significantly higher among these patients compared to the patients not on hydroxyurea (median 12.7% vs 7.4%; respectively. $P < 0.0001$).

Table 7: Unadjusted odd ratios for associations with spleen visualisation or non-visualisation on ultrasonography

Variables	**Spleen not visualized. (n=97)**	**Spleen visualized (n=107)**	**OR (95% CI)**	**P value**
Male, n/n (%)	42/97 (43.3)	59/107 (55.1)	0.62 (0.36 -1.08)	0.092
Laboratory parameters				
WBC count, $\times 10^3$ /µL, mean (SD)	13.4 (3.5)	13.8 (5.2)	1.02 (0.96 - 1.09)	0.454
Hb, g/dl, mean (SD)	7.0 (1.6)	7.8 (1.7)	1.38 (1.14 - 1.66)	0.001*
MCV, fl, mean (SD)	84.1 (8.5)	81.9 (9.7)	0.97 (0.94 -1.00)	0.115
MCH, pg, mean (SD)	29.5 (3.5)	27.9 (3.6)	0.88 (0.80- 0.96)	0.006*
MCHC, g/dl, mean (SD)	35.1 (1.7)	34.1 (1.7)	0.70 (0.58 - 0.85)	0.0001*
Platelets, $\times 10^6$/µL, mean (SD)	445.5 (163.3)	348.8 (161.9)	0.99 (0.99 - 0.99)	0.0001*
Reticulocytes, %, mean (SD)	8.7 (4.7)	8.0 (5.2)	0.97 (0.92 -1.02)	0.364
HbF level, %, mean (SD)	7.5 (5.2)	12.2 (7.4)	1.13 (1.06 - 1.19)	0.0001*
HbA_2 level, %, mean (SD)	3.6 (1.5)	3.2 (0.8)	0.67 (0.46 - 0.98)	0.039*
Malaria parasite positive, n/n (%)	14/68 (20.6)	35/90 (38.9)	2.45 (1.19 - 5.07)	0.015*
Clinical parameters				
Proportion of patients on hydroxyurea, n/n (%)	9/97 (9.3)	23/107 (21.5)	2.68 (1.17 - 6.12)	0.020*
Hospitalization over last 12 months, mean (SD)	0.38 (0.8)	0.63 (0.9)	1.42 (0.99 -2.03)	0.057

Febrile episodes, mean (SD)	2.68 (2.3)	2.52 (1.7)	0.96 (0.84 - 1.01)	0.573
Vaso occlusive crisis, mean (SD)	2.43 (2.7)	2.52 (2.7)	1.01 (0.91 - 1.12)	0.811

Continuous variables are presented as mean ± standard deviation (SD);while categorical variables are presented as absolute (n) and relative (%) frequency. WBC white blood cell; Hb haemoglobin; MCV mean corpuscular haemoglobin; MCH mean corpuscular haemoglobin concentration; MCHC mean corpuscular haemoglobin concentration; SD standard deviation. CI confidence interval: OR indicates the odds ratio for patients with spleen visualized or not visualized on scan. Patients with non-visualized spleens were used as the reference category. *All P values were less than .05.

Table 8: Adjusted odd ratios for associations with spleen visualisation or non-visualisation on ultrasonography .

Parameter	**Co-efficient (Standard error)**	**Adjusted OR (95% CI)**	**P value**
Hb, g/dl	0.07 (0.2)	1.07 (0.78 – 1.46)	0.675
MCH, g/dl	-0.16 (0.1)	0.86 (0.72 – 1.02)	0.077
MCHC, pg	-0.37 (0.2)	0.69 (0.47 – 0.99)	0.049*
Platelets, $\times 10^6/\mu L$	-0.00 (0.0)	0.99 (0.99 -1.00)	0.274
HbF level, %	0.15 (0.1)	1.17 (1.05 -1.29)	0.003*
HbA_2 level, %	-0.29 (0.4)	0.75 (0.35 – 1.58)	0.447
Malaria parasite positive	0.94 (0.9)	2.55 (0.96 - 6.75)	0.060
Hydroxyurea intake	0.61 (0.7)	1.84 (0.42 – 7.99)	0.418

Hb haemoglobin; MCH mean corpuscular haemoglobin; MCHC mean corpuscular haemoglobin concentration; CI confidence interval: OR indicates the odds ratio for patients with spleen visualized or not visualized on scan. Patients with non-visualized spleens were used as the reference category. *All P values were less than .05

5.6 Discussion

5.6.1 Overview of findings

We have documented the visualisation or non-visualisation of the spleen across various age groups among SCD patients using ultrasonography. Factors associated with preservation of the spleen were also explored. In our study, the spleen was visualised on ultrasonography among all the children less than five years, but declined in prevalence with successively older age groups, so that only about a quarter of patients had their spleens visualised on ultrasonography after the age of 15 years. A similar pattern was noted recently among a large Jamaican birth cohort of SCD patients (n=2138); non-visualised spleens increased with age from 34% in those aged 6.0-7.9 years to 72% in those aged 24 years and above (Walker *et al.*, 2022). In contrast, a study among SCD children in the United Kingdom (n=100), showed that only 5.6% of children aged 6–10 years and 19.4% of children aged 11–16 years had no visible spleen on ultrasonography (Nardo-Marino *et al.*, 2022). Our current observation that the spleen was visualised in some patients and not in others within each of the age groups indicates the variability in the rate of progression of splenic injury amongst SCD patients.

The overall prevalence of patients with non-visualised spleens in our study was 47.2 %. This is similar to previous reports from Northern Nigeria (32% to 36%) (Luntsi *et al.*, 2018; G B Inah and Ekanem, 2018) and Sudan (47.8%) (Attalla, 2010a), but contrasts with the lower rates reported from South-East (23% to 33%) (Akpan, 2015; Eze *et al.*, 2013; Ezeike, 2019) and South-West Nigeria (0 -11 %) (Olatunji and Olatunji, 2001; Okongwu *et al.*, 2018; OT Ojo, WA Shokunbi and Agunloye, 2014), and among SCD patients in the Democratic Republic of Congo (6.7%) (Banza *et al.*, 2019). Frequency of non-visualised spleens in our study was also similar to those

reported in SCD patients in Turkey (42.9%) (Balci *et al.*, 2008) and the USA (35.7%) (Gale *et al.*, 2016). Lower frequencies have been found in Asia (11%) (Kaushal *et al.*, 2014), the Middle-East (6.1%) (Al-Salem *et al.*, 1998a) and the USA (9%) (Nardo-Marino *et al.*, 2022). The variability of splenic non-visualisation on ultrasonography in the different geographic location may partly be explained by the presence of genetic factors known to inhibit sickling such as alpha thalassaemia and HbF (Walker *et al.*, 2022; Serjeant, Hambleton and Serjeant, 2021), improvement in clinical care and outcomes in SCD (Gale *et al.*, 2016; Nardo-Marino *et al.*, 2022), and also environmental factors that results in the frequent exposure to organisms known to cause spleen enlargement including malaria and bacterial infections (Adekile *et al.*, 1988; Kizito *et al.*, 2007).

5.6.2 Splenic parenchymal appearances

An abnormal pattern and splenic nodules were more frequent among our patients in the age groups 5-9 years and 10-14 years; discreet hypoechoic nodules were seen in 5.6% and in some cases, the nodules were multiple. Splenic infarcts may appear wedge-shaped or as rounded hypoechoic areas on ultrasonography (Lonergan, Cline and Abbondanzo, 2001). None of the patients had symptoms related to the spleen at the time of our examination. A similar age pattern has been observed in children and adolescents with SCD in Nigeria (Eze *et al.*, 2015) and USA (Gale *et al.*, 2016). An abnormal splenic echotexture may represent the severity and frequency of vaso-occlusion (Lonergan, Cline and Abbondanzo, 2001). Similar lesions have been reported at comparable rates among SCD patients in Sudan (7.8%) (Attalla, 2010b) and in Turkey (6.0%) (Balci *et al.*, 2008).

5.6.3 Clinical and laboratory factors associated with splenic visualisation on ultrasonography.

HbF was associated with preservation of the spleen among our SCD patients; however, this association was only significant in patients less than 10 years. HbF was significantly higher among patients whose spleens were visualized compared to those without splenic visualisation. It has been suggested that the high HbF level inhibits HbS polymerisation and consequently reduces the number of irreversibly sickled cells (Serjeant, 1970). This slows down the rate of splenic fibrosis. Only a few studies have evaluated the association of preserved spleens on ultrasonography and level of HbF. One of these studies from South-West Nigeria among children with SCD demonstrated higher levels of HbF in children with visualised spleens compared to those without visible spleens (P = 0.012) (Akinlosotu *et al.*, 2018). Similarly, studies among SCD patients in Saudi Arabia and Jamaica have demonstrated high HbF levels in patients with visualised spleens compared to those without visible spleens on ultrasonography (Al-Salem *et al.*, 1998a; Walker *et al.*, 2022). Other studies from Africa (Durosinmi *et al.*, 2005; Diagne *et al.*, 2010; Mpalampa *et al.*, 2012) which all used abdominal palpation to detect splenomegaly, found no relationship between the presence of palpable spleens and HbF. Manual palpation is less sensitive than ultrasonography for evaluating the presence or absence of the spleen; this may be because the spleen is not palpable until it is two to three times its normal size (Zhang and Lewis, 1989)

The mean corpuscular haemoglobin concentration (MCHC) showed borderline association with visualisation of the spleen in our study; values were lower in patients with spleens that were visualised on ultrasonography. This is similar to a previous study among SCD in South-West Nigeria (F.A. Fasola and Adekanmi, 2019). Concurrent alpha thalassemia may be partly responsible for the low MCHC observed

in our study population, however, this could not be verified because the study was not designed to assess the co-inheritance of alpha thalassemia. Alpha thalassemia trait is common (36% to 54% of the population) in individuals of West and East African origin (Mockenhaupt *et al.*, 1999; Franklin *et al.*, 2011; Wambua *et al.*, 2006). Furthermore, studies among SCD patients in Africa have shown a high prevalence of alpha thalassemia trait (37% and 46%) (Falusi *et al.*, 1987; Rumaney *et al.*, 2014; Olatunya *et al.*, 2019). The effect of reduced alpha globin production is manifest by reduced mean corpuscular volume (MCV) and MCHC both of which are likely to reduce intravascular sickling (Steinberg *et al.*, 1984). The mean MCHC varied considerably among SCD patients in a Jamaican cohort, with lowest values occurring in those with homozygous (α–/α–) alpha thalassemia 2 and high values in those without the trait. The low MCHC inhibits HbS polymerisation; consequently, there is lower rate of haemolysis, higher haemoglobin level and persistence of splenomegaly (Higgs *et al.*, 1982). In another study, patients with homozygous (α–/α–) alpha thalassemia 2 had significantly lower MCHC, MCH, MCV, decreased levels of markers of haemolysis and more had splenomegaly than patients with a normal alpha-globin-gene complement (αα/αα); heterozygotes (α–/αα) for alpha thalassemia had intermediate values (Stevens *et al.*, 1986).

Studies examining the relationship between malaria parasitaemia and splenomegaly in SCD patients have produced contradictory results. In our study, significantly more SCD patients with visualised spleens had asymptomatic *Plasmodium falciparum* parasitaemia than those whose spleens were not visualised ($P = 0.015$), but this association just failed to reach statistical significance after adjusting for other laboratory parameters ($P = 0.060$). The use of regular anti-malaria chemoprophylaxis and bed nets among our SCD patients was high and had no effect on the frequency of

malaria parasitaemia (data not shown). A study among children with SCD from South-East Nigeria found a higher malaria parasite density among patients with splenomegaly and normal spleen sizes compared to those whose spleens were not visualised on ultrasonography (Awotua-Efebo, Alikor and Nkanginieme, 2004). The significant reduction in spleen size following treatment with antimalarial over a 6-month period among children with SCD in South-West Nigeria suggests that malaria may contribute to more frequent and marked splenomegaly ($P = 0.01$) (Adekile *et al.*, 1988). In contrast, a study among children from South-West Nigeria found no significant relationship between the spleen size on ultrasonography and malaria parasitaemia ($P = 0.469$) (Akinlosotu *et al.*, 2018). Other reports among SCD patients in Nigeria (Durosinmi *et al.*, 2005), Kenya (Sadarangani *et al.*, 2009) and Tanzania (Makani *et al.*, 2010a) found no relationship between spleen size on palpation and malaria parasitaemia. In view of the possibility of multiple and interrelated factors that may act as confounders, the exact role of malaria in influencing spleen size still remains to be elucidated in SCD patients.

No significant difference was observed in the frequency of febrile episodes and vaso occlusive crises among patients with visualised spleens compared to those with non-visualised spleens in our study. This is unexpected because with fibrosis of the spleen, and associated hyposplenism, there is increased susceptibility to infections which would be likely to increase febrile episodes, painful crisis, hospitalisation, and blood transfusion. Rather, we noted more patients with visualised spleens had been admitted to the hospital over the last 12 months compared with patients with non-visualised spleens, although the difference just failed to reach statistical significance ($P = 0.057$). It is possible that frequent infections (including malaria) may cause hyperplasia of the reticulo-endothelial system of the spleen and subsequent splenic enlargement.

Only a few patients were on hydroxyurea (15%) in our study, we noted a higher HbF level among these patients and a trend towards increased visualisation of the spleen compared to the non-hydroxyurea group, however, this did not reach statistical significance, potentially due to a lack of power. Hydroxyurea induces production of HbF and has been used as a disease modifying agent in SCD over the past three decades (Rogers *et al.*, 2011). In view of the limited effect of HbF on the presence of the spleen beyond 10 years of age observed in this study, commencing hydroxyurea early may be beneficial for preservation of the spleen. Hydroxyurea is included in the WHO Model Lists of Essential Medicines for the treatment of SCD (Organization, 2017), however, in a recent cross-sectional study involving physicians, nurses/counselors, patients and caregivers at 13 different health facilities in Nigeria, the authors demonstrated that the uptake of hydroxyurea was limited by provider prescription practices and patient adherence (Okocha *et al.*, 2022). The issue of cost and availability also played an important role in the utilisation of the drug; the cost for a pack of 100 tablets of hydroxyurea (~$40) cannot be afforded by most Nigerians (Okocha *et al.*, 2022). Therefore, subsidising the cost of hydroxyurea or providing it free of charge may pave way for wider access and may provide a simple and inexpensive oral medication that can alter the progressive splenic damage associated with SCD and help preserve the spleen.

5.6.4 Limitation

This study was limited by being a single-centre, hospital-based study which may affect generalisability of our findings as some patients from the rural community may not have been included into the study. Although, we have obtained data on the prevalence of patients with persistent spleens among SCD patients across various age groups, it is difficult to infer on factors that are causally related to preservation of the spleen

without longitudinal data. Moreover, there is a difference of splenic parameters between the Northern and Southern parts of Nigeria. This calls for more national and international collaborative studies to investigate the modifying factors.

5.7. Conclusion

Failure to visualise the spleen was not observed in patents with SCD less than 5 years old but was progressively more frequent among older age groups. HbF was significantly associated with preservation of the spleen before 10 years of age. Since early administration of hydroxyurea will increase Hb F level, we expect that it would help to preserve the spleen.

CHAPTER 6: EVALUATION OF TWO RED CELL INCLUSION STAINING METHODS FOR ASSESSING SPLEEN FUNCTION AMONG SICKLE CELL DISEASE PATIENTS IN NORTH-EAST NIGERIA

6.1 Chapter overview

In the preceding chapters, I noted some SCD still had their spleen retained up to adulthood, however, it was unclear if these spleens were still functioning. Hence, this chapter focuses on the laboratory aspect of assessing spleen function. I studied two methods of assessing spleen function to determine which method may be more suitable in resource -limited settings.

Chapter 6 was published in PLOS Global Health and can be assessed using the link: https://doi.org/10.1371/journal.pgph.0001552

6.2 Abstract

Introduction

The loss of splenic function is associated with an increased risk of infection in sickle cell disease (SCD); however, spleen function is rarely documented among SCD patients in Africa, due partly to the non-availability of sophisticated techniques such as scintigraphy. Methods of assessing splenic function which may be achievable in resource-poor settings include counting red blood cells (RBC) containing Howell Jolly Bodies (HJB) and RBC containing silver-staining (argyrophilic) inclusions (AI) using a light microscope. We evaluated the presence of HJB - and AI - containing RBC as markers of splenic dysfunction among SCD patients in Nigeria.

Methods

We prospectively enrolled children and adults with SCD in steady state attending outpatient clinics at a tertiary hospital in North-East Nigeria. The percentages of HJB

- and AI-containing red cells were estimated from peripheral blood smears and compared to normal controls.

Results

There were 182 SCD patients and 102 healthy controls. Both AI- and HJB-containing red cells could be easily identified in the participants blood smears. SCD patients had a significantly higher proportion of red cells containing HJB (1.5%; IQR 0.7% - 3.1%) compared to controls (0.3%; IQR 0.1% - 0.5%) (P <0.0001). The AI red cell counts were also higher among the SCD patients (47.4%; IQR 34.5% - 66.0%) than the control group (7.1%; IQR 5.1% - 8.7%) (P < 0.0001). The intra-observer reliability for assessment of HJB- (r = 0.92; r^2 = 0.86) and AI- containing red cells (r = 0.90; r^2 = 0.82) was high. The estimated intra-observer agreement was better with the HJB count method (95% limits of agreement, -4.5% to 4.3%; P = 0.579).

Conclusion

I have demonstrated the utility of light microscopy in the assessment of red cells containing - HJB and AI inclusions as indices of splenic dysfunction in Nigerian SCD patients. These methods can be easily applied in the routine evaluation and care of patients with SCD to identify those at high risk of infection and initiate appropriate preventive measures.

6.3 Introduction

The spleen plays an important role in protection against infection and individuals with loss of spleen function are at an increased risk of infection with encapsulated microorganisms and systemic spread (William and Corazza, 2007). In sickle cell disease (SCD), the spleen undergoes a sequence of pathological changes that ultimately result in loss of splenic function (Diggs, 1935), and this accounts for most of the early morbidity and mortality associated with this disorder (Pearson *et al.*, 1979).

The tests often used to assess spleen function are based on scintigraphy, haematological, and immunological techniques (de Porto *et al.*, 2010; Lammers *et al.*, 2012). The scintigraphy method is the gold standard and assesses the ability of the spleen to filter blood of abnormal cells and particles. However, this method is expensive and involves injecting radio-labelled substances into patients (Lammers *et al.*, 2012; Adekile *et al.*, 2002b; Rogers *et al.*, 2011; Al-Jam'a *et al.*, 2000). The haematological methods reflect the inability of the spleen to phagocytose poorly deformable red blood cells (RBCs) or those containing inclusions. Splenic dysfunction is usually evaluated on the basis of increased numbers of such abnormal RBCs in circulation. The presence of Howell-Jolly bodies (HJB) is one such example; percentages of HJB red cells can be estimated from peripheral blood smears using the classical May-Grunwald Giemsa stain (MGG) (Corazza *et al.*, 1990; Lammers *et al.*, 2012), by flow cytometry or imaging flow cytometry methods (El Hoss *et al.*, 2018; Harrod *et al.*, 2007; Peretz *et al.*, 2022; Pourdieu *et al.*, 2023). In patients with diminished splenic function, the membrane of the red blood cells contains surface indentations referred to as ‘pits’ when viewed using contrast-enhancing microscopy techniques such as interference phase-contrast (Adekile *et al.*, 1993; Pearson *et al.*, 1979; Al-Awamy, Wilson and Pearson, 1984) and differential interference contrast (DIC) microscopy (Lammers *et al.*, 2012; Serjeant, Hambleton and Serjeant, 2021; Casper *et al.*, 1976). Recently, a new fluorescence-based method and an automated deep neural network for quantifying pitted red cell counts have been developed (Sissoko *et al.*, 2022; Nardo-Marino *et al.*, 2022). The pitted red cells count method represents a sensitive technique for evaluating splenic function; however, it requires specialised equipment and personnel. Another method of evaluating spleen function is counting red cells containing silver-stained (argyrophilic) inclusions (AI). The silver

stain was originally developed to study the nucleolar organizer regions (NORs) of chromosomes and to evaluate their function (Howell and Black, 1980; Ploton *et al.*, 1986). In addition to staining the argyrophilic proteins associated with NORs, the stain can detect non-NOR silver-staining cytoplasmic granules and inclusions such as hemosiderin (appear as dark-brown to black), calcium and phosphate when applied to marrow smears (Tham and Cousar, 1993; Nikicicz and Norback, 1990). The silver stain technique was first employed to assess splenic function by a group of investigators in the USA, following their observation that red cells from SCD patients and splenectomised individuals contained large numbers of AI (Tham *et al.*, 1996). The AI count method is non-invasive and requires only a light microscope.

Spleen function has not been well studied in SCD patients residing in Africa (Ladu *et al.*, 2021), the region with more than two thirds of the global burden of SCD (Piel *et al.*, 2013a). This is mainly because most of the methods mentioned above are not readily available in most low-income settings of Africa as evidenced by the scarcity of data in the literature (Adekile *et al.*, 1991; Adekile *et al.*, 1993). The HJB and AI methods may be suitable but have not been widely used in Africa; both tests require only a light microscope and therefore are feasible in Nigeria and most low-to-middle-income countries. They can be used to facilitate the measurement of splenic function in SCD patients where resources for spleen scintigraphy and interference contrast microscopy are absent. Improvement in spleen function has been reported following chronic hyper-transfusion (Barrios *et al.*, 1993; Pearson *et al.*, 1970) and following hydroxyurea therapy; the splenic filtration function was preserved after three years in a third of the patients following hydroxyurea therapy (Hankins *et al.*, 2008; Nottage *et al.*, 2014). Therefore, assessment of splenic function among SCD patients in low-

income countries can play a vital role in identifying those patients who can benefit from such therapy.

The objective of the current study was to assess splenic function by comparing the frequencies of red cells containing HJB and AI in SCD patients with those of normal controls. I also evaluated which of the method was associated with the least intra-observer variation. If splenic function could be assessed in this manner, these techniques might be utilised for early identification of SCD patients at risk of developing severe infection because of reduced or absent splenic function, who may benefit from more intensive infection prevention measures.

6.4 Methods

6.4.1 Study participants

See section 3.3.3

6.4.2 Laboratory analysis

See section 3.4

6.4.2.1 Blood smears

Two thin blood smears, each for AI and HJB red cells estimation were made from blood samples collected by venepuncture from the study participants within 4 hours of collection and allowed to air dry. Smears for AI red cells were fixed for 3 minutes in a solution made by diluting 150 ml ethanol to 450 ml formalin (37% formaldehyde), washed in distilled water, and allowed to dry thoroughly before proceeding to staining. Smears for HJB red cells were fixed in absolute methanol for one minute and allowed to air dry before staining with May-Grunwald Giemsa (MGG) as described below. All fixed smears were stored in slide boxes if staining was not performed immediately.

6.4.2.2 Method for AI count using silver stain

The silver stain method was based on the technique described previously (Tham *et al.*, 1996), with some modifications (Appendix 22). A pilot study to assess and refine the test performance was performed using samples from controls and SCD patients. We conducted a pre-test run to check the effect of staining duration, temperature, the constitution of eosin, and pre-treatment of slides with potassium iodide on optimal staining conditions. As a result, the staining duration was reduced to 20 minutes in the final study protocol, as a longer duration was associated with over-stained slides that could not be interpreted (Appendix 29). The eosin (Thermo Fisher scientific) was constituted using an aqueous solution as this provided better counterstaining ability, compared to an alcohol-based formulation. The reduction of slides with potassium iodide did not produce any added effect; therefore, this step was not included in the final protocol. Furthermore, two different brands of silver nitrate salts (50%; Honeywell Fluka and FujiFilm) and Gelatin powder (500g: Sigma-Aldrich and Riedel De Haen) were tested and both gave the same staining quality. Finally, not more than 3 ml of working solution was prepared per staining session, as the stain began to deteriorate after about 3 minutes of preparation (Appendix 30). The slides were stained using silver stain at 38°C in dark for 20 minutes as described (Appendix 22). All stained slides were mounted with coverslips (22 x 40 mm; thickness 0.13 to 0.16 mm) using a permanent mount (DPX); this improved the clarity of microscopy and reduced the effect of reflection observed with unmounted slides.

6.4.2.3 Method for quantitative HJB count using May-Gruenwald Giemsa (MGG) stain

See section 3.4.3 and appendix 23.

6.4.2.4 Microscopy

Microscopy was performed with an upright light microscope (Leica DM 750, equipped with a ICC50 E colour camera). Smears were examined using the x 40/0.65 and x 100/0.80 (oil immersion) HI PLAN objectives. For the AI count, a minimum of 500 consecutive RBCs per smear were examined for the presence of one or more distinct black granules. Red cells with diffuse or fine reticular pattern of brown staining were not regarded as positive (Tham *et al.*, 1996). For the HJB count, 400 RBCs were counted per smear for the presence of a distinct purple to dark-purple dot. Red cells with more than one dots were not counted. The AI and HJB counts were expressed as percentages of the total red cells counted. Two blood smears each for AI and HJB red cells from each study participant when available were examined separately for the percentages of cells of interest; the mean of the two counts observed was then calculated. All counts were performed directly in the microscope eyepiece. I had undergone prior training and quality checks during the pilot phase of the project. To remove intra-observer variation and improve the precision of the microscopy results, I performed all the counts myself.

6.4.3 Statistical analysis

The data were entered into Excel spread sheet for cleaning and sorting and exported into Statistical Package for the Social Sciences (SPSS) (version 25; SPSS, Chicago, IL, USA) and MedCalc v.20.114 (MedCalc Statistical Software Ltd, Ostend, Belgium) for analysis. Categorical data were summarised using frequency and proportions while continuous data were expressed as means, median and interquartile range as

appropriate. For the AI and HJB methods, the intra-observer reliability and agreement for paired measurements performed for an individual sample were assessed. Firstly, scatter plots were drawn to assess the relationship between the two separate readings. The Pearson's correlation analysis was used to assess the intra-observer reliability for the paired readings obtained using each method. The Bland Altman analysis was used to measure the limit of agreement between the two separate readings (Bland and Altman, 1986); individual values were expressed relative to the geometric mean of the first and second counts for that sample. A non-parametric test was used to compare results between SCD patients and controls as appropriate. The level of significance was set at the two-tailed P-value <0.05.

6.4.4 Ethical considerations

See section 3.6.4

6.5 Results

6.5.1 General characteristics

Blood smears for identification of red cells containing AI and HJB were obtained from 182 SCD patients (175 Hb SS, 5 Hb SC, and 2 Hb SB thal) and 102 controls (93 Hb AA, 7 Hb AS, and 2 Hb AC). The median ages for the SCD group and controls were 11.0 years (range 1 - 45 years) and 12.0 years (range 1 - 32 years) respectively.

6.5.2 Identification of argyrophilic inclusion-positive (AI) and HJB red cells

Both AI- and HJB-containing red cells could be easily identified in the participants; representative smears for AI and HJB are shown below (Figs.13 A&B). The AI inclusions occurred either as single or multiple inclusions within a red cell (Fig.13A).

The HJB appeared as single, spherical inclusion often situated close to the cell periphery (Fig. 13B).

The distribution of HJB and AI red cells across the study population is shown in table 8. We compared the range of results obtained for red cell inclusions using both the AI and HJB methods. It was observed that the results were always higher with the silver stain method for AI red cells. Among the SCD group, the AI red cell counts ranged from 34.5% to 66.0% and HJB counts ranged from 0.7% to 3.1%. Among the controls, the AI red cells count ranged from 5.1% to 8.7% whereas the HJB counts ranged from 0.1% to 0.5%.

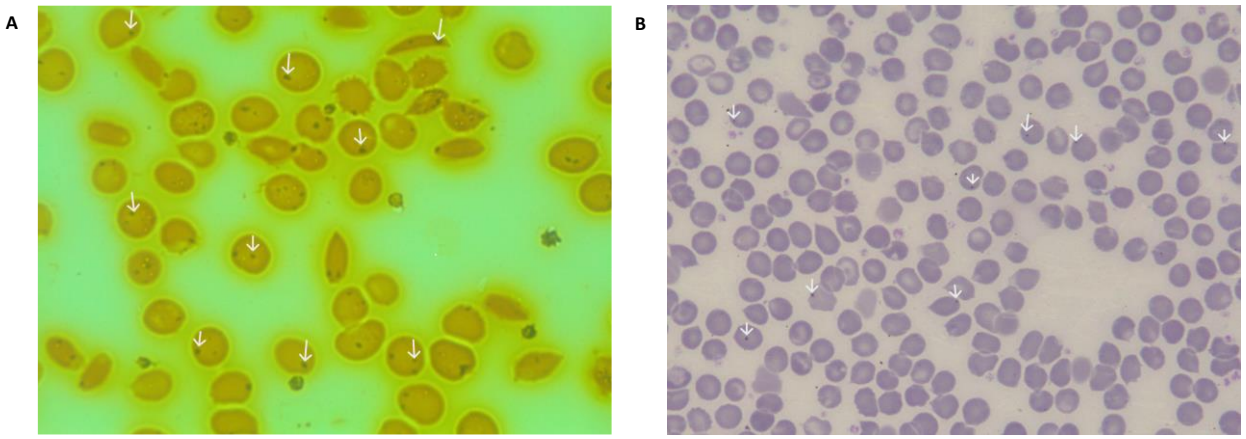


Figure 13: Detection of argyrophilic inclusion (AI) and Howell Jolly body (HJB) containing red cells

Legend: A) Silver-stained blood smear (x100/ 0.80) from a 16-year-old male HbSS patient. Some examples of red cells containing black granules corresponding to the argyrophilic inclusions (white arrows). The inclusions varied in size and numbers. Several sickled red cells also noted. B) May-Grunwald Giemsa (MGG) stained blood smear (x40 / 0.65) from a 23-year-old female HbSS patient showing some examples of red cells with HJB (white arrows).

Table 9: Distribution of AI and HJB red cell counts across study population

Hb phenotype	**N (%)**	**Sex (m), n (%)**	**%HJB RBC**	**%AI RBC**
			Median (IQR)	Median (IQR)
Hb SS	175 (95.9)	87 (49.7)	1.4 (0.8 - 3.1)	46.3 (35.0 - 66.0)
Hb SC	5 (2.9)	4 (80.0)	2.2 (1.2 - 3.1)	62.5 (27.0 - 74.0)
Hb SB Thal	2 (1.1)	2 (100.0)	1.2 (0.7 - *)	35.2 (16.0 - *)
All	**182 (100)**	**93 (51.1)**	**1.5 (0.7 - 3.1)**	**47.4 (34.5 - 66.0)**
Hb AA	93 (91.1)	56 (60.2)	0.3 (0.1 - 0.5)	6.9 (5.1 - 8.6)
Hb AS	7 (6.9)	6 (85.7)	0.3 (0.0 -0.9)	7.5 (4.0 - 13.1)
Hb AC	2 (2.0)	0 (0.0)	0.4 (0.2 - *)	8.9 (7.6 - *)
All	**102 (100)**	**62 (60.8)**	**0.3 (0.1 - 0.5)**	**7.1 (5.1 - 8.7)**

AI: Argyrophilic inclusions; HJB: Howell Jolly Bodies; RBC red blood cells; Hb: haemoglobin. * 75th centile was not generated because there were only two data points in the group

6.5.3 Comparison of AI and HJB red cells count among the study population.

The median percentage of AI red cells in the SCD group (47.4%; IQR 34.5% - 66.0%) was significantly higher than those of the control group (7.1%; IQR 5.1% - 8.7%) ($P < 0.0001$) (Fig.14A). Similarly, the median percentage of HJB red cells in the SCD group (1.5%; IQR 0.7% - 3.1%) was greater than those of the control group (0.3%; IQR 0.1% - 0.5%) ($P < 0.0001$) (Fig. 14B). Analysing the results according to Hb phenotype (Kruskal- Wallis test), within the SCD group, the HJB and AI red cell counts tended to be higher in the HbSS population compared to the other compound heterozygotes, however, the results were not statistically significant either for the HJB ($P = 0.883$) or AI counts ($P = 0.534$). The small number of the latter group (5 Hb SC and 2 Hb SB Thal) may have affected the power to detect any statistical significance

among the SCD group. Among the control group, the median percentage of AI red cells for those with Hb AA was not significantly different from those with Hb AS and Hb AC (*P* = 0.296). Similarly, the frequency of HJB red cells in the Hb AA controls was comparable to those of Hb AS and Hb AC controls (*P* = 0.946).

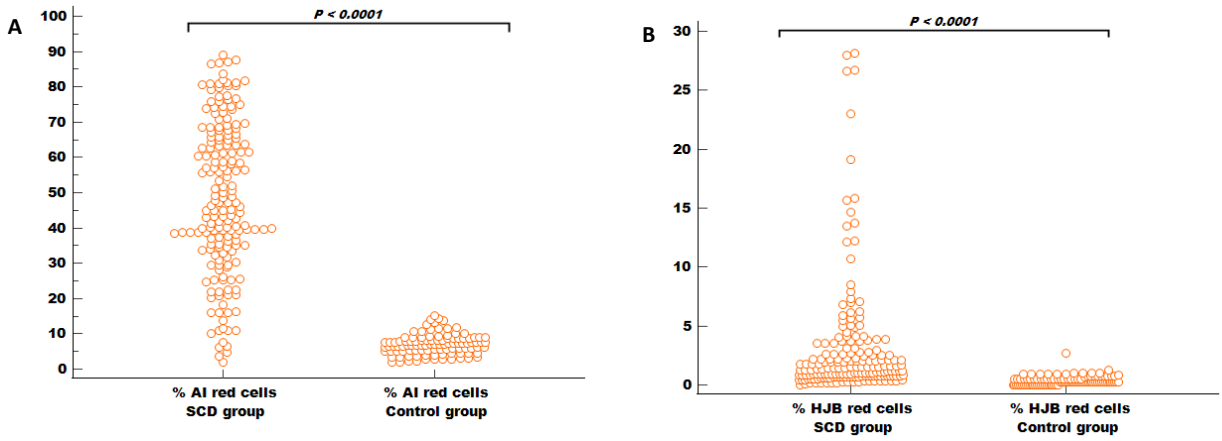


Figure 14: Comparison of AI and HJB red cells count among the study participants.

Legend: Plots showing significantly high proportion of AI red cells (A) and HJB (B) red cells among the SCD patients (n=182) compared to the control group (n=102). Mann-Whitney U test was used (***: P < 0.0001)

6.5.4 Comparison of frequency of AI and HJB red cells based on spleen size on ultrasonography

The SCD patients were divided into two groups based on whether the spleen was visible (n=99) or not visible (n=73) on abdominal ultrasonography. The median percentage of AI red cells was significantly higher in the group without visible spleens (56.0%; IQR 38% - 73%) compared to those whose spleens were visible on ultrasonography (44.9%; IQR 29% - 61%) (*P* = 0.011) (Fig.15A). Similarly, the median percentage of HJB red cells in the group without visible spleens on ultrasonography was higher (2.2%; IQR 1.5% - 5.1%) compared to those whose spleens were visible (0.9%; IQR 0.6% - 2.0%) (*P* = 0.0001) (Fig.15B). Furthermore,

among the SCD patients with visible spleens, the spleens were classified as small (n=18), normal (n=62), and enlarged (n = 19) using spleen length of the controls as reference. The frequencies of AI and HJB red cells were compared across subgroup of spleen sizes. The proportion of AI red cells showed an inverse relationship with spleen size among the whole study cohort (Fig. 15C). The proportion of HJB red cells was however not different among SCD patients with small, normal, or enlarged spleens (Fig.15D)

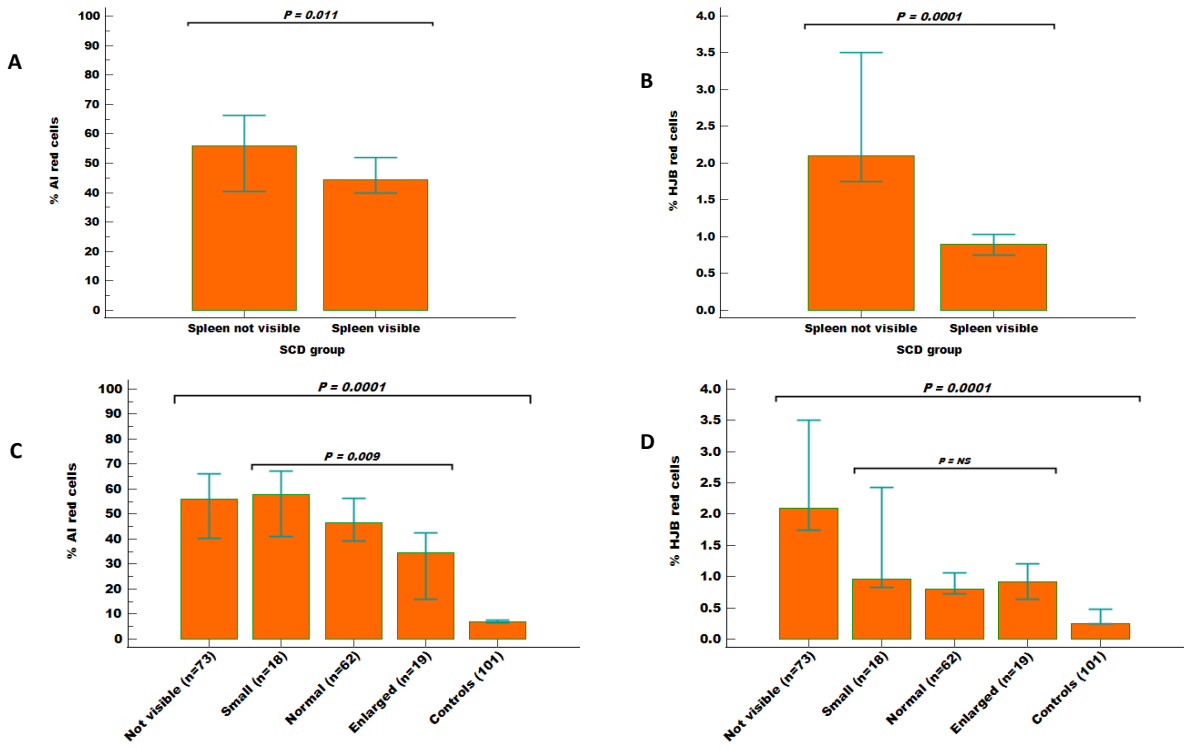


Figure 15: Comparison of AI and HJB red cells among the study participants based on spleen size

Legend: Bar charts showing significant difference in the proportion of AI (A) and HJB (B) red cells between SCD patients with visible spleens (n=99) and those without visible spleens (n=73) on ultrasonography. Mann-Whitney U test was used. Further categorization of AI and HJB red cell counts based on different spleen sizes among the SCD group (i.e not visible, small, normal, and enlarged) and the controls was made (C and D). The proportion of AI red cells (C) showed an inverse relationship with spleen sizes among the SCD (P = 0.009). The proportion of HJB red cells (D) was not significantly across the different spleen sizes. Error bars represent the 95% Confidence

interval for median. Kruskal-Wallis and Dunn's multiple comparison tests were used for the analysis between the SCD and control group.

6.5.5 Intra-observer reliability and agreement between counts obtained for the AI and HJB method.

Scatter plots for paired readings obtained from the same sample for both the AI and HJB methods are shown below (Figs: 14A - D). The Pearson's correlation (r) for the AI and HJB counts was 0.90 (r^2 = 0.82; P <0.001) and 0.92 (r^2 = 0.86; P <0.001) respectively. Plots of the paired measurements of the AI and HJB counts on the original scale (Fig. 16 A and C respectively) shows a cluster of points around the lower part of the graphs and a few outlying values. Use of log scales for the AI and HJB counts (Figs. 16 B and D respectively) results in a more uniform scattering of the points on the graph and a scatter that was roughly constant with increasing count. The corresponding correlation coefficient (r) was 0.86 for the AI and 0.70 for the HJB red cell counts.

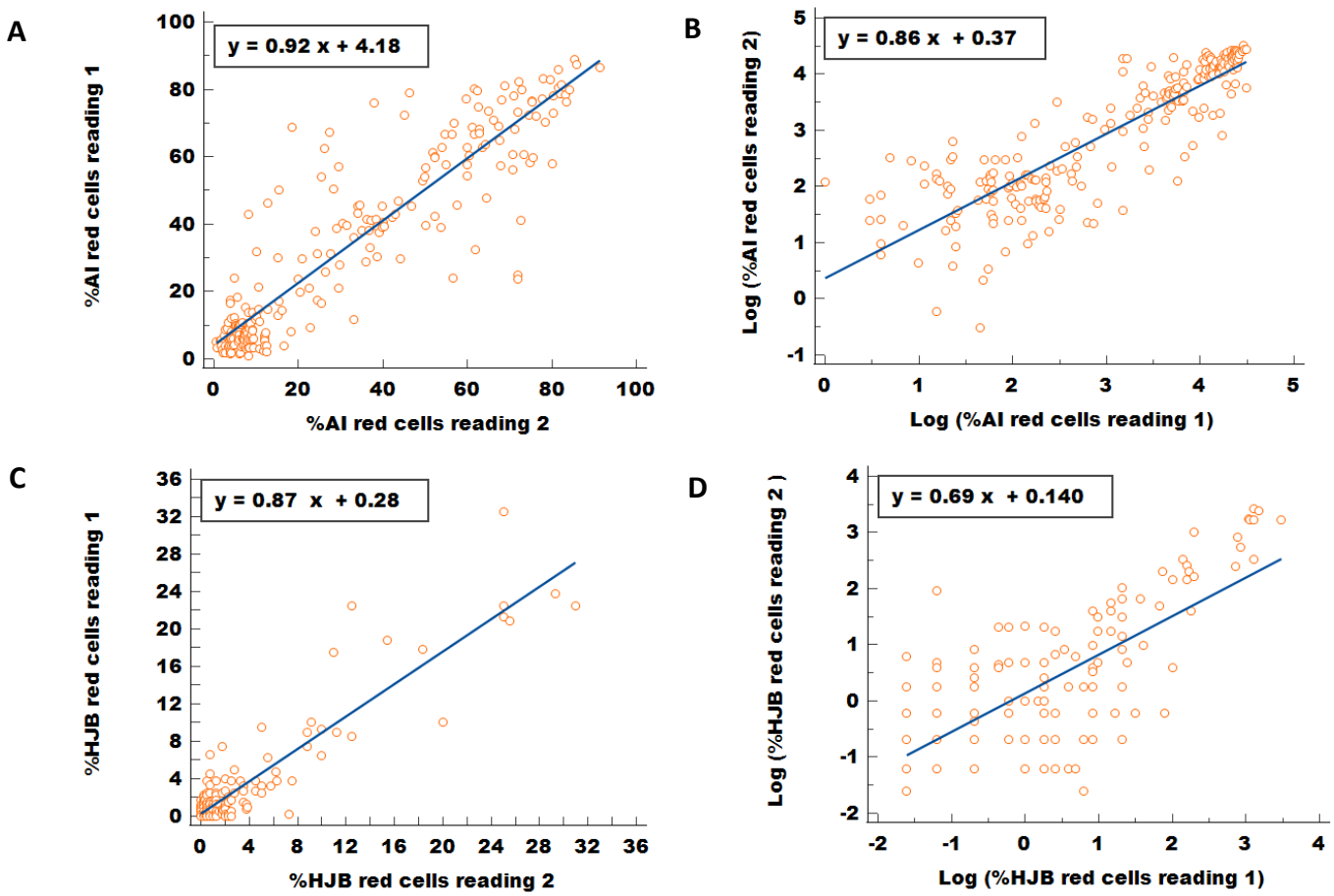


Figure 16: Graphs showing intra-observer reliability for the AI and HJB counts

Legend: A) Scatter plot for paired measurements for the AI red cell count (y = 0.924x + 4.18) (r = 0.90). A total of 233 paired samples (both SCD and controls) were analysed. B) Plot showing the log transformed AI red cell count from A above (y = 0.858x + 0.370) (r =0.86). C) Scatter plot for paired measurements obtained for the HJB red cell counts (y = 0.867x + 0.277) (r = 0.92). A total of 174 paired samples (SCD patients only) were analysed. D) Plots for the log transformed paired measurements of HJB red cells count from C above (n=138; HJB values zero or below were not log transformed and were excluded (y =0.687x + 0.140) (r = 0.70)

6.5.6 Intra-observer agreement between counts obtained for the AI and HJB method.

The Bland-Altman test was used to evaluate the intra-observer agreement for the two methods. The results of the AI count showed a weak agreement between the two sets of counts when all the data sets were analysed (i.e., both SCD and control data) (Fig.17A). The mean difference between the two sets of readings was high (difference 1.7%; 95% Confidence interval (CI): 0.2% to 3.3%) (P = 0.03). The upper limit of agreement (LOA) was 25.6% (95% CI: 22.9% to 28.3%) and the lower LOA -22.1% (95% CI: -24.8% to -19.4% (Fig.17A). Nineteen measurements (8.2%) fell outside the LOAs; all were from samples with AI count of > 20%. However, when only the data sets from the control population were analysed, there was good agreement between the two sets of reading evidenced by the significantly low mean difference of 0.35 (95% CI: -0.7% to 1.4%) (P = 0.526) (Fig 17B). The upper LOA was 10.6% (95% CI: 8.8% to 12.5%) and the lower LOA -9.9% (95% CI: -11.8% to -8.1%). The mean difference between the two sets of HJB readings was low (difference -0.1%; 95% CI: -0.4% to 0.2%) (P = 0.579) (Fig.17C); although, the points appeared clustered towards the lower end, they were scattered relatively evenly above and below the mean line of equality. The upper limit of agreement (LOA) was 4.3% (95% CI: 3.7% to 4.9%) and the lower LOA -4.5% (95% CI: -5.1% to -3.9%). Eleven measurements (6.3%) fell out of the LOAs, and the majority were from samples with a HJB count of >5%.

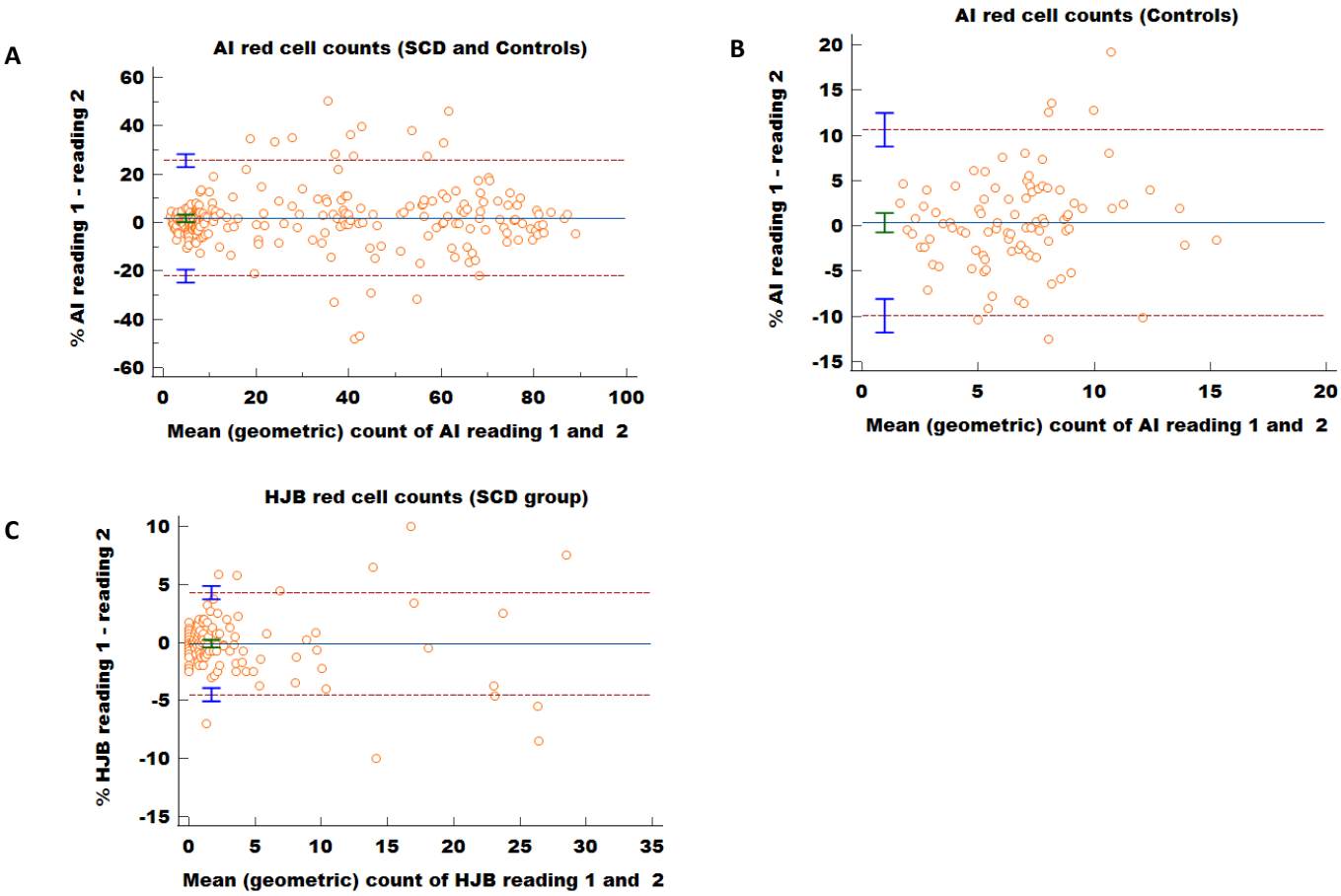


Figure 17: Bland Altman (BA) plot comparing paired measurements obtained for both the HJB and AI counts

Legend: (A) BA plot of paired AI measurements in 233 SCD and control subjects. Mean difference between measurements = 1.7% (95% CI: 0.17% to 3.3%, horizontal middle line). Upper limit of agreement = 25.6% (95% CI: 22.9% to 28.3% upper dashed line). Lower limit of agreement = −22.1% (95% CI: −24.8% to −19.4%, lower dashed line). (B) BA plot of paired AI measurements in 93 controls; mean difference between measurements = 0.35% (95% CI: -0.7% to 1.4%, horizontal middle line). The uniform distribution of the points around the mean suggest they agree. Upper limit of agreement = 10.6% (95% CI: 8.8% to 12.5%, upper dashed line). Lower limit of agreement = −9.9% (95% CI: −11.8% to −8.1%, lower dashed line). (C) BA plot of 174 paired measurements for HJB count from SCD patients only. Mean difference between measurements = - 0.1% (95% CI: −0.4% to 0.2%, horizontal middle line). Majority of the points centred around the mean and were clustered towards the lower end; there was an increasing variability with higher counts to the right. Upper limit of agreement = 4.3% (95% CI: 3.7% to 4.9%, upper dashed line). Lower limit of agreement = −4.5% (95% CI: −5.1% to −3.9%, lower dashed line).

6.6. Discussion

6.6.1 Overview of findings

Spleen function is usually evaluated by the spleen's ability to remove abnormal cells from circulation. SCD patients develop progressive splenic dysfunction and quantification of red cells containing two types of inclusions - HJB and AI, was employed in the current study to evaluate their splenic function. The silver stain method used in the current study to evaluate AI-containing red cells as a marker of spleen function was based on that first described by Tham et al. (Tham *et al.*, 1996). The exact nature of the AI inclusions is unclear; however, studies have demonstrated the presence of vacuoles and inclusions among circulating red cells (Kent *et al.*, 1966; Holroyde and Gardner, 1970). The inclusions were increased in individuals without spleens or in those with abnormalities of erythropoiesis. They were more abundant in splenectomised persons with concurrent hematologic disorders such as thalassemia and haemolytic anaemia. Their increased presence in abnormalities of erythropoiesis may be readily explained by the larger amount of product of haemolysis to be disposed of in these conditions. Their increase however in splenectomised individuals, with otherwise normal erythropoiesis, suggests an intact spleen is required for their elimination. In one study, the authors noted inclusions in 54.3% of red cells from 20 splenectomised individuals, using differential interference contrast microscopy (Holroyde and Gardner, 1970). These inclusions are probably equivalent to the AI inclusions observed in the current study using the silver stain, in close agreement with our observed mean value of about 50% for the AI red cells. The authors noted the inclusions neither stained nor looked like conventional red cell inclusions, such as HJB, Heinz bodies or siderotic bodies. Morphologically, the inclusions were suggestive of haemoglobin degeneration. They concluded that mature normal red cells

continually form inclusions which may reflect the degradative cellular process consequent upon cell aging. Kent and colleagues also observed, by electron microscopy, the presence of autophagic vacuoles in mature red cells and reticulocytes; the inclusions contained a variety of materials including haemoglobin and altered cytoplasmic organelles such as ribosomes, mitochondria, and smooth membranes (Kent *et al.*, 1966). The inclusions appeared to be instrumental in the disposal of those organelles or other materials not required by the fully mature red cells. The increased presence of these inclusions in splenectomised subjects indicates that, although developing human red cells are capable of eliminating their digestive residues, the presence of the spleen is required to fulfil this task adequately.

The use of MGG to demonstrate HJB in red cells is a long-established method of evaluating splenic dysfunction (O'Brien *et al.*, 1976; Davis, 1976; Pearson *et al.*, 1979). HJB are small (approximately 1 micron) red cell DNA inclusions that result from cytogenetic damage. Normally, HJB-containing red cells are formed at low frequency and are quickly removed by an intact spleen, thus their presence in peripheral smears is an indirect evidence of splenic dysfunction (Pearson, Spencer and Cornelius, 1969; Davis, 1976). Our comparison of results obtained using HJB and AI inclusions as markers of splenic dysfunction showed the AI in red cells were always higher than the HJB counts. This indicates the two tests identify different intra-cellular structures. While the silver stain picks up all intracellular argyrophilic particles, MGG stains only HJB, which are usually formed at low frequency. The argyrophilic inclusions varied in size and number within the red cells, whereas the HJB always appeared as a single inclusion. The higher AI count may also be related to the increased erythropoiesis seen in haemolytic disorders like SCD. Kent et al. found increased red cells with inclusions in patients with haematological disorders and reticulocytosis but

intact spleens; a high proportion of the reticulocytes contained inclusions (Kent *et al.*, 1966). Recent studies have demonstrated significantly high fraction of RBCs retaining-mitochondria in circulation among SCD patients (Moriconi *et al.*, 2022; Jagadeeswaran *et al.*, 2017); the abnormal presence of mitochondria was thought to be related to the stress erythropoiesis observed in SCD. Aside its utility in assessing argyrophilic nucleolar organising regions, the silver stain can stain other elements including iron, calcium, and phosphate (Tham and Cousar, 1993). Application of the silver stain to a series of marrow particle sections showed the silver stain was specific for identifying abnormal mitochondrial "iron" deposits in ringed sideroblast (Tham and Cousar, 1993). Given the heterogenous nature of red cell vacuoles described above (Holroyde and Gardner, 1970; Kent *et al.*, 1966), the sensitive but non-specific nature of the silver stain may allow it to stain a wide range of cellular content. Whatever their nature, it appears the AI are found in increased numbers in SCD patients compared to HJB. The former, although more sensitive in picking up red cells inclusions, may be influenced by both splenic dysfunction and haemolysis related abnormalities.

6.6.2 Comparison between SCD patients and controls

To our knowledge, this is the first detailed study of splenic function among SCD patients in an African setting. Our study found significant differences in the frequencies of AI and HJB red cells between the SCD patients and controls, which indicates the presence of splenic dysfunction in the former. The percentage of AI red cells in our SCD population was significantly higher than those of the controls; such a finding using the AI red cells has been reported in the US (Tham *et al.*, 1996), though the study only included 9 SCD patients and 45 controls. The results obtained for the AI red cells in our SCD patients (IQR 34.5% - 66.0%) and controls (IQR 5.1% - 8.7%)

were higher than the data from the SCD patients (11.8% - 52.7%) and controls (0.3% - 3.0%) in the American study (Tham *et al.*, 1996). The SCD patients in the present study, demonstrated a higher frequency of HJB red cells compared to the controls. The range of HJB red cells obtained among our SCD population was higher (IQR 0.7% - 3.1%) than results obtained among SCD patients (n = 12; age range 5 months to 39 years) in the United States (HJB% range 0.0% - 1.1%) (Casper *et al.*, 1976), and among children (n = 20; age range 5 years to 22 years) with SCD in Brazil (HJB% range 0.0% - 1.4%) (Zago and Bottura, 1983). The small sample size from both studies compared to our sample population (n=182) may account for the differences compared to our results.

6.6.3 Comparison of AI and HJB red cells with spleen size on ultrasonography

Both the AI and HJB red cell counts from SCD patients without visible spleens (i.e autosplenectomy) were higher than patients whose spleens were visualised on ultrasonography, especially for the HJB red cell counts. This is not unexpected as the spleen is the site of removal of red cell inclusions, therefore patients with autosplenectomy are likely to have higher numbers of inclusions within their red cells than those with intact spleens (Peretz *et al.*, 2022; Sissoko *et al.*, 2022). However, despite the presence of spleens among some of the SCD patients, the frequency of red cell inclusions was still higher than in the controls. This suggests the presence of functional hyposplenism among SCD patients, whereby despite having the spleen present, it may not be effectively eliminating the inclusions (Pearson, Spencer and Cornelius, 1969). Previous studies have shown that function can be restored among such group of patients using transfusion or disease modifying agents like hydroxyurea (Barrios *et al.*, 1993; Pearson *et al.*, 1970; Nottage *et al.*, 2014). Furthermore, among

SCD patients whose spleens were still visible on ultrasonography, the AI and HJB counts appeared variable among those with small, normal, and enlarged spleens, with a tendency of been lower in those with enlarged spleens. The implication of this finding is that the spleen function may not be completely lost. A recent study demonstrated that spleen function may be preserved even among adults patients with SCD as long sinus structures still persist in the preserved or partially damaged spleen (Sissoko *et al.*, 2022). It would be interesting to follow up the group of patients whose spleens are still visible on ultrasonography with disease modifying therapy like hydroxyurea to determine if this will have any effect on reducing the frequency of the red cell inclusions.

6.6.4 Reliability and agreement analysis for the HJB and AI methods

To evaluate which of the method would give the least intra-observer variation and be useful for the longitudinal follow up of our patients, we evaluated the intra-observer reliability and agreement of both methods. Our data demonstrated good intra-observer correlation for paired readings obtained with the AI test ($r = 0.90$; $r^2 = 0.82$), in keeping with an earlier report ($r=0.736$; $P < 0.001$) (Tham *et al.*, 1996). While correlation measures the linear relationship between two sets of measurement, agreement on the other hand assesses the equality of the individual values between two sets of measurement (Berchtold, 2016). Since a perfect positive correlation is not proof of equal responses between two testing occasions (Watson, 2004), we tested results obtained for both the AI and HJB methods for agreement using the Bland Altman analysis (Bland and Altman, 1986). A good agreement was observed for the AI counts among the controls ($P=0.499$), but at higher counts obtained among the SCD patients, the AI method did not produce such a good agreement between two readings taken on different occasions. This finding reflects the character of the AI test; the difference

between two separate readings taken on the same sample is related to the value of the measurement, larger measurements among the SCD patients imply a larger average error between both readings and hence the significant difference between these counts (*P*=0.03).

Our data also showed a good limit of agreement for counts obtained using the HJB method (95% CI, -4.5% to 4.3%; *P* = 0.579). Several investigators have shown the HJB count can be reliably used to assess spleen function. Corazza et al. found a significant correlation between HJB red cell counts obtained by the classical MGG and the pitted red cells counts (P < 0.0001); they noted that pitted red cells count above 8% was always associated with increasing HJB red cells count (Corazza *et al.*, 1990). Serial measurements of HJB red cells using the manual estimation were reliably used to monitor spleen function before and after bone marrow transplantation in SCD patients over a period of 15 years (Bernaudin *et al.*, 1993a; Bernaudin *et al.*, 2022a). In a similar, but small study, the frequency of HJB red cells declined progressively following bone marrow transplantation, and together with scintigraphy, both techniques were used to monitor spleen function in these patients (Ferster *et al.*, 1993). Also, a robust correlation between the MGG method and a newly-developed imaging flow cytometry method for evaluating HJB has been demonstrated (El Hoss *et al.*, 2018; Pourdieu *et al.*, 2023). A few studies however have reported a lack of correlation between the MGG HJB method with other methods of assessing spleen function (Casper *et al.*, 1976; Zago and Bottura, 1983; Lammers *et al.*, 2012).

6.6.5 Clinical implication

A prospective study of SCD patients (n=12) identified at birth in a screening programme in the USA, demonstrated that splenic dysfunction is an acquired defect occurring as early as five months of age; the onset of splenic dysfunction documented

by serial splenic scintigraphy correlated with the appearance of HJB in peripheral smears (O'Brien *et al.*, 1976). This suggests that the presence of HJB can be used as a reliable indicator of the onset of splenic dysfunction. Of note however, none of the studies that have evaluated HJB manually as a marker of splenic dysfunction have indicated what percentage of red cells containing HJB indicates hyposplenism. Using flow cytometry, quantitative HJB values of $>300/10^6$ red cells (i.e 0.03%) have been suggested to indicate loss of splenic filtration among SCD (Harrod *et al.*, 2007). We have included a control group in our study and our result for the percentage of HJB among the controls obtained with the manual count (median 0.3%; IQR 0.1% - 0.5%) is comparable to that obtained from children and adult healthy controls (0.3%; range, 0.01%-0.6%) in studies using flow cytometry counts of HJB (El Hoss *et al.*, 2018; Pourdieu *et al.*, 2023). This indicate that the measurement of HJB by the MGG technique using light microscopy may allow accurate evaluation of splenic function among SCD patients in low resource setting.

The 97.5^{th} centile (non-parametric upper reference limit) for HJB counts (0.9%) generated from our controls could be considered as upper limit of normal and values above this may be used to indicate presence of splenic dysfunction. However, for the longitudinal follow-up of patients, simple comparison to a particular threshold value may be overly simplistic, because splenic dysfunction among SCD is known to be progressive (Pearson *et al.*, 1979; Pearson *et al.*, 1985), and there is great inter-individual variability in the HJB counts among children and adults with SCD (Pourdieu *et al.*, 2023; Harrod *et al.*, 2007; Davis, 1976). Rather, identifying a baseline value for an individual patient may aid the early detection of splenic dysfunction when a considerable increase from the baseline HJB level occur (Davis, 1976; El Hoss *et al.*, 2019). Also, while some studies have used the appearance of HJB to identity the onset

of splenic dysfunction (Pearson *et al.*, 1979; O'Brien *et al.*, 1976), other studies have used disappearance or decreasing levels of HJB to monitor response to therapy such as bone marrow transplant and hydroxyurea (Ferster *et al.*, 1993; Bernaudin *et al.*, 2022a; Nottage *et al.*, 2014) since it indicates the return of splenic function among SCD patients. Therefore, in clinical practice serial monitoring of an individual's HJB level could help in monitoring spleen function and guide the appropriate timing of interventions rather than comparison to a threshold. Compared to other currently available methods of assessing spleen function, such as spleen scintigraphy, pitted red cell counts and flow cytometry, counting of HJB red cells by the MGG method provides a simple and reliable technique that can easily be used in most laboratories in the African settings. HJB was easy to perform, simpler than the AI count method and can be used to evaluate splenic function at regular intervals. This will be valuable in identifying early the onset of splenic dysfunction, which is associated with increased susceptibility to overwhelming infections.

6.6.6 Limitation

To determine estimates of diagnostic accuracy such as sensitivity and specificity, we would have compared our findings with those of a reference standard such as the spleen scintigraphy or pitted red cell count (Cohen *et al.*, 2016). Since these methods are not available in Nigeria, we have compared our findings to those in the published literature.

6.7 Conclusion

Determination of red cells with AI and HJB enables the assessment of splenic function in SCD patients in low-resource settings. Both markers can serve as indicators of splenic dysfunction in SCD patients as reflected by the high percentages of circulating

levels compared to controls. Their presence was easily demonstrable, and both showed good intra-observer reliability. Although the red cells inclusions were readily demonstrable using the AI method, the silver stain deteriorates rapidly. The HJB method is simpler and requires fewer reagents than the AI method. Moreover, the higher AI counts in patients with haemolytic anaemias such as SCD may be due to both the hematologic disturbance and abnormal splenic function, therefore, further validation in larger studies may be required for the AI method before the generalisability of its efficacy in assessing spleen function in SCD can be ascertained.

CHAPTER 7: CLINICAL AND LABORATORY FACTORS ASSOCIATED WITH SPLENIC DYSFUNCTION AMONG SICKLE CELL DISEASE PATIENTS

7.1 Chapter overview

The focus of this study was on the clinical aspect of splenic function. Building on from the information obtained from the study in Chapter 6, the data were further analysed to identify clinical and laboratory parameters that may influence spleen function.

Chapter 7 has been published by Transaction of the Royal Society of Tropical Medicine and can be accessed by the link below:

https://doi.org/10.1093/trstmh/trad059

7.2 Abstract

Background

Although loss of splenic function is the expected natural course for individuals with sickle cell disease (SCD), factors such as high HbF and coexistence of alpha thalassemia may ameliorate this process. We evaluated factors associated with two surrogate markers of spleen dysfunction - Howell-Jolly bodies (HJB) and argyrophilic inclusion (AI) red cell counts among SCD patients.

Methods

Cross-sectional data of 182 SCD patients (age 11 years;1- 45 years) and 102 normal controls (age 12 years;1-34 years) were evaluated. Blood tests including full blood count, serum chemistry and HPLC were performed. The HJB and AI red cell counts were performed on peripheral blood smears.

Results

The percentages of HJB- and AI- red cells rose significantly with increasing age in the SCD group. On regression analysis, frequency of HJB red cells associated positively with MCH (β = 0.289; *P* = 0.001) and negatively with HbF (β = -0.259; *P* = 0.002). The AI red cell counts also associated positively with MCH (β = 0.321; *P*=0.000) and negatively with HbF (β = -0.242; *P* = 0.020).

Conclusion

Data from this study indicates that the negative association of HbF with both markers of splenic dysfunction among our SCD patients residing in a malaria-endemic region is similar to findings elsewhere of the ameliorating effect of HbF on splenic dysfunction.

7.3 Introduction

The spleen plays an important role in the defence against infections and is one of the earliest organs to be affected in sickle cell disease (SCD). This occurs as a result of repeated cycles of ischemia and infarction within the organ (Diggs, 1935). Observations among patients with SCD residing in the Western hemisphere indicate that splenic dysfunction starts as early as 6 months of age, and the majority of patients are affected by two years of life (El Hoss *et al.*, 2019; Rogers *et al.*, 2011; O'Brien *et al.*, 1976). The experience is however different in SCD patients in Africa, Asia, and the Middle East where splenic dysfunction starts much later (Mallouh *et al.*, 1984; Al-Jam'a *et al.*, 2000; Wali *et al.*, 2002; Adekile *et al.*, 1991; Serjeant, Hambleton and Serjeant, 2021). The age variability at which splenic dysfunction starts, the rate of progression, and consequently the stage at which it becomes clinically significant can be related to several factors including the coexistence of alpha thalassemia, high level of HbF, and malaria infection (Wali *et al.*, 2002; Nottage *et al.*, 2014; Adekile *et al.*,

1991). Supportive therapies including the use of hydroxyurea, chronic red cell transfusions and stem cell therapy have also been associated with reversal or prolongation of spleen function among SCD patients (Bernaudin *et al.*, 1993b; Ferster *et al.*, 1993; Hankins *et al.*, 2008; Nottage *et al.*, 2014). The widespread use of these therapies may therefore influence the rate of splenic dysfunction among patients.

Given the role the spleen plays in protection against infections, splenic dysfunction increases vulnerability to invasive infections with encapsulated bacteria and parasitic infections (Booth, Inusa and Obaro, 2010; Lenti *et al.*, 2022); however, the prevalence of splenic dysfunction and factors associated with its development are largely unknown in Sub-Saharan Africa , the region where the majority of SCD patients reside (Piel *et al.*, 2013a). This is because most of the tests used to assess spleen function, such as radionuclide scans and percentage of pitted red cells using contrast-enhancing microscopy are not readily available in most African countries. Thus, factors associated with splenic dysfunction among SCD patients in Africa have not yet been fully investigated.

We recently employed the presence of two red cell containing inclusions - Howell-Jolly bodies (HJB) and argyrophilic (silver staining) inclusion (AI) red cells - to assess splenic dysfunction among our SCD patients (Ladu *et al.*, 2023). In the present study, we aimed to investigate the variation in both markers of splenic dysfunction (i.e HJB and AI) with age among the SCD patients and compare results with those of healthy controls. Also, the relationship between the markers of splenic dysfunction with clinical outcomes and laboratory variables among the SCD patients were explored.

7.4 Methods

7.4.1 Study participants

See section 3.3.1

7.4.2 Data collection

See section 3.3.3.

7.4.3 Statistical analysis

The data were analysed using Statistical Package for the Social Sciences (SPSS) (version 25; SPSS, Chicago, IL, USA). Categorical data were summarised using frequencies and proportions, while continuous data were summarised using descriptive statistics. Comparisons of HJB and AI red cell counts across the age groups of SCD patients and controls were performed using Mann-Whitney test. Factors potentially associated with both markers of splenic dysfunction were analysed individually using univariate regression analysis. The goal of the analysis was to identify clinical and laboratory factors associated with increased levels of both markers. The presence of collinearity among the independent factors was explored and all variables with a variance inflation factor of > 10 excluded. A full model analysis containing all the significant variables was performed using the backward elimination method to evaluate the independent effects of each covariate by controlling the effects of other variables. The adjusted odds ratios and 95% confidence intervals (CIs) were computed. A p-value < 0.05 was considered statistically significant.

7.4.4 Ethical considerations

See section 3.6.4

7.5 Results

7.5.1 Clinical characteristics of study participants

The study consisted of 182 SCD patients (median age 11 years; range 1 to 45 years) and 102 controls (median age 12 years; range 1 to 32 years). The Hb phenotypes of the SCD patients consisted of 175 homozygous sickle cell disease (Hb SS) (96.2%), five sickle-haemoglobin C disease (Hb SC) (2.7%), and two sickle cell β-thalassaemia (Hb Sβ) (1.1%). The majority of the SCD patients reported one or more episodes of fever (89%) and painful crises (77%) over the last 12 months, and 63 (34.6%) patients required in-patient hospitalisation. The majority of parents and guardians of the younger patients admitted having completed routine childhood immunisation for their children, however, most of the older SCD patients were unsure of their childhood immunisation status. Only two patients were on penicillin prophylaxis. Twenty-nine (15.9%) patients were on regular hydroxyurea (HU) treatment. None of the patients were on chronic transfusion treatment, however, the majority (n=119/182; 65.4%) have been transfused once or more in the past (mean lifetime transfusion 3.7 (SD 8.1). None of the patients in our cohort had received a stem cell therapy.

7.5.2 Comparison of AI and HJB red cell counts across age groups between SCD patients and controls

Distribution of HJB and AI red cells showed consistently high levels of both markers in SCD patients compared to controls across all age groups (Table 10). The progression of the frequencies of both markers with age differed between patients and controls (Figs.18A –D). Within the SCD population, the median percentage of HJB red cells rose steadily with increasing age, rising from 0.7% in children less than five, to 1.6% in those above 10 years and reaching 2.5% in those older than 15 years. The median

percentage of AI red cells was lowest in children less than five years (median 40%) and rose to 50.0 - 57.5% in those over 5 years, including into adulthood. Within the control population, the opposite trend was noted. The AI red cell count decreased with increasing age from 7.5% in children less than five years, to 5.6% in the adult group. The frequency of HJB red cells showed little variability with age among the controls.

Table 10: Comparison of AI and HJB red cell counts across age groups between SCD patients and controls

Variable	**SCD patients (n=182)**			**Controls (n=102)**			**P value**
	N	Median (IQR)	Min-Max	N	Median (IQR)	Min-Max	
%HJB RBCs	39	0.7 (0.4 -1.1)	0.0 – 5.5	21	0.3 (0.0 -0.7)	0.0 - 1.2	0.002*
< 5 years	37	0.9 (0.5 -2.4)	0.1- 23.0	21	0.5 (0.2 -0.6)	0.0 - 0.9	0.001*
5 - 9 years	34	1.6 (0.9- 2.5)	0.2 – 8.0	19	0.2 (0.2 – 0.5)	0.0 - 2.6	0.001*
10 - 14 years	72	2.5 (1.4 -5.5)	0.4 - 28.2	41	0.2 (0.0 -0.5)	0.0 - 0.9	0.001*
≥15 years	182	1.5 (0.7-3.1)	0.0 - 28.2	102	0.3 (0.1 - 0.5)	0.0 – 2.7	0.001*
All							
%AI RBCs							
< 5 years	39	40 (22-40)	3.7 - 81.2	21	7.5 (6.3-9.6)	4.5 -13.9	0.001*
5 - 9 years	37	50 (40-50)	25.5 - 82.1	21	7.8 (6.3-9.1)	2.8 -15.3	0.001*
10 - 14 years	34	58 (39-58)	2.0 - 87.5	19	7.2 (5.7-8.0)	2.0 -11.3	0.001*
≥15 years	72	52 (34-50)	4.9 - 88.9	41	5.6 (3.6 -8.3)	2.1- 14.4	0.001*
All	182	47 (35 - 66)	2.0 – 88.9	102	7.1(5.1 - 8.7)	2.0 -15.2	0.001*

*Significant P value by Mann Whitney U test for within age group analysis. AI argyrophilic inclusion; HJB Howell Jolly bodies; IQR interquartile range

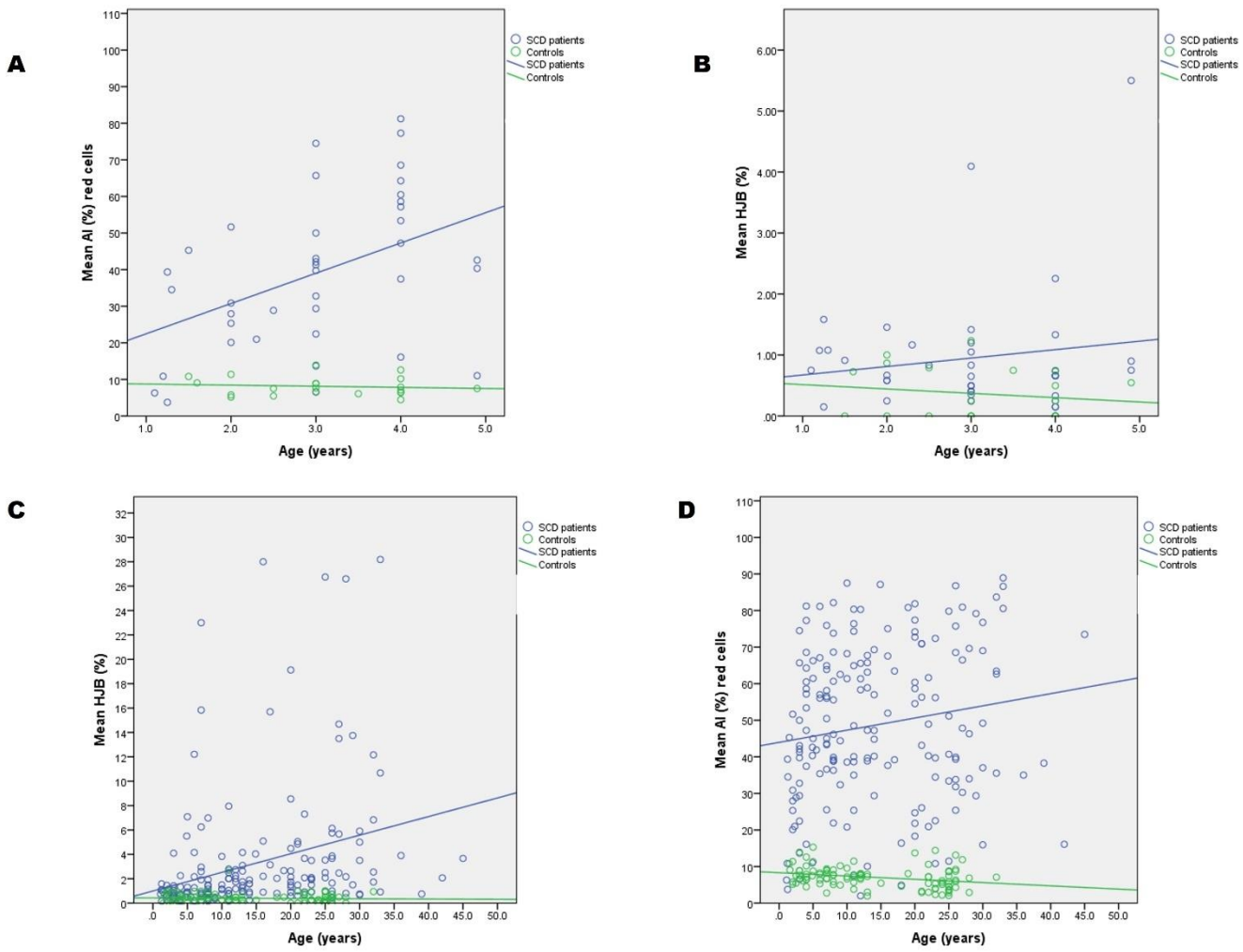


Figure 18: Scatter plot showing the relationship between age and frequency of AI and HJB red cells among the study participants

Legend: (A) Among the less than five years old group, the AI red cell counts were higher among the SCD patients (n=37) and showed an increasing trend with age, whereas levels among the controls (n=22) were low and showed little variability with age. (B) Similarly, while the values of HJB increased steadily with age in the SCD group, there was an opposite trend among the less than five years old in the controls. (C) Among the whole study population, the values of AI red cells were generally high across the SCD population with little variability across the age groups, whereas the values showed a progressive decline with age among the controls. (D) There was an increasing trend for HJB red cell counts with age among the SCD patients; however, considerable variability was noted, as several younger children had elevated HJB red cells, while several older children had near-normal values. The levels of HJB red cells were generally low across all age groups of the controls (D).

7.5.3 Distribution of AI and HJB red cell counts based on hydroxyurea (HU) treatment and phenotype.

The median age of SCD patients on hydroxyurea (HU) treatment was 11 years (IQR 16.6) and 12 years (IQR 16.0) in the non-HU group. The level of HbF was significantly higher among the SCD patients on HU therapy (median 13.6%; IQR 9.2) compared to the non-HU group (median 8.1%; IQR 8.5) ($P < 0.001$). However, levels of HJB red cells among the HU group were not significantly different compared to the non-HU group (median 1.2% vs 1.5%) ($P= 0.389$). Similarly, levels of AI red cells were not significantly different between the HU group compared to the non-HU group (median 56.2% vs 46.1%) ($P = 0.789$).

Among the SCD phenotype, the median HJB red cell counts were 1.4% (IQR 0.8 - 3.1) in HbSS, 2.2% (IQR 1.2 -3.1) in HbSC and 1.2% in Hb Sβ patients. The median AI red cell counts were 46.3% in HbSS (IQR 35 - 66), 62.5% in HbSC (IQR 27 - 47) and 35.2% in Hb Sβ patients (IQR -not computed as only two patients). Further comparison based on phenotype was not performed because of the small number of HbSC (n=5) and Hb Sβ (n=2) of patients. However, the mean age of the HbSC (mean 20 years) and Hb Sβ (mean 25 years) patients appeared higher those of the HbSS (mean 14 years).

7.5.4 Association between AI and HJB red cell counts with clinical events and laboratory factors.

To determine factors associated with the frequency of AI and HJB red cells, we analysed several clinical and laboratory parameters using univariate regression analysis (Table 11). None of the clinical events over the 12 months preceding the study including in-patient hospitalisation ($R = 0.034$; $P = 0.650$), febrile episodes ($R = 0.063$; $P = 0.398$), and painful crises ($R = -0.044$; $P = 0.558$) were associated with the percentage of AI red cells. Similarly, the percentage of HJB red cells showed no association with in-patient hospitalisation ($R = -0.010$; $P = 0.894$), febrile episodes (R

= 0.029; *P* = 0.695), or painful crises (R = -0.185; *P* = 0.252). The association of laboratory factors with the percentage of AI red cells was explored. Few factors retained significance in the final regression model (R = 0.489; R^2 = 0.239; Adj. R^2 0.223) (Table 12). The AI red cell counts associated positively with MCH (β = 0.321; *P* = 0.0001) but negatively with HbF (β = -0.242; *P* = 0.020). On exploration of the laboratory factors associated with frequency of HJB red cells, the final regression model (R=0.364, R^2 = 0.132; Adj. R^2 = 0.118) showed a positive association with MCH (β = 0.289; *P* = 0.001), but a negative association with HbF (β = -0.259; p=0.002) (Table 12).

Table 11: Linear regression analysis for factors associated with AI and HJB red cell counts

Variable	Mean (SD)	Univariate analysis for AI red cells		Univariate analysis for HJB red cells	
		Standardized co-efficient β	P value	Standardized co-efficient β	P value
WBC (x 10^3 /µL)	13.8 (4.6)	0.071	0.344	0.045	0.545
Hb, g/dl	7.4 (1.7)	-0.185	**0.013***	-0.234	**0.002***
Platelets, count (x 10^6 /µL)	391 (166)	0.070	0.346	-0.025	0.741
MCV (fl)	82.8 (9.6)	0.237	**0.001***	0.145	0.052
MCH (pg)	28.6 (3.7)	0.298	**0.0001***	0.207	**0.005***
MCHC (g/dl)	34.5 (1.8)	0.244	**0.0001***	0.188	**0.011***
ANC (%)	5.8 (2.6)	0.153	**0.041***	0.049	0.514
Reticulocyte (%)	8.3 (4.9)	0.235	**0.001***	0.163	**0.029***
Bilirubin total (umol/l)	35.1 (24.2)	0.100	0.205	0.248	**0.001***
ASAT (IU/l)	21.0 (16.2)	0.137	0.081	0.007	0.931
Hb F (%)	10.4 (7.1)	-0.258	**0.001***	-0.202	**0.010***
HbA_2 (%)	3.3 (1.3)	0.065	0.468	0.152	0.088
Hb S (%)	78.8 (9.6)	0.330	**0.0001***	0.254	**0.001***
Hospitalisation over last 12 months	0.51 (0.9)	-0.034	0.650	-0.010	0.894
Febrile episodes over last 12 months	2.4 (1.9)	0.063	0.398	0.029	0.695
Painful crises over last 12 months	2.4 (2.9)	-0.044	0.558	-0.185	0.252

Footnote: WBC white blood cells; Hb haemoglobin; MCV mean corpuscular volume; MCH mean corpuscular haemoglobin; MCHC mean corpuscular haemoglobin concentration; ANC absolute neutrophil count; ASAT aspartate amino transferase; SD standard deviation; AI argyrophilic inclusion; HJB Howell Jolly bodies *All P values were less than .05

Table 12: Multi-linear regression analysis for laboratory factors associated with AI and HJB red cells

Variable	Co-efficient	Standard Error	Standardized co-efficient β	P value*
Dependent variable: AI red cells				
MCH (pg)	1.77	0.41	0.321	0.000*
Hb F (%)	-0.72	0.31	-0.242	0.020*
Hb S (%)	0.41	0.23	0.184	0.073
Dependent variable: HJB red cells				
MCH (pg)	0.336	0.09	0.289	0.001*
Hb F (%)	-0.160	0.05	-0.259	0.002*

Footnote: WBC white blood cells; Hb haemoglobin; MCV mean corpuscular volume; MCH mean corpuscular haemoglobin; MCHC mean corpuscular haemoglobin concentration; ANC absolute neutrophil count; ASAT aspartate amino transferase; AI argyrophilic inclusion; HJB Howell Jolly bodies *All P values were less than .05.

Variables consecutively removed from the model by the backward elimination:

AI red cells model: MCV, ANC, MCHC, reticulocyte count and haemoglobin.

HJB red cells model: Hb S, Bilirubin (total), reticulocyte count, MCHC and haemoglobin.

*Analysis was restricted to SCD patients with complete clinical and laboratory data for the AI (n=146) and HJB red cell counts (n=129)

7.6 Discussion

7.6.1 Overview of findings

In the current study, levels of both AI and HJB red cells were significantly higher across all age groups of SCD patients compared to the controls, indicating the presence of splenic dysfunction among the patient group. This is in keeping with previous reports of raised AI (Tham *et al.*, 1996) and HJB red cell inclusions among SCD patients (Corazza *et al.*, 1990; O'Brien *et al.*, 1976; Pearson *et al.*, 1979; Bernaudin *et al.*, 2022c). The effect of age produced different patterns on the two markers between the SCD patients and controls. The frequency of red cell inclusions increased steadily with increasing age among the SCD population; this was especially marked for the HJB red cell counts, corroborating previous studies from non-malaria regions (El Hoss *et al.*, 2019; Bernaudin *et al.*, 2022b; Pourdieu *et al.*, 2023; O'Brien *et al.*, 1976). The findings are also similar to reports from studies that shows increasing frequencies of red cell inclusions such as the pitted red cells with aging among SCD patients (Rogers, 1982; Pearson *et al.*, 1979; Serjeant, Hambleton and Serjeant, 2021{Nardo-Marino, 2022 #5093; Nardo-Marino *et al.*, 2022; Adekile *et al.*, 1991). Our results suggests that splenic dysfunction begins early in life in SCD and continues to deteriorate into adulthood. Early loss of splenic function renders SCD patients prone to infection (Rogers, Vaidya and Serjeant, 1978; Pearson *et al.*, 1979; Rogers, Serjeant and Serjeant, 1982) and this has implications for clinical management, especially among children less than five years, which is the age associated with a high risk for bacterial infections with encapsulated organisms (namely *Neisseria meningitidis, Streptococcus pneumoniae, Haemophilus influenzae type b*). Management strategies are aimed at preventing severe sepsis and its related morbidity; this includes patient's education, prompt and aggressive treatment of fever, vaccination prophylaxis and antibiotic

chemoprophylaxis, as recommended for all individuals with impaired splenic function at risk of fulminant infection (Lenti *et al.*, 2022). Splenic dysfunction also results in poor clearance of malaria-infected red cells (Luzzatto, 2012), thus malaria preventative measures, such as use of insecticide treated bed nets and malaria chemoprophylaxis according to national guidelines are recommended in SCD residing in malaria endemic countries (Galadanci *et al.*, 2014).

The levels of both markers were also high among patients with compound heterozygotes for SCD in this study; because of the small number of this group (HbSC, n=5 and Hb Sβ, n=2), we could not analyse the effect of phenotype on both markers of splenic dysfunction. However, the observed raised AI and HJB red cell counts among the HbSC patients in this study contrasts with findings from previous studies of preserved splenic function among HbSC patients (Lane *et al.*, 1995; Pourdieu *et al.*, 2023). The older age of the HbSC patients (mean 20 years; range 9- 45 years) in the current study may account for this observation. The influence of age on both markers produced an opposite trend in the control group compared to the SCD patients. In the controls, the AI red cell count declined with increasing age; this may be explained by the progressive and physiological increase in spleen size of normal individuals (Pelizzo *et al.*, 2018), thus the ability to more effectively clear out inclusions. The HJB red cell count in controls was not affected by increasing age; the median value remained stable across all age groups (median 0.3%; $P = 0.834$). HJB are formed at a very low frequency and are rarely encountered in normal individuals because of the high efficiency of removal by the healthy spleen (Dertinger *et al.*, 2003). Our findings are similar to previous reports of HJB estimated by flow cytometry in normal individuals (El Hoss *et al.*, 2018; Harrod *et al.*, 2007).

We also sought to determine laboratory factors associated with the HJB and AI red cell inclusions Both the frequencies of AI and HJB red cells showed a significant positive association with mean corpuscular haemoglobin (MCH) and a negative association with HbF level. This suggests that both markers maybe influenced by the same process in the spleen. We noted both markers correlated more strongly with the MCH (P = 0.0001) than with HbF (AI count, P = 0.020; HJB count, P = 0.002), indicating that MCH may be the more important association with splenic dysfunction than HbF among our patient population. It is not known if the observed association with MCH is due to the presence of alpha thalassemia trait among our SCD population, a condition known to ameliorate splenic dysfunction (Adekile *et al.*, 2002b; Adekile *et al.*, 1996; Wali *et al.*, 2002; Serjeant, Hambleton and Serjeant, 2021; Al-Jam'a *et al.*, 2000). Alpha thalassemia is highly prevalent (36% to 54%) among individuals of West and East African origin due to its protective effect on malaria (Wambua *et al.*, 2006; Mockenhaupt *et al.*, 1999; Falusi *et al.*, 1987). The MCH is a measure of the average amount of haemoglobin in the red cells; a high level favours polymerisation of HbS thereby inducing intravascular sickling of the red cells (Mozzarelli, Hofrichter and Eaton, 1987). The splenic environment also favours the process of red cells sickling; the blood within the splenic cords has a high haematocrit and stagnation of red cells within the cords leads to hypoxia and acidosis, all of which promote red cell sickling (Wolf and Neiman, 1989). Therefore, a combination of a splenic hostile environment and high MCH is likely to favour the sickling process in patients with SCD and to result in accelerated splenic dysfunction.

The negative association of HbF level with increased frequencies of AI and HJB red cell inclusions in our study is in keeping with the influence of HbF in preserving spleen function. High levels of HbF are associated with reduced intracellular HbS

polymerisation, hence preservation of spleen function in SCD patients as evidenced in reports from the USA (Nottage *et al.*, 2014; Pearson *et al.*, 1979; Rogers *et al.*, 2011) and India (Serjeant, Hambleton and Serjeant, 2021). Normal or near normal spleen function has also been reported in the majority of SCD patients with high HbF from the Middle East (Al-Awamy, Wilson and Pearson, 1984; Babiker *et al.*, 1985; Wali *et al.*, 2002). The protective effect of HbF on splenic function has however not been confirmed in all studies (Al-Jam'a *et al.*, 2000; Nardo-Marino *et al.*, 2022). We found that a high level of HbS was associated with increased AI red cell counts. The high level of HbS favours the process of polymerisation and ultimately impacts on the frequency of intravascular sickling of red cells sickling which worsens spleen function.

None of the clinical parameters analysed in the current study showed an association with both markers of splenic dysfunction. Nevertheless, the majority SCD patients reports having at least one episodes of fever over the preceding year and about a third have required hospitalisation; thus, it is reasonable to recommend anti-pneumococcal prophylaxis and anti-malaria prophylaxis especially for the less than five years old. Only a few of our SCD patients (15.9%) were on hydroxyurea treatment, this may have affected our ability to detect any significant association with both markers of splenic dysfunction. However, as expected, a higher HbF among the SCD patients on hydroxyurea treatment compared to the non- hydroxyurea group was noted. Hydroxyurea induces production of HbF and has been used as a disease modifying agent in SCD over the past three decades (Rogers *et al.*, 2011). Previous studies have shown increase in the level of HbF was associated with splenic preservation (Nottage *et al.*, 2014; Claster and Vichinsky, 1996); other studies have not observe improvement in splenic function among their hydroxyurea treated patients (Olivieri and Vichinsky, 1998; Santos *et al.*, 2002). Despite the disparity in reports, given the progressive

increase noted with age with the markers of splenic dysfunction in this study, starting hydroxyurea treatment early may be beneficial, as the associated increase in HbF level may allow for the maintenance or restoration of splenic function before irreversible fibrosis and loss of function occurs.

7.6.2 Limitation

The cross-sectional design of the study meant we could not monitor longitudinally the changing levels of markers of splenic dysfunction with age and therefore identify the exact onset of splenic dysfunction among our patients. However, the paired assessment of markers of splenic dysfunction between comparable age groups of SCD and healthy controls enabled us to document the changing pattern across a broad spectrum of age.

7.7 Conclusion

An age-related increase in markers of splenic dysfunction (i.e. HJB and AI red cell inclusions) occurred among our SCD patients. Although both markers of hyposplenism increased with increasing age among the SCD patients in our study, there was less variability after five years with the AI red cell counts. The steady increase with age observed with the HJB red cell counts indicates that this would be the preferred method to track splenic function in each individual. We observed a negative association between HbF and levels of both markers of splenic dysfunction. Although, we found no association between hydroxyurea therapy and both markers - possible due to the small number of patients on hydroxyurea, the high HbF among patients on hydroxyurea and the known protective effect of HbF on splenic function suggests that early administration of hydroxyurea may be beneficial in ameliorating the course of splenic dysfunction among our SCD patients.

CHAPTER 8: MALARIA INFECTION IN PATIENTS WITH SICKLE CELL DISEASE IN NIGERIA: ASSOCIATION WITH MARKERS OF HYPOSPLENISM

8.1 Chapter overview

The focus of this chapter was on malaria infection, which was the first sub-study within the spleen and infection study. Previous studies have attributed the high mortality observed in SCD patients to malaria infection. In this study, I evaluated malaria infection in SCD patients and normal control to determine if there is associated increased risk. The study also examined if hyposplenism is associated with the increased risk of malaria among acutely ill SCD patients.

Chapter 8 was submitted to Hemoglobin on the 16th of May 2023 and the reviewers have recommended for publication upon completion of minor revisions (1/11/23).

8.2 Abstract

Background

Malaria is considered an important cause of morbidity and mortality among people living with sickle cell disease (SCD). This has partly been attributed to the loss of splenic function that occurs early in the disease process. We aimed to study the prevalence of malaria infection among Nigerian SCD patients and explore the association with spleen size and function.

Method

This was a hospital-based, cross-sectional study performed at the University of Maiduguri Teaching Hospital in North-Eastern Nigeria from October 2020 to November 2021. Giemsa-stained blood smears for malaria parasites, Howell-Jolly body (HJB) red cells enumeration for spleen function evaluation and ultrasonography for spleen size assessment, were performed in acutely-ill SCD patients. Results of

malaria parasitaemia and parasite density were compared with those of steady-state SCD patients and non-SCD controls.

Results

A total of 394 participants consisting of 119 acutely-ill SCD patients, 167 steady-state SCD controls and 108 non-SCD controls were studied. The prevalence of *P. falciparum* parasitaemia was 51.3% in acutely-ill SCD patients, 31.7% in steady-state SCD controls and 13.0% in the non-SCD controls. In the SCD group, the mean parasite density was significantly higher among the acutely-ill SCD patients than the steady-state SCD controls (29,747 vs 18,563 parasites / ul; *P = 0.001*). Although parasitaemia prevalence was lower among the non-SCD controls, parasite density was significantly higher compared to both SCD groups (*P = 0.0001*). Among the acutely-ill SCD patients, the prevalence of clinical malaria and severe malaria anaemia were highest among children less than 5 years of age. Prevalence of parasitaemia (*P = 0.540*) and parasite density (*P = 0.975*) among acutely-ill SCD patients with visualised spleens on ultrasonography were not statistically different compared to those with absent spleens. Similarly, the frequency of HJB red cells among patients with parasitaemia was not significantly different compared to patients without parasitaemia (*P = 0.183*).

Conclusion

This study highlights the frequency and role of malaria infection in acutely-ill SCD patients, especially in those younger than five years. Although I have found no evidence of an increased risk of malaria parasitaemia or parasite density with markers of hyposplenism, the role played by an underlying immunity to malaria among SCD patients is not clear. Further studies are required to elucidate the role of hyposplenism and malaria in SCD patients in malaria-endemic regions.

8.3 Introduction

More than 300,000 infants are born worldwide with sickle cell disease (SCD) annually, and about two-thirds of these are in Sub-Saharan Africa (SSA) where malaria infection is also endemic (Piel *et al.*, 2013b). Malaria has widely been considered a major cause of morbidity and mortality among SCD patients in SSA (Flening, 1989; Cornille-Brøgger *et al.*, 1979; Serjeant, 2005; Konotey-Ahulu, 1971). The heterozygous form of the sickle gene (Hb AS) confers both innate and acquired protection against malaria infection (Gong *et al.*, 2012; Billo *et al.*, 2012). Hb AS red cells sickle preferentially when they are infected with *P. falciparum*, the infectious agent causing malaria; the parasitized sickled cells are phagocytosed by macrophages of the reticulo-endothelial system in the spleen and other organs (Luzzatto, 2012). The clinically-relevant consequence of this process is that the parasite level is relatively low in Hb AS heterozygotes. It has been suggested that the degree of protection against malaria infection might be correlated with the intracellular concentration of HbS, thus the protection should be greater among the homozygotes (Cholera *et al.*, 2008). However, clinical studies of malaria among SCD patients have shown differing outcomes. A lower prevalence and density of malaria parasitaemia among individuals with Hb SS compared to normal controls was reported in studies from Kenya (Albert *et al.*, 2009; Aluoch, 1997; McAuley *et al.*, 2010), Tanzania (Makani *et al.*, 2010b) and Nigeria (Okuonghae, Nwankwo and Offor, 1992; Awotua-Efebo, Alikor and Nkanginieme, 2004), suggesting that SCD patients experience some degree of resistance to the infection than heterozygotes. Other studies have shown that homozygotes (HbSS) can develop severe and fatal complications following infection with *P. falciparum* (Aloni *et al.*, 2013; Ambe, Fatunde and Sodeinde, 2001; Akinyanju and Johnson, 1987);

malaria infection was a common precipitating factor for both vaso-occlusive and haemolytic crisis.

The presence of splenic dysfunction which occurs early in the disease process through recurrent vaso-occlusion may make malaria more severe among SCD individuals compared to normal individuals (Williams and Obaro, 2011). The spleen plays an important role in the control of *P. falciparum* parasite load even in the presence of pre-existing antibody-acquired immunity (Buffet *et al.*, 2011; Chotivanich *et al.*, 2002). The absence of the spleen could thus affect an individual's ability to clear parasitised red cells from the circulation; other organs such as the liver may take over the role of parasite clearance, but they are less efficient than the spleen (Buffet *et al.*, 2011). Cohort studies among SCD patients residing in the Northern hemisphere show that loss of splenic function occurs as early as 6 months of age and a majority of children would have reduced or absent spleen function by the age of two years (Rogers *et al.*, 2011; El Hoss *et al.*, 2019). There are few data on splenic function in the African context (Adekile *et al.*, 1991; Adekile *et al.*, 1993), thus little is known about the increased risk of malaria associated with hyposplenism among our SCD patients (Ladu *et al.*, 2021). While SCD patients are at an increased risk of malaria infection from hyposplenism, individuals resident in endemic regions often develop immunity following repeated episodes of infection (Day and Marsh, 1991; Snow *et al.*, 1994). A large number of the general population in malaria-endemic regions carry malaria parasites throughout life but are asymptomatic (Smith *et al.*, 1999), however, the relevance of asymptomatic parasite carriage among SCD patients is not very clear (Oyetunji *et al.*, 2020; Kotila, Okesola and Makanjuola, 2007). The aim of this study was to determine the frequency of malaria parasitaemia and parasite density among acutely-ill SCD patients and compare it with those of steady-state SCD controls and

non-SCD controls from the same environment to determine the role malaria plays in crisis. The relationships between parasitaemia prevalence and parasite density with spleen size and function among the acutely-ill patients were also evaluated to ascertain if hyposplenism is associated with increased risk of malaria.

8.4 Methods

8.4.1 Study design, setting and period.

This was a hospital-based, cross-sectional study conducted at the University of Maiduguri Teaching Hospital, North-Eastern Nigeria from October 2020 to November 2021. It is a tertiary facility of 500-bed capacity, which serves as a referral hospital to Maiduguri and the surrounding township. Maiduguri is the largest city in North-East Nigeria, with an estimated population of about one million people (Bell and Card, 2021). It is located in the Sahel-Savanna and is characterized by three climate patterns - a harmattan or cool-dry season (October–February), a hot season (March–May/June) and a rainy season (June or July–September) (Waziri, 2012). Malaria is meso-endemic in Maiduguri with a seasonal transmission of 4 - 6 months during the rainy season. The prevalence of *P. falciparum,* from previous studies, ranges from 22.6% to 36.0% among febrile children and adults (Ayanugwo and Kalu, 1997; Balogun *et al.*, 2019), with the highest prevalence during the rainy seasons of August and September (74% to 84%) (Balogun *et al.*, 2019). Part of the routine care of individuals living with SCD in Nigeria is the administration of malaria chemoprophylaxis using proguanil (Galadanci *et al.*, 2014).

8.4.2 Study participants and data collection

All febrile and/or acutely-ill SCD patients who attended the hospital during the study period were invited to participate. The patients were divided into four age groups 1:

Less than 5 years ; 2: 5–9 years; 3: 10–14 years and group 4: 15 years and above. To put our findings into context, we compared them with two control groups. The first consisted of SCD patients considered to be in steady state (Ballas, 2012), on follow-up at the outpatient paediatric and adult haematology clinics during the study period. The second group included healthy individuals (i.e., with no SCD) consisting of medical students, children of hospital personnel and paediatric patients on post-op follow-up in the surgical clinic. A case report form was used to obtain demographic characteristics and self-reported medical history from the patients (or their carers), including the use of insecticide-treated bed nets, anti-malaria prophylaxis, and the regular use of hydroxyurea in the 12 months preceding the study. Axillary temperature was measured in all patients. Malaria among the acutely-ill SCD patients was classified into three categories: (1) parasitaemia, a positive blood film (2) clinical malaria, a positive blood film in the presence of fever (defined as an axillary temperature of >37.5°C); and (3) severe malarial anaemia (SMA), haemoglobin of less than 5 g/dL in the presence of malaria parasitaemia (Albert *et al.*, 2009; Makani *et al.*, 2010a)

8.4.3 Routine blood counts

See section 3.4

8.4.4 Blood smears for malaria parasite analysis

Giemsa-stained thin smears for malaria parasites detection were made from EDTA blood samples not more than 4 hours after collection, air-dried and fixed in 100% methanol. Slides were then stained in 20% Giemsa (diluted in a buffer with a pH of 7.2) for 20 min. *P. falciparum* densities were assessed by counting number of asexual-stage parasites per 500 red blood cells (RBC) and expressed as parasites per microliter

of whole blood. The density of parasites was calculated using each participant's RBC count for the SCD population, or an average count of 4000 RBCs / ul of blood for the controls for whom no RBC counts were performed (O'Meara *et al.*, 2006; Komba *et al.*, 2009)

8.4.5 Blood smears for spleen function analysis

See section 3.4.3

8.4.6 Ultrasonography for assessment of the spleen

See section 3.4.6

8.5 Results

A total of 140 acutely-ill SCD patients (median age 13.0 years; IQR 16.0 years) were enrolled. Blood smears for malaria parasite counts were available for 119 (85%); the remaining 21 samples were excluded because of poor quality. Blood smears for malaria parasite count were available for 167 steady-state SCD controls and 108 non-SCD controls. The baseline characteristics of the study population are shown in Table 13. The use of insecticide-treated bed nets ($P = 0.859$), malaria chemoprophylaxis ($P=0.350$) and hydroxyurea ($P=0.508$) were not significantly different between the acutely-ill SCD patients and steady-state SCD controls.

Table 13: Baseline characteristics of the study population

Parameters	Acutely-ill SCD patients	Steady-state SCD controls	Non-SCD controls
Number	119	167	108
Age (years)	13.0 (16.0)	11 (15.0)	12.0 (17.8)
Age groups, n (%) Less than 5 years 5 – 9 years 10 - 15 years ≥15 years	 27 (22.7) 20 (16.8) 27 (22.7) 45 (46.2)	 37 (22.0) 35 (20.8) 31 (21.4) 60 (35.7)	 20 (18.5) 22 (20.4) 21 (19.4) 45 (41.7)
Sex (male)	55 (46.2)	81 (48.2)	66 (61.1)
Use of insecticide treated nets, n (%)	103 (86.6)	146 (87.4)	NR
Malaria prophylaxis, n (%)	101 (84.9)	134 (80.2)	NR
Hydroxyurea therapy, n (%)	16 (13.4)	28 (16.8)	NR

NR- not recorded.

8.5.1 Prevalence of malaria parasitaemia and parasite density among the study participants

The prevalence of *P. falciparum* parasitaemia was 51.3% (95% Confidence interval (CI): 42% - 60%) in acutely-ill SCD patients, 31.7% (95% CI: 25% - 39%) in steady-state SCD controls and 13.0% (95% CI: 7% - 19%) in the non-SCD controls. Compared to the non-SCD control group, the odds ratio (OR) for *P. falciparum* parasitaemia was significantly higher for both the steady-state SCD controls (OR 3.12; 95% CI: 1.63 - 5.98) and acutely-ill SCD patients (7.06; 95% CI, 3.63 -13.7). Furthermore, the OR for *P. falciparum* parasitaemia was significantly higher among the acutely-ill SCD patients than the steady-state SCD controls (OR 2.26; 95% CI: 1.39 - 3.67).

Among those infected with *P. falciparum* parasites, the mean parasite density was significantly higher among the non-SCD controls ($P = 0.0001$) (Table 14). In the SCD group, the mean parasite density was significantly higher among the acutely- ill SCD patients than the steady-state SCD controls (29,747 vs 18,563 parasites/ul; $P = 0.001$).

Table 14: Comparison of parasite density across the study population

Variable	Geometric mean (parasite / ul)	95% CI	P value (& post hoc analysis)
Parasite density SCD patients, n=61 SCD-controls, n=51 Non-SCD controls, n=14	 29,747 18,563 100,512	 19,655 – 45,202 13,317 – 25,769 50,845 – 198,693	**0.0001***

*Kruskal Wallis analysis; CI confidence interval; Post hoc analysis: acute-ill SCD patients vs SCD-control: 0.001; acutely-ill SCD patients vs non-SCD control: 0.0001; SCD-controls vs non-SCD controls: 0.012

8.5.2 Frequency of malaria parasitaemia and parasite density across age groups among the study participants

Among the acutely-ill SCD patients, the frequency of malaria parasitaemia ($P = 0.731$) and parasite density ($P = 0.533$) was not significantly different across the age groups (Table 15). Among the steady-state SCD controls, individuals aged 15 years and above were less likely to have parasitaemia compared to those less than five years (7.5% vs 22.6%; OR 0.14; 95% CI: 0.04 - 0.49) ($P = 0.002$); however, parasite density among those with parasites detected was found to be significantly higher among the older age groups ($P = 0.008$). In contrast, among the non-SCD controls, the frequency of malaria parasitaemia and parasite density tended to be higher among the older individuals 10 years and above, although the difference did not reach statistical significance for either parasitaemia ($P = 0.312$) or parasite density ($P = 0.582$).

Table 15: Comparison of malaria parasitaemia and parasite density across age groups among the study population

Variables	Parasitaemia present		Parasite density	
	Number (%)	P values	GM (95% CI)	P values
Acutely-ill SCD patients, n=61				
1 - <5 years	16 (26.2)	0.731	20,737 (8449 - 50,897)	0.533
5 - <10years	11 (18.0)		39,134 (13,285 - 115,284)	
10 - <15 years	13 (21.3)		22,862 (10,388 - 50,320)	
≥15 years	21(34.4)		39,922 (17,850 - 89,285)	
Steady-state SCD controls, n=53				
1 - <5 years	12 (22.6)	0.0001**	9363 (6777 - 12,936)	0.008*
5 - <10years	24 (45.3)		13,402 (9285 - 19,347)	
10 - <15 years	13 (24.5)		51, 249 (21,537 -121,593)	
≥15 years	4 (7.5)		37,623 (4666 - 303,329)	
Non- SCD controls, n=14				
1 - <5 years	1 (7.1)	0.312	96,000^^	0.582
5 - <10years	1 (7.1)		192,000^^	
10 - <15 years	4 (28.6)		183,411 (94,128-357,381)	
≥15 years	8 (57.2)		69,019 (20,848 - 230,706)	

*Sig.P value <0.05 - Kruskal Wallis test for parasite density across the age groups. **Sig.P value <0.05 – Logistic regression analysis, the 1-<5 years age group was used as the reference group; ^^CI not generated; sample size = 1. GM Geometric mean; CI Confidence interval

8.5.3 Severe malaria episodes across age groups among acutely-ill SCD patients

The distribution of severe malaria episodes across age groups among the acute-ill SCD patients is shown in Table 16. Nineteen (16.0%) had clinical malaria, 15 (12.6%) had severe malaria anaemia (SMA) and seven (5.9%) had both clinical malaria and SMA. Clinical malaria was less common among older patients 15 years and above when compared to those younger than 5 years (21.1% vs 42.1%; OR 0.81; 95% CI: 0.67 - 0.98) (P = 0.034); however, the prevalence of SMA was not significantly different between these two age groups (33.3% vs 26.7%; OR 0.72; 95% CI: 0.18 - 2.95) (P = 0.646). Combined clinical malaria and SMA occurred most frequently in patients less than five-year-old (57.1%).

Table 16: Summary of malaria episodes across age groups among acutely-ill SCD patients

Age group	Clinical malaria*	Severe malaria anaemia**	Clinical malaria and SMA
1 - <5 years, n (%)	8 (42.1)	4 (26.7)	4 (57.1)
5 - <10 years, n (%)	2 (10.5)	2 (13.3)	1 (14.3)
10 - <15 years, n (%)	5 (26.3)	4 (26.7)	2 (28.6)
≥15 years, n (%)	4 (21.1)	5 (33.3)	0 (0)
Total, n (%)	**19 (16.0)**	**15 (12.6)**	**7 (5.9)**

*Malaria parasitaemia and temperature above 37.5° C

** Malaria parasitaemia and haemoglobin level less than 5 g/dl

8.5.4 Association of malaria parasitaemia with splenic parameters among acutely-ill SCD patients

Ultrasonography was available for 58 out of the 61 acutely-ill SCD patients with malaria parasitaemia. There was a tendency towards a higher prevalence of malaria parasitaemia among patients with visible spleens (n=34/58; 58.6%) (Fig. 19A) compared to those with absent spleens (n=24/58; 41.4%), however, the difference was not significant (*P = 0.540*). Similarly, the geometric mean parasite density in patients with visible spleens (23,100/ul) was not statistically different with those patients with absent spleens (24,300/ul) (*P =0.975*) (Fig.19B). The frequency of HJB red cells among patients with parasitaemia (median 1.7%, IQR 3.4) was not significantly

different compared to patients without parasitaemia (median 1.5%, IQR 6.2) (*P = 0.183*).

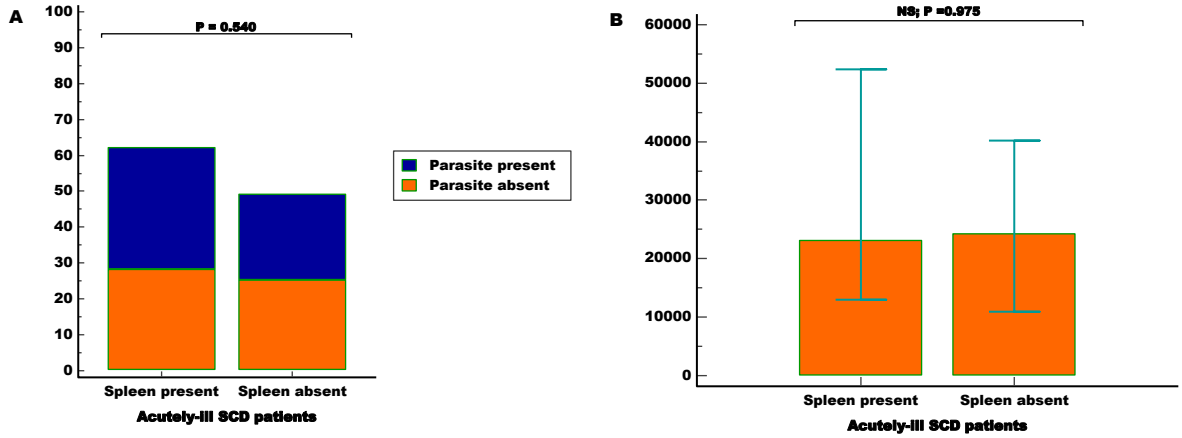


Figure 19: Distribution of malaria parasitaemia and parasite density based on spleen ultrasonography among the acutely-ill SCD patients.

Legend: Prevalence of malaria parasitaemia (A) (P = 0.540; Logistic regression analysis) or parasite density (B) (P=0.975; Kruskal Wallis test) was not significantly different between patients with visible or absent spleens on ultrasonography.

8.5.5 Association of malaria parasitaemia with clinical and laboratory parameters among acutely-ill SCD patients

On physical examination, pallor and jaundice were recorded in 49 (41.2%) and 42 (35.3%) patients respectively. The liver was palpable in 10 (8.4%) patients. The frequencies of a palpable liver or the presence of jaundice and pallor were not significantly different among the acutely-ill SCD patients with parasitaemia and those without (Table 17). Furthermore, the use of bed nets, antimalarial prophylaxis and hydroxyurea showed no significant difference in the prevalence of parasitaemia among the two groups. None of the laboratory parameters differed among patients with parasitaemia and those without parasitaemia.

Table 17: Univariate analysis for factors associated with malaria parasitaemia among acutely ill SCD patients.

Clinical feature	No malaria parasitemia [n=58 (48.7)]	Malaria parasitemia [n=61 (51.3)]	OR (95% CI)	P
Male, n/n (%)	32/58 (55.2)	31/61(50.8)	1.28 (0.62 – 2.63)	0.507
Age, median, IQR	13.0 (15.3)	13.0 (16.3)	0.99 (0.95 – 1.02)	0.493
Physical signs				
Temperature, median, IQR	37.2 (2.0)	37.1 (1.0)	0.79 (0.54 – 1.16)	0.222
Pallor present, n/n (%)	27/58 (46.6)	22/61 (36.1)	0.65 (0.39 – 1.35)	0.246
Jaundice present, n/n (%)	24/58 (41.4)	18/61 (29.5)	0.59 (0.28 – 1.27)	0.177
Liver palpable on examination, n/n (%)	3/58 (5.2)	7/61 (11.5)	2.38 (0.58 – 9.67)	0.227
Regular antimalaria prophylaxis intake, n/n (%)	45/57 (78.9)	55/61 (90.2)	2.44 (0.85- 7.02)	0.097
Use of bed net, n/n (%)	52/58 (89.7)	51/61 (83.6)	0.59 (0.19 – 1.74)	0.337
Regular hydroxyurea intake, n (%)	7/58 (12.1)	9/61 (14.8)	1.26 (0.44 – 3.64)	0.668
Laboratory parameters				
WBC (10^9/l)	16.5 (10.9)	18.4 (11.4)	1.05 (0.99 – 1.10)	0.053
Haemoglobin, g/dl	6.7 (3.5)	6.1 (3.0)	0.97 (0.81 – 1.17)	0.781
Platelets (10^9/l)	390.0 (292)	366.0 (225)	1.00 (0.99 -1.00)	0.930
Reticulocyte %	3.9 (8)	6.1 (11)	1.03 (0.97 – 1.10)	0.291
Bilirubin (total)	21.0 (24.0)	20.0 (26.3)	0.99 (0.98 -1.01)	0.852
ASAT, (iu/l)	19.0 (27)	17.5 (15.5)	0.99 (0.97 -1.01)	0.457
HbF %	6.4 (9.2)	7.7 (7.1)	0.99 (0.92 – 1.06)	0.700

ASAT aspartate amino transferase; WBC white blood cell count. OR odds ratio; CI confidence interval; IQR inter quartile range.

8.6 Discussion

Reports in the literature suggest that malaria is a major precipitant of crisis among SCD patients. However, since these studies were conducted in areas of high malaria transmission, where asymptomatic parasitaemia is common, and where infections from other organisms is common, the presence of parasitaemia cannot be categorically

stated as the cause of the crisis (Ambe, Fatunde and Sodeinde, 2001; Juwah, Nlemadim and Kaine, 2003; Akinyanju and Johnson, 1987; Ibidapo and Akinyanju, 2000). The current study provided us an opportunity to evaluate the possible role of malaria among our SCD patients. We have studied malaria parasitaemia and parasite density in asymptomatic and symptomatic SCD patients and compared our results with healthy controls from the same environment. Although parasitaemia prevalence was lower among the non-SCD controls compared to both SCD groups, parasite density was significantly higher in the non-SCD controls. This may suggest a higher resistance to the parasites amongst the SCD population and maybe attributed to the high concentration of HbS in the red cells of SCD patients, which have been shown to confer protection (Cholera *et al.*, 2008; Pasvol, Weatherall and Wilson, 1978). However, among the SCD population, *P. falciparum* prevalence and parasites densities were higher among the acutely-ill SCD patients than steady-state SCD-controls indicating that malaria infection is an important contributor to crises. Other studies from low malaria transmission regions have shown an increased prevalence of malaria during hospitalisation compared to patients in out-patient clinics (Albert *et al.*, 2009; Makani *et al.*, 2010a).

8.6.1 Malaria burden among acutely-ill SCD patients

In malaria-endemic regions, there is a large variation in the clinical severity of *P. falciparum* infection. The malaria spectrum includes asymptomatic infection (parasitaemia), febrile illness, severe illness (including profound anaemia and cerebral complications) and death at the extreme end (Network, 2008). Only a small proportion of *P. falciparum* infections progress to severe malaria. The probability of a child progressing from one event to another is as follows: asymptomatic parasitaemia (50% of each year), malaria fever twice per year; severe malaria - 3% per year, and death -

1% probability per year (Network, 2008). This trend was observed in our study; we noted fewer episodes of clinical malaria (16%) compared to asymptomatic parasitaemia (51.3%). Few patients had severe malaria (SMA) (12.5%), and fewer had a combination of clinical malaria and SMA (5.9%). This observation is similar to an earlier report from Tanzania (Makani *et al.*, 2010a). These complications were more common in the acutely-ill SCD patients less than five years of age, correlating with the high level of parasitaemia seen among the steady-state SCD controls. This finding underscores the susceptibility of the less than five years of age SCD patients to both malaria and exacerbation of their pre-existing anaemia (Juwah, Nlemadim and Kaine, 2003), and highlights the importance of prompt and effective management of malaria among acutely-ill SCD patients to prevent progression to severe complications. Like any other infection, malaria can trigger an acute crisis in an individual with SCD. Studies have shown that both severe anaemia and death are considerably more common among SCD than non-SCD patients who were hospitalised with malaria (McAuley *et al.*, 2010; Makani *et al.*, 2010a).

8.6.2 Prevalence of parasitaemia and parasite density among steady-state SCD controls and non-SCD controls

Malaria parasitaemia was present in both our steady-state SCD controls and non-SCD controls, which may imply the presence of some degree of immunity to the parasite in the population. A high prevalence of parasitaemia was also noted among the younger (less than 10 years) steady-state SCD controls compared to the older patient population. In contrast, among the non-SCD controls, the frequency of malaria parasitaemia and parasite density showed an increasing frequency with increasing age; the prevalence was higher in patients aged 15 years and above compared to the younger patients aged five years and below. Similarly, parasite density was higher among the

older population. The small number of those less than five (n=1) and less than 10 (n=1) years of age may have affected the power to detect any statistical significance among the non-SCD controls. Nevertheless, age has been shown to be an important factor that determines immunity to malaria in an endemic region (Day and Marsh, 1991); when a child becomes infected, he or she usually develops symptoms of the disease including fever but eventually recovers. Repeated episodes of infection among older children and adults result in the development of clinical immunity to malaria. Therefore, adults are likely to develop asymptomatic infection (Network, 2008; Snow *et al.*, 1994; Day and Marsh, 1991). As asymptomatic subjects do not seek treatment, they may serve as reservoirs of the parasites, thus contributing to the perpetual spread of the parasites (Smith *et al.*, 1999).

8.6.3 Association of malaria parasitaemia with splenic parameters among acutely-ill SCD patients

The clinical pattern and outcome of infection with *P. falciparum* may be impacted by several factors including prior exposure and immunity, intensity of transmission in the area, age, and the ability of the spleen to clear parasitised red cells. The spleen plays an important role in the control of *P. falciparum* parasite load (Buffet *et al.*, 2011; Safeukui *et al.*, 2008; Chotivanich *et al.*, 2002). In endemic areas, fever and parasitaemia were significantly more frequent in splenectomised patients compared to subjects with intact spleen; severity and fatality of *P. falciparum* infection was increased in such patients but not to a large extent (Bach *et al.*, 2005; Boone and Watters, 1995). Mature forms of the parasite express an adhesion protein (*P. falciparum* erythrocytes membrane proteins - PfEMP1), which allows them to adhere to several cells within small vessels; by so doing, the parasites escape retention and destruction by the spleen. The PfEMP1 is a highly immunogenic molecule, and with

repeated exposures, children residing in endemic areas progressively acquire antibodies against it (Smith *et al.*, 1999). In immune patients with a spleen, as well as in splenectomised patients, antibodies to PfEMP1 prevent the sequestration of mature forms of the parasite in the microcirculation, thereby preventing severe complications. In patients with intact spleens, the mature forms are cleared by the spleen; however, in the immune individual without a spleen, the parasites are less efficiently cleared either because the microcirculation of other organs are unable to mechanically retain mature forms of the parasites or opsonisation is less efficient in the sinusoids of these organs as compared to the splenic cords (Buffet *et al.*, 2011). In our study, no significant association between the presence or absence of the spleen on ultrasonography with prevalence of parasitaemia or parasite density was noted among our acutely-ill SCD patients. Also, clinical and laboratory parameters were not different among the group with parasitaemia and those without parasitaemia. It is unclear if the presence of immunity to the parasite among our patient population accounts for this finding. The current study is the first to describe the association between malaria and splenic function (i.e. HJB) among SCD patients residing in a malaria-endemic region. The frequency of HJB red cells was not significantly different between the group with parasitaemia and those without parasitaemia. Splenic dysfunction has widely been reported to be associated with increased susceptibility to infections including malaria. What is not clear is whether acute malaria infection reduces spleen function as in other febrile conditions, or if it is the loss of splenic function in SCD that predisposes to the malaria infection.

8.6.4 Association of malaria parasitaemia with clinical parameters among acutely-ill patients

Given the previous reports from early studies (Luzzatto, 2012; Konotey-Ahulu, 1971) about the fatal consequences of malaria in SCD patients residing in malaria-endemic regions, they are routinely placed on lifelong antimalaria prophylaxis. Despite the high intake of antimalaria prophylaxis (84.9%) and use of bed nets (86.6%) among the acutely-ill SCD patients, the prevalence of malaria parasitaemia was still high. About half of the patients using bed nets (49.5%) and antimalaria prophylaxis (55%) were positive for *P. falciparum*. This is similar to the observations in several studies, where despite regular prophylaxis (with proguanil in most cases), SCD patients still developed malaria infection (Ambe, Fatunde and Sodeinde, 2001; Awodu, Wagbatsoma and Enosolease, 2008; Kotila, Okesola and Makanjuola, 2007), calling into question the efficacy of bed nets and chemoprophylaxis. A recent Cochrane review on malaria chemoprophylaxis in SCD patients concluded it was beneficial to provide routine malaria chemoprophylaxis in SCD patients in malaria-endemic areas (Oniyangi and Omari, 2006), however, decision on malaria chemoprophylaxis remains challenging (Aneni, Hamer and Gill, 2013). Other anti-malaria agents including mefloquine, amodiaquine, halofantrine and artemether-lumefantrine may be suitable for chemoprophylaxis, but they will need to be evaluated in future studies in order to provide data that can be used to develop consensus guidelines for malaria chemoprophylaxis in SCD patients (Aneni, Hamer and Gill, 2013).

8.6.5 Limitations

Our study has some limitations. Firstly, the single centre and hospital-based nature of the study conducted in an area with high malaria transmissions may affect the generalisability of our results to the general population with SCD. Secondly, we have

used thin blood smears to evaluate malaria parasitaemia as opposed to thick blood films which are routinely used. The thin blood has been shown to be less sensitive at low parasitaemia (Bowers *et al.*, 2009), and may have under-estimated the prevalence of parasitaemia among our study population, especially the low rate reported among our non-SCD controls when compared to the high value reported from a previous report in the study area (Balogun *et al.*, 2019). However, an acceptable agreement between thin and thick film density measurements has been reported (Planche *et al.*, 2001). Moreover, studies comparing parasite densities observed in thick films to those observed in thin films have reported that a large percentage of parasites can be obscured in the thick film or lost during staining and lysis of red blood cells (O'Meara *et al.*, 2006; Bejon *et al.*, 2006).

8.7 Conclusion

In our study conducted in an area of high malaria transmission, we found that malaria parasitaemia and parasite density were more common among acutely-ill than steady-state SCD controls. We also found that severe malaria events were significantly higher among under-five than in older acutely-ill SCD patients. There was no association of malaria parasitaemia and parasite density with spleen size on ultrasonography or with splenic function.

CHAPTER 9: BACTERAEMIA AMONG PATIENTS WITH SICKLE CELL DISEASE IN NIGERIA: ASSOCIATION WITH SPLEEN SIZE AND FUNCTION

9.1 Chapter overview

This chapter covers the second sub-study in the spleen and infection study and focuses on bacterial infection among SCD patients. Bacterial infection has been linked to a high risk of morbidity and mortality among SCD, hence in this study, I studied the prevalence of bacterial infection and its relationship with splenic parameters, that is, both spleen size and function.

Chapter 9 has been published as a scientific letter in the Mediterranean Journal of Haematology and Infectious disease and can be accessed using the link below.

https://doi.org/10.4084/MJHID.2023.054

9.2 Introduction

In Sub-Saharan Africa, infections are a leading cause of morbidity among individuals with sickle cell disease (SCD). The causes of the increased risk of infection are poorly documented, but the loss of splenic function is important. Previous studies have documented increased susceptibility to bacterial infections among SCD patients, evidenced by increasing markers of splenic dysfunction (Pearson *et al.*, 1979; Rogers, Serjeant and Serjeant, 1982); however, there are no data on the association between bacterial infections and splenic function among the SCD population in Sub-Saharan Africa, partly because most of the techniques required to assess splenic function are not readily available (Ladu *et al.*, 2021). We recently employed the presence of two red cell containing inclusions - Howell-Jolly bodies (HJB) and argyrophilic (silver staining) inclusion (AI) red cells - to assess splenic dysfunction among our SCD

patients (Ladu *et al.*, 2023). In the present study, we aimed to determine the prevalence and pattern of organisms causing bacteraemia among our acutely-ill SCD patients and to describe any association between bacteraemia with splenic status on ultrasonography and two markers of splenic dysfunction (i.e HJB and AI red cells).

9.3 Methods

This was a hospital-based, cross-sectional study conducted at the University of Maiduguri Teaching Hospital, North-Eastern Nigeria from October 2020 to May 2021. All febrile and/or acutely-ill SCD patients presenting to the adult or paediatric emergency unit during the study period were invited to take part. A case report form was used to obtain baseline clinical characteristics from the patients (or their carers). Under aseptic conditions, between 3 and 8 ml of venous blood for cultures were collected directly into appropriate BACTEC plus bottle and incubated manually at 37°C. Positive blood cultures were sub-cultured on standard media with the use of routine microbiological techniques. Non-pathogenic organisms commonly associated with contaminated blood cultures such as coagulase-negative *staphylococci*, *Acinetobacter* species, *Bacillus* species, *Corynebacterium* species, *Micrococcus* species and non-meningitidis *Neisseria* species were considered as contaminants (Doern *et al.*, 2019). Splenic function was assessed by manual estimation of Howell-Jolly bodies (HJB) and argyrophilic inclusion (AI) containing red cells from blood smears as previously described (Ladu *et al.*, 2023). The splenic status was assessed using ultrasonography. Data were analysed using Statistical Package for the Social Sciences (SPSS) (version 25; SPSS, Chicago, IL, USA). The data were summarised using descriptive statistics. The prevalence of bacteraemia was defined as the proportion of positive cultures in all the blood cultures taken. Clinical and laboratory

features of patients with bacteraemia were compared to those without bacteraemia, using non-parametric analysis.

9.3.1 Ethical considerations

See section 3.6.4

9.4 Results

Over the 7-month study period, a total of 162 febrile episodes involving 140 SCD patients (median age 13.0 years; IQR 5.0 - 21.0) occurred during visits to the adult and paediatric emergency units. The baseline clinical characteristics of the patients is shown in table 18. The predominant haemoglobin phenotype was HbSS (98.1%); only two patients (1.9%) had HbSC phenotype. A total of 113 (69.8%) blood cultures were obtained. Bacteraemia occurred in six (5.2%, 95% CI, 1% to 10%) of the culture samples. Two cultures from children aged 2 and 3 years grew *Salmonella* species. The remaining four positive cultures were among the adult patients (one culture each grew *Serratia Marcescens, Citrobacter spp, Enterobacter spp* and an unidentified Gram-negative bacillus). The antibiotic sensitivity for the isolates is shown in table 19. Four other positive cultures were considered contaminants and excluded during the analysis. The clinical characteristics and splenic status on ultrasonography of patients with bacteraemia are shown in table 20. The spleen was present in three patients (2 children and 1 adult) and absent in the remaining three (i.e autosplenectomy). The presence of bacteraemia was not significantly different in SCD patients with spleen present or absent spleen on ultrasonography (P = 0.87). Comparison of clinical and laboratory parameters between patients with and without bacteraemia are shown in table 21. The median HJB and AI red cell counts were 1.8% (IQR 0.5% - 7.3%) and 48.2% (IQR 27.2% - 63.5%) respectively among the study participants. There was a trend towards

higher counts of red cells with HJB (median 2.4% vs 1.9%) and AI (62.4% vs 43.4%) in patients with bacteraemia compared to those without bacteraemia respectively; however, the result was not significant for either the HJB (P = 0.744) or AI red cell counts (P = 0.075) (Table 21). Patients with bacteraemia had significantly higher white blood cell counts (mean 34.9 vs 21.6; P = 0.018) and raised neutrophil counts (19.4 vs 11.3; P = 0.006) in comparison to patients without bacteraemia. All other clinical and laboratory parameters were not significantly different between the two groups.

Table 18:Baseline characteristics of the acutely-ill SCD patients

Variables	**Median (IQR)/Frequency, %**	**Number of observations**
Age (years)	13.0 (5.0 - 21.0)	140
Male, n, %	70 (50)	140
SCD treatment		
Proportion of patients on: Folate supplementation, n, % Malaria chemoprophylaxis, n, % Oral penicillin prophylaxis, n, % Hydroxyurea, n, %	 143 (88.3) 137 (84.6) 3 (2.1) 20 (14.3)	140
Routine vaccination completed, n, % Yes No Not sure	 74 (52.9) 18 (12.9) 48 (34.3)	140
Previous history of blood transfusion, n, % Yes No	 99 (70.7) 41 (29.3)	140
Clinical features on presentation		
Pain, n, %	100 (61.7)	162
Anaemia, n,%	55 (34)	162
Fever, (>37.5°C), n,%	55 (34)	162
Pallor, n, %	68 (42.0)	162
Jaundice, n, %	59 (36.4)	162
Clinical outcomes of SCD, n,%		
Pain crises over last 12 months None 1-3 >3	 54 (33.4) 42 (25.9) 66 (40.7)	162
Fever episodes over last 12 months None 1 -3 > 3	 18 (11.1) 66 (40.7) 78 (48.2)	162
Hospitalization over last 12 months None 1-3 >3	 83 (51.2) 73 (45.1) 6 (3.7)	162
Use of over-the-counter antibiotics prior to presentation	47 (29.0)	162
Laboratory parameters		
White blood cell count (10^9/l)	17.8 (11.9 - 23.9)	154
Haemoglobin (g/dl)	6.4 (4.9 - 7.7)	154
Platelets (10^9/l)	370.0 (254.0 – 502.0)	153
ANC (10^9/l)	8.3 (5.9 - 13.6)	153
MCV (fl)	82.2 (76.6 - 88.5)	154
MCH (pgl)	27.8 (26.1 - 30.2)	154
MCHC (g/dl)	34.0 (32.3 - 34.1)	154

Reticulocytes (%)	5.0 (1.5 -10.6)	143
Bilirubin (total)(umol/l)	21.0 (13.0 - 39.0)	122
ASAT (iu/l)	13.0 (8.0 - 27.0)	123
HbF (%)	7.4 (3.9 - 11.4)	108
HbS (%)	79.3 (71.7 - 83.7)	108
HbA_2 (%)	3.0 (2.6 - 3.5)	77

ASAT aspartate amino transferase; ANC absolute neutrophil count; Hb haemoglobin; MCV mean corpuscular volume; MCH mean corpuscular haemoglobin; MCHC mean corpuscular haemoglobin concentration.; IQR interquartile range.

Table 19: Antibiotics sensitivity pattern

Isolates	Sensitive	Resistant
Salmonella typhi	Ciprofloxacin, Co-trimoxazole, Clindamycin and Ceftriaxone + Sulbactam	Nil
Salmonella typhi	Ciprofloxacin, Ceftriaxone, Gentamicin	Clindamycin, Augmentin
Enterococci sp	Ciprofloxacin, Vancomycin, Linezolid	Amoxicillin, Erythromycin
Serratia marcescens	Ciprofloxacin, Nitrofurantoin	Gentamicin, Augmentin and CAZ
Citrobacter	Ciprofloxacin, Gentamicin and Ceftriaxone	Augmentin and Nitrofurantoin
GNB (unidentified)	Ceftriaxone + Sulbactam, Ciprofloxacin, Co-trimoxazole	Augmentin and Nitrofurantoin

Table 20: Clinical characteristics of SCD patients with positive blood cultures

Isolates	Sex	Age (years)	Temp	Ill looking	WBC	Prior antibiotics use*	Immunization completed	% AI red cells (median)	% HJB red cells (median)	Spleen size on ultrasound
Salmonella typhi	F	2	38.5	Yes	34.7	Yes	Yes	65.6%	1.3%	5.6 cm
Salmonella typhi	F	3	37.8	Yes	30.0	Yes	Yes	45.%	0.6%	8.2 cm
Enterococci sp	F	16	37.2	Yes	33.5	No	No	61.1%	26.0%	6.8 cm
Serratia marcescens	F	22	37.2	Yes	18.1	No	Yes	73.0%	3.5%	Autosplenectomy*
Citrobacter	M	25	37.8	Yes	26.7	No	Not sure	74.6%	NR	Autosplenectomy
GNB (unidentified)	M	17	36.0	Yes	22.8	Yes	Yes	53.2%	2.9%	Autosplenectomy

AI argyrophilic inclusion; HJB Howell-Jolly bodies. M: male; F: female. Temp: temperature. GNB: Gram-negative bacillus WBC: white blood cell count; NR not reported; * spleen was not visualized on ultrasound.* refers to use of antibiotics for treatment of symptoms before presenting to the hospital.

Table 21: Association of clinical and laboratory parameters with bacteraemia

	Bacteraemia absent [n = 107/113 (94.8)]	**Bacteraemia present [n = 6/113 (5.2)]**	***P***
Clinical parameters			
Age, years, mean (SD)	12.8 (8.9)	17.1 (11.5)	0.565
Male, n/n (%)	56/107 (52.3)	2/6 (33.3)	0.611
Temperature, mean (SD)	37.5 (0.9)	37.5 (1.0)	0.938
Jaundice, n/n (%)	36/106 (34.0)	3/6 (50.0)	0.418
Pallor, n/n (%)	50/106 (47.2)	4/6 (66.7)	0.426
Immunisation completed	58/107 (54.2)	3/6 (50)	0.340
Spleen parameters			
Spleen status on ultrasound: n/n (%) Spleen present. Spleen absent**	 53/99 (53.5) 46/99 (46.5)	 3/6 (50) 3/6 (50)	 0.87
% AI red cells, median (IQR)	43.4 (35.5)	62.4 (22)	0.075
% HJB red cells, median (IQR)	1.9 (6.7)	2.4 (19.7)	0.744
Laboratory parameters			
White blood cell count (10^6/l, mean (SD)	21.6 (17.3)	34.9 (20.0)	0.018*
Hb (g/dl) mean (SD)	6.3 (2.1)	7.3 (2.1	0.209
Platelets (10^9/l), mean (SD)	418 (210)	516 (241)	0.274
ANC (10^9/l) mean (SD)	11.3 (8.8)	19.4 (6.0)	0.006*
Reticulocyte count (%), mean (SD)	5.7 (6.3)	10.2(7.2)	0.148
Bilirubin (total)(umol/l), mean (SD)	28.7 (27.7)	22.0 (10.4)	0.881
ASAT (iu/l) mean (SD)	17.5 (16.3)	30.8 (29.6)	0.218
Haemoglobin F, %, mean (SD)	8.3 (5.9)	6.9 (1.9)	0.172

AI argyrophilc inclusion; ASAT aspartate amino transferase; ANC absolute neutrophil count; HJB Howell-Jolly body; Hb haemoglobin; IQR inter quartile range; SD standard deviation; *Significant P value by Mann Whitney U test. **autosplenectomy

9.5 Discussion

9.5.1 Overview of findings

In the present study we determined the prevalence and pattern of bacteraemia among our patients with SCD. The observed prevalence rate of 5.2% in our study is comparable to previous studies across Africa, which ranged between 4.0% and 9.7% (Alima Yanda *et al.*, 2017; Makani *et al.*, 2015; Williams *et al.*, 2009). Our prevalence rate is also similar to studies beyond Africa including 6.1% in Jamaica (Wierenga *et al.*, 2001), 5.2% in the USA (Zarkowsky *et al.*, 1986) and 3.4% in the United Kingdom (Morrissey *et al.*, 2015). Though our finding is comparable to other studies, it is not clear if the use of over-the-counter antibiotics may have contributed to the low rates observed in studies from Africa where there is unrestricted access to over-the-counter antibiotics (Kizito *et al.*, 2007; Alima Yanda *et al.*, 2017); more than a quarter of our patients admitted using antibiotics for their symptoms prior to presentation at the health facility. The majority of isolates cultured in this study were Gram-negative organisms. This is similar to reports among SCD patients in Nigeria (Brown *et al.*, 2017; Akinyanju and Johnson, 1987). In contrast, while some studies from Africa have reported Gram-positive organism like *Staphylococcus aureus* as the predominant bacteria isolated s (Kizito *et al.*, 2007; Akuse, 1996), other studies from Africa (Williams *et al.*, 2009), and Western countries have identified the Gram-positive organism, *Streptococcus pneumonia,* as the major bacterial pathogen implicated in infection among their SCD patients (Ellison *et al.*, 2013; Zarkowsky *et al.*, 1986). Patients with SCD are susceptible to infection with encapsulated organisms (*S. pneumonia*, *H.influenza*) and *Salmonella* species due to their underlying splenic dysfunction (Booth, Inusa and Obaro, 2010). The prevalence and pattern of pathogens implicated in infections in SCD may also be influenced by their varying epidemiology

in different geographical settings, availability of infection preventative strategies, including vaccination and antibiotics prophylaxis (Obaro and Tam, 2016). Furthermore, the frequency with which specific pathogens cause infections has been shown to follow age-specific pattern. *Salmonella* bacteraemia is common among younger patients with SCD, with a peak incidence between 2 and 10 years and can be associated with an increased risk of osteomyelitis (Zarkowsky *et al.*, 1986; Wright, Thomas and Serjeant, 1997). In the current study, *Salmonella sp* was isolated in two of the children less than five years old, one of whom had a previous history and management for osteomyelitis of both femuri. The expanded bone marrow in patients with SCD, with its sluggish blood flow is vulnerable to thrombosis, infarction, and fibrosis; this can result in ischaemic foci which allows for localisation of *salmonellae.* Proliferation of previously dormant foci of infection can accompany a sickle crisis, and with local bone changes, can result in the passage of the organisms into the blood stream (Anand and Glatt, 1994).

Despite the high morbidity and mortality among SCD patients attributed to loss of splenic function, there are no studies evaluating the presence of bacterial infection and markers of splenic dysfunction in SCD patients in SSA (Ladu *et al.*, 2021). We have recently used two markers of splenic dysfunction that required simple techniques and thus can easily be performed in most resource-poor settings; the proportion of both markers, HJB- and AI-containing red cells were higher in patients with autosplenectomy than those with visible spleens (Ladu *et al.*, 2023). In the current study, we noted that SCD patients with bacteraemia were more likely to have higher AI and HJB red cell counts, although the difference for both markers failed to reach statistical significance. The small number of patients with bacteraemia (n=6), may have affected the power to detect any significant relationship. This limits our ability to

make concrete conclusions regarding the relationship between these markers and the risk of bacteraemia. Furthermore, although a high count of markers of splenic dysfunction is expected to be associated with an increased risk of bacterial infections, the ability of the spleen to filter the blood of pathogens depends on several other mechanisms including complement activation, humoral and cellular immune responses (Booth, Inusa and Obaro, 2010), therefore, it is not clear whether the HJB or AI counts alone can accurately reflect the spleen-related risk of bacterial infection. A larger study will be useful in providing more insight into the relationship between bacteraemia and splenic parameters.

9.5.2 Limitation

This study has some limitations. The small number of patients with bacteraemia (5.2%) may have affected the power to identify an association with splenic parameters; the low prevalence of bacteraemia observed could be due to prior use of antibiotics by the patients. The use of one culture bottle per set rather than two for adults and the use of a manual incubator instead of the standard BAC/Alert system for our bacterial detection may have affected identification of fastidious organisms and highlights the difficulties in performing clinical research in laboratories with limited resources characterising the conditions in most Sub-Saharan African countries.

CHAPTER 10: GENERAL DISCUSSION

10.1 Chapter overview

In this chapter, I consider how the results from the different sub-studies aligned to address the main aim of the thesis: to evaluate the spleen size and function and the risk associated with infections. At the start of my research, I performed a systematic review of published literature to determine the spectrum of splenic complications in SCD in Africa (Chapter 2). Based on the gaps and information identified in the review, I assessed baseline spleen sizes among steady-state SCD patients using ultra-sonography (Chapter 4) and explored factors associated with preservation of the spleen (Chapter 5). Next, I evaluated two methods of assessing spleen function (Chapter 6) and explored factors associated with splenic dysfunction among the steady-state SCD patients (Chapter 7). Finally, I determined the prevalence of malaria (Chapter 8) and bacteraemia (Chapter 9) among acutely-ill SCD patients and explored their association with spleen size and function. In this chapter I summarise the key findings, and highlight the strengths, challenges, and limitations of the project. This chapter concludes with recommendations and direction for future studies.

10.2 Summary of project findings

To determine the relationship between the spleen and SCD in the Africa context, I performed a systematic search of published literature including 55 studies from 14 African countries (Chapter 2). The review showed the difference in frequency of splenic complications in SCD patients in Africa when compared with their counterparts in the United States and Europe. While several studies (n=45) from Africa described splenomegaly with a prevalence of 12% to 73% among children, and 4% to 50% among adults with SCD, only two (3.7%) studies provided data on spleen

function. The review also showed a conflicting pattern amongst studies that evaluated the relationship between splenomegaly and the presence of bacterial and malaria infections. A higher malaria parasite density in patients with normal sized or enlarged spleens compared to those with autosplenectomy was reported from one study (Awotua-Efebo, Alikor and Nkanginieme, 2004); other studies found no association between the frequency of malaria infections and spleen size (Durosinmi *et al.*, 2005; Sadarangani *et al.*, 2009; Makani *et al.*, 2010a).

Assessment of spleen size

Assessment of spleen size using ultrasonography was the focus of Chapters 4 and 5. Chapter 4 focused on identifying baseline spleen sizes among the SCD participants. About half of the SCD patients had no visible spleens on ultrasonography (47%). Among the remaining patients whose spleens were visualised (53%), age-specific reference ranges for splenic length and volume obtained among the healthy controls were used to classify the spleen size as small, normal, or enlarged for the age of the patients; the majority of patients had normal sized spleens, while enlarged and small sized spleens were found in 19.7% and 16.4% respectively. Our data on splenic length among the controls were similar to previous findings among paediatric (Tsehay *et al.*, 2021; Ezeofor *et al.*, 2014; Eze *et al.*, 2013) and adult population in Africa (Agwu and Okoye, 2005; Mustapha *et al.*, 2010; Ehimwenma and Tagbo, 2011). In contrast, the upper limit of spleen length in our controls were smaller when compared among a normal population from previous studies in the United states and Europe (Rosenberg *et al.*, 1991; Pelizzo *et al.*, 2018; Hosey *et al.*, 2006). Other studies from Nigeria have made similar observations of smaller spleen sizes among their population compared to published data among the White population (Agwu and Okoye, 2005; Mustapha *et al.*, 2010; Ehimwenma and Tagbo, 2011). This underscores the importance of using

population-specific reference values in classifying spleen sizes among individuals with SCD and other disease conditions affecting the spleen.

Of note, the spleen size in SCD patients during the first two years of life was three-fold higher than in controls, thereafter, there was a progressive age-related decline in size. Also, the majority of our older SCD patients had no visible spleen on ultrasonography, however, the spleens among a quarter (n=5/20) of those with visualised spleens were found to be markedly enlarged. Patients with enlarged spleens may be prone to complications related to splenomegaly including sub-clinical sequestration and hypersplenism resulting in worsening anaemia, splenic infarction, and splenic abscess (Diagne *et al.*, 2010); therefore close monitoring by ultrasonography may be required to identify those patients with sub-acute sequestration, especially that splenomegaly was detected less often with manual palpation (5%) compared to ultrasonography (23%). This is particularly important given that subtle splenic enlargement and acute splenic sequestration crisis could be missed on clinical examination (Casey, Kinney and Ware, 1994).

The focus of Chapter 5, the second spleen size assessment study, was to investigate factors associated with splenic visualisation or non-visualisation on ultrasonography. The spleen was visualised on ultrasonography among all the children less than five years, but declined in prevalence with successively older age groups, so that only about a quarter of patients had their spleens visualised on ultrasonography after the age of 15 years. A similar pattern was noted recently among a large Jamaican birth cohort of SCD patients (Walker *et al.*, 2022). In contrast, a study among SCD children in the United Kingdom , showed that only 5.6% of children aged 6–10 years and 19.4% of children aged 11-16 years had no visible spleen on ultrasonography (Nardo-Marino *et al.*, 2022). Our current observation that the spleen was visualised in some patients and

not in others within each of the age groups indicates the variability in the rate of progression of splenic injury amongst SCD patients (Serjeant, 2002; Rogers *et al.*, 2011).

I also assessed laboratory factors that could potentially influence preservation of the spleen among SCD patients and may be useful in their management. HbF was significantly higher among patients whose spleens were visualised compared to those without splenic visualisation. It has been suggested that the high HbF level inhibits HbS polymerisation and consequently reduces the number of irreversibly sickled cells; this slows down the rate of splenic fibrosis (Serjeant, 1970). Similar studies among SCD patients in Nigeria (Akinlosotu *et al.*, 2018), Saudi Arabia (Al-Salem *et al.*, 1998a) and Jamaica (Walker *et al.*, 2022) have demonstrated high HbF levels in patients with visualised spleens compared to those without visible spleens on ultrasonography. Furthermore, only few patients (15%) were on hydroxyurea in this study, however, a higher HbF level among these patients and a trend towards increased visualisation of the spleen compared to the non-hydroxyurea group was observed. Hydroxyurea induces production of HbF and has been used as a disease modifying agent in SCD over the past three decades (Rogers *et al.*, 2011). In view of the limited effect of HbF on the presence of the spleen beyond 10 years of age observed in this study, and a trend towards autosplenectomy after 5 years of age, my study confirms findings of previous studies that commencing hydroxyurea as early as first year of life may be beneficial for preservation of the spleen (Claster and Vichinsky, 1996; Nottage *et al.*, 2014). Moreover, most of the older patients may have developed fibrotic areas in their spleens which may not be reversible with hydroxyurea (Olivieri and Vichinsky, 1998; Santos *et al.*, 2002; Hankins *et al.*, 2008).

Spleen function studies

Based on evidence from the systematic review (Chapter 2), spleen function has rarely been documented among SCD patients in Africa, due partly to the non-availability of sophisticated techniques such as scintigraphy and pitted red cell counts. Therefore, I evaluated two methods of assessing splenic function that required only a light microscope and may be achievable in resource-poor settings. Chapter 6 focused on the laboratory aspects of evaluating spleen function. Splenic function is usually evaluated by the spleen's ability to remove abnormal cells from circulation (de Porto *et al.*, 2010; Lammers *et al.*, 2012; Lenti *et al.*, 2022). Therefore, I assessed the presence of two red cell inclusions - Howell-Jolly Bodies (HJB) and argyrophilic (silver staining) inclusions as markers of splenic dysfunction among the SCD patients and compared the findings with those of normal controls. Both HJB and AI- containing red cells could easily be identified in the participants' blood smears and both counts were higher in SCD patients than controls, thus indicating presence of splenic dysfunction. The range of HJB red cells obtained among our SCD population was higher than results obtained from studies among SCD patients in the United States (Casper *et al.*, 1976) and Brazil (Zago and Bottura, 1983); however, the small sample size from both studies (n=9 and n=12 respectively) compared to our sample population (n=182) may account for the differences compared to our results.

I noted the HJB and AI counts were higher in patients without visible spleens than patients whose spleens were visualised on ultrasonography. This was not unexpected as the spleen is the site of removal of red cell inclusions, therefore patients with autosplenectomy are likely to have higher numbers of inclusions within their red cells than those with intact spleens (Peretz *et al.*, 2022; Sissoko *et al.*, 2022). Furthermore, among the SCD patients whose spleens were still visualised on ultrasonography, the

AI and HJB counts appeared variable among those with small, normal, and enlarged spleens, with a tendency to be lower in those with enlarged spleens, which may indicate some degree of splenic preservation among those with large spleens. A recent study demonstrated that spleen function may be preserved even among adults patients with SCD as long sinus structures still persist in the preserved or partially damaged spleen (Sissoko *et al.*, 2022).

Overall, the findings demonstrate the utility of light microscopy in the assessment of red cells containing inclusions as an index of splenic dysfunction in resource-low settings as reflected by the high percentages of circulating levels compared to controls. Their presence was easily demonstrable, and both showed good intra-observer reliability and agreement. Although the red cell inclusions were readily demonstrable using the AI method, the silver stain deteriorates rapidly. The HJB method is simpler and requires fewer reagents than the AI method. Also, the higher AI counts in SCD patients may partly be due to the underlying haematological disturbance; an increase in the percentage of red cell inclusions in patients with haematological disorders such as thalassemia and haemolytic anaemia but intact spleens have been reported (Kent *et al.*, 1966; Tham *et al.*, 1996; Moriconi *et al.*, 2022; Jagadeeswaran *et al.*, 2017). Therefore, further validation in larger studies may be required for the AI method before the generalisability of its efficacy in assessing spleen function in SCD can be ascertained.

Chapter 7 focused on the clinical aspects of splenic dysfunction. Because of the age variability at which splenic dysfunction starts, the rate of its progression, and consequently the stage at which it becomes clinically significant, data obtained from the lab study (i.e Chapter 6) were explored further to determine the association between the two markers of splenic dysfunction (i.e HJB and AI red cells) with clinical

and laboratory factors. The percentages of HJB- and AI- red cells rose significantly with increasing age in the SCD patients, in corroboration with findings from previous studies (Bernaudin *et al.*, 2022c; Nardo-Marino *et al.*, 2022; Rogers, 1982; Pearson *et al.*, 1979). This observation also parallels the finding of progressive loss of splenic visualisation with age observed among the SCD patients as highlighted in Chapters 4 and 5. Although I observed both markers of hyposplenism increased with increasing age in this study, there was less variability after five years with the AI red cell counts; the steady increase with age observed with the HJB red cell counts indicates that this would be the preferred method to track splenic function in each individual.

Furthermore, the frequencies of both AI and HJB red cells showed a significant positive association with the mean corpuscular haemoglobin (MCH) and a negative association with HbF level. These findings were interesting as earlier on, I had noted that visualisation of the spleen on ultrasonography (Chapter 5) was associated with high level of HbF and low mean corpuscular haemoglobin concentration (MCHC). This suggests a link between preservation of spleen size and spleen function with increased level of HbF and concur with findings in the literature of HbF and splenic preservation (Adekile *et al.*, 1996; Nottage *et al.*, 2014; Al-Jam'a *et al.*, 2000). Following birth, as the level of HbF decreases to less than 15% to 20%, the corresponding increase in Hb S results in haemoglobin polymerisation and red cells sickling, hence a progressive dysfunction of the spleen (Claster and Vichinsky, 1996). Also, it is not known if the observed association of both markers of hyposplenism with MCH (and splenic visualisation on ultrasonography with MCHC observed in Chapter 5) is due to the presence of alpha-thalassemia trait among our SCD population, a condition known to ameliorate splenic dysfunction (Adekile *et al.*, 2002b; Adekile *et al.*, 1996; Wali *et al.*, 2002; Serjeant, Hambleton and Serjeant, 2021; Al-Jam'a *et al.*,

2000). Alpha thalassemia is highly prevalent (36% to 54%) among individuals of West and East African origin due to its protective effect on malaria (Wambua *et al.*, 2006; Mockenhaupt *et al.*, 1999; Falusi *et al.*, 1987). Further studies are required to elucidate this finding.

Spleen and infection studies

Given the important role the spleen plays in protection against infection and the fact that loss of splenic function occurs early in life in SCD patients, it was evident from my systematic review that, due to the paucity of data on splenic function in the African context, little is known about the increased risk of malaria or bacterial infection associated with splenic dysfunction among our SCD patients, hence, the aim of Chapters 8 and 9 were on malaria and bacterial infection and their relationship with splenic parameters.

The focus of chapter 8 was on malaria infection among the SCD patients. The frequency of malaria parasitaemia and parasite density among acutely-ill SCD patients were determined and compared with those of steady-state SCD and non-SCD controls. Although parasitaemia prevalence was lower among the non-SCD controls compared to both SCD groups, parasite density was significantly higher in the non-SCD controls. This may suggest a higher resistance to the parasites amongst the SCD population and may be attributed to the high concentration of HbS in the red cells of SCD patients, which have been shown to confer protection (Cholera *et al.*, 2008; Pasvol, Weatherall and Wilson, 1978). However, among the SCD population, parasitaemia prevalence and parasites densities were higher among the acutely-ill SCD patients than steady-state SCD-controls, indicating that malaria infection is an important contributor to crises as shown from previous studies (Ambe, Fatunde and Sodeinde, 2001). Studies have

shown that both severe anaemia and death are considerably more common among SCD than non-SCD patients who were hospitalised with malaria (McAuley *et al.*, 2010; Makani *et al.*, 2010a).

The clinical pattern and outcome of infection with *P. falciparum* may be impacted by several factors including prior exposure and immunity, intensity of transmission in the area, age, and the spleen. Mature forms of the parasite express an adhesion protein (*P. falciparum* erythrocytes membrane proteins - PfEMP1), a highly immunogenic molecule, and with repeated exposures, children residing in endemic areas progressively acquire antibodies against the parasite (Smith *et al.*, 1999). The finding of parasitaemia in both steady-state SCD controls and non-SCD controls may therefore imply the presence of some degree of immunity to the parasite in both populations.

There was no significant association between the visualisation or non-visualisation of the spleen on ultrasonography with prevalence of parasitaemia or parasite density among the SCD patients. This finding is similar to an earlier report from South-West Nigeria (Akinlosotu *et al.*, 2018). Other studies have also indicated no relationship between the frequency of malaria parasitaemia or parasite density, clinical malaria and spleen size (Durosinmi *et al.*, 2005; Sadarangani *et al.*, 2009; Makani *et al.*, 2010a); however, these studies have used manual palpation to assess spleen size, which is less sensitive compared to ultrasonography for assessing spleen size. The current study is the first to describe the association between malaria and splenic function (i.e. HJB) among SCD patients residing in a malaria-endemic region, however, the frequency of HJB red cells was not significantly different between the group with parasitaemia and those without parasitaemia. Given the multiple and inter-related factors that may impact outcome with malaria infection, further studies with larger sample may be helpful in throwing more light on the risk of malaria infection associated with splenic

dysfunction. Although, I have found no evidence of an increased risk of malaria parasitaemia or parasite density with markers of hyposplenism, the role played by an underlying immunity to malaria among SCD patients residing in malaria-endemic region is not clear and will need further studies with a larger sample size. The definition of severe malaria anaemia used in this research was based on previous studies among SCD (Albert *et al.*, 2009; Makani *et al.*, 2010a) consistent with the criteria used by WHO; however, given that SCD are already significantly anaemic, it's unclear the usefulness of defining severe malaria anaemia using a threshold Hb of less than 5g/dl. Further studies to identify an appropriate threshold of haemoglobin level that may be useful in identifying patients with severe malaria more promptly.

Chapter 9 focused on bacterial infection. The overall prevalence of bacteraemia among the SCD patients was low (5.2%). A low prevalence of bacteraemia (3.9% to 9.7%) among SCD patients have been reported in other studies from Africa (Adekile *et al.*, 1993; Williams *et al.*, 2009; Makani *et al.*, 2015; Alima Yanda *et al.*, 2017; Kazadi *et al.*, 2019). It is not clear if prior use of over-the-counter antibiotics may have contributed to the low rates observed in these studies from Africa where there is unrestricted access to over-the-counter antibiotics. A quarter of patients in my study admitted using antibiotics prior to presentation at the health facility. Also, the use of one set of culture bottle instead of the recommended two for the adult participants may have impacted on the yield (Doern *et al.*, 2019). The majority of isolates cultured in this study were Gram-negative organisms; this agrees with reports among SCD patients in Nigeria (Akinyanju and Johnson, 1987{Okuonghae, 1993 #5042; Aken'Ova, Bakare and Okunade, 1998; Brown *et al.*, 2017) and Uganda (Kizito *et al.*, 2007). In contrast, other studies from Africa (Williams *et al.*, 2009; Bello *et al.*, 2018), the USA (Ellison *et al.*, 2013; Rogers, 1982) and Jamaica (Zarkowsky *et al.*, 1986)

have identified the Gram-positive organism, *Streptococcus pneumonia,* as the major bacterial pathogen implicated in infection among their SCD patients. While this disparity may be due to the varying epidemiology of bacterial agents in different geographical settings, it may also be due to the difficulty in isolating fastidious organisms such as *S. pneumoniae* in resource-poor settings; for example the lack of BACTEC machine meant that I had to do a blind sub-culture and therefore I could have missed those organisms that are usually identified in the first 24-48 hours.

I observed that SCD patients with bacteraemia were more likely to have high levels of markers of splenic dysfunction, AI and HJB red cell counts, although the difference for both markers failed to reach statistical significance. The small number of patients with bacteraemia, and the fact that assessment for markers of splenic dysfunction was only available for five of these patients, may have affected the power to detect any significant relationship. Although, a high count of markers of splenic dysfunction have been shown to be associated with an increased risk of bacterial infections (Zarkowsky *et al.*, 1986; Rogers, Serjeant and Serjeant, 1982), the ability of the spleen to filter the blood of pathogens depends on several other mechanisms including complement activation, humoral and cellular immune responses (Booth, Inusa and Obaro, 2010; Obaro and Tam, 2016). A larger study will be useful in providing more insight into the relationship between bacteraemia and splenic parameters, and the role other mechanisms play in the increased risk to bacterial infection among SCD patients.

10.3 Strengths of study

10.3.1Assuring quality of study

- Data collection

I recruited participants with no restrictions on age, gender and ethnicity. Also, data for the controls were obtained from participants in the same environment. Data collection

was thorough, and effort was made to collect all information relevant to answering the research questions. Participants with incomplete information were followed up with a phone call to fill in the missing information. Data entered in excel were validated regularly to ensure information entered matched what was entered in the case report forms.

- Sample processing

Blood samples were processed on the same day they were collected to reduce the possibility of sample number mix-ups and to preserve the quality of the samples. Blood smears were fixed in appropriate fixatives, labelled with study ID, and stored in suitable slide jackets. Blood samples in EDTA and plain bottles were centrifuged, and the resulting plasma and serum transferred into cryotubes which were carefully labelled. To ensure the correct samples went into the correct tubes, samples numbers were double checked before transferring the plasma or serum into the cryotubes. Blood samples for high performance liquid chromatography were kept in a refrigerator for not more than 72 hours before being transferred to Aminu Kano Teaching Hospital for processing. The samples were transported in a suitable ice container. Blood cultures were transferred to the incubator immediately after collection.

10.3.2 Laboratory testing

Despite working in a resource-limited setting, laboratory testing for all the tests carried out in the project were comprehensive and performed strictly following the respective SOPs developed prior to the start of the project. A pilot study to assess and refine testing parameters for the spleen function tests (i.e HJB and AI red cell counts) was performed using samples from controls and SCD patients. I conducted a pre-test run to check the effect of staining duration, temperature, the constitution of eosin, and pre-treatment of slides with potassium iodide on optimal staining conditions. This

provided me the opportunity to improve my ability in identifying both red cell inclusions accurately. Also, I had prior training in the basics of ultrasonography using an online course. I undertook training on how to analyse thin blood films for malaria parasites and as well as training in medical bacteriology under the diploma in tropical medicine and hygiene course at LSTM. This provided me with the experience required to work in the microbiology lab. Although thick films were also prepared and analysed by two independent research assistants; the reports showed poor quality and failed assurance; hence, malaria reports were based on the thin films. All tests were repeated at any point when an inconclusive result was obtained.

Demonstrating that splenic function can be assessed using a simple technique like HJB among our SCD patients was a key methodological strength of this study. The ultrasonography was performed by a single radiologist which brought consistency and accuracy to the spleen size data. Generating reference ranges and percentile curves for various splenic dimensions in children and adults that can serve as comparison for SCD population was another key methodological strength of this study.

10.3.3 Supervision

This project was supervised by renowned researchers from the United Kingdom. I was also supported by a senior researcher from Nigeria based in Kuwait. These supervisors have long term research experience in SCD and the spleen in Sub-Saharan Africa. Bringing together their rich and varying expertise enriched the output of the project.

10.4 Limitations of study

The project had the following limitations.

10.4.1 Study design

Although, I have obtained data on the factors associated with spleen preservation in SCD patients across various age groups, it is difficult to infer factors that are causally related to preservation of the spleen without longitudinal data.

10.4.2 Study method

Tests for diagnostic accuracy such as sensitivity and specificity for the red cell inclusions (i.e spleen function test) was not performed because of the absence test of a reference standard, such as spleen scintigraphy or pitted red cell counts in Nigeria. Despite this limitation, the two methods used showed good intra-observer reliability and agreement for repeated measurements in an individual sample; the results were comparable with those of published studies including those using flow cytometry counts of HJB (Pourdieu *et al.*, 2023; El Hoss *et al.*, 2019; Tham *et al.*, 1996). The use of one culture bottle per set rather than two for adults and the use of a manual incubator instead of the standard BAC/Alert system for our bacterial detection may have affected identification of fastidious organisms including *S.Pneumonia*. Furthermore, the thin blood has been shown to be less sensitive at low parasitaemia (Bowers *et al.*, 2009), and may have under-estimated the prevalence of parasitaemia among our study population, especially the low rate reported among our non-SCD controls when compared to the high value reported from a previous report in the study area (Balogun *et al.*, 2019).

10.4.3 Sample size

At the start of my research, one of the primary objectives was to determine the prevalence of enlarged spleens among SCD patients, hence, the sample size calculation was defined based on a binary outcome, i.e. prevalence of splenomegaly. However, prior to the field work and commencement of data collection, the aim of the research was modified instead to look at baseline spleen sizes among SCD patients (continuous variables). This represented a disconnect between the final aim of the project and the choice of parameter used in the sample size calculation; the sample size was calculated based on the formular for a cross sectional study using prevalence of splenomegaly from a previous study in the same hospital (section 3.3.5.1). However, the use of a margin of error (i.e precision) of 5% provided me with a reasonable sample size (n= 214) for the spleen baseline study that allowed me to answer the research questions including spleen sizes across the various age groups. Furthermore, the controls were not considered in the sample size calculations because at the start of the project, I did not plan to compare results between the SCD population and controls. However, realizing I needed data from normal individuals to aid the interpretation of findings among the SCD, I applied for approval for a modification of protocol from LSTM and UMTH Ethics which was granted. Because of time and financial constraints, I decided to enroll one control for every two SCD patients (a ratio of 2:1) (section 3.3.5.3). The sample size for the controls used to generate the reference range meant that the number within each age group was small.

For the spleen infection study, the sample size was also calculated based on the formular for a cross sectional study using a prevalence rate of bacteremia from a previous study in Nigeria (section 3.3.5.2); however, the small number of patients with bacteraemia affected the ability to analyse the relationship with splenic parameters.

This limited my ability to state the risk associated with spleen size and spleen function tests. However, the prevalence of patients with malaria parasitaemia was high and yet there was no association with spleen size and function parameters. Whether other factors besides splenic dysfunction contribute to the increased risk of infections among SCD patients may need further study.

10.5 Challenges

10.5.1 Covid 19

As a result of the COVID-19 pandemic, I experienced some delay in data collection because I could not travel to Nigeria for the fieldwork as outlined in my original project timeline. Also, the pandemic affected recruitment into the project significantly, as most patients were reluctant to visit the hospital even when ill, because of the perception and fear that they may pick up COVID-19 infection in the hospital environment. Also, I had to temporarily halt data collection for some weeks because I came down with COVID-19 and I needed some time off because of the physical weakness that persisted after the initial phase of the infection.

10.5.2 Power outage

Due to the ongoing Boko haram crisis in Maiduguri, the light supply to the city was vandalised and there was power outage for more than 18 months including during the time of my data collection. The hospital relied on a diesel generator and later on solar to generate electricity to run the hospital. Because of rationing of the light supply to different parts of the hospital, I could only perform microscopy for a limited period of time. Because of the large number of blood smears, I had generated from the study, it was not possible to finish the microscopy in Nigeria. I had to bring some slides back to the UK to complete the analysis. This extended the period of the laboratory work.

The incubator used for blood culture in the microbiology laboratory was not affected as there was a dedicated standby generator attached to the laboratory.

10.5.3 Blood culture

There were problems with media contamination despite the strictness in checking sterility of newly prepared media. This cost me more since the whole batch of media had to be discarded when any among the batch fails the sterility test. This meant I incurred additional costs since the whole batch of media had to be discarded when any among the batch fails the sterility test. The lack of BACTEC machine meant that I had to do a blind sub-culture and I could therefore have missed those organisms that are usually fastidious including *strep pneumonia*. Also, due to fund constraints, only one set of samples was obtained from the adult patients and may have affected the yield of bacteraemia.

10.6 Implication for further research

Spleen size study

Longitudinal study among SCD patients less than five years old to track if change in spleen sizes is followed by changing HbF level would help to gain further insights into factors that may affect spleen preservation. Low MCH and MCHC were associated with splenic preservation in this project; it's not clear if the concurrent presence of alpha-thalassemia among the study population is responsible for this finding. Further studies to assess the effect of genetic factors known to inhibit sickling such as alpha-thalassemia will help provide more insights into the determinants of sickling and consequently splenic atrophy.

Spleen function

Improvement in spleen function has been reported following chronic hyper-transfusion and following hydroxyurea therapy, given the trend towards splenic visualisation among the few patients in this study, it may be beneficial to start SCD patients as early as first year of life on hydroxyurea and follow them over time to determine any change in baseline spleen parameters. Also, it would be interesting to follow up the group of patients whose spleens are still visible on ultrasonography with disease modifying therapy like hydroxyurea to determine if this will have any effect on reducing the frequency of the red cell inclusions.

Spleen and infection studies

The underlying role of immunity to malaria infection among SCD patients in endemic regions and the presence of splenic dysfunction needs further exploration in order to understand if the two are linked and how they affect SCD patients' risk to malaria.

10.7 Recommendations for clinical practice and health system strengthening in Nigeria and other low resource settings.

Based on findings from my research, I have generated some recommendations to improve clinical practice for SCD patients, health system strengthening and research relating to public health systems. These may be useful in Nigeria and in other low-middle income countries where SCD is common.

10.7.1 Recommendation for clinical practice

From the spleen size sub-study

- Regular spleen scans to assess changes in spleen size and enlarged spleens can help identify SCD patients at risk of splenomegaly complications including subclinical acute sequestration and hypersplenism.
- Early administration of hydroxyurea from the age of one year and above may be helpful in increasing HbF and consequently in preserving spleen function. However, the majority of the SCD patients in this study (and in my clinical experience) had no prior knowledge about hydroxyurea; thus, education on hydroxyurea for patients and clinicians will also be needed to improve hydroxyurea uptake. An audit to survey prescribing practise in relation to hydroxyurea and SCD and changes in the number of patients on treatment would help to determine the success of such education, or indicate if more education would be needed.
- An additional public health intervention could be to teach parents how to palpate for the spleen in their child with SCD and what action to take quickly to prevent the consequences of ASSC.

From the spleen function sub-study

Identifying a baseline value of the markers of splenic dysfunction, in particular, HJB, for an individual patient and then following any changes over time would aid the early detection of splenic dysfunction and may indicate a need for more frequent clinic visits or more intensive measures to mitigate risks from infections.

From the spleen and infection sub-study

Although I found no association between spleen size and spleen function with the risk of malaria, parasitaemia and parasite density were higher among the acutely-ill patients. Therefore, it is reasonable to recommend the use of bed nets, chemoprophylaxis, and other malaria measures (including vaccination), as these measures are likely to be important in reducing infection and illness especially in the younger patients aged five years and below. Furthermore, a recent international trial of hydroxyurea among SCD patients across four SSA countries (REACH trial) {McGann, 2016 #263}indicated that hydroxyurea use reduces the incidence of infections including malaria, hence supports the need for wider access to hydroxyurea among SCD patients.

10.7.2 Recommendation for health system strengthening

The recommendations for health system strengthening as related to the hospital, the Northeast region/Nigeria and Africa are described below:

1. **The hospital**

a. Cost exemptions for SCD patients: because SCD is a chronic and lifelong condition, and many patients must pay for their health care, providing appropriate clinical care may be challenging. Government or state exemptions for health care for SCD patients - or at discounted rates - may facilitate access to routine monitoring and treatments including blood transfusion.

b. Shared learning between the paediatric and adult haematology units: this will help facilitate continuity of care when patients transit from the paediatric to the adult care which is often a difficult transition for SCD patients.

c. Laboratory Quality assurance (QA): engagement in internal and external QA scheme will help improve the quality and standard of laboratory within and among diagnostic laboratories in Nigeria.

d. Training of microbiology staff: contamination was a major problem in the bacteriology lab. Periodic training of staff will ensure those involved in the process of media preparation have the necessary skills.

e. Improved laboratory diagnostic capability for service delivery: provision of a BACTEC machine for blood culture would help overcome the suboptimal isolation and high rates of contamination associated with manual methods. An HPLC service in Borno state (rather than having to send samples to Kano) would provide local capability for quantification of haemoglobin variants and enable monitoring of SCD patients' HbF levels. The need to send samples to Kano for HPLC adds to the cost for the patient and results in delays in getting results back. My research generated excellent rapport across AKTH with the diagnostic services and with senior hospital leaders which may help to pave way for introducing additional tests that are not currently available in our center.

f. Uptake of new services: my research has shown that routine HJB count can be performed in UMTH and is a useful, low-cost way to monitor spleen function in SCD patients and other conditions that may be associated with splenic dysfunction. I will therefore continue to advocate for the introduction of HJB as a routinely available haematology test.

2. **The Northeastern region and Nigeria**

a. Expansion of neonatal screening programme nationally: Most SCD patients enrolled in my research were diagnosed with the SCD following symptomatic presentation during childhood. Neonatal screening for SCD is not yet universal

in many SSA countries including Nigeria but is being widely promoted because detection of SCD at birth will allow for early intervention including education, prompt treatment of infections and adherence to routine immunization. These measures have been shown to contribute to improving the quality of life for people living with SCD. The findings from my research add weight to the importance of early diagnosis, preferably through universal newborn screening, and the enrolment of those identified with SCD in a follow-up clinic. I am already part of our national team involved in scaling up neonatal SCD screening and will continue to advocate actively for this.

b. Most of our patients pay for their health care from their pockets; providing access to the National Insurance health scheme free of charge for SCD patients will improve their access to health care.

c. Hydroxyurea utilisation was very low among our SCD patients. Providing Hydroxyurea at subsidised rate may help improve access and uptake. The drug should be made readily available in all sickle cell clinics and registered pharmaceutical outlets, and the paediatric formulation should also be made readily available.

d. Extend the coverage of pneumococcal vaccines to older children and adults with SCD: currently, only children less than five years are eligible to free treatment.

3. Africa

- To better understand the spleen in children and adults with SCD, national and international collaborative studies to investigate other modifying factors such as ethnicity and genetic factors may provide further insight. To achieve this requires the collective efforts of clinicians and relevant healthcare team. The

collaborative landscape in Nigeria has improved over the last decade with different research groups collaborating both at national and international level. Organizations such as the Nigerian Sickle Cell Support Society Network (**SCSSN**) which works with the government and professional groups for the control of SCD in Nigeria, and research bodies such as the Sickle Pan African Research Consortium (**SPARCo) and** African Research and Innovative Initiative for Sickle cell Education (**ARISE), that are** researching to have a better understanding of SCD, **are already facilitating such collaborative efforts.** Being a member of these bodies provides me with a platform through which findings from my project can be used for future research projects that can translate to better clinical care for SCD patients. I intend to use the opportunity to present findings from my research during meetings or workshops and to share publications obtained from my research study with these groups.

10.7.3 Recommendations to improve knowledge and uptake of hydroxyurea among clinicians and patients.

A. Physicians are generally under-prescribing hydroxyurea for various reasons including lack of information on how to prescribe it, concern about its safety and patients' ability to afford it. This situation could be improved by:

a. Providing information (lectures, seminars etc) as part of physicians' in-service training about the effectiveness and safety profile of hydroxyurea among SCD patients from Nigerian and international trials.
b. Training on hydroxyurea prescribing for clinicians.
c. Perform periodic national or hospital-based audits on hydroxyurea utilisation.

d. Generate local data supporting the safety and usefulness of hydroxyurea among our patients.

B. Recommendation to improve parents/patient's adherence to hydroxyurea: The lack of information, fear of side effects (especially among the older patients), cost, unavailability of hydroxyurea in an appropriate formulation for children and accompanying laboratory monitoring were some of the barriers to hydroxyurea uptake and patient adherence. The following recommendations may improve patient's adherence to hydroxyurea:

a. Education on hydroxyurea given the potential benefits of hydroxyurea in ameliorating the course of SCD, education can help provide patients with the necessary information and help guide their choices.
b. Education/training of health care providers on how to counsel patients about hydroxyurea including its benefits. This will include understanding and addressing their concerns and fears about toxicity and malignancy.
c. Make available hydroxyurea in appropriate formulation for children to ease its use. Currently, our hospital pharmacy compounds paediatric formulation as a syrup which has a short shelf life, necessitating the parents/guardians of SCD patients on hydroxyurea to have to pick up new supplies every two weeks. This can discourage long term adherence.
d. The cost of clinical and laboratory monitoring of hydroxyurea is also expensive and unaffordable for many patients; cost exemption (see 1 above) by the hospital may solve this problem.

10.8 Conclusion

By combining six sub-studies that explored spleen size, spleen function and infections risk related to splenic parameters, my research has provided new insights into the spleen and SCD among patients in the African setting. I have shown that the spleen can be visualised among almost all SCD patients till the age of five when autosplenectomy starts to occur and that these young children with SCD have larger spleens than their non-SCD counterparts. I have provided evidence that assessment of spleen function is feasible among SCD patients even in resource-limited settings. Importantly, I have provided evidence about the consistency of HbF as predictor of both spleen size preservation and function among our SCD patients. Lastly, evidence regarding the role of the spleen in malaria and bacterial infection was obtained, thereby filling an important gap in knowledge regarding the role of the spleen and risk of infection.

References

1. Abdullahi, S. U., Hassan-Hanga, F. and Ibrahim, M. (2014) 'Ultrasonographic spleen size and haematological parameters in children with sickle cell anaemia in Kano, Nigeria', *The Nigerian Postgraduate Medical Journal,* 21(2), pp. 165-170.
2. Abjah, U. M. and Aken'Ova, Y. A. (2003) 'Levels of malaria specific immunoglobulin G in Nigerian sickle cell disease patients with and without splenomegaly', *Nigerian Journal Of Medicine: Journal Of The National Association Of Resident Doctors Of Nigeria,* 12(1), pp. 32-38.
3. Adamkiewicz, T., Driss, A., Hyacinth, I. H., Hibbert, J. and Stiles, J. K. (2013) 'Determinants Of Mortality and Survival In Children With Sickle Cell Disease (SCD) In Sub Saharan Africa', *Blood,* 122(21).
4. Adegoke, S. A., Adeodu, O. O. and Adekile, A. D. (2015) 'Sickle cell disease clinical phenotypes in children from South-Western, Nigeria', *Nigerian Journal Of Clinical Practice,* 18(1), pp. 95-101.
5. Adekile, A., Akar, N., Ayyash, K., Saldanha, W. and Prakash, B. (2002a) 'Indications for splenectomy among children with sickle cell disease in Kuwait', *Kuwait Medical Journal,* 34(1), pp. 33-35.
6. Adekile, A., Tuli, M., Haider, M., Al-Zaabi, K., Mohannadi, S. and Owunwanne, A. (1996) 'Influence of α-thalassemia trait on spleen function in sickle cell anemia patients with high HbF', *American journal of hematology,* 53(1), pp. 1-5.
7. Adekile, A. D., Adeodu, O. O., Jeje, A. A. and Odesanmi, W. O. (1988) 'Persistent gross splenomegaly in Nigerian patients with sickle cell anaemia: relationship to malaria', *Annals of Tropical Paediatrics,* 8(2), pp. 103-107.
8. Adekile, A. D., Adeodu, R. O., Johnson, W. and Dairo, B. A. (1991) 'Pitted red cell counts in Nigerian children with sickle cell anaemia: correlation with age and splenic size', *West African Journal Of Medicine,* 10(2), pp. 137-140.
9. Adekile, A. D., McKie, K. M., Adeodu, O. O., Sulzer, A. J., Liu, J. S., McKie, V. C., Kutlar, F., Ramachandran, M., Kaine, W., Akenzua, G. I. and et, a. (1993) 'Spleen in sickle cell anemia: comparative studies of Nigerian and U.S. patients', *American Journal Of Hematology,* 42(3), pp. 316-321.
10. Adekile, A. D., Owunwanne, A., Al-Za'abi, K., Haider, M. Z., Tuli, M. and Al-Mohannadi, S. (2002b) 'Temporal sequence of splenic dysfunction in sickle cell disease', *Am J Hematol,* 69(1), pp. 23-7.
11. Adeodu, O. O. and Adekile, A. D. (1990) 'Clinical and laboratory features associated with persistent gross splenomegaly in Nigerian children with sickle cell anaemia', *Acta Paediatrica Scandinavica,* 79(6-7), pp. 686-690.
12. Agrawal, R. K., Patel, R. K., Nainiwal, L. and Trivedi, B. (2014) 'Hydroxyurea in sickle cell disease: drug review', *Indian Journal of Hematology and Blood Transfusion,* 30(2), pp. 91-96.
13. Agwu, K. and Okoye, I. (2005) 'Sonographic splenic sizes in normal adult Nigerian population', *West African Journal of Radiology*.
14. Ahmed, S. G. and Ibrahim, U. A. (2017) 'A compendium of pathophysiologic basis of etiologic risk factors for painful vaso-occlusive crisis in sickle cell disease', *Nigerian Journal of Basic and Clinical Sciences,* 14(2), pp. 57.

15. Aken'Ova, Y., Bakare, R. and Okunade, M. (1998) 'Septicaemia in sickle cell anaemia patients: the Ibadan experience', *The Central African Journal of Medicine,* 44(4), pp. 102-104.
16. Akinlosotu, M., Adeodu, O., Adegoke, S., Oseni, S. and Ayoola, O. (2018) 'Fetal hemoglobin level and its relationship with spleen size and malaria parasite density in Nigerian children with sickle cell anemia', *Annals of Tropical Medicine & Public Health,* 11(4), pp. 133-139.
17. Akinola, N. O., Bolarinwa, R. A. and Faponle, A. F. (2009) 'The import of abdominal pain in adults with sickle cell disorder', *West African Journal Of Medicine,* 28(2), pp. 83-86.
18. Akinyanju, O. and Johnson, A. (1987) 'Acute illness in Nigerian children with sickle cell anaemia', *Annals of tropical paediatrics,* 7(3), pp. 181-186.
19. Akpan, E. A. (2015) Abdominal Ultrasound Findings In Clinically Stable Patients With Sickle-Cell Anaemia In The University Of Port Harcourt Teaching Hospital, Port Harcourt. Fellowship In Radiology Part II Dissertation Submitted To The National Postgraduate Medical College Of Nigeria University Of Port Harcourt Teaching Hospital, Port Harcourt, Nigeria.
20. Akuse, R. M. (1996) 'Variation in the pattern of bacterial infection in patients with sickle cell disease requiring admission', *Journal of tropical pediatrics,* 42(6), pp. 318-323.
21. Al-Awamy, B., Wilson, W. A. and Pearson, H. A. (1984) 'Splenic function in sickle cell disease in the Eastern Province of Saudi Arabia', *The Journal Of Pediatrics,* 104(5), pp. 714-717.
22. Al-Jam'a, A. H., Al-Dabbous, I. A., Chirala, S. K., Al-Majid, H. and Al-Ali, J. (2000) 'Splenic function in sickle cell anemia patients in Qatif, Saudi Arabia', *American Journal Of Hematology,* 63(2), pp. 68-73.
23. Al-Salem, A. H. (2006) 'Indications and complications of splenectomy for children with sickle cell disease', *Journal of Pediatric Surgery,* 41(11), pp. 1909-1915.
24. Al-Salem, A. H. (2011) 'Splenic complications of sickle cell anemia and the role of splenectomy', *ISRN Hematol,* 2011, pp. 864257.
25. Al-Salem, A. H., Al-Aithan, S., Bhamidipati, P., Al Jam'a, A. and Al Dabbous, I. (1998a) 'Sonographic assessment of spleen size in Saudi patients with sickle cell disease', *Annals of Saudi medicine,* 18(3), pp. 217-220.
26. Al-Salem, A. H., Qaisaruddin, S., Al Jam'a, A., Al-Kalaf, J. and El-Bashier, A. M. (1998b) 'Splenic abscess and sickle cell disease', *American Journal Of Hematology,* 58(2), pp. 100-104.
27. Albert, N. K., Julie, M., Manish, S., Tolu, A.-A., James, A. B., Charles, R. J. C. N., Kevin, M. and Thomas, N. W. (2009) 'Malaria as a Cause of Morbidity and Mortality in Children with Homozygous Sickle Cell Disease on the Coast of Kenya', *Clinical Infectious Diseases,* 49(2), pp. 216.
28. Alima Yanda, A. N., Nansseu, J. R., Mbassi Awa, H. D., Tatah, S. A., Seungue, J., Eposse, C. and Koki, P. O. (2017) 'Burden and spectrum of bacterial infections among sickle cell disease children living in Cameroon', *BMC Infect Dis,* 17(1), pp. 211.
29. Aloni, M. N., Tshimanga, B. K., Ekulu, P. M., Ehungu, J. L. G. and Ngiyulu, R. M. 2013. Malaria, clinical features and acute crisis in children suffering from

sickle cell disease in resource-limited settings: a retrospective description of 90 cases.
30. Alsultan, A., Alabdulaali, M. K., Griffin, P. J., AlSuliman, A. M., Ghabbour, H. A., Sebastiani, P., Albuali, W. H., Al-Ali, A. K., Chui, D. H. and Steinberg, M. H. (2014) 'Sickle cell disease in S audi A rabia: the phenotype in adults with the A rab-I ndian haplotype is not benign', *British journal of haematology,* 164(4), pp. 597-604.
31. Alsultan, A., Solovieff, N., Aleem, A., AlGahtani, F. H., Al-Shehri, A., Elfaki Osman, M., Kurban, K., Bahakim, H., Kareem Al-Momen, A. and Baldwin, C. T. (2011) 'Fetal hemoglobin in sickle cell anemia: Saudi patients from the Southwestern province have similar HBB haplotypes but higher HbF levels than African Americans', *American journal of hematology,* 86(7), pp. 612-614.
32. Alufohai, E. and Odusanya, O. O. (2006) 'Splenectomy in a rural surgical practice', *Nigerian Journal Of Clinical Practice,* 9(1), pp. 81-83.
33. Aluoch, J. (1997) 'Higher resistance to Plasmodium falciparum infection in patients with homozygous sickle cell disease in western Kenya', *Tropical medicine & international health,* 2(6), pp. 568-571.
34. Ambe, J. P., Fatunde, J. O. and Sodeinde, O. O. (2001) 'Associated morbidities in children with sickle-cell anaemia presenting with severe anaemia in a malarious area', *Tropical Doctor,* 31(1), pp. 26-27.
35. Anand, A. J. and Glatt, A. E. 'Salmonella osteomyelitis and arthritis in sickle cell disease'. *Seminars in arthritis and rheumatism*: Elsevier, 211-221.
36. Aneni, E. C., Hamer, D. H. and Gill, C. J. (2013) 'Systematic review of current and emerging strategies for reducing morbidity from malaria in sickle cell disease', *Tropical Medicine and International Health,* 18(3), pp. 313-327.
37. Anthony Chukwuka, U., Suleiman Tanimu, S., Emmanuel Ayuba, B., Saleh, Y. and Alhaji Modu, A. (2018) 'Sonographic determination of liver and spleen sizes in patients with sickle cell disease at Gombe, Nigeria', *CHRISMED Journal of Health and Research,* (3), pp. 182.
38. Arora, N., Sharma, P., Sahai, A. and Singh, R. (2010) 'Sonographic measurements of the spleen in relation to age; a prospective study in north Indian adults', *Journal of Anatomical Society of India,* 59(2), pp. 177-181.
39. Attalla, B. I. (2010a) 'Abdominal Sonographic Findings in Children With Sickle Cell Anemia', *Journal of Diagnostic Medical Sonography,* 26(6), pp. 281-285.
40. Attalla, B. I. (2010b) 'Sonographic Findings in Sudanese Children With Sickle Cell Anemia', *Journal of Diagnostic Medical Sonography,* 26(6), pp. 276-280.
41. Awodu, O. A., Wagbatsoma, V. A. and Enosolease, M. E. (2008) 'Malaria parasitemia and antimalaria prophylaxis in sickle cell anemia patients in steady state', *Orak hücreli anemi hastalarında malarya parazitemisi ve anti-malarya profilaksisi.,* 25(1), pp. 8.
42. Awotua-Efebo, O., Alikor, E. A. D. and Nkanginieme, K. E. O. (2004) 'Malaria parasite density and splenic status by ultrasonography in stable sickle-cell anaemia (HbSS) children', *Nigerian Journal Of Medicine: Journal Of The National Association Of Resident Doctors Of Nigeria,* 13(1), pp. 40-43.
43. Ayanugwo, M. and Kalu, A. (1997) 'Variations in the prevalence of malaria in northern east Nigeria: studies on Maiduguri metropolis', *Ann. Borno,* 13(14), pp. 320-320.

44. Aygun, B. and Odame, I. (2012) 'A global perspective on sickle cell disease', *Pediatric Blood & Cancer,* 59(2), pp. 386-390.
45. B. J. Brown, O. J. F., O. Sodeinde 'Correlates of Steady-State Haematocrit and Hepatosplenomegaly in Children with Sickle Cell Disease in Western Nigeria', *West African journal of medicine,* 31 (2), pp. 86–91.
46. Babadoko, A. A., Ibinaye, P. O., Hassan, A., Yusuf, R., Ijei, I. P., Aiyekomogbon, J., Aminu, S. M. and Hamidu, A. U. (2012) 'Autosplenectomy of sickle cell disease in Zaria, Nigeria: an ultrasonographic assessment', *Oman Medical Journal,* 27(2), pp. 121-123.
47. Babiker, M. A., el-Hazmi, M. A., Al-Jobori, A. M., Obeid, H. and Bahakim, H. M. (1985) 'Splenic function in children with sickle cell disease: two different patterns in Saudi Arabia', *Scandinavian Journal Of Haematology,* 35(2), pp. 191-193.
48. Bach, O., Baier, M., Pullwitt, A., Fosiko, N., Chagaluka, G., Kalima, M., Pfister, W., Straube, E. and Molyneux, M. (2005) 'Falciparum malaria after splenectomy: a prospective controlled study of 33 previously splenectomized Malawian adults', *Trans R Soc Trop Med Hyg,* 99(11), pp. 861-7.
49. Bain, B. J., Bates, I. and Laffan, M. A. (2016) *Dacie and lewis practical haematology e-book.* Elsevier Health Sciences.
50. Balci, A., Karazincir, S., Sanguen, O., Gali, E., Daplan, T., Cingiz, C. and Egilmez, E. 2008. Prevalence of abdominal ultrasonographic abnormalities in patients with sickle cell disease, Diagn Interv Radiol 2008; 14:133-137
51. Ballas, S. K. (2012) 'More definitions in sickle cell disease: steady state v base line data', *American journal of hematology,* 87(3), pp. 338-338.
52. Balogun, S. T., Sandabe, U. K., Okon, K. O., Akanmu, A. O. and Fehintola, F. A. (2019) 'Malaria burden and pre-hospital medication among subjects with malaria in Maiduguri, Northeast Nigeria', *Heliyon,* 5(8), pp. e02280-e02280.
53. Banza, M. I., Mulefu, J. P., Lire, L. I., N'Dwala Y, T. B., Tshiamala, I. B. and Cabala, V. P. K. (2019) 'Digestives diseases associated to sickle cell anemia in Lubumbashi: epidemiological and clinical aspects', *Pan Afr Med J,* 33, pp. 253.
54. Barrios, N., Livaudais, F., McNeil, J., Humbert, J. and Corrigan, J. (1993) 'Reversible splenic hypofunction in hypertransfused children with homozygous sickle cell disease', *Journal of the National Medical Association,* 85(9), pp. 677.
55. Bates, I. and Bedu-Addo, G. (1997) 'Review of diagnostic criteria of hyper-reactive malarial splenomegaly', *The Lancet,* 349(9059), pp. 1178.
56. Bates, I., Bedu-Addo, G., Rutherford, T. and Bevan, D. (1997) 'Circulating villous lymphocytes—a link between hyperreactive malarial splenomegaly and splenic lymphoma', *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 91(2), pp. 171-174.
57. Bayoumi, R. A., Abu Zeid, Y. A., Abdul Sadig, A. and Awad Elkarim, O. (1988) 'Sickle cell disease in Sudan', *Transactions Of The Royal Society Of Tropical Medicine And Hygiene,* 82(1), pp. 164-168.
58. Bedu-Addo, G. and Bates, I. (2002) 'Causes of massive tropical splenomegaly in Ghana', *The Lancet,* 360(9331), pp. 449-454.
59. Bejon, P., Andrews, L., Hunt-Cooke, A., Sanderson, F., Gilbert, S. C. and Hill, A. V. (2006) 'Thick blood film examination for Plasmodium falciparum malaria

has reduced sensitivity and underestimates parasite density', *Malaria journal,* 5(1), pp. 1-4.
60. Belhani, M., Morle, L., Godet, J., Bachir, D., Henni, T., Zerhouni, F., Bensenouci, A. and Colonna, P. (1984) 'Sickle cell beta-thalassaemia compared with sickle cell anaemia in Algeria', *Scandinavian Journal Of Haematology,* 32(4), pp. 346-350.
61. Bell, M. and Card, K. (2021) 'Maiduguri: city scoping study', *Manchester: African Cities Research Consortium*.
62. Bello, N., Kudu, A. T. D., Adetokun, A. B., Taura, D. W., Jobbi, Y. D., Umar, M. and Yusuf, I. (2018) 'Characterization and Antimicrobial Susceptibility Profile of Bacteraemia Causing Pathogens Isolated from Febrile Children with and without Sickle Cell Disease in Kano, Nigeria', *Mediterr J Hematol Infect Dis,* 10(1), pp. e2018016.
63. Benesch, R. E., Kwong, S. and Benesch, R. (1982) 'The effects of α chain mutations cis and trans to the β 6 mutation on the polymerization of sickle cell haemoglobin', *Nature,* 299(5880), pp. 231-234.
64. Berchtold, A. (2016) 'Test–retest: agreement or reliability?', *Methodological Innovations,* 9, pp. 2059799116672875.
65. Bernaudin, F., Arnaud, C., Kamdem, A., Pondarré, C., Hau, I., Bayani, N., Kuentz, M., Gluckman, E., de Latour, R. P. and Dhédin, N. (2022a) 'P1469: Longitudinal sickle cell disease cohort-study: in chidren with splenic sequestration history or hypersplenism, partial vs total splenectomy before stem cell transplantation preserves spleen function', *HemaSphere,* 6, pp. 1351-1352.
66. Bernaudin, F., Arnaud, C., Kamdem, A., Pondarré, C., Hau, I., Lelong, F., Dalle, J., de Latour, R. P. and Lezeau, H. (2022b) ' Comparative score of howell-jolly bodies in sickle cell disease patients according to genotype, age, splenectomy or stem cell transplantation history', *hemasphere,* 6(suppl), pp. 7.
67. Bernaudin, F., Souillet, G., Vannier, J. P., Plouvier, E., Lemerle, S., Michel, G., Bordigoni, P., Lutz, P. and Kuentz, M. (1993a) 'Bone-marrow transplantation (bmt) in 14 children with severe sickle-cell disease (scd) - the french experience', *Bone Marrow Transplantation,* 12, pp. 118-121.
68. Bernaudin, F., Souillet, G., Vannier, J. P., Plouvier, E., Lemerle, S., Michel, G., Bordigoni, P., Lutz, P. and Vernant, J. P. (1993b) 'Treatment of severe forms of sickle-cell-anemia by allogeneic bone-marrow transplantation - report on 15 cases in france', *Nouvelle Revue Francaise D Hematologie,* 35(3), pp. 319-323.
69. Bezerra, A. S., D'Ippolito, G., Faintuch, S., Szejnfeld, J. and Ahmed, M. (2005) 'Determination of splenomegaly by CT: is there a place for a single measurement?', *American Journal of Roentgenology,* 184(5), pp. 1510-1513.
70. Billo, M. A., Johnson, E. S., Doumbia, S. O., Poudiougou, B., Sagara, I., Diawara, S. I., Diakité, M., Diallo, M., Doumbo, O. K., Tounkara, A., Rice, J., James, M. A. and Krogstad, D. J. (2012) 'Sickle cell trait protects against Plasmodium falciparum infection', *American Journal of Epidemiology,* 176(Suppl. 7), pp. S175-S185.

71. Bland, J. M. and Altman, D. (1986) 'Statistical methods for assessing agreement between two methods of clinical measurement', *The lancet,* 327(8476), pp. 307-310.
72. Boone, K. E. and Watters, D. A. (1995) 'The incidence of malaria after splenectomy in Papua New Guinea', *Bmj,* 311(7015), pp. 1273.
73. Booth, C., Inusa, B. and Obaro, S. K. (2010) 'Infection in sickle cell disease: a review', *International Journal Of Infectious Diseases,* 14(1), pp. e2-e12.
74. Bowdler, A. J. (2001) *The complete spleen: structure, function, and clinical disorders.* Springer Science & Business Media.
75. Bowers, K. M., Bell, D., Chiodini, P. L., Barnwell, J., Incardona, S., Yen, S., Luchavez, J. and Watt, H. (2009) 'Inter-rater reliability of malaria parasite counts and comparison of methods', *Malaria journal,* 8, pp. 1-9.
76. Brousse, V., Buffet, P. and Rees, D. (2014) 'The spleen and sickle cell disease: the sick(led) spleen', *British journal of haematology,* 166, pp. 165-176.
77. Brousse, V., Elie, C., Benkerrou, M., Odièvre, M. H., Lesprit, E., Bernaudin, F., Grimaud, M., Guitton, C., Quinet, B. and Dangiolo, S. (2012) 'Acute splenic sequestration crisis in sickle cell disease: cohort study of 190 paediatric patients', *British journal of haematology,* 156(5), pp. 643-648.
78. Brown, B., Dada-Adegbola, H., Trippe, C. and Olopade, O. (2017) 'Prevalence and Etiology of Bacteremia in Febrile Children with Sickle Cell Disease at a Nigeria Tertiary Hospital', *Mediterr J Hematol Infect Dis,* 9(1), pp. e2017039.
79. Brown, B. J., Fatunde, O. J. and Sodeinde, O. (2012) 'Correlates of steady-state haematocrit and hepatosplenomegaly in children with sickle cell disease in Western Nigeria', *West African Journal Of Medicine,* 31(2), pp. 86-91.
80. Buffet, P. A., Safeukui, I., Deplaine, G., Brousse, V., Prendki, V., Thellier, M., Turner, G. D. and Mercereau-Puijalon, O. (2011) 'The pathogenesis of Plasmodium falciparum malaria in humans: insights from splenic physiology', *Blood, The Journal of the American Society of Hematology,* 117(2), pp. 381-392.
81. Bukhari, M. H., Niazi, S., Khan, S. A., Hashmi, I., Perveen, S., Qureshi, S. S., Chaudhry, N. A., Qureshi, G. and Hasan, M. (2007) 'Modified method of AgNOR staining for tissue and interpretation in histopathology', *International journal of experimental pathology,* 88(1), pp. 47-53.
82. Casey, J. R., Kinney, T. R. and Ware, R. E. (1994) 'Acute splenic sequestration in the absence of palpable splenomegaly', *The American journal of pediatric hematology/oncology,* 16(2), pp. 181-182.
83. Casper, J. T., Koethe, S., Rodey, G. E. and Thatcher, L. G. (1976) 'A new method for studying splenic reticuloendothelial dysfunction in sickle cell disease patients and its clinical application: a brief report', *Blood,* 47(2), pp. 183.
84. Catalano, O., Sandomenico, F., Matarazzo, I. and Siani, A. (2005) 'Contrast-enhanced sonography of the spleen', *American Journal of Roentgenology,* 184(4), pp. 1150-1156.
85. Cholera, R., Brittain, N. J., Gillrie, M. R., Lopera-Mesa, T. M., Diakité, S. A., Arie, T., Krause, M. A., Guindo, A., Tubman, A. and Fujioka, H. (2008) 'Impaired cytoadherence of Plasmodium falciparum-infected erythrocytes containing sickle hemoglobin', *Proceedings of the National Academy of Sciences,* 105(3), pp. 991-996.

86. Chopra, R., Al-Mulhim, A. R. and Al-Baharani, A. T. (2005) 'Fibrocongestive splenomegaly in sickle cell disease: a distinct clinicopathological entity in the Eastern province of Saudi Arabia', *American journal of hematology,* 79(3), pp. 180-186.
87. Chotivanich, K., Udomsangpetch, R., McGready, R., Proux, S., Newton, P., Pukrittayakamee, S., Looareesuwan, S. and White, N. J. (2002) 'Central role of the spleen in malaria parasite clearance', *Journal of Infectious Diseases,* 185(10), pp. 1538-1541.
88. Claster, S. and Vichinsky, E. (1996) 'First report of reversal of organ dysfunction in sickle cell anemia by the use of hydroxyurea: splenic regeneration', *Blood,* 88(6), pp. 1951-1953.
89. Cohen, J. F., Korevaar, D. A., Altman, D. G., Bruns, D. E., Gatsonis, C. A., Hooft, L., Irwig, L., Levine, D., Reitsma, J. B. and De Vet, H. C. (2016) 'STARD 2015 guidelines for reporting diagnostic accuracy studies: explanation and elaboration', *BMJ open,* 6(11), pp. e012799.
90. Corazza, G. R., Ginaldi, L., Zoli, G., Frisoni, M., Lalli, G., Gasbarrini, G. and Quaglino, D. (1990) 'Howell-Jolly body counting as a measure of splenic function. A reassessment', *Clinical And Laboratory Haematology,* 12(3), pp. 269-275.
91. Cornille-Brøgger, R., Fleming, A. F., Kagan, I., Matsushima, T. and Molineaux, L. (1979) 'Abnormal haemoglobins in the Sudan savanna of Nigeria. II. Immunological response to malaria in normals and subjects with sickle cell trait', *Annals Of Tropical Medicine And Parasitology,* 73(2), pp. 173-183.
92. da Guarda, C. C., Yahouédéhou, S. C. M. A., Santiago, R. P., Neres, J. S. d. S., Fernandes, C. F. d. L., Aleluia, M. M., Figueiredo, C. V. B., Fiuza, L. M., Carvalho, S. P. and Oliveira, R. M. d. (2020) 'Sickle cell disease: a distinction of two most frequent genotypes (HbSS and HbSC)', *PLoS One,* 15(1), pp. e0228399.
93. Daniel, W. W. (1999) *Biostatistics: a foundation for analysis in the health sciences. Wiley series in probability and statistics. Applied probability and statistics section* New York: Wiley.
94. Darko, R., Rodrigues, O. P., Oliver-Commey, J. O. and Kotei, C. N. (2005) 'Gallstones in Ghanaian children with sickle cell disease', *West African Journal Of Medicine,* 24(4), pp. 295-298.
95. Davis, L. (1976) 'Changing blood picture in sickle-cell anaemia from shortly after birth to adolescence', *Journal of clinical pathology,* 29(10), pp. 898-901.
96. Day, K. P. and Marsh, K. (1991) 'Naturally acquired immunity to Plasmodium falciparum', *Parasitology today,* 7(3), pp. 68-71.
97. de Porto, A. P. N. A., Lammers, A. J. J., Bennink, R. J., ten Berge, I. J. M., Speelman, P. and Hoekstra, J. B. L. (2010) 'Assessment of splenic function', *European Journal Of Clinical Microbiology & Infectious Diseases,* 29(12), pp. 1465-1473.
98. Dertinger, S. D., Chen, Y., Miller, R. K., Brewer, K. J., Smudzin, T., Torous, D. K., Hall, N. E., Olvany, K. A., Murante, F. G. and Tometsko, C. R. (2003) 'Micronucleated CD71-positive reticulocytes: a blood-based endpoint of cytogenetic damage in humans', *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 542(1-2), pp. 77-87.

99. Diagne, I., Diagne-Gueye, N. R., Fall, A. L., Deme, I., Sylla, A., Coly, J. I., Camara, B. and Sow, H. D. (2010) 'Epidemiology and course of splenomegaly in children and adolescents with sickle cell disease in Senegal', *Arch Pediatr,* 17(7), pp. 1017-25.
100. Diggs, L. (1935) 'Siderofibrosis of the spleen in sickle cell anemia', *Journal of the American Medical Association,* 104(7), pp. 538-541.
101. Doern, G. V., Carroll, K. C., Diekema, D. J., Garey, K. W., Rupp, M. E., Weinstein, M. P. and Sexton, D. J. (2019) 'A comprehensive update on the problem of blood culture contamination and a discussion of methods for addressing the problem', *Clin Microbiol Rev,* 33(1), pp. e00009-19.
102. Doumbo, O., Toure, A., Coulibaly, B., Koita, O., Traore, B., Dolo, A., Diallo, M., Diallo, A. N. and Quilici, M. (1992) 'Incidence of malaria and haemoglobin S in a paediatric hospital in Bamako, Mali. *Médecine Tropicale,* 52(2), pp. 169-174.
103. Durosinmi, M. A., Salawu, L., Ova, Y. A., Lawal, O. O. and Fadiran, O. A. (2005) 'Haematological parameters in sickle cell anaemia patients with and without splenomegaly', *The Nigerian Postgraduate Medical Journal,* 12(4), pp. 271-274.
104. Edelstein, S. J., Telford, J. N. and Crepeau, R. H. (1973) 'Structure of fibers of sickle cell hemoglobin', *Proceedings of the National Academy of Sciences,* 70(4), pp. 1104-1107.
105. Ehimwenma, O. and Tagbo, M. T. (2011) 'Determination of normal dimension of the spleen by ultrasound in an endemic tropical environment', *Nigerian Medical Journal,* 52(3), pp. 198.
106. El Hoss, S. and Brousse, V. (2019) 'Considering the spleen in sickle cell disease', *Expert Rev Hematol,* 12(7), pp. 563-573.
107. El Hoss, S., Cochet, S., Marin, M., Lapoumeroulie, C., Dussiot, M., Bouazza, N., Elie, C., de Montalembert, M., Arnaud, C., Guitton, C., Pellegrino, B., Odievre, M. H., Moati, F., Le Van Kim, C., Aronovicz, Y. C., El Nemer, W. and Brousse, V. (2019) 'Insights into determinants of spleen injury in sickle cell anemia', *Blood Adv,* 3(15), pp. 2328-2336.
108. El Hoss, S., Dussiot, M., Renaud, O., Brousse, V. and El Nemer, W. (2018) 'A novel non-invasive method to measure splenic filtration function in humans', *Haematologica,* 103(10), pp. e436-e439.
109. Ellison, A. M., Ota, K. V., McGowan, K. L. and Smith-Whitley, K. (2013) 'Epidemiology of bloodstream infections in children with sickle cell disease', *Pediatr Infect Dis J,* 32(5), pp. 560-3.
110. Emond, A. M., Collis, R., Darvill, D., Higgs, D. R., Maude, G. and Serjeant, G. (1985) 'Acute splenic sequestration in homozygous sickle cell disease: natural history and management', *The Journal of pediatrics,* 107(2), pp. 201-206.
111. Eze, C. U., Agwu, K., Ezeasor, D., Ochie, K., Aronu, A., Agwuna, K. and Nwadike, I. (2013) 'Sonographic biometry of spleen among school age children in Nsukka, Southeast, Nigeria', *African health sciences,* 13(2), pp. 384-392.
112. Eze, C. U., Offordile, G. C., Agwuna, K. K., Ocheni, S., Nwadike, I. U. and Chukwu, B. F. (2015) 'Sonographic evaluation of the spleen among sickle cell

disease patients in a teaching hospital in Nigeria', *Afr Health Sci,* 15(3), pp. 949-58.

113. Ezeike, C. I. (2019) 'Ultrasonographic Assessment of the Splenic Size in Sickle Cell Anemia: Single Splenic Span Measurement Vs Splenic Volume *International Journal of Research and Scientific Innovation (IJRSI),* 6(12), pp. 77-83.

114. Ezeofor, S., Obikili, E., Anyanwu, G., Onuh, A. and Mgbor, S. (2014) 'Sonographic assessment of the normal limits of the spleen in healthy school children in South-East Nigeria', *Nigerian Journal of Clinical Practice,* 17(4), pp. 484-488.

115. F.A. Fasola and Adekanmi, A. J. (2019) 'Haematological profile and blood transfusion pattern of patients with sickle cell anaemia vary with spleen size', *Annals of Ibadan Postgraduate Medicine,* 17(1), pp. 30-38.

116. Falusi, A., Esan, G., Ayyub, H. and Higgs, D. (1987) 'Alpha-thalassaemia in Nigeria: Its interaction with sickle-cell disease', *European journal of haematology,* 38(4), pp. 370-375.

117. Fatunde, O. J. and Scott, R. B. (1986) 'Pitted red cell counts in sickle cell disease. Relationship to age, hemoglobin genotype, and splenic size', *The American journal of pediatric hematology/oncology,* 8(4), pp. 329-333.

118. Ferster, A., Bujan, W., Corazza, F., Devalck, C., Fondu, P., Toppet, M., Verhas, M. and Sariban, E. (1993) 'Bone marrow transplantation corrects the splenic reticuloendothelial dysfunction in sickle cell anemia'.

119. Flening, A. (1989) 'The presentation, management and prevention of crisis in sickle cell disease in Africa', *Blood reviews,* 3(1), pp. 18-28.

120. Franceschi, L. d., Sada, S., Andreoli, A., Angheben, A., Marocco, S. and Bisoffi, Z. (2005) 'Sickle cell disease and hyperreactive malarial splenomegaly (HMS) in young immigrants from Africa', *Blood,* 106(13), pp. 4415-4417.

121. Franklin, K., Opoku-Okrah, C., Obiri-Danso, K., Owiredu, W. and Annan, A. (2011) 'The effect of alpha (+)-Thalassaemia on P. falciparum Malaria parasitaemia in children attending Komfo Anokye Teaching Hospital', *Inter J Biomed Lab Sc,* 1(1), pp. 7-14.

122. G B Inah and Ekanem, E. E. (2018) 'Ultrasonographically determined autosplenectomy rates in Nigerian sicklers and the predictors running title: splenic changes in Nigerian sicklers', *IOSR Journal of Dental and Medical Sciences (IOSR-JDMS),* 17(7(13)), pp. 61-64.

123. Galadanci, N., Wudil, B. J., Balogun, T. M., Ogunrinde, G. O., Akinsulie, A., Hasan-Hanga, F., Mohammed, A. S., Kehinde, M. O., Olaniyi, J. A., Diaku-Akinwumi, I. N., Brown, B. J., Adeleke, S., Nnodu, O. E., Emodi, I., Ahmed, S., Osegbue, A. O., Akinola, N., Opara, H. I. O., Adegoke, S. A., Aneke, J. and Adekile, A. D. (2014) 'Current sickle cell disease management practices in Nigeria', *International Health (RSTMH),* 6(1), pp. 23-28.

124. Gale, H. I., Bobbitt, C. A., Setty, B. N., Sprinz, P. G., Doros, G., Williams, D. D., Morrison, T. C., Kalajian, T. A., Tu, P., Mundluru, S. N. and Castro-Aragon, I. (2016) 'Expected Sonographic Appearance of the Spleen in Children and Young Adults With Sickle Cell Disease An Update', *.J Ultrasound Med,* 35, pp. 1735-1745.

125. Gassaye, D., Ibara, J. R., Mbongo, G. A., Okouo, M., Mambouana, N. P., Ntari, B. and Itoua-Ngaporo, A. (2000) 'Splenic abscess: report of 13 cases. / Les abcés de la rate. A propos de 13 cas', *Médecine d'Afrique Noire,* 47(1), pp. 41-43.
126. Gluckman, E., Cappelli, B., Bernaudin, F., Labopin, M., Volt, F., Carreras, J., Pinto Simões, B., Ferster, A., Dupont, S. and de La Fuente, J. (2017) 'Sickle cell disease: an international survey of results of HLA-identical sibling hematopoietic stem cell transplantation', *Blood, The Journal of the American Society of Hematology,* 129(11), pp. 1548-1556.
127. Gnassingbe, K., Akakpo-Numado, G. K., Attipou, K., Gbadoe, A. and Tekou, H. (2007) 'Prophylactic splenectomy to prevent complications of splenomegaly in children with sickle cell anemia?', *Sante (Montrouge, France),* 17(4), pp. 207-211.
128. Gomez-Perez, G. P., van Bruggen, R., Grobusch, M. P. and Dobano, C. (2014) 'Plasmodium falciparum malaria and invasive bacterial co-infection in young African children: the dysfunctional spleen hypothesis', *Malar J,* 13, pp. 335.
129. Gong, L., Maiteki-Sebuguzi, C., Rosenthal, P. J., Hubbard, A. E., Drakeley, C. J., Dorsey, G. and Greenhouse, B. (2012) 'Evidence for both innate and acquired mechanisms of protection from Plasmodium falciparum in children with sickle cell trait', *Blood,* 119(16), pp. 3808-3814.
130. Goussanou, S., Alihonou, E., Rahimy, M. C., Gangbo, A., Ahouignan, G., Adjou, R. and Deguenon, C. (2003) 'Effect of a comprehensive clinical care program on disease course in', *Blood, 102, pp. 834-838.*
131. Grosse, S. D., Odame, I., Atrash, H. K., Amendah, D. D., Piel, F. B. and Williams, T. N. (2011) 'Sickle cell disease in Africa: a neglected cause of early childhood mortality', *American Journal Of Preventive Medicine,* 41(6 Suppl 4), pp. S398-S405.
132. Gulbis, B., Haberman, D., Dufour, D., Christophe, C., Vermylen, C., Kagambega, F., Corazza, F., Devalck, C., Dresse, M.-F. and Hunninck, K. (2005) 'Hydroxyurea for sickle cell disease in children and for prevention of cerebrovascular events: the Belgian experience', *Blood,* 105(7), pp. 2685-2690.
133. Hankins, J. S., Helton, K. J., McCarville, M. B., Li, C. S., Wang, W. C. and Ware, R. E. (2008) 'Preservation of spleen and brain function in children with sickle cell anemia treated with hydroxyurea', *Pediatric blood & cancer,* 50(2), pp. 293-297.
134. Harrod, V. L., Howard, T. A., Zimmerman, S. A., Dertinger, S. D. and Ware, R. E. (2007) 'Quantitative analysis of Howell-Jolly bodies in children with sickle cell disease', *Exp Hematol,* 35(2), pp. 179-83.
135. Higgs, D. R., Aldridge, B. E., Lamb, J., Clegg, J. B., Weatherall, D. J., Hayes, R. J., Grandison, Y., Lowrie, Y., Mason, K. P. and Serjeant, B. E. (1982) 'The interaction of alpha-thalassemia and homozygous sickle-cell disease', *New England Journal of Medicine,* 306(24), pp. 1441-1446.
136. Hirst, C. and Owusu-Ofori, S. (2014) 'Prophylactic antibiotics for preventing pneumococcal infection in children with sickle cell disease', *Cochrane database of systematic reviews,* (11).

137. Hoban, M. D., Orkin, S. H. and Bauer, D. E. (2016) 'Genetic treatment of a molecular disorder: gene therapy approaches to sickle cell disease', *Blood, The Journal of the American Society of Hematology,* 127(7), pp. 839-848.
138. Hoffbrand, A. V., Catovsky, D. and Tuddenham, E. G. D. (2005) *Postgraduate haematology.* 5th ed. edn. Oxford: Blackwell.
139. Holroyde, C. P. and Gardner, F. H. (1970) 'Acquisition of autophagic vacuoles by human erythrocytes physiological role of the spleen', *Blood,* 36(5), pp. 566-575.
140. Hosey, R. G., Mattacola, C. G., Kriss, V., Armsey, T., Quarles, J. and Jagger, J. (2006) 'Ultrasound assessment of spleen size in collegiate athletes', *British journal of sports medicine,* 40(3), pp. 251-254.
141. Howell, W. t. and Black, D. (1980) 'Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method', *experientia,* 36(8), pp. 1014-1015.
142. Hulbert, M. L. and Shenoy, S. (2018) 'Hematopoietic stem cell transplantation for sickle cell disease: Progress and challenges', *Pediatric blood & cancer,* 65(9), pp. e27263.
143. Hyacinth, H. I., Adekeye, O. A. and Yilgwan, C. S. (2013) 'Malnutrition in sickle cell anemia: implications for infection, growth, and maturation', *Journal of social, behavioral and health sciences,* 7(1).
144. Ibidapo, M. and Akinyanju, O. (2000) 'Acute sickle cell syndromes in Nigerian adults', *Clinical & Laboratory Haematology,* 22(3), pp. 151-155.
145. JA Olaniyi, U. A. (2007) 'Frequency of hepatomegaly and splenomegaly in Nigerian patients with sickle cell disease', *West African journal of medicine,* 26(4), pp. 274-277.
146. Jagadeeswaran, R., Lenny, H., Vazquez, B., Muniz, J., Schad, A., Jain, S., Gowhari, M., Lavelle, D., Diamond, A. and Molokie, R. E. (2017) 'The abnormal presence of mitochondria in circulating red blood cells cause an increased oxygen consumption rate, ROS generation and hemolysis in patients with sickle cell disease', *Blood,* 130, pp. 2237.
147. Jama, A. H. A., Salem, A. H. A. and Dabbous, I. A. A. (2002) 'Massive splenic infarction in Saudi patients with sickle cell anemia: a unique manifestation', *American journal of hematology,* 69(3), pp. 205-209.
148. Jebbin, N. J. and Adotey, J. M. (2011) 'Acute abdominal conditions in people with sickle cell disease: a 10-year experience in Port Harcourt, Nigeria', *Annals Of African Medicine,* 10(2), pp. 165-170.
149. Juwah, A., Nlemadim, A. and Kaine, W. (2003) 'Clinical presentation of severe anemia in pediatric patients with sickle cell anemia seen in Enugu, Nigeria', *American journal of hematology,* 72(3), pp. 185-191.
150. Kaine, W. N. (1982) 'Sickle cell anaemia in children in Eastern Nigeria: a detailed analysis of 210 cases', *East African Medical Journal,* 59(11), pp. 742-749.
151. Kassim, A. A. and Sharma, D. (2017) 'Hematopoietic stem cell transplantation for sickle cell disease: the changing landscape', *Hematology/oncology and stem cell therapy,* 10(4), pp. 259-266.

152. Kato, G. J., Piel, F. B., Reid, C. D., Gaston, M. H., Ohene-Frempong, K., Krishnamurti, L., Smith, W. R., Panepinto, J. A., Weatherall, D. J. and Costa, F. F. (2018) 'Sickle cell disease', *Nature Reviews Disease Primers,* 4(1), pp. 1-22.
153. Kaushal, D. L., Verma, D. V. K., Ahirwar, D. C. P., Patil, D. A. and Pratap Singh, D. S. (2014) 'Sonographic evaluation of abdominal organs in a sickle cell disease patient', *International Journal of Medical Research and Review,* 2(3), pp. 202-208.
154. Kazadi, A. L., Ngiyulu, R. M., Gini-Ehungu, J. L., Mbuyi-Muamba, J. M. and Aloni, M. N. (2019) 'The clinical characteristics of Congolese children and adolescents suffering from sickle-cell anemia are marked by the high frequencies of epistaxis compared to Western series', *Pediatric Hematology And Oncology,* 36(5), pp. 267-276.
155. Kent, G., Minick, O., Volini, F. and Orfei, E. (1966) 'Autophagic vacuoles in human red cells', *The American journal of pathology,* 48(5), pp. 831.
156. Kizito, M. E., Mworozi, E., Ndugwa, C. and Serjeant, G. R. (2007) 'Bacteraemia in homozygous sickle cell disease in Africa: is pneumococcal prophylaxis justified?', *Arch Dis Child,* 92(1), pp. 21-3.
157. Komba, A. N., Makani, J., Sadarangani, M., Ajala-Agbo, T., Berkley, J. A., Newton, C. R. J. C., Marsh, K. and Williams, T. N. 2009. Malaria as a Cause of Morbidity and Mortality in Children with Homozygous Sickle Cell Disease on the Coast of Kenya.Clinical Infectious Diseases, Volume 49, Issue 2, 15 July 2009, Pages 216–222.
158. Konotey-Ahulu, F. (1971) 'Malaria and sickle-cell disease', *British Medical Journal,* 2(5763), pp. 710.
159. Kotila, R., Okesola, A. and Makanjuola, O. (2007) 'Asymptomatic malaria parasitaemia in sickle-cell disease patients: how effective is chemoprophylaxis?', *Journal of Vector Borne Diseases,* 44(1), pp. 52-55.
160. Kotila, T. R., Fawole, O. I. and Shokunbi, W. A. (2000) 'Haemoglobin F and clinical severity of sickle cell anaemia among Nigerian adults', *African Journal Of Medicine And Medical Sciences,* 29(3-4), pp. 229-231.
161. Ladu, A., Satumari, N., Abba, A., Abulfathi, F., Jeffery, C., Adekile, A. and Bates, I. 2023. Evaluation of two red cell inclusion staining methods for assessing spleen function among sickle cell disease patients in North-East Nigeria. medRxiv.
162. Ladu, A. I., Aiyenigba, A. O., Adekile, A. and Bates, I. (2021) 'The spectrum of splenic complications in patients with sickle cell disease in Africa: a systematic review', *British Journal of Haematology,* 193(1), pp. 26-42.
163. Lamb, P., Lund, A., Kanagasabay, R., Martin, A., Webb, J. and Reznek, R. (2002) 'Spleen size: how well do linear ultrasound measurements correlate with three-dimensional CT volume assessments?', *The British journal of radiology,* 75(895), pp. 573-577.
164. Lammers, A. J. J., de Porto, A. P. N. A., Bennink, R. J., van Leeuwen, E. M. M., Biemond, B. J., Goslings, J. C., van Marle, J., ten Berge, I. J. M., Speelman, P. and Hoekstra, J. B. L. 2012. Hyposplenism: Comparison of different methods for determining splenic function. American Journal of Hematology. 2012;87(5):484-9.

165. Lane, P. A., O'Connell, J. L., Lear, J. L., Rogers, Z. R., Woods, G. M., Hassell, K. L., Wethers, D. L., Luckey, D. W. and Buchanan, G. R. (1995) 'Functional asplenia in hemoglobin SC disease', *Blood* 85 (8), pp. 2238 -2244.
166. Lenti, M. V., Luu, S., Carsetti, R., Osier, F., Ogwang, R., Nnodu, O. E., Wiedermann, U., Spencer, J., Locatelli, F. and Corazza, G. R. (2022) 'Asplenia and spleen hypofunction', *Nature Reviews Disease Primers,* 8(1), pp. 1-18.
167. Lesher, A. P., Kalpatthi, R., Glenn, J. B., Jackson, S. M. and Hebra, A. (2009) 'Outcome of splenectomy in children younger than 4 years with sickle cell disease', *J Pediatr Surg,* 44(6), pp. 1134-8.
168. Lesprit, E. and Lesprit, P. (2004) 'Infection in sickle cell disease', *La Revue du praticien,* 54(14), pp. 1574-1577.
169. Liberati, A., Altman, D. G., Tetzlaff, J., Mulrow, C., Gøtzsche, P. C., Ioannidis, J. P., Clarke, M., Devereaux, P. J., Kleijnen, J. and Moher, D. (2009) 'The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration', *Journal of clinical epidemiology,* 62(10), pp. e1-e34.
170. Lindner, L. E. (1993) 'Improvements in the silver-staining technique for nucleolar organizer regions (AgNOR)', *Journal of Histochemistry & Cytochemistry,* 41(3), pp. 439-445.
171. Lonergan, G. J., Cline, D. B. and Abbondanzo, S. L. (2001) 'Sickle cell anemia', *Radiographics,* 21(4), pp. 971-994.
172. Luna, A., Ribes, R., Caro, P., Luna, L., Aumente, E. and Ros, P. R. (2006) 'MRI of focal splenic lesions without and with dynamic gadolinium enhancement', *American Journal of Roentgenology,* 186(6), pp. 1533-1547.
173. Luntsi, G., Eze, C. U., Ahmadu, M. S., Bukar, A. A. and Ochie, K. (2018) 'Sonographic Evaluation of Some Abdominal Organs in Sickle Cell Disease Patients in a Tertiary Health Institution in Northeastern Nigeria', *J Med Ultrasound,* 26(1), pp. 31-36.
174. Luzzatto, L. (2012) 'Sickle Cell Anaemia and Malaria', *Mediterranean Journal of Hematology & Infectious Diseases,* 4(1), pp. 1-6.
175. Luzzatto, L. and Makani, J. (2019) 'Hydroxyurea - An Essential Medicine for Sickle Cell Disease in Africa', *New England Journal of Medicine,* 380(2), pp. 187-189.
176. Ma'aji, S., Jiya, N., Saidu, S., Danfulani, M., Yunusa, G., Sani, U., Jibril, B., Musa, A., Gele, H., Baba, M. and Bello, S. 2012. Transabdominal ultrasonographic findings in children with sickle cell anemia in Sokoto, North-Western Nigeria. Nigerian Journal of Basic and Clinical Sciences; 9(1), pp. 14.
177. Madani, G., Papadopoulou, A., Holloway, B., Robins, A., Davis, J. and Murray, D. (2007) 'The radiological manifestations of sickle cell disease', *Clinical radiology,* 62(6), pp. 528-538.
178. Magnus, S. A., Hambleton, I. R., Moosdeen, F. and Serjeant, G. R. (1999) 'Recurrent infections in homozygous sickle cell disease', *Archives of Disease in Childhood,* 80(6), pp. 537-541.
179. Makani, J., Komba, A. N., Cox, S. E., Oruo, J., Mwamtemi, K., Kitundu, J., Magesa, P., Rwezaula, S., Meda, E. and Mgaya, J. (2010) 'Malaria in patients

with sickle cell anemia: burden, risk factors, and outcome at the outpatient clinic and during hospitalization', *Blood,* 115(2), pp. 215-220.

180. Makani, J., Mgaya, J., Balandya, E., Msami, K., Soka, D., Cox, S. E., Komba, A. N., Rwezaula, S., Meda, E., Muturi, D., Kitundu, J., Fegan, G., Kirkham, F. J., Newton, C. R., Snow, R. W. and Lowe, B. 2015. Bacteraemia in sickle cell anaemia is associated with low haemoglobin: a report of 890 admissions to a tertiary hospital in Tanzania.
181. Makani, J., Ofori-Acquah, S. F., Nnodu, O., Wonkam, A. and Ohene-Frempong, K. (2013) 'Sickle cell disease: new opportunities and challenges in Africa', *Thescientificworldjournal,* 2013, pp. 193252-193252.
182. Makani, J., Williams, T. N. and Marsh, K. (2007) 'Sickle cell disease in Africa: burden and research priorities', *Annals Of Tropical Medicine And Parasitology,* 101(1), pp. 3-14.
183. Mallouh, A., Burke, G. M., Salamah, M. and Ahmad, M. S. (1984) 'Splenic function in Saudi children with sickle cell disease', *Annals of Tropical Paediatrics,* 4(2), pp. 87-91.
184. McAuley, C. F., Webb, C., Makani, J., Macharia, A., Uyoga, S., Opi, D. H., Ndila, C., Ngatia, A., Scott, J. A. G., Marsh, K. and Williams, T. N. (2010) 'High mortality from Plasmodium falciparum malaria in children living with sickle cell anemia on the coast of Kenya', *Blood,* 116(10), pp. 1663.
185. McCarville, M. B., Luo, Z., Huang, X., Rees, R. C., Rogers, Z. R., Miller, S. T., Thompson, B., Kalpatthi, R., Wang, W. C. and Investigators, B. H. (2011) 'Abdominal ultrasound with scintigraphic and clinical correlates in infants with sickle cell anemia: baseline data from the BABY HUG trial', *American Journal of Roentgenology,* 196(6), pp. 1399-1404.
186. Mebius, R. E. and Kraal, G. (2005) 'Structure and function of the spleen', *Nature reviews immunology,* 5(8), pp. 606-616.
187. Mockenhaupt, F. P., Falusi, A. G., May, J., Ademowo, O. G., Olumese, P. E., Meyer, C. G. and Bienzle, U. (1999) 'The contribution of α+–thalassaemia to anaemia in a Nigerian population exposed to intense malaria transmission', *Tropical Medicine & International Health,* 4(4), pp. 302-307.
188. Moola, S., Munn, Z., Tufanaru, C., Aromataris, E., Sears, K., Sfetcu, R., Currie, M., Qureshi, R., Mattis, P. and Lisy, K. (2017) 'Chapter 7: Systematic reviews of etiology and risk', *Joanna Briggs Institute Reviewer's Manual. The Joanna Briggs Institute*, pp. 2019-05.
189. Moriconi, C., Dzieciatkowska, M., Roy, M., D'Alessandro, A., Roingeard, P., Lee, J. Y., Gibb, D. R., Tredicine, M., McGill, M. A. and Qiu, A. (2022) 'Retention of functional mitochondria in mature red blood cells from patients with sickle cell disease', *British Journal of Haematology,* 198(3), pp. 574-586.
190. Morrissey, B. J., Bycroft, T. P., Almossawi, O., Wilkey, O. B. and Daniels, J. G. (2015) 'Incidence and predictors of bacterial infection in febrile children with sickle cell disease', *Hemoglobin,* 39(5), pp. 316-319.
191. Mouélé, R., Boukila, V., Fourcade, V., Feingold, J. and Galactéros, F. (1999) 'Sickle-cell disease in Brazzaville, Congo: genetical, hematological, biochemical and clinical aspects', *Acta Haematologica,* 101(4), pp. 178-184.

192. Mozzarelli, A., Hofrichter, J. and Eaton, W. A. (1987) 'Delay time of hemoglobin S polymerization prevents most cells from sickling in vivo', *Science,* 237(4814), pp. 500-506.
193. Mpalampa, L., Ndugwa, C. M., Ddungu, H. and Idro, R. (2012) 'Foetal haemoglobin and disease severity in sickle cell anaemia patients in Kampala, Uganda', *BMC Hematol, 12(11).*
194. Mustapha, Z., Tahir, A., Tukur, M., Bukar, M. and Lee, W.-K. (2010) 'Sonographic determination of normal spleen size in an adult African population', *European journal of radiology,* 75(1), pp. e133-e135.
195. Nagel, R. L. and Fleming, A. F. (1992) 'Genetic epidemiology of the beta s gene', *Baillieres Clin Haematol,* 5(2), pp. 331-65.
196. Nardo-Marino, A., Braunstein, T. H., Petersen, J., Brewin, J. N., Mottelson, M. N., Williams, T. N., Kurtzhals, J. A., Rees, D. C. and Glenthøj, A. (2022) 'Automating pitted red blood cell counts using deep neural network analysis: a new method for measuring splenic function in sickle cell Anaemia', *Frontiers in Physiology,* 13.
197. Nardo-Marino, A., Glenthøj, A., Brewin, J. N., Petersen, J., Braunstein, T. H., Kurtzhals, J. A., Williams, T. N. and Rees, D. C. (2022) 'The significance of spleen size in children with sickle cell anemia', *American Journal of Hematology,* 97(12), pp. 1520-1528.
198. Network, T. M. G. E. (2008) 'A global network for investigating the genomic epidemiology of malaria', *Nature,* 456(7223), pp. 732.
199. Nikicicz, E. and Norback, D. (1990) 'Argyrophilic nucleolar organiser region (AgNOR) staining in normal bone marrow cells', *Journal of clinical pathology,* 43(9), pp. 723-727.
200. Nottage, K. A., Ware, R. E., Winter, B., Smeltzer, M., Wang, W. C., Hankins, J. S., Dertinger, S. D., Shulkin, B. and Aygun, B. (2014) 'Predictors of splenic function preservation in children with sickle cell anemia treated with hydroxyurea', *European journal of haematology,* 93(5), pp. 377-383.
201. O'Brien, R. T., McIntosh, S., Aspnes, G. T. and Pearson, H. A. (1976) 'Prospective study of sickle cell anemia in infancy', *The Journal of Pediatrics,* 89(2), pp. 205-210.
202. O'Meara, W. P., Barcus, M., Wongsrichanalai, C., Muth, S., Maguire, J. D., Jordan, R. G., Prescott, W. R. and McKenzie, F. E. (2006) 'Reader technique as a source of variability in determining malaria parasite density by microscopy', *Malaria journal,* 5, pp. 1-7.
203. Obaro, S. K. and Tam, P. Y. I. (2016) 'Preventing Infections in Sickle Cell Disease: The Unfinished Business', *Pediatric Blood & Cancer,* 63(5), pp. 781-785.
204. Ogunsola, J. and Antia, R. (2018) 'Adaptation of the agyrophil technique for nucleolar organizer regions to canine peripheral blood smears', *Open veterinary journal,* 8(2), pp. 182-185.
205. Ojo, O., Ibijola, A., Shokunbi, W., Busari, O., Olatunji, P. and Ganiyu, A. (2018) 'Correlation between splenic size and CD4+ T lymphocytes in sickle cell anaemia patients in a Tertiary Hospital', *The Egyptian Journal of Haematology,* 43(2).

206. Okocha, E. C., Gyamfi, J., Ryan, N., Babalola, O., Etuk, E.-A., Chianumba, R., Nwegbu, M., Isa, H., Madu, A. J. and Adegoke, S. (2022) 'Barriers to therapeutic use of hydroxyurea for sickle cell disease in Nigeria: a cross-sectional survey', *Frontiers in Genetics,* 12, pp. 2834.
207. Okongwu, C. I., Fasola, F. A., Adekanmi, A. J. and Onifade, A. A. (2018) 'Morbidity pattern and interferon gamma level in sickle cell anemia patients with autosplenectomy', *Nigerian Journal Of Clinical Practice,* 21(12), pp. 1615-1621.
208. Okoro, B. A., Kaine, W. N. and Okeahialam, T. C. (1989) 'Splenectomy in Nigerian children with sickle cell anaemia', *Tropical And Geographical Medicine,* 41(2), pp. 123-127.
209. Okpala, I., Thomas, V., Westerdale, N., Jegede, T., Raj, K., Daley, S., Costello-Binger, H., Mullen, J., Rochester-Peart, C. and Helps, S. (2002) 'The comprehensive care of sickle cell disease', *European Journal of Haematology,* 68(3), pp. 157-162.
210. Okuonghae, H., Nwankwo, M. and Offor, E. (1992) 'Malarial parasitaemia in febrile children with sickle cell anaemia', *Journal of tropical pediatrics,* 38(2), pp. 83-85.
211. Olatunji, A. A. and Olatunji, P. O. (2001) 'Splenic size determination in sickle cell anaemia: an ultrasonographic study', *East African Medical Journal,* 78(7), pp. 366-369.
212. Olatunya, O. S., Albuquerque, D. M., Adekile, A. and Costa, F. F. (2019) 'Influence of alpha thalassemia on clinical and laboratory parameters among nigerian children with sickle cell anemia', *Journal of clinical laboratory analysis,* 33(2), pp. e22656.
213. Olivieri, N. F. and Vichinsky, E. P. (1998) 'Hydroxyurea in children with sickle cell disease: impact on splenic function and compliance with therapy', *Journal of pediatric hematology/oncology,* 20(1), pp. 26-31.
214. Oniyangi, O. and Omari, A. A. (2006) 'Malaria chemoprophylaxis in sickle cell disease', *Cochrane Database of Systematic Reviews*, pp. N.PAG-N.PAG.
215. Opoka, R. O., Ndugwa, C. M., Latham, T. S., Lane, A., Hume, H. A., Kasirye, P., Hodges, J. S., Ware, R. E. and John, C. C. (2017) 'Novel use Of Hydroxyurea in an African Region with Malaria (NOHARM): a trial for children with sickle cell anemia', *Blood,* 130(24), pp. 2585-2593.
216. Organization, W. H. (2017) 'WHO model list of essential medicines, 20th list (March 2017, amended August 2017)'.
217. OT Ojo, WA Shokunbi and Agunloye, A. (2014) 'Splenic Size in Sickle Cell Anaemia Patients in A Tertiary Hospital ', *Nigerian Hospital Practice* 13(5-6).
218. Oyetunji, I. A., Amoo, A. J., Adeyemo, T. A. and Ojewunmi, O. O. (2020) 'Asymptomatic malaria infection among children with Sickle Cell Anaemia: The role of IL-10 and possible predisposing factors', *PAMJ-One Health,* 2(7).
219. Padmos, M., Sackey, K., Roberts, G., Kulozik, A., Bail, S., Morris, J., Serjeant, B. and Serjeant, G. (1991) 'Two different forms of homozygous sickle cell disease occur in Saudi Arabia', *British journal of haematology,* 79(1), pp. 93-98.

220. Pasvol, G., Weatherall, D. and Wilson, R. (1978) 'Cellular mechanism for the protective effect of haemoglobin S against P. falciparum malaria', *Nature,* 274(5672), pp. 701-703.
221. Pearson, H. A., Cornelius, E. A., Schwartz, A. D., Zelson, J. H., Wolfson, S. L. and Spencer, R. P. (1970) 'Transfusion-reversible functional asplenia in young children with sickle-cell anemia', *New England Journal of Medicine,* 283(7), pp. 334-337.
222. Pearson, H. A., Gallagher, D., Chilcote, R., Sullivan, E., Wilimas, J., Espeland, M., Ritchey, A. K. and Disease, C. S. o. S. C. (1985) 'Developmental pattern of splenic dysfunction in sickle cell disorders', *Pediatrics,* 76(3), pp. 392-397.
223. Pearson, H. A., McIntosh, S., Ritchey, A. K., Lobel, J. S., Rooks, Y. and Johnston, D. (1979) 'Developmental aspects of splenic function in sickle cell diseases', *Blood,* 53(3), pp. 358-365.
224. Pearson, H. A., Spencer, R. P. and Cornelius, E. A. (1969) 'Functional asplenia in sickle-cell anemia', *New England Journal of Medicine,* 281(17), pp. 923-926.
225. Pelizzo, G., Guazzotti, M., Klersy, C., Nakib, G., Costanzo, F., Andreatta, E., Bassotti, G. and Calcaterra, V. (2018) 'Spleen size evaluation in children: Time to define splenomegaly for pediatric surgeons and pediatricians', *PloS one,* 13(8), pp. e0202741.
226. Peretz, S., Livshits, L., Pretorius, E., Makhro, A., Bogdanova, A., Gassmann, M., Koren, A. and Levin, C. (2022) 'The protective effect of the spleen in sickle cell patients. A comparative study between patients with asplenia/hyposplenism and hypersplenism', *Frontiers in Physiology*, pp. 1756.
227. Piel, F. B., Hay, S. I., Sunetra, G., Weatherall, D. J. and Williams, T. N. (2013a) 'Global burden of sickle cell anaemia in children under five, 2010-2050: modelling based on demographics, excess mortality, and interventions', *PLoS Medicine,* 10(7), pp. e1001484-e1001484.
228. Piel, F. B., Patil, A. P., Howes, R. E., Nyangiri, O. A., Gething, P. W., Dewi, M., Temperley, W. H., Williams, T. N., Weatherall, D. J. and Hay, S. I. (2013b) 'Global epidemiology of sickle haemoglobin in neonates: a contemporary geostatistical model-based map and population estimates', *Lancet,* 381(9861), pp. 142-151.
229. Piel, F. B., Patil, A. P., Howes, R. E., Nyangiri, O. A., Gething, P. W., Williams, T. N., Weatherall, D. J. and Hay, S. I. (2010) 'Global distribution of the sickle cell gene and geographical confirmation of the malaria hypothesis', *Nature communications,* 1(1), pp. 104.
230. Planche, T., Krishna, S., Kombila, M., Engel, K., Faucher, J., Ngou-Milama, E. and Kremsner, P. (2001) 'Comparison of methods for the rapid laboratory assessment of children with malaria', *The American journal of tropical medicine and hygiene,* 65(5), pp. 599-602.
231. Ploton, D., Menager, M., Jeannesson, P., Himber, G., Pigeon, F. and Adnet, J. (1986) 'Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level', *The Histochemical Journal,* 18(1), pp. 5-14.

232. Pourdieu, C., El Hoss, S., Le Roux, E., Pages, J., Koehl, B., Missud, F., Holvoet, L., Ithier, G., Benkerrou, M. and Haouari, Z. (2023) 'Relevance of Howell-Jolly body counts for measuring spleen function in sickle cell disease', *American journal of hematology,* 98(5), pp. E110-E112.
233. Powars, D. R. (1975) 'Natural history of sickle cell disease. The first ten years', *Seminars in hematology,* 12(3), pp. 267.
234. Powell, R. W., Levine, G. L., Yang, Y.-M. and Mankad, V. N. (1992) 'Acute splenic sequestration crisis in sickle cell disease: early detection and treatment', *Journal of pediatric surgery,* 27(2), pp. 215-219.
235. Quinn, C. T. (2016) 'Minireview: Clinical severity in sickle cell disease: the challenges of definition and prognostication', *Experimental biology and medicine,* 241(7), pp. 679-688.
236. Quinn, C. T., Rogers, Z. R., McCavit, T. L. and Buchanan, G. R. (2010) 'Improved survival of children and adolescents with sickle cell disease', *Blood,* 115(17), pp. 3447-3452.
237. Ramakrishnan, M., Moisi, J. C., Klugman, K. P., Feris Iglesias, J. M., Grant, L. R., Mpoudi-Etame, M. and Levine, O. S. 2010. Increased risk of invasive bacterial infections in African people with sickle-cell disease: a systematic review and meta-analysis.
238. Rees, D. C., Williams, T. N. and Gladwin, M. T. (2010) 'Sickle-cell disease', *The Lancet,* 376(9757), pp. 2018-2031.
239. Riva, M. A., Ferraina, F., Paleari, A., Lenti, M. V. and Di Sabatino, A. (2019) 'From sadness to stiffness: the spleen's progress', *Internal And Emergency Medicine,* 14(5), pp. 739-743.
240. Rogers, D., Serjeant, B. and Serjeant, G. (1982) 'Early rise in the" pitted" red cell count as a guide to susceptibility to infection in childhood sickle cell anaemia', *Archives of Disease in Childhood,* 57(5), pp. 338-342.
241. Rogers, D. W. (1982) *The spleen in sickle cell anaemia during early childhood.* Masters Electronic Thesis or Dissertation, University of Oxford, 1982
242. Rogers, D. W., Vaidya, S. and Serjeant, G. R. (1978) 'Early splenomegaly in homozygous sickle-cell disease: an indicator of susceptibility to infection', *The Lancet,* 312(8097), pp. 963-965.
243. Rogers, Z. R., Wang, W. C., Luo, Z., Iyer, R. V., Shalaby-Rana, E., Dertinger, S. D., Shulkin, B. L., Miller, J. H., Files, B., Lane, P. A., Thompson, B. W., Miller, S. T., Ware, R. E. and Investigators, B. H. (2011) 'Biomarkers of splenic function in infants with sickle cell anemia: baseline data from the BABY HUG Trial', *Blood,* 117(9), pp. 2614-2617.
244. Rosenberg, H., Markowitz, R., Kolberg, H., Park, C., Hubbard, A. and Bellah, R. (1991) 'Normal splenic size in infants and children: sonographic measurements', *AJR. American journal of roentgenology,* 157(1), pp. 119-121.
245. Rosse, W. F., Narla, M., Petz, L. D. and Steinberg, M. H. (2000) 'New views of sickle cell disease pathophysiology and treatment', *ASH Education Program Book,* 2000(1), pp. 2-17.
246. Rumaney, M. B., Ngo Bitoungui, V. J., Vorster, A. A., Ramesar, R., Kengne, A. P., Ngogang, J. and 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), pp. e100516.

247. Sadarangani, M., Makani, J., Komba, A. N., Ajala-Agbo, T., Newton, C. R., Marsh, K. and Williams, T. N. (2009) 'An observational study of children with sickle cell disease in Kilifi, Kenya', *British Journal Of Haematology,* 146(6), pp. 675-682.

248. Safeukui, I., Correas, J. M., Brousse, V., Hirt, D., Deplaine, G., Mule, S., Lesurtel, M., Goasguen, N., Sauvanet, A., Couvelard, A., Kerneis, S., Khun, H., Vigan-Womas, I., Ottone, C., Molina, T. J., Treluyer, J. M., Mercereau-Puijalon, O., Milon, G., David, P. H. and Buffet, P. A. (2008) 'Retention of Plasmodium falciparum ring-infected erythrocytes in the slow, open microcirculation of the human spleen', *Blood,* 112(6), pp. 2520-8.

249. Santos, A., Pinheiro, V., Anjos, C., Brandalise, S., Fahel, F., Lima, M., Etchebehere, E., Ramos, C. and Camargo, E. E. (2002) 'Scintigraphic follow-up of the effects of therapy with hydroxyurea on splenic function in patients with sickle cell disease', *Eur J Nucl Med Mol Imaging,* 29(4), pp. 536-41.

250. Serjeant, B., Hambleton, I. and Serjeant, G. (2021) 'Retained splenic function in an Indian population with homozygous sickle cell disease may have important clinical significance', *Indian Journal of Community Medicine: Official Publication of Indian Association of Preventive & Social Medicine,* 46(4), pp. 715.

251. Serjeant, G. R. (1970) 'Irreversibly sickled cells and splenomegaly in sickle-cell anaemia', *British journal of haematology,* 19(5), pp. 635-641.

252. Serjeant, G. R. (2002) 'The spleen in sickle cell disease', *The Complete Spleen: Structure, Function, and Clinical Disorders*, pp. 251-257.

253. Serjeant, G. R. (2005) 'Mortality from sickle cell disease in Africa: interventions used to reduce mortality in non-malarial areas may be inappropriate', *BMJ: British Medical Journal (International Edition),* 330(7489), pp. 432-433.

254. Shongo, M. Y. P., Mukuku, O., Lubala, T. K., Mutombo, A. M., Kanteng, G. W., Umumbu, W. S., Lukamba, R. M., Wembonyama, S. O. and Luboya, O. N. (2014) 'Sickle cell disease in Lubumbashi children aged 6-59 months in stationary phase: epidemiological and clinical profiles, *Pan African Medical Journal,* 19, pp. 71-71.

255. Singhal, A., Thomas, P., Kearney, T., Venugopal, S. and Serjeant, G. (1995) 'Acceleration in linear growth after splenectomy for hypersplenism in homozygous sickle cell disease', *Archives of disease in childhood,* 72(3), pp. 227-229.

256. Sissoko, A., Fricot-Monsinjon, A., Roussel, C., Manceau, S., Dumas, L., Capito, C., Allali, S., Yekkache, N., Dussiot, M. and Nguyen, Y. (2022) 'Erythrocytic vacuoles that accumulate a fluorescent dye predict spleen size and function in sickle cell disease', *American journal of hematology,* 97(11), pp. E385-E388.

257. Slovis, C. M., Talley, J. D. and Pitts, R. B. (1986) 'Non relationship of climatologic factors and painful sickle cell anemia crisis', *Journal of chronic diseases,* 39(2), pp. 121-126.

258. Smith, T., Felger, I., Tanner, M. and Beck, H.-P. (1999) '11. Premunition in Plasmodium falciparum infection: insights from the epidemiology of multiple infections', *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 93(Supplement_1), pp. 59-64.
259. Snow, R., De Azevedo, I. B., Lowe, B., Kabiru, E., Nevill, C., Mwankusye, S., Kassiga, G., Marsh, K. and Teuscher, T. (1994) 'Severe childhood malaria in two areas of markedly different falciparum transmission in east Africa', *Acta tropica,* 57(4), pp. 289-300.
260. Statistics, N. B. o. (2016) *Population 2006-2016*. Available at: https://nigerianstat.gov.ng/elibrary/read/474 (Accessed: 15/10/2023.
261. Steinberg, M. H. (2008) 'Sickle cell anemia, the first molecular disease: overview of molecular etiology, pathophysiology, and therapeutic approaches', *The Scientific World Journal,* 8, pp. 1295-1324.
262. Steinberg, M. H., Rosenstock, W., Coleman, M. B., Adams, J. G., Platica, O., Cedeno, M., Rieder, R. F., Wilson, J. T., Milner, P. and West, S. (1984) 'Effects of thalassemia and microcytosis on the hematologic and vasoocclusive severity of sickle cell anemia'.
263. Stevens, M. C., Maude, G. H., Beckford, M., Grandison, Y., Mason, K., Taylor, B., Serjeant, B. E., Higgs, D. R., Teal, H. and Weatherall, D. J. (1986) 'a Thalassemia and the hematology of homozygous sickle cell disease in childhood', *Blood,* 67(2), pp. 411-414.
264. Tham, K. and Cousar, J. (1993) 'Combined silver Perls's stain for differential staining of ringed sideroblasts and marrow iron', *Journal of clinical pathology,* 46(8), pp. 766-768.
265. Tham, K. T., Teague, M. W., Howard, C. A. and Chen, S. Y. (1996) 'A simple splenic reticuloendothelial function test: counting erythrocytes with argyrophilic inclusions', *American Journal Of Clinical Pathology,* 105(5), pp. 548-552.
266. Thiam, L., Dramé, A., Coly, I. Z., Diouf, F. N., Seck, N., Boiro, D., Ndongo, A. A., Basse, I., Niang, B., Deme/Ly, I., Sylla, A., Diagne, I. and Ndiaye, O. (2017) 'Epidemiological, clinical and hematological profiles of homozygous sickle cell disease during the intercritical period among children in Ziguinchor, Senegal', *The Pan African Medical Journal,* 28, pp. 208-208.
267. Thomson, A. M., McHugh, T. A., Oron, A. P., Teply, C., Lonberg, N., Tella, V. V., Wilner, L. B., Fuller, K., Hagins, H. and Aboagye, R. G. (2023) 'Global, regional, and national prevalence and mortality burden of sickle cell disease, 2000–2021: a systematic analysis from the Global Burden of Disease Study 2021', *The Lancet Haematology*.
268. Thuilliez, V., Ditsambou, V., Mba, J. R., Mba Meyo, S. and Kitengue, J. (1996) 'Current aspects of sickle cell disease in children in Gabon', *Archives De Pediatrie: Organe Officiel De La Societe Francaise De Pediatrie,* 3(7), pp. 668-674.
269. Tluway, F. and Makani, J. (2017) 'Sickle cell disease in Africa: an overview of the integrated approach to health, research, education and advocacy in Tanzania, 2004-2016', *British Journal of Haematology,* 177(6), pp. 919-929.

270. Tolo-Diebkile, A., Koffi, K. G., Nanho, D. C., Sawadogo, D., Kouakou, B., Siransy-Bogui, L., Sekongo, Y. M., N'Dhatz, E., Meite, N., Ayemou, R. and Sanogo, I. (2010) 'Homozygous sickle cell disease in Ivory Coast adults', *Sante,* 20(2), pp. 63-7.
271. Topley, J. M., Rogers, D., Stevens, M. and Serjeant, G. R. (1981) 'Acute splenic sequestration and hypersplenism in the first five years in homozygous sickle cell disease', *Archives of Disease in Childhood,* 56(10), pp. 765-769.
272. Tsehay, B., Shitie, D., Afenigus, A. and Essa, M. (2021) 'Sonographic evaluation of spleen size in apparently healthy children in north-west Ethiopia, 2020: time to define splenomegaly', *BMC pediatrics,* 21(1), pp. 1-7.
273. Tshilolo, L., Mukendi, R. and Girot, R. (1996) '[Sickle cell anemia in the south of Zaire. Study of two series of 251 and 340 patients followed-up 1988-1992]', *Archives De Pediatrie: Organe Officiel De La Societe Francaise De Pediatrie,* 3(2), pp. 104-111.
274. Tshilolo, L., Tomlinson, G., Williams, T. N., Santos, B., Olupot-Olupot, P., Lane, A., Aygun, B., Stuber, S. E., Latham, T. S., McGann, P. T. and Ware, R. E. (2019) 'Hydroxyurea for children with sickle cell anemia in sub-Saharan Africa', *New England Journal of Medicine,* 380(2), pp. 121-131.
275. Tubman, V. N. and Makani, J. (2017) 'Turf wars: exploring splenomegaly in sickle cell disease in malaria-endemic regions', *British Journal Of Haematology,* 177(6), pp. 938-946.
276. Ugwu, A. C., Saad, S. T., Buba, E. A., Yuguda, S. and Ali, A. M. (2018) 'Sonographic determination of liver and spleen sizes in patients with sickle cell disease at Gombe, Nigeria', *CHRISMED Journal of Health and Research,* 5(3), pp. 182.
277. Vincent, O., Oluwaseyi, B., James, B. and Saidat, L. (2016) 'Coinheritance of B-Thalassemia and Sickle Cell Anaemia in Southwestern Nigeria', *Ethiopian journal of health sciences,* 26(6), pp. 517-522.
278. Wali, Y. A., Al-Lamki, Z., Hussein, S. S., Bererhi, H., Kumar, D., Wasifuddin, S., Zachariah, M. and Ghosh, K. (2002) 'Splenic function in Omani children with sickle cell disease: correlation with severity index, hemoglobin phenotype, iron status, and alpha-thalassemia trait', *Pediatr Hematol Oncol,* 19(7), pp. 491-500.
279. Walker, T. and Serjeant, G. (1993) 'Focal echogenic lesions in the spleen in sickle cell disease', *Clinical radiology,* 47(2), pp. 114-116.
280. Walker, T. M., Hambleton, I. R., Mason, K. P. and Serjeant, G. (2022) 'Spleen size in homozygous sickle cell disease: TRENDS in a birth cohort using ultrasound', *The British Journal of Radiology*, pp. 20220634.
281. Walters, M. C., De Castro, L. M., Sullivan, K. M., Krishnamurti, L., Kamani, N., Bredeson, C., Neuberg, D., Hassell, K. L., Farnia, S. and Campbell, A. (2016) 'Indications and results of HLA-identical sibling hematopoietic cell transplantation for sickle cell disease', *Biology of Blood and Marrow Transplantation,* 22(2), pp. 207-211.
282. Wambua, S., Mwangi, T. W., Kortok, M., Uyoga, S. M., Macharia, A. W., Mwacharo, J. K., Weatherall, D. J., Snow, R. W., Marsh, K. and Williams, T. N. (2006) 'The effect of α+-thalassaemia on the incidence of malaria and other

diseases in children living on the coast of Kenya', *PLoS medicine,* 3(5), pp. e158.
283. Ware, R. E. 2015. Optimizing hydroxyurea therapy for sickle cell anemia. *ASH Education Program Book,* 2015, pp.436-43
284. Ware, R. E. and Aygun, B. (2009) 'Advances in the use of hydroxyurea', *ASH Education Program Book,* 2009(1), pp. 62-69.
285. Watson, D. (2004) 'Stability versus change, dependability versus error: Issues in the assessment of personality over time', *Journal of Research in Personality,* 38(4), pp. 319-350.
286. Wayne, P. (2008) 'CLSI Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory-Approved Guideline', *CLSI Document EP28-A3C. Third edition. Available online: http://shop. (accessed on 19 October 2010)*.
287. Waziri, M. (2012) 'Spatial Pattern of Maiduguri City', *Researchers' Guide. Kano: Adamu Joji Publishers*, pp. 17-29.
288. Wierenga, K. J., Hambleton, I. R., Wilson, R., Alexander, H., Serjeant, B. E. and Serjeant, G. R. (2001) 'Significance of fever in Jamaican patients with homozygous sickle cell disease', *Archives of disease in childhood,* 84(2), pp. 156-159.
289. William, B. M. and Corazza, G. R. (2007) 'Hyposplenism: a comprehensive review. Part I: basic concepts and causes', *Hematology (Amsterdam, Netherlands),* 12(1), pp. 1-13.
290. Williams, T. N. and Obaro, S. K. (2011) 'Sickle cell disease and malaria morbidity: a tale with two tails', *Trends in parasitology,* 27(7), pp. 315-320.
291. Williams, T. N., Uyoga, S., Macharia, A., Ndila, C., McAuley, C. F., Opi, D. H., Mwarumba, S., Makani, J., Komba, A., Ndiritu, M. N., Sharif, S. K., Marsh, K., Berkley, J. A. and Scott, J. A. G. (2009) 'Bacteraemia in Kenyan children with sickle-cell anaemia: a retrospective cohort and case-control study', *Lancet,* 374, pp. 1364-1370.
292. Wolf, B. and Neiman, R. (1989) 'Non-neoplastic disorders of erythrocytes, granulocytes, and platelets', *Disorders of the Spleen. Philadelphia, PA, Saunders*, pp. 115-128.
293. Wright, J., Thomas, P. and Serjeant, G. R. (1997) 'Septicemia caused by Salmonella infection: an overlooked complication of sickle cell disease', *The Journal of pediatrics,* 130(3), pp. 394-399.
294. Yakubu CI, I. N., Olowoselu FO, Ezesobor C, Temiye E O, Awosanya GOG (2017) 'Sonographic Determination Of Splenic Volume In Children With Sickle Cell Anaemia In Steady State', *West African Journal of Ultrasound* 18 (2), pp. 34-40.
295. Yetunde, A. and Anyaegbu, C. C. (2001) 'Profile of the Nigerian sickle cell anaemia patients above 30 years of age', *The Central African Journal Of Medicine,* 47(4), pp. 108-111.
296. Zago, M. and Bottura, C. (1983) 'Splenic function in sickle-cell diseases', *Clinical Science,* 65(3), pp. 297-302.
297. Zarkowsky, H. S., Gallagher, D., Gill, F. M., Wang, W. C., Falletta, J. M., Lande, W. M., Levy, P. S., Verter, J. I., Wethers, D. and Disease, C. S. o. S. C.

(1986) 'Bacteremia in sickle hemoglobinopathies', *The Journal of pediatrics,* 109(4), pp. 579-585.

298. Zhang, B. and Lewis, S. (1989) 'A study of the reliability of clinical palpation of the spleen', *Clinical & Laboratory Haematology,* 11(1), pp. 7-10.

Appendix 1: Search terms and Boolean Operators used

Medline search: results 115

SCD	Spleen	Africa
Key words: "Sickle cell" OR "Sickle cell anemia" OR "sickle cell anaemia" OR "Sickle cell disorder" OR haemoglobinopathies OR hemoglobinopathies OR "hemoglobin SS disease" OR "hemoglobin SC disease" OR "hemoglobin S B thalassemia" **MESH words**: ("Anemia, sickle cell+") OR (MH "Hemoglobin, sickle") OR (MH "Hemoglobin SC disease") OR (MH "hemoglobinopathies") **Search:** Add KW plus Mesh using OR	**Key words:** Spleen OR Hypersplenism OR hyposplenism OR "functional asplenia" OR Splenectomy OR Splenomegaly OR "enlarged spleen" OR Splenic* OR "Splenic function" OR "Splenic Infarction" OR "Splenic dysfunction" OR "splenic sequestration" **Mesh words:** (MH "splenomegaly") OR (MH "spleen") OR (MH "splenectomy") OR (MH" Splenic infarction") OR (MH "hypersplenism") OR (MH "splenic rupture") **Search:** Add KW plus mesh using OR	**Keywords:** "sub-Saharan Africa" OR "northern Africa" **Mesh word:** (MH "Africa south of the Sahara+") OR (MH "Africa, Northern+") **Search:** Add Key words and mesh words using OR

CINAHL search: results 15

Sickle cell disease	Spleen	Africa
Key words: "Sickle cell" OR "Sickle cell anemia" OR "sickle cell anaemia" OR "Sickle cell disorder" OR haemoglobinopathies OR hemoglobinopathies OR "hemoglobin SS disease" OR "hemoglobin SC disease" OR "hemoglobin S B thalassemia" **Subject heading:** (MH "anemia, sickle cell" +) OR (MH "hemoglobinopathies" +) **Search:** Add KW plus subject heading using OR	**Key words:** Spleen OR Hypersplenism OR hyposplenism OR "functional asplenia" OR Splenectomy OR Splenomegaly OR "enlarged spleen" OR Splenic* OR "Splenic function" OR "Splenic Infarction" OR "Splenic dysfunction" OR "splenic sequestration" **Subject heading:** (MH "Spleen") OR (MH "splenic rupture") OR (MH "splenic disease") OR (MH "Splenomegaly") OR (MH "hypersplenism") OR (MH "splenectomy") **Search:** Add KW plus subject heading using OR	**Keywords:** "sub-Saharan Africa" OR "northern Africa" **Subject heading:** (MH "Africa south of the Sahara+") OR (MH "Africa, Northern+") **Search:** Add KW plus subject heading using OR

Global health search: results 45

Sickle cell disease	Spleen	Africa
Key words: "Sickle cell" OR "Sickle cell anemia" OR "sickle cell anaemia" OR "Sickle cell disorder" OR haemoglobinopathies OR hemoglobinopathies OR "hemoglobin SS disease" OR "hemoglobin SC disease" OR "hemoglobin S B thalassemia" **Thesaurus heading:** (DE "sickle cell anaemia" +) OR (DE "hemoglobinopathies" +) **Search:** Add KW plus thesaurus heading using OR	**Key words:** Spleen OR Hypersplenism OR hyposplenism OR "functional asplenia" OR Splenectomy OR Splenomegaly OR "enlarged spleen" OR Splenic* OR "Splenic function" OR "Splenic Infarction" OR "Splenic dysfunction" OR "splenic sequestration" **Thesaurus heading:** (DE "Spleen") OR (DE "splenic disease") OR (DE "Splenomegaly") OR (DE "malaria splenomegaly") (DE "splenectomy") **Search:** Add KW plus thesaurus heading using OR	**Keywords:** "sub-Saharan Africa" OR "northern Africa" **Thesaurus heading:** (DE "Africa south of the Sahara+") OR (DE "Central Africa") OR (DE "East Africa") OR (DE "West Africa") OR (DE "Sahel" OR DE "Southern Africa") (DE "North Africa" OR DE "Maghreb" OR DE "Libya" OR "DE Egypt") **Search:** Add KW plus thesaurus heading using OR

Web of Science search: results 27

Sickle cell disease	Spleen	Africa
Key words: "Sickle cell" OR "Sickle cell anemia" OR "sickle cell anaemia" OR "Sickle cell disorder" OR haemoglobinopathies OR hemoglobinopathies OR "hemoglobin SS disease" OR "hemoglobin SC disease" OR "hemoglobin S B thalassemia"	**Key words:** Spleen OR Hypersplenism OR hyposplenism OR "functional asplenia" OR Splenectomy OR Splenomegaly OR "enlarged spleen" OR Splenic* OR "Splenic function" OR "Splenic Infarction" OR "Splenic dysfunction" OR "splenic sequestration"	**Keywords:** "sub-Saharan Africa" OR "northern Africa"

Grey literature search

Date searched	Name of source	Search terms	Number retrieved	Type
13/2/2020	Ethos library	Sickle cell, spleen, Africa Sickle cell, spleen,	0 2	Thesis 0
13/2/2020	BASE search engine	Sickle cell, spleen, Africa	9	Journals 7 book 2
14/2/2020	Networked digital library of thesis and dissertations (NDLTD)	,,	0	0
14/2/2020	Google scholar	‘’	13	Thesis 2, journal 11

Appendix 2: Quality assessment of studies included in the review

1. **PROSPECTIVE CROSS-SECTIONAL STUDIES = 34**

Author	Year	Description of selection criteria	Description of study participants	Definition of exposure variables	Measurement of spleen size using standard method	Strategies to deal with confounding factors	Definition of outcomes	Use of appropriate statistical analysis	Score	Grade
Adegoke	2015	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7	A
Adekile et al	1993	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7	A
Akinlosotu et al	2016	Yes	Yes	Yes	Yes	No	Yes	Yes	6	B
Akpan, E. A	2015	Yes	Yes	Unclear	Yes	No	Yes	No	4	C
Ambe et al	2001	Yes	Yes	Yes	No	No	Yes	Yes	5	B
Awotua-Efebo	2004	Yes	Yes	Yes	Yes	No	Yes	No	5	B
Bayoumi et al	1988	Yes	Yes	Yes	No	No	Yes	Unclear	4	C
Bedu-Addo et al	2002	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7	A
Darko et al	2005	Yes	Yes	Yes	Yes	No	Yes	No	5	B
Doumbo et al	1992	Yes	Yes	Yes	Yes	No	Yes	No	5	B
Eze et al	2015	Yes	Yes	Yes	Yes	No	Yes	Unclear	5	B
Fasola et al	2019	Yes	Yes	Yes	Yes	No	Yes	Yes	6	B
Inah	2018	Yes	Yes	Yes	Yes	No	Yes	No	5	B
Olaniyi et al	2007	Yes	Yes	Yes	No	No	Yes	No	4	C
Goussanou et al	2003	Yes	Yes	Yes	No	No	Yes	Yes	5	B
Kaine	1982	Yes	Yes	No	Yes	No	Yes	Yes	5	B
Kazadi et al	2019	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7	A

Kizito et al	2007	Yes	Yes	Yes	yes	No	Yes	Yes	6	B
Kotila et al	2000	Unclear	Yes	Yes	Unclear	No	Yes	Yes	4	C
Luntsi et al	2018	Yes	Yes	NA	Yes	No	Yes	Yes	5	B
Ma'aji et al	2012	No	Yes	Yes	Yes	No	Yes	No	4	C
Makani	2010	Yes	Yes	Yes	No	Yes	Yes	Yes	6	B
Mouélé et al	1999	Yes	Yes	Yes	Yes	Yes	Yes	No	6	B
Mpalampa et al	2012	Yes	Yes	Yes	Unclear	No	Yes	Yes	5	B
Ojo et al	2018	No	Yes	Yes	Yes	No	Yes	Yes	5	B
Okongwu et al	2018	Yes	Yes	Yes	Yes	No	Yes	No	5	B
Okoro et al	1989	Yes	Yes	Unclear	Yes	No	Yes	Yes	5	B
Sadarangani	2009	Yes	Yes	Yes	Yes	No	Yes	Yes	6	B
Shongo et al	2014	Yes	Yes	Unclear	Yes	No	Yes	Yes	5	B
Tshilolo et al	1996	Yes	Yes	Yes	Yes	No	Yes	No	4	C
Thuilliez et al	1996	Yes	Yes	No	Yes	No	Yes	Yes	4	C
Ugwu et al	2018	Yes	Yes	Yes	Yes	No	Yes	Yes	6	B
Yakubu et al	2017	Yes	Yes	Yes	Yes	No	Yes	No	4	C
Yetunde and Anyaegbu	2001	Yes	Yes	Yes	No	No	Yes	Yes	5	B

2. **RETROSPECTIVE CROSS-SECTIONAL STUDIES = 8**

Author	**Year**	**Description of selection criteria**	**Description of study participants**	**Definition of exposure variables**	**Measurement of spleen size using standard method**	**Strategies to deal with confounding factors stated**	**Definition of outcomes**	**Use of appropriate statistical analysis**	**Score**	**Grade**
Akinola et al	2009	Yes	Yes	No	No	No	Yes	Yes	4	C
Aloni et al	2013	Unclear	Yes	Yes	No	No	Yes	Yes	5	B
Alufohai et al	2006	Yes	Yes	Yes	NA	No	Yes	Yes	5	B

Brown et al	2012	Yes	Yes	Yes	Yes	No	Yes	Yes	6	B
Banza et al	2019	Yes	Yes	No	Yes	No	Yes	No	4	C
Diagne et al	2010	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7	A
Thiam et	2017	Yes	Yes	No	Yes	No	Yes	Yes	5	B
Tolo-Diebkilé,	2010	No	Yes	No	Yes	Yes	Yes	Yes	6	B

3. **CASE CONTROL STUDIES = 10**

Author	**Year**	**Description of selection criteria for cases and controls**	**Description of study participants**	**Definition of exposure variables for cases & controls**	**Measurement of spleen size using standard method**	**Strategies to deal with confounding factors stated**	**Definition of outcomes**	**Use of appropriate statistical analysis**	**Score**	**Grade**
Abdullahi et al	2014	Yes	Yes	No	Yes	No	Yes	Yes	5	B
Abjah et al	2003	Yes	Yes	Yes	Yes	No	Yes	No	5	B
Adekile et al	1988	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7	A
Adekile et al	1991	Yes	Yes	Yes	Yes	No	Yes	Yes	5	B
Adeodu et al	1990	Yes	Yes	Yes	Yes	Yes	Yes	Yes	4	A
Babadoko et al	2012	Yes	Yes	Yes	Yes	No	Yes	Unclear	5	B
Belhani et al	1984	Yes	Yes	Yes	Unclear	No	Yes	No	4	C
Durosinmi et al	2005	Yes	Yes	Yes	Unclear	No	Yes	Yes	5	B
Ezeike	2019	Unclear	Unclear	Yes	Yes	No	Yes	No	3	C
Olatunji et al.	2001	Yes	Yes	Yes	Yes	No	Yes	No	5	B

4. **CASE STUDIES = 3**

Author	Year	Description of inclusion criteria	Was there consecutive & complete inclusion of participants?	Description of study population	Clear definitions of splenic complication (s)	Clear reporting of clinical information of participants	Outcomes or follow up results stated	Use of appropriate statistical analysis	Score	Grade
Gassaye et al	2000	Yes	Yes	Yes	Yes	Yes	No	NA	5	B
Gnassingbe et al	2007	Yes	Yes	Yes	Yes	Yes	Yes	NA	6	B
Jebbin et al	2011	Yes	No	Yes	Yes	Yes	Yes	Yes	6	B

NA:Not applicable

Appendix 3: Summary of studies included in the review

1.Studies involving children only (N=23)									
First author (publication year)	Country	Study size	Hb Phenotype	Mean age (SD, range)	Hb, g/dl / PCV, (%) Mean (SD)	HbF level, (%) Mean (SD)	Spleen size assessment	Splenomegaly (%)	Study Design
Belhani **(1984)**	Algeria	84	HbS-β-thal^0 HbS-β-thal$^+$ HbSS	NR (NR, <15)	Hb: HbS-β-thal^0 8.2(1.7) HbS-β-thal$^+$ 10.2(1.7) HbSS 7.7(1.5)	HbS-β-thal^0 : 14.3 (8.9) HbS-β-thal$^+$: 6.8(3.7) HbSS:8.5 (8.3)	Manual palpation	HbS-β-thal^0 : 92.0% HbS-β-thal$^+$: 88.0% HbSS: 34.0%	Case control study
Adekile et al **(1988)**	Nigeria	139	HbSS, HbAA	7.1 (4.2, 0.5-15)	NA	NA	Manual palpation	33.8%	Case control study
Okoro **(1989)**	Nigeria	4359	HbSS	NRC	NA	NA	Manual palpation	NRC	Cross sectional
Adeodu **(1990)**	Nigeria	25	HbSS	11.3 (2.9, 8 -15)	PCV: 20.5(2.4)	NA	Manual palpation	HbSS with PGS (15) and without PGS	Case control study
Adekile et al **(1991)**	Nigeria	54	HbSS, HbAS, HbAA	8.9 (NR, 3 -7)	NA	NA	Manual palpation	25.0%	Case control study
Doumbo **(1992)**	Mali	236	HbSS, HbAA	-	NA	NA	Manual palpation	12.0%	Cross sectional

Thuilliez **(1996)**	Gabon	302	HbSS	NR	Hb: 7.0 (NR)	NA	Manual palpation	33.0%	Cross sectional
Mouélé **(1999)**	Republic of the Congo	116	HbSS	9.4 (5.3, 1- 32)	Hb: 6.6 (1.4)	8.8 (5.8)	Manual palpation	40%	Cross sectional
Ambe **(2001)**	Nigeria	104	HbSS	NR (NR, 0.5 -15)	NA	NA	Manual palpation=	NA	Cross sectional
Goussanou **(2003)**	Republic of Benin	236	HbSS	Median 2.9 (0.6-12)	Hb: 7.8 (1.1) PCV: 25.3(3.7)	10.1(5.8)	Manual palpation	NR	Cross sectional
Awotua-Efebo **(2004)**	Nigeria	100	HbSS, HbAA	NR (NR, 0.5 -15)	NA	NA	Ultra-sound scan	27.0%	Comparative, cross sectional
Darko **(2005)**	Ghana	315	HbSS, HbSC, HbS-β-thal^{+}	NR (NR, 2 - 13)	NA	NA	Ultra-sound scan	NR	Cross sectional
Gnassingbe **(2007)**	Togo	8	HbSS, HbSC	NR (NR, 8 - 13)	NA	NA	Manual palpation	Yes, 5 (HbSS 3, HbSC 2)	Retrospective review over 17 years
Kizito **(2007)**	Uganda	155	HbSS	4.4 (NR, 0.3 -14.8)	NA	NA	Manual palpation	36.0%	Cross sectional
Sadarangani et al **(2009)**	Kenya	124	HbSS	6.3 (NR, 0.8 -13.7)	NA	NA	Manual palpation	33.0%	Cross sectional
Diagne **(2010)**	Senegal	698	HbSS, HbSC HbS-β-thal^{+}	12.2(NR, 0.7-24)	Hb: 8.0 (1.1)	8.3 (7.9)	Manual palpation	HbSS: 20.1% HbSC: 41.9% Hb S-β-thal^{+}: 57.1%	Retrospective review over 15 years

Brown **(2012)**	Nigeria	415	HbSS, HbSC	7.3 (4.4, 0.5 -17)	PCV HbSS: 24(3.7), HbSC: 28(4.5)	NA	Manual palpation	HbSS: 31.7% HbSC: 33.3%	Retrospective review over 10 years
Aloni **(2013)**	DRC	108	HbSS	Median 5.4 (0.5 - 13)	NA	NA	Manual palpation	37.8%	Retrospective review over 10 years
Abdullahi **(2014)**	Nigeria	300	HbSS, HbAA	NR (NR, 0.5 -15)	Hb: 7.3 (1.3)	4.6 (1.7)	Ultra-sound scan	35.3%	Case control study
Shongo **(2014)**	DRC	205	HbSS	3.2 (1.4, NR)	NA	NA	Manual palpation	73.2%	Cross sectional
Adegoke **(2015)**	Nigeria	240	HbSS,HbSC	5.9 (3.7, 0.5 -15)	PCV:18.7 (7.8)	NA	Manual palpation	HbSS: 12.5%, HbSC: 4.3%	Cross sectional
Yakubu **(2017)**	Nigeria	200	HbSS	7.9 (4, 1 - 15)	NA	NA	Ultra-sound scan	53.5%	Cross sectional
Akinlosotu **(2018)**	Nigeria	105	HbSS	7.3 (3.6, NR)	Hb: 7.7 (1.1) PCV:23.4 (2.2)	9.9 (6)	Combined: Manual, USS	26.0%	Cross sectional
2.Studies involving children and adults (N=19)									
First author (publication year)	Country	Study size	Hb Phenotype	Mean age (SD, range)	Hb, g/dl / PCV, (%) Mean (SD)	HbF level, (%) Mean (SD)	Spleen size assessment	Splenomegaly (%)	Study Design
Kaine **(1982)**	Nigeria	210	HbSS	6.1 (NR, 0.8 -19)	NA	NA	Manual palpation	55% <5yrs 45% 5-10yrs 18% >10yrs	Cross sectional

Bayoumi **(1988)**	Sudan	50	HbSS, HbS-β-thal^{+}	6.4 (NR, 0.5-38)	Hb: 7.3 (NR)	7.0 (NR)	Manual palpation	42.0%**	Descriptive cross sectional
Adekile **(1993)**	Nigeria	410	HbSS, HbAS, HbAA	9.7 (0.3,1 - 25)	Hb: 7.6 (NR)	9.3 (NR)	Manual palpation	23.3%	Comparative cross sectional
Tshilolo **(1996)**	DRC	591	HbSS	NR, (NR, 3 - 12)	NA	NA	Manual palpation	44.4%	Cross sectional
Olatunji **(2001)**	Nigeria	98	HbSS, HbAA	13.9 (7.4, 3 - 47)	NA	NA	Ultra-sound scan	NR	Case control study
Alufohai **(2006)**	Nigeria	17	HbSS	11.8 (NR,10 - 15)	NA	NA	Manual palpation	NA	Retrospective review over 12 years
Makani **(2010a)**	Tanzania	2305	HbSS	Median 11 (0.3 - 47)	NA	NA	Manual palpation	10.0%	Cross sectional
Jebbin **(2011)**	Nigeria	6	HbSS	NR(NR,1 1-20)	NA	NA	NA	NA	Case-studies
Ma'aji **(2012)**	Nigeria	71	HbSS	NA	NA	NA	Ultra-sound scan	21.1%	Cross sectional
Mpalampa **(2012)**	Uganda	216	HbSS	9.3 (4.8, 1 -18)	NA	NA	Manual palpation	24.0%	Cross sectional
Akpan **(2015)**	Nigeria	220	HbSS	12.1 (8.3,1 - 41)	NA	NA	Ultra-sound scan	2.3%	Cross sectional
Eze **(2015)**	Nigeria	104	HbSS, HbSC, HbAA	NR (NR, 2 - 58)	NA	NA	Ultra-sound scan	33.0%**	Comparative cross sectional

Thiam **(2017)**	Senegal	46	HbSS	8, (NR, 0.92 - 21)	Hb: 8.6 (5)	4.0 (NR)	Manual palpation	21.7%	Retrospective review over 2 years
Inah **(2018)**	Nigeria	120	HbSS	Median 14.5 (6 - 25)	NA	NA	Ultra-sound scan	0.83%	Cross sectional
Luntsi **(2018)**	Nigeria	126	HbSS	18 (6.3, 3 - 38)	NA	NA	Ultra-sound scan	50.0%	Cross sectional
Ugwu **(2018)**	Nigeria	237	HbSS	Median 9.8, (1 - 49)	NA	NA	Ultra-sound scan	NR	Cross sectional
Banza **(2019)**	DRC	206	HbSS	11.8 (21.9, 1.1 - 38)	NA	NA	Ultra-sound scan	13.1%	Retrospective, 3 years review
Ezeike **(2019)**	Nigeria	100	HbSS, HbAA	NR (NR, 0 - 30)	NA	NA	Ultra-sound scan	31.0%	Case control study
Kazadi **(2019)**	DRC	256	HbSS	8.4 (4.9, 0.5 - 24)	Hb: 7.4(1.5) PCV: 23.3(4.5)	NA	Manual palpation	41.7%	Cross sectional
3.Studies involving adults only (N=13)									
First author (publication year)	Country	Study size	Hb Phenotype	Mean age (SD, range)	Hb, g/dl / PCV, (%) Mean (SD)	HbF level, (%) Mean (SD)	Spleen size assessment	Splenomegaly (%)	Study Design
Bedu-addo **(1997)**	Ghana	221	HbAA ,HbSS, HbSC, Other*	Median 31(8 - 75)	NA	NA	Manual palpation	Yes, all 6 with HbSS(n=2) and HbSC(n=4)	Descriptive cross sectional

Gassaye **(2000)**	Republic of Congo	13	HbSS	NR (NR, 4 - 62)	NA	NA	Ultra-sound scan	NA	Retrospective review over 5 years
Yetunde **(2001)**	Nigeria	98	HbSS	NR, (NR, 30 - 52)	PCV: 24 (NR)	NA	Manual palpation	35.0%	Cross sectional
Abjah **(2003)**	Nigeria	70	HbSS, HbSC, HbAA	NR (NR,15 - 54)	NA	NA	Manual palpation	HbSS: 50% HbSC: 67%	Case control study
Durosinmi **(2005)**	Nigeria	71	HbSS	Median 21 (16 - 48)	PCV: 24 (0.1)	4.3 (NR)	Manual palpation	26.8%	Case-control study
JA Olaniyi **(2007)**	Nigeria	220	HbSS, HbSC	24.7 (8.7, 12 - 60)	PCV:22.3 (5.2)	NA	Manual palpation	HbSS: 20.2% HbSC: 25.9%	Cross sectional
Kotila **(2007)**	Nigeria	50	HbSS	20 (NR, NR)	NA	7.4 (3.6)	Manual palpation	16%	Cross sectional
Akinola **(2009)**	Nigeria	154	HbSS, HbSC	22.5 (7.3, NR)	PCV: HbSS: 23(3.7) HbSC: 29(3.8)	NA	Manual palpation	HbSS: 17.2% HbSC: 19.2%	Retrospective review
Tolo-Diebkilé **(2010)**	Ivory coast	48	HbSS	26.1 (NR, 21 - 56)	Hb: 9.5 (NR)	10.6 (NR)	Manual palpation	0%	Retrospective review
Babadoko **(2012)**	Nigeria	74	HbSS, HbAA	23.3 (5.3, NR)	PCV: 25.9(3.9)	NA	Ultra-sound scan	4.1%	Case control study
Ojo **(2018)**	Nigeria	40	HbSS, HbAA	25.2 (2.2, 16 -40)	NA	NA	Ultra-sound scan	15.0%	Cross sectional

Okongwu **(2018)**	Nigeria	40	HbSS	29.3 (8,17 - 51)	NA	NA	Ultra-sound scan	12.5%	Cross sectional
Fasola **(2019)**	Nigeria	42	HbSS, HbAA	29 (8.1, NR)	Hb: 7.7 (15.9) PCV:25.5 (5.3)	NA	Ultra-sound scan	10.0%	Case control study

DRC: Democratic Republic of Congo; Hb: Haemoglobin; HbF: Haemoglobin F; PCV: packed cell volume; PGS: Persistent

Appendix 4: Summary of studies that reported on the variation in spleen size across different ages in the review

Country	Study year	Study size	Prevalence of splenomegaly	Summary of findings	Reference
1 Studies involving children only.					
Gabon	1996	302	33.0%	A third of the SCD patients still had splenomegaly beyond five years of age.	Thuilliez et al
Kenya	2009	124	33.0%	The peak prevalence of splenomegaly occurred in the 6-8 years group. The largest mean spleen size of 4.8 cm was seen in the 8 to 10 years age-group. However, there were no significant relationship between spleen size and age	Sadarangani et al
Senegal	2010	698	HbSS - 20.1% HbSC - 41.9% HbS-β-thal - 57.0%	The prevalence of splenomegaly showed a steady increase from infancy up to the age of 10 years before it started decreasing	Diagne et al
Nigeria	2012	415	HbSS - 31.7% HbSC - 33.3%	A relatively stable prevalence of palpable spleen, occurring in about a third of patients until 15 years of age was observed before it started dropping. The HbSC patients had a higher frequency of splenomegaly between the ages of 5 to 14 years	Brown et al
DRC	2013	90	37.8%	Splenomegaly was more common in those under 5 years (61.7%) of age compared to those above 5 years (11.6%)	Aloni et al
Nigeria	2014	150	35.3%	Splenomegaly persisted into older age patients. The prevalence of splenomegaly	Abdullahi et al

				among children with SCD who were older than 10 years was 11.3%	
Nigeria	2018	105	26.0%	All patients younger than 8 years had their spleen detectable on USS, while 4 out of the remaining 44 patients older than 8 years had autosplenectomy.	Akinlosotu et al
Nigeria	2017	200	53.5%	The splenic volume was higher in the age group 12 years and above and lowest in the 1-2 years age group.	Yakubu et al
2 Studies involving children and adults					
Nigeria	1982	210	55.0%	The spleen size decreased progressively with increasing age: 55% for <5 years, 45% for 5 -10years and 18% for >10yrs	Kaine
Nigeria	1993	310	23.0%	Frequency of splenomegaly across the age groups was: 20.3% for < 5 years; 25.7% for 6 to 10 years; 23.7% for 11 to 15 years; and 17.9% for > 15 years. No significant differences in the mean ages of those with or without splenic enlargement was observed	Adekile et al
DRC	1996	591	44.0%	Splenomegaly rarely occurred before three years of age in the SCD patients compared with normal control, but persisted longer in the SCD group compared with normal control	Tshilolo et al
Nigeria	2001	91	NR	The longitudinal spleen length increased continuously up to the age of 30 years. A reduction in the coronal diameter after the age of 30 years was the only indicator of splenic size reduction.	Olatunji et al
Nigeria	2015	220	2.3%	Splenomegaly was confined to children less than 10 years of age. 1/3rd of the	Akpan

				study group had normal spleen and most belonged to the under 5 group, while 1/3rd of patients had autosplenectomy and most belonged to the adolescent group.	
Nigeria	2015	104	33.1%	The spleen of SCD subjects generally had undulating variations in size increasing rapidly from 2 years of age to the childhood / adulthood transitional age of 18 years. Beyond 18 years, there was a mixture of sharp reduction and increase in spleen size in an undulating form up to the age of 58 years.	Eze et al
Nigeria	2019	237	NR	The mean spleen length of SCD patients in this study was enlarged in the age group of 1–10 years; a progressive decrease was observed with each successive age group afterwards.	Ugwu et al
Nigeria	2019	100	31.0%	The largest spleen length and volume was recorded in the age group of 5 to 9, being twice the dimension compared to the 0 to 4 age group.	Ezeike
3Studies involving adults only					
Nigeria	2001	98	35.0%	More than one third of the study population had palpable spleen despite the average age of 30 years and above.	Yetunde et al

DRC: Democratic republic of Congo; NR: Not reported; USS: ultrasound scan

Appendix 5: Case report form for steady state SCD patients

Case report form for steady state patients			
		Study ID = DATE	
Demographic data	Remark	Medical hx	Remark
Age		Frequency of day care unit or emergency department visits over the last one year?	Specify [ ] Can't recall [ ]
DOB [Day/ Month/ Year]		Any hx of previous hospitalization?	Yes [ ] No [ ]
Sex	Male [ ] Female [ ]…… …… …… ……	Average duration of previous hospitalization?	Specify [ ] Not applicable [ ]
Religion	Islam [ ] Christianity [ ] Others [ ]	Frequency of in-hospital admission over the last 12months?	
Education level (Patients / Parents / Guardian)	None [ ] Primary [ ] Secondary[ ] Tertiary [ ] Informal [ ]	Total lifetime transfusion	Specify [ ] Can't recall [ ]
State of origin		Last transfusion episode?	Specify [ ] Can't recall [ ] Not applicable [ ]
Marital status	Single [ ] Married [ ] Divorced [ ] Widowed [ ]	Previous history of left abdominal pain	Yes [ ] No [ ]

	Not applicable (child) [ ]		
Tribe		Past hx of acute left abdominal swelling (splenic sequestration)	Yes [ ] No [ ]
Phone number (Patients / Parents / Guardian)		Past hx of hypersplenism	Yes [ ] No [ ] Don't know [ ]
Alternative phone number		Past history of (tick all applicable)	Bacteremia [ ] Septicaemia [ ] Meningitis [ ] Osteomyelitis [ ] Pneumonia [ ] None of above [ ]
Occupation (Patients / Parents / Guardian)	Civil servant [ ] Student [ ] Self-employed [ ] Unemployed [ ]	If yes record age of occurrence	
Disease severity	**Remark**	**Drug / immunization Hx**	**Remark**
Age at diagnosis of SCA		Do you use a bed net?	Yes [ ] No [ ]
Mode of diagnosis	Symptomatic diagnosis [ ] Family screening [ ] New-born screening [ ]	Are you regular on antimalaria prophylaxis	Yes [ ] No [ ]: Why
Frequency of febrile attack over the last 12 months?		Are you regular on Folic acid prophylaxis ?	Yes [ ] No [ ]: Why

Frequency of acute malaria over the last 12 months?	**Specify [ ] Can't recall [ ]**	**Are you on Hydroxyurea**	**Yes [ ] No [ ] Never heard of it [ ]**	
Number of antimalaria treatment received over the last 12 months?	**Specify [ ] Can't recall [ ]**	**Are you on regular IM Penicillin prophylaxis (Paediatrics)**	**Yes [ ] No [ ]: Why**	
Number of antibiotics treatment received over the last 12 months?	**Specify [ ] Can't recall [ ]**	**Are you on regular oral Penicillin prophylaxis (Paediatrics)**	**Yes [ ] No [ ]: Why**	
Average number of painful crises per year?	**Specify [ ] Can't recall [ ]**	**Has your child completed their routine vaccination program?**	**Yes [ ] No [ ]: Why Not sure [ ]**	
Average duration of crisis episodes		**If yes, how long since completion.**	**Specify [ ] Can't recall [ ]**	
HBSag status HIV status	**Positive [ ] Neg [ ] Not tested [ ] Positive [ ] Neg [ ] Not tested [ ]**	**Have you received the following vaccines?**	**1.Pneumococcal: Yes [ ] No [ ] Don't know 2.Meningococcal: Yes [ ] No [ ] Don't know 3.Haemophilus infleunza type b: Yes [ ] No [ ] Don't know [ ] 4.Hepatitis B: Yes [ ] No [ ] Don't know [ ]**	
	Remark			

Examination		Action	Date
Weight	Kg	Appointment given for Ultrasound scan. If yes specify date…… ……	
Height / Length	M	Appointment given for HPLC sampling ? If yes specify date	
Mid upper circumference (Paediatric)	Cm	Appointment given for repeat spleen function. If yes specify date	
Triceps thickness (Paediatric)		Review form to ensure all information are collected	
Pallor	Yes [ ] No [ ]		
Jaundice	Yes [ ] No [ ]		
Temperature	Celsuis		
SPO^2	%		
Respiratory rate	/ Min		
Pulse rate	/ Min		
Blood pressure (adults)	mm/Hg		
Palpable spleen	cm		
Palpable liver	cm		

Appendix 6: Case report for acutely-ill SCD patients

Case report form for acutely- ill SCD patients			
		Study ID = DATE	
Demographic data	**Remark**	**Medical hx**	**Remark**
Age		Frequency of day care unit or emergency department visits over the last one year?	Specify [ ] Can't recall [ ]
DOB [Day/ Month/ Year]		Any hx of previous hospitalization?	Yes [ ] No [ ]
Sex	Male [ ] Female [ ] …… ……	Average duration of previous hospitalization?	Specify [ ] Not applicable [ ]
Religion	Islam [ ] Christianity [ ] Others [ ]	Frequency of in-hospital admission over the last 12months?	
Education level (Patients / Parents / Guardian)	None [ ] Primary [ ] Secondary[ ] Tertiary [ ] Informal [ ]		
State of origin		Total lifetime transfusion	Specify [ ] Can't recall [ ]
Marital status	Single [ ] Married [ ] Divorced [ ] Widowed [ ] Not applicable (child) [ ]	Last transfusion episode?	Specify [ ] Can't recall [ ] Not applicable [ ]
Tribe		Previous history of left abdominal pain	Yes [ ] No [ ]
Phone number (Patients / Parents / Guardian)		Past hx of acute left abdominal swelling (splenic sequestration)	Yes [ ] No [ ]
Alternative phone number		Past hx of hypersplenism	Yes [ ] No [ ] Don't know [ ]

Occupation (Patients / Parents / Guardian)	Civil servant [ ] Student [ ] Self-employed [ ] Unemployed [ ]	Past history of (tick all applicable)	Bacteremia [ ] septicaemia [ ] Meningitis [ ] Osteomyelitis [ ] Pneumonia [ ] None of above [ ]
		If yes record age of occurrence	
Disease severity	**Remark**	**Drug / immunization Hx**	**Remark**
Age at diagnosis of SCA		Do you use a bed net?	Yes [ ] No [ ]
Mode of diagnosis	Symptomatic diagnosis [ ] Family screening [ ] NBS [}	Are you regular on antimalaria prophylaxis	Yes [ ] No [ ]: Why
Frequency of febrile attack over the last 12 months?		Are you regular on Folic acid prophylaxis ?	Yes [ ] No [ ]: Why
Frequency of acute malaria over the last 12 months?	Specify [ ] Can't recall [ ]	Are you on Hydroxyurea	Yes [ ] No [ ] Never heard of it [ ]
Number of antimalaria treatment received over the last 12 months?	Specify [ ] Can't recall [ ]	Are you on regular IM Penicillin prophylaxis (Paediatrics)	Yes [ ] No [ ]: Why
Number of antibiotics treatment received over the last 12 months?	Specify [ ] Can't recall [ ]	Are you on regular oral Penicillin prophylaxis (Paediatrics)	Yes [ ] No [ ]: Why

Average number of painful crises per year?	**Specify [ ] Can't recall [ ]**	**Has your child completed their routine vaccination program?**	**Yes [ ] No [ ]: Why Not sure [ ]**
Average duration of crisis episodes		**If yes, how long since completion.**	**Specify [ ] Can't recall [ ]**
HBSag status **HIV status**	**Positive [ ] Neg [ ] Not tested [ ]** **Positive [ ] Neg [ ] Not tested [ ]**	**Have you received the following vaccines?**	**1.Pneumococcal vaccine: Yes [ ] No [ ] Don't know 2.Meningoccocal vaccine: Yes [ ] No [ ] Don't know [ ] 3.Haemophilus infleunza type b vaccine: Yes [ ] No [ ] Don't know [ ] Hepatitis B: Yes [ ] No [ ] Don't know [ ]**
Current symptoms		**Clinical diagnosis for index presentation**	
None		**Vaso-occlusive crisis**	**Yes [ ] No [ ]**
Bone pain:	**Mild [ ] Moderate [ ] Severe [ ]**	**Anaemia/anaemic crisis**	**Yes [ ] No [ ]**
Symptomatic anaemia (easy fatiguability, palpitation, dizziness)	**Yes [ ] No [ ]**	**Mixed crisis**	**Yes [ ] No [ ]**
Fever	**Yes [ ] No [ ]**	**Acute splenic sequestration**	**Yes [ ] No [ ]**

Cough	Yes [ ] No [ ]	IS CRISIS ABOVE ASSOCIATED WITH ANY OF THE CLINICAL INFECTIONS LISTED BELOW ?	Tick all applicable:
Abdominal pain	Yes [ ] No [ ]	Acute Malaria	Yes [ ] No [ ]
Others	Specify	Abscess	Yes [ ] No [ ]
		Bacteraemia	Yes [ ] No [ ]
		Cellulitis	Yes [ ] No [ ]
Examination		Diarrhoea	Yes [ ] No [ ]
Weight	Kg	Dysentery	Yes [ ] No [ ]
Height / Length	M	Meningitis/Encephalitis	Yes [ ] No [ ]
Mid upper circumference (Paediatric)	Cm	Osteomyelitis	Yes [ ] No [ ]
Triceps thickness (Paediatric)		Pharyngitis/Tonsilitis	Yes [ ] No [ ]
Pallor	Yes [ ] No [ ]	Pneumonia/Acute chest syndrome	Yes [ ] No [ ]
Jaundice	Yes [ ] No [ ]	Sepsis of unknown source	Yes [ ] No [ ]
Temperature	Celsius	Sinusitis (acute)	Yes [ ] No [ ]
SPO^2	%	Urinary tract infection	Yes [ ] No [ ]
Respiratory rate	/ Min	Acute upper respiratory tract infection	Yes [ ] No [ ]
Pulse rate	/ Min	Otitis media	Yes [ ] No [ ]
Blood pressure (adults)	mm/Hg	Others (specify)	
Palpable spleen	cm		
Palpable liver	cm		
Action	**Date**		
Appointment given for			

Ultrasound scan. If yes specify date			
Appointment given for HPLC sampling ? If yes specify date			
Appointment given for repeat spleen function. If yes specify date			
Review form to ensure all information are collected			

Appendix 7: Case report form for controls

Data capture sheet for non-sickle cell disease participants			
Title: **A study to assess the spleen size and function in sickle cell disease and the link with disease-causing agents**			
Study ID			
Study date			
Sex	Male		Female
Date of birth			
Age (years)			
Hb phenotype	Hb SS	Hb AS	Don't know
Weight (kg)			
Height (meters)			
Participants phone number: **Alternative phone number:**			

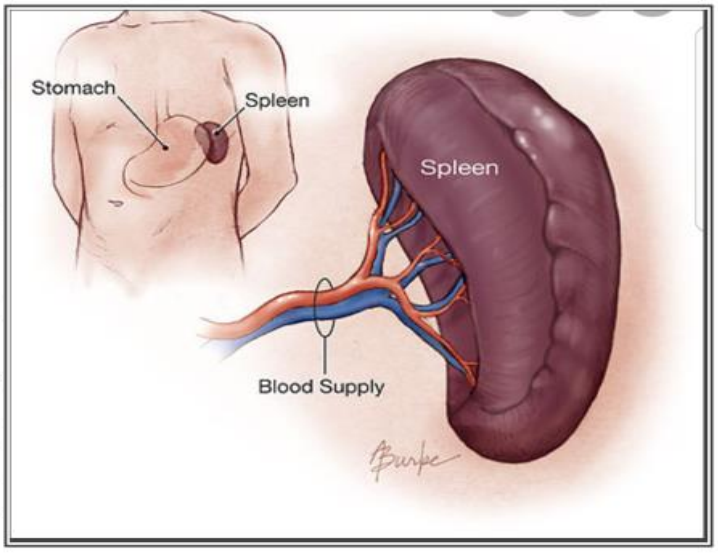
Appendix 8: Patient information sheet for steady state patients (>18 years)

Title: 'A study to assess the spleen size and function in sickle cell disease and the link with disease-causing agents'

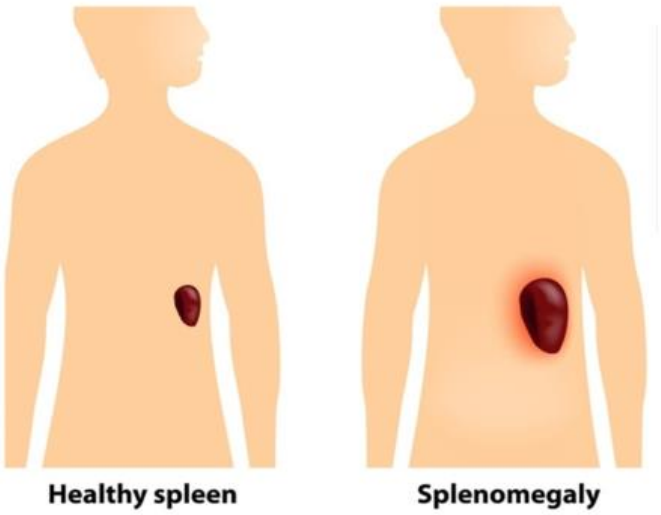
My name is ..... You are being invited to take part in a research study. Before you make your decision, I would like to go through this information sheet with you and answer any question you may have. If you decide to take part, you will be given this information sheet to keep and will be asked to sign a consent form.

Purpose of the study

- We would like to find out information regarding one of the organs in your tummy known as the spleen
- This is located on the left upper part of the belly under the ribcage as shown in this picture.
- The spleen acts like a filter in the body. It clears out old and damaged cells from the body.
- The spleen also helps get rids of germs like malaria. It contain certain type of cells called the white cells which attack and destroys germs and remove them from the body.



- Unfortunately, the size and function of the spleen can become affected by the disease process of sickle cell anaemia (SCA).
- In some it may become small
- While in others it may increase in size, a process known as 'splenomegaly'
- Both changes may interfere with the ability of the spleen to filter the blood from old cells and germs



Why are we asking you to take part?

Patients with SCA been managed at UMTH are being invited to take part in the study, therefore, your participation is being requested. The study will involve patients with SCA who are in stable condition.

What will be involved if I agree to participate?

You will be asked questions about your health including frequency of your painful crises, hospitalization, blood transfusion and drug history. You will undergo a general examination including your tummy. You will be offered ultrasound scan of your tummy in order to check your spleen. The procedure is safe and painless and will be performed in the presence of a chaperone. During the scan, you will be lying on a couch and a probe will be placed on your skin over the part of the tummy to be examined. A gel will be applied on to the skin so that the probe makes good contact with your body. The probe takes live pictures of the spleen and enables us to identify the presence of a problem.

You will also be required to provide blood sample; this will be used for running some tests as follow:

1. **Complete blood count (CBC)**: this will provide us information about the level of your blood.

2. **Blood chemistry**: this will provide us information regarding some chemicals call LDH, ASAT and bilirubin, which indicates how much red cells your body is breaking down.
3. **Spleen function test**: this will be used to count the number of some abnormal cells in blood and will give an indication of whether the spleen is working properly or not.
4. **Malaria parasite test:** this will test if the germ is present and their numbers, because some individuals may be carrying the germ and still have no symptoms.
5. **HPLC**: this is a special test that is currently not available here at UMTH. The sample will be sent securely to a laboratory in Aminu Kano teaching hospital (AKTH). The test will help us measure the level of a blood protein known as haemoglobin F (HbF), which is important in maintaining the function of the spleen.

All the above tests will require about **8 mls** of blood to be taken. If you are having blood taken in the clinic today for any of these tests, the research team can run them for you and provide your doctor with a copy of the results. Any remaining blood samples will be stored securely here at UMTH and with your permission, further test can be carried out on these samples in the future.

Do I have to participate?

You can choose if you want to take part, and if you want more time to decide, you can take up to 24 hours to do so. If you do not want to take part, you do not have to give reason, and this will not affect your medical care in anyway. Also, if you change your mind at any point later, you can withdraw from the study without giving reasons.

Are there any costs to the study?

The blood test will be performed free of charge, and if you are happy to have the ultrasound scan, we will book you an appointment for the procedure. If you need help with transport costs to attend for the appointment, this will be taken care of too.

What are the benefits and risks of taking part in the study?

If you agree to take part, the information obtained from the blood results and ultrasound scan will provide us important information about the size and function of the spleen in SCA patients in this environment and can help us identify those individuals who are more at risk of getting germs in their body. It will also assist the ministry of health and other organization in planning for prevention of such germs using certain drugs like antibiotics and antimalaria.

We do not think there is any major risk to you from participating in the study. You may experience some discomfort or mild bruising from the process of blood collection; however, these are minor risk and we do not think they may cause any harm to you.

Right to anonymity & confidentiality

Your name, answers and test results will be treated with privacy and confidentiality and will not be given to people not involved in the study or in your medical care. Information will be kept on forms which will be kept securely.

With your consent, information from this study may sometimes be shared with other research teams if it will answer important questions. We will also tell other doctors about the results using reports and presentation, but no one will be able to identify you from this.

Who is (are) responsible for the study and funding?

The study is sponsored by Liverpool School of Tropical Medicine (LSTM), United Kingdom (UK). The principal investigator is Dr. Adama Ladu, who is currently a PhD student at LSTM under the Commonwealth scholarship. She is also a registered doctor and haematologist with the Medical and Dental Council of Nigeria.

Who has approved the study?

The Health and Research Ethics Committee (REC) of the University of Maiduguri Teaching Hospital, Nigeria, and the REC committee of Liverpool School of Tropical Medicine (LSTM), UK have given approval for the conduct of the study.

Who to contact for further information?

You are free to ask any question regarding the study. If you need any further information, please contact the PI using the details below:

Dr Adama Isah Ladu: Department of haematology and blood transfusion, University of Maiduguri Teaching Hospital. Borno state. Telephone number: +234 80 37720130

What if I am unhappy or if there is problem?

Should you wish further information about your rights, safety and well-being in the research, or if you have a problem, you feel you cannot come to any of the study team members, please contact the UMTH REC secretariat using the following details :

Health and Research Ethics Committee (HREC) Secretariat,

University of Maiduguri Teaching Hospital,

Or You can also contact the chairperson of the Liverpool School of Tropical Medicine Research and Ethics committee: Email lstmrec@lstmed.ac.uk

Postal address: Liverpool School of Tropical Medicine,

Pembroke Place, Liverpool L3 5QA, UK.

Thank you very much for taking time to read/listen to this information.

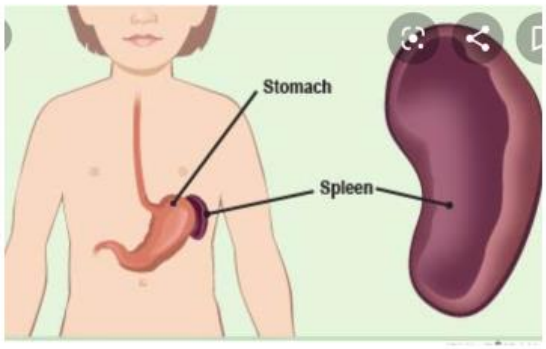
Appendix 9: Patient information sheet for steady state patients (<18 years)

Title: 'A study to assess the spleen size and performance in sickle cell disease and the link with disease-causing agents'

My name is ....., I would like to invite you to take part in a research study. This sheet is to explain what this involves and to try and answer any question you might have.

What is the research study for?

- We would like to find out information regarding one of the organs in your tummy known as the spleen
- This is located on the left upper part of the belly under the ribcage as shown in this picture.
- The spleen acts like a filter in the body. It clears out old and damaged cells from the body.
- The spleen also helps get rids of germs like malaria from the body.



However, in individuals with sickle cell anaemia (SCA) the size and function of the spleen can become altered by the disease process and this may interfere with the ability of the spleen to filter the blood from old cells and germs. In this study, I would like to look at the size and performance of the spleen. This will help us identify individuals who have problem with their spleen and can help us plan on how to look after them properly in the future

Why am I asking you to help?

I am asking you to take part because you have got SCA and this study involves only people with this condition. The study will be conducted in two parts: The first part will involve patients who are in stable condition, while the second part will involve those in painful crisis. In the current part, only patients in **steady**

state condition are invited to take part. However, you may be invited to take part in the second part if you do develop acute crisis during the period of the study.

What happens if I want to help?

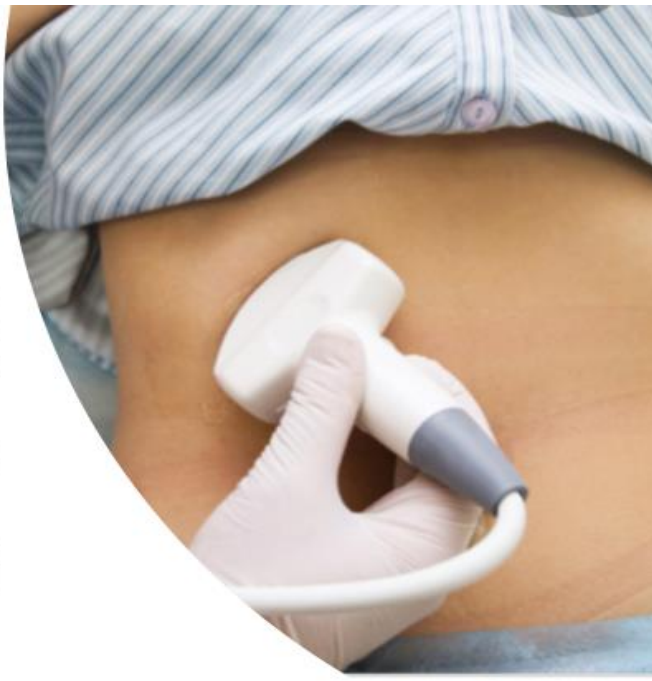
I will ask you some questions about your health and one of my colleagues will examine you.

We will take some blood samples from you just the way we would normally do during your regular checkup.

This help tell us the level of your blood.

You will also have an ultrasound scan of your tummy. The test is safe and painless and will help us take pictures of your spleen.Your parents can also be around during the procedure if you want them to.

- Before the exam, you will change into a hospital gown and you will most likely be lying down on a table with a section of your tummy exposed for the test.
- A special jelly will be applied to your skin, this prevents friction when the ultrasound instrument is applied to your skin.
- The instrument enable live pictures of your spleen to be taken
- After the procedure, the gel will be cleaned off your body and you will be free to go home immediately



Do I have to help?

You can choose if you want to take part. If you don't want to take part that is also okay, and it will not affect the care you are getting from us. We have discussed this with your parents, and they know we are asking for your agreement.

How will this help me?

The study will provide us with information about the spleen in SCA patients in this environment that will help us look after them in a better way.

Are there any risk to me taking part in the study?

We don't expect anything bad or dangerous to happen to you. You may feel some discomfort during the blood collection, we will apply a little cream over the area to minimize this. The tummy scan won't hurt too because of the special gel we will use during the test.

Will anyone else know about this?

We won't tell anyone else that you are in this research and information collected about you from the research will be put away safely so that only team members involve in the study can see them. When the study is finished, we

may share the results with other doctors during meetings and presentations, but no one will be able to identify you from this.

What if a problem comes up during the study?

If you are not satisfied or any problem arise, you can tell you mum or dad and they can inform any member of the study team, or you can tell us yourself.

What happens now?

If you feel you have understood what the study is all about and what will happen, and want to take part, I will provide you with a form to either write your name or use a thumbprint at the end. You will be given a copy to keep in addition to this information leaflet. You mum or dad or guardian will need to sign a similar form to agree to you taking part.

Thank you very much for taking time to read/listen to this information.

Appendix 10: Patient information sheet for acutely-ill SCD patients (>18 years)

Title: Evaluation of spleen size and function and the relationship with malaria and bacterial infections among sickle cell anaemia patients.

You have been invited to participate in a research project titled: 'Evaluation of spleen size and function and the relationship with malaria and bacterial infections among sickle cell anaemia patients. Before you make your decision to participate, I would like to go through this information sheet with you and answer any question you may have. If you decide to take part, you will be given this information sheet to keep and will be asked to sign a consent form.

Purpose of the study: I would like to find out information regarding one of your abdominal organs known as the spleen, this is located on the left side of the abdomen and is responsible for providing defense against infections such as malaria and blood borne bugs (bacteria). Unfortunately, the size and function of this organ can become distorted from the disease process of sickle cell anaemia (SCA).

I would like to carry out this study to find out if the level of function of the spleen and its size in patients with SCA in this environment is altered. Also, whether because of this change they are likely to develop infection with malaria parasite or blood borne bugs more frequently. The study will be conducted in two parts:

1) The first part will involve patients in their stable condition. The size of their spleen will be measured using ultrasound scan and the function will be measured using a drop of blood on a glass slide. This information will give us an idea of how many people have abnormal sized spleen, and how many have abnormal functioning spleen. Also, the level of a protein call HbF which help to preserve the function of the spleen will be measured.
2) In the second part of the study, patients presenting with bone crisis, fever, symptoms of shortage of blood or yellow discoloration of their eyes will have their blood sample taken to test for the presence of malaria infection or blood bugs. The size of their spleen and the function will also be measured. This information will help us understand if the change in the size and function of their spleen is responsible for the frequency of their symptoms.

Participants: Patients with SCA being managed at the UMTH. Therefore, you are being requested to participate in (tick ☐ appropriate): **Part 1= Part 2 =**

Do I have a choice to participate or not?

Your participation in this study is voluntary. You are also free to withdraw from participating at any time without giving reason and this will not affect your medical care in anyway.

Procedures if you agree to participate.

You will be asked questions about your health including frequency of your painful crises, hospitalization, blood transfusion and drug history. You will also undergo a general physical examination and abdominal examination.

Blood samples for testing for your blood level, and the level of a blood protein called HbF will be collected and sent to a teaching hospital in Kano state (AKTH) for analysis, because currently there is no facility for such test at UMTH. Also, a drop of the blood will be put onto a glass slide and used for testing the function of the spleen. Part of this test will be performed here at UMTH, and another part at LSTM, UK to double check the results.

Blood sample to check for presence of malaria and blood bugs will be collected. You will also be offered abdominal ultrasound in order to estimate the size of your spleen.

Are there any costs to the study?

The blood test and ultrasound scan will be performed free of charge.

What are the benefits and risks of the study?

The information obtained from the blood test and ultrasound scan will provide us important information about the size and function of the spleen in SCA in this environment and help us identify those individuals who are more at risk of developing infection. It will also assist the ministry of health and other organization in planning for prevention of infections using certain antibiotics and provide more support for the existing antimalaria prevention program.

Apart from the discomfort and the time spent coming to the hospital and conducting an abdominal scan test, we do not think there is any harm from the study.

Who is (are) responsible for the study and funding?

Dr. Adama Ladu is the principal investigator. She is a registered doctor and a haematologist with the Medical and Dental Council of Nigeria. She is currently doing a PhD with the Liverpool School of Tropical Medicine, UK. Professor

Imelda Bates is the overall supervisor and the guarantor for the study. The Commonwealth Scholarship Commission UK is funding the research.

Right to anonymity & confidentiality

Your name, answers and test results will be treated with privacy and confidentiality and will not be given to people not involved in the study or your medical care. Information will be kept on forms which will be kept securely. With your consent, information from this study may sometimes be shared with other research teams if it will answer important questions. We will also tell other doctors about the results using reports and presentation, but no one will be able to identify you from this**.**

Who has approved the study?

The Health and Research Ethics Committee (REC) of the University of Maiduguri Teaching Hospital, Borno State, Nigeria, and the REC committee of Liverpool school of tropical Medicine (LSTM), UK have given approval for the conduct of the study.

Who to contact for further information?

You are free to ask any question regarding the study. If you need any further information:

Dr Adama Isah Ladu, department of Haematology and blood transfusion, University of Maiduguri Teaching Hospital. Borno state (+234 80 37720130)

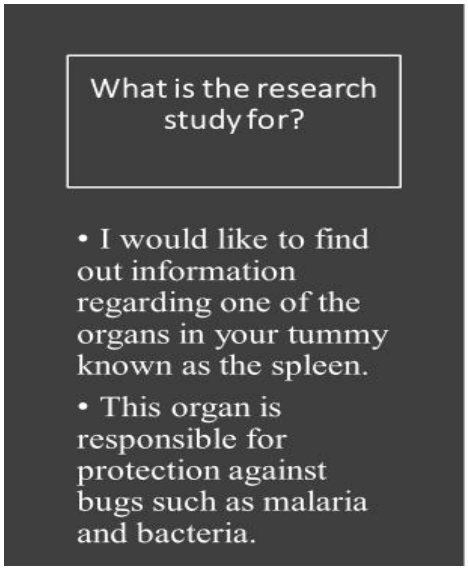
Appendix 11: Patient information sheet for acutely-ill SCD patients (< 18 years)

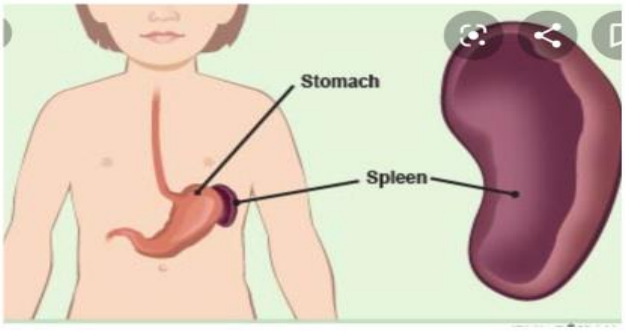
Title: Evaluation of splenic size and function and the relationship with malaria and bacterial infections among sickle cell anaemia patients.

I would like to invite you to take part in a research study. This sheet is to explain what this involves and to try and answer any question you might have.

Who is doing this research?

I (Dr Ladu) am conducting this study as part of my PhD research at the Liverpool School of Topical Medicine (LSTM), UK. In total I plan to include 140 adults and children for the study.





However, in individuals with sickle cell anaemia (SCA) this function may become affected because of the on and off episodes of painful crisis this condition causes, as a result such individual may likely suffer from frequent bugs' attack.

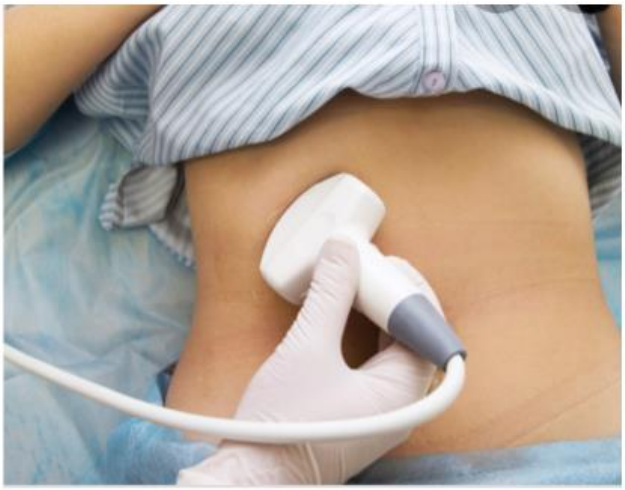
In this study, I would like to look at the size of the spleen and check the function as well in patients with SCA in this environment. This will help us identify individuals who have problem with their spleen and can help us plan on how to look after them properly in the future.

Why am I asking you to help?

I am asking you to take part because you have got SCA, and this study involves only people with this condition.

- **What happens if I want to help?**
- **I will ask you some questions about your health and one of my colleagues will examine you.**
- **We will take some blood samples from you like how blood is normally taken during your regular checkup**

- You will also have an abdominal ultrasound scan.
- This will help us take image of your spleen
- The test is safe and painless
- Your parents can also be around during the procedure if you want them to.



Do I have to help?

You can choose if you want to take part. If you don't want to take part that is also okay. Also, if you say no, it will not affect the care you are getting from us.

We have discussed this with your parents, and they know we are asking for your agreement.

How will this help me?

The study will provide us with information that will help us look after children with this condition in a better way.

Are there any risks to me taking part in the study?

We don't expect there to be anything bad or dangerous that could happen to you. The scan won't hurt, a gel will be applied onto your tummy before the procedure, this help cool the area of the scan

Will anyone else know about this?

We won't tell anyone else that you are in this research and information about you collected from the research will be put away safely so that only team members involve in the study can see it. When the study is finished, we may share the results with other doctors during meetings and presentations, but no one will be able to identify you from this.

What if a problem comes up during the study?

If you are not satisfied or any problem arise, you can tell you mum or dad and they can inform me or a member of the study team, or you can tell us yourself.

What happens now?

If you feel you have understood what the study is all about and what will happen, and want to take part, I will provide you with a form to either write your name or use a thumbprint at the end. You will be given a copy to keep in addition to this information leaflet. You mum or dad or guardian will need to sign a similar form to agree to you taking part.

Appendix 12: Consent form for steady state SCD patients (>18 years)

Title: 'A study to assess the spleen size and function in sickle cell disease and the link with disease-causing agents'

Participant ID number: ----------

Please initial box

1. I confirm that I have read and understood the information sheet dated ……. for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered to my satisfaction.

2. I understand that participation in this study is voluntary, and I am free to withdraw my consent at any time, without giving any reason, without any penalty.

3. I understand that data collected during this study may be looked at by individuals from LSTM, UK and other regulatory authorities. I give permission for these individuals to have access to my records.

4. I understand that some of my samples will be analysed in laboratories in AKTH, Kano Nigeria and LSTM, UK for those test that cannot be performed here in UMTH. I consent for my samples to be analysed in these facilities.

5. I hereby declare that I have not been subjected to any form of coercion in giving consent to the above.

6. I agree / do NOT agree for blood samples and data about me collected in this study being stored for further use in the future.

7. I agree to take part in this study.

8.

Signing this declaration does not affect your right to decline to take part in any future study.

.…….	……	……..
Name of participant	Date	Signature
……	…….	……
Name of Witness	Date	Signature
……	…….	……
Name of Researcher	Date	Signature

Thank you.

Appendix 13: Consent form for parents/guardian of steady state SCD patients (<18 years)

Title: 'A study to assess the spleen size and function in sickle cell disease and the link with disease-causing agents'

Participant ID number: ----------

Please initial box

1. I confirm that I have read and understood the information sheet dated ……. of the above study. I have had the opportunity to consider the information, ask questions, and have had these answered to my satisfaction.

2. I understand that participation of my **Child / Relation** in this study is voluntary, and I am free to withdraw consent at any time, without giving any reason and without any penalty.

3. I understand that data collected during this study may be looked at by individuals from LSTM, UK and other regulatory authorities. I give permission for these individuals to have access to my **Child / Relation** records.

4. I understand that some samples will be analysed in laboratories in AKTH, Kano Nigeria and LSTM, UK for those test that cannot be performed here in UMTH. I consent for my **Child / Relation** samples to be analysed in these facilities.

5. I hereby declare that I have not been subjected to any form of coercion in giving consent to the above.

6. I understand that only study approved staff will have access to information that can identify me or my **Child / Relation**

7. I agree / do NOT agree for blood samples and data about my **Child / Relation** collected in this study being stored for further use in the future.

8. I agree for my **Child / Relation** to take part in this study.

Signing this declaration does not affect your right to decline to take part in any future study.

…….
Name of participating child

……	…….	……
Name of Parent/Guardian	Date	Signature

If no signature from parent or guardian, a witness is required.

……..	…….	……
Name of Witness	Date	Signature

……	…….	……
Name of Researcher	Date	Signature

Thank you

Appendix 14: Consent form for acute-ill SCD patients (> 18 years)

Title of Project: Evaluation of spleen size and function and the relationship with malaria and bacterial infections amongst sickle cell anaemia patients

Participant ID number: ----------

Please initial box

9. I confirm that I have read and understood the information sheet dated ……. for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered to my satisfaction.
10. I understand that participation in this study is voluntarily, and I am free to withdraw consent at any time, without giving a reason, without any penalties.
11. I understand that data collection during this study may be looked at by individuals from LSTM, UK and from regulatory authorities. I give permission for these individuals to have access to my records.
12. I understand that some of my samples will be analysed in laboratories in AKTH, Kano Nigeria and LSTM, UK for those test that cannot be performed currently here in UMTH. I consent for my samples to be analysed in these facilities.
13. I hereby declare that I have not been subjected to any form of coercion in giving consent to the above.
14. I agree / do NOT agree for blood samples and data about me collected in this study being stored for further use in the future.
15. I agree to take part in this study.

Signing this declaration does not affect your right to decline to take part in any future study.

…….	……	……..
Name of participant	Date	Signature
……	…….	……
Name of Witness	Date	Signature
……	…….	……
Name of Researcher	Date	Signature

Appendix 15: Assent form for acutely-ill SCD (<18 years)

Assent form 12- 18years		
If you will like to part in the study, please fill and sign the form below		
Has somebody explained the study to you?	Yes	No
Do you understand what this study is about?	Yes	No
Have you asked all the questions you want ?	Yes	No
Have your questions been answered in a way you understand?	Yes	No
Do you understand its ok to stop taking part at any time?	Yes	No
Are you happy to join in?	Yes	No
I have read the information sheet for the above study dated …	Yes	No
I agree to take part in the spleen study	Yes	No
STUDY ID		
Name of child	Date	Signature or thumbprint
If no signature from the child, a witness is required		
Name of Witness	Date	Signature or thumbprint
Name of Researcher	Date	Signature or thumbprint
THANK YOU		

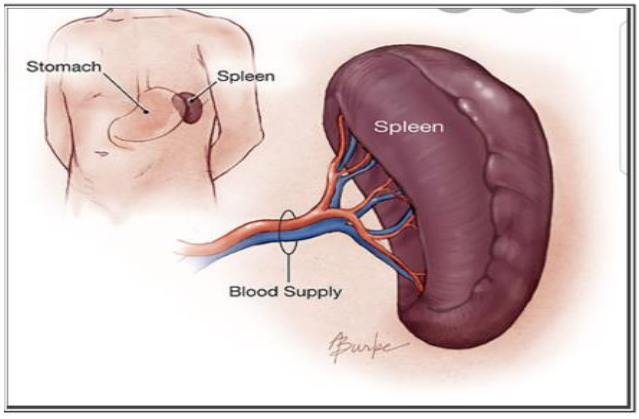
Appendix 16: Patient information sheet for controls (>18 years)

Title: 'A study to assess the spleen size and function in sickle cell disease and the link with disease-causing agents'

My name is Dr Adama Isah Ladu. I am a student at the Liverpool School of Tropical Medicine working with Prof. Imelda Bates. We would like to invite you to take part in our research study. Before you decide, we would like you to understand why the research is being done and what it would involve for you. I will go through the information sheet with you and answer any questions you have. If you decide to take part, you will be given this information sheet to keep and will be asked to sign a consent form.

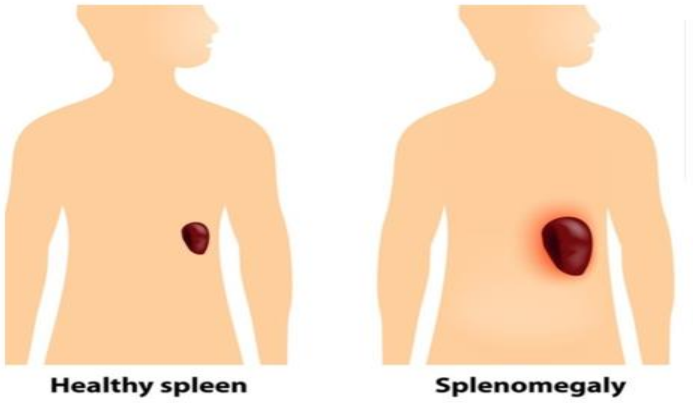
Purpose of the study

We would like to find out information regarding one of the organs in your tummy known as the spleen. This is located on the left upper part of the belly as shown in the picture below. The spleen helps clear the body from old and damaged cells, and germs like malaria.



However, in individuals with sickle cell disease (SCD), the spleen size can become altered by the disease process and this may interfere with its ability to filter the blood from old cells and germs. In some it may become enlarged, while in others, it may become small or even absent. In this study, we would like to look at the size and performance of the spleen in SCD patients. This will

help us identify those who have problem with their spleen and can help us plan on how to look after them properly in the future.



Why are we asking you to take part?

We are inviting you to participate because we want to compare the spleen size in persons with sickle cell disease and those without the disease.

What will be involved if I agree to participate?

Information about your age, haemoglobin genotype, weight and height will be collected. You will be offered ultrasound scan of your tummy to check your spleen. The procedure is safe and painless and will be performed in the presence of a chaperone. During the scan, you will be lying on a couch and a probe will be placed on your skin over the part of the tummy to be examined. A gel will be applied on to the skin so that the probe makes good contact with your body. The probe takes live pictures of the spleen and enables us to determine the size.

You will also be required to provide blood sample for a test call **HPLC**. The test will help us measure the level of a blood protein known as haemoglobin F (HbF), which is important in maintaining the size and function of the spleen. The test will require about 1 **ml** of blood to be taken.

Do I have to participate?

You can choose if you want to take part, and if you want more time to decide, you can take up to 24 hours to do so. If you do not want to take part, you do

not have to give reason. Also, if you change your mind at any point later, you can withdraw from the study without giving reasons.

Are there any costs to the study?

No, if you are happy to have the ultrasound scan, we will book you an appointment for the procedure. If you need help with transport costs to attend for the appointment, this will be taken care of too.

What are the benefits and risks of taking part in the study?

If you agree to take part, the information obtained from the blood test and ultrasound scan will provide us important information about the size and function of the spleen in in normal individuals in this environment which can used to compare with individuals with sickle cell disease.

We do not think there is any major risk to you from participating in the study. You may experience some discomfort or mild bruising from the process of blood collection; however, these are minor risk and we do not think they may cause any harm to you.

Right to anonymity & confidentiality

Your name, answers and test results will be treated with privacy and confidentiality and will not be given to people not involved in the study. Information will be kept on forms which will be kept securely.

With your consent, information from this study may sometimes be shared with other research teams if it will answer important questions. We will also tell other doctors about the results using reports and presentation, but no one will be able to identify you from this.

Who is (are) responsible for the study and funding?

The study is sponsored by Liverpool School of Tropical Medicine (LSTM), United Kingdom (UK). The principal investigator is Dr. Adama Ladu, who is currently a PhD student at LSTM under the Commonwealth scholarship. She is also a registered doctor and haematologist with the Medical and Dental Council of Nigeria.

Who has approved the study?

The Health and Research Ethics Committee (REC) of the University of Maiduguri Teaching Hospital, Nigeria, and the REC committee of Liverpool School of Tropical Medicine (LSTM), UK have given approval for the conduct of the study.

Who to contact for further information?

You are free to ask any question regarding the study. If you need any further information, please contact the PI using the details below:

Dr Adama Isah Ladu: Department of haematology and blood transfusion, University of Maiduguri Teaching Hospital. Borno state. Telephone number: +234 80 37720130

What if I am unhappy or if there is problem?

Should you wish further information about your rights, safety and well-being in the research, or if you have a problem, you feel you cannot come to any of the study team members, please contact the UMTH REC secretariat using the following details:

Health and Research Ethics Committee (HREC) Secretariat,

University of Maiduguri Teaching Hospital,

Or You can also contact the chairperson of the Liverpool School of Tropical Medicine Research and Ethics committee: Email lstmrec@lstmed.ac.uk

Postal address: Liverpool School of Tropical Medicine,

Pembroke Place, Liverpool L3 5QA, UK.

Thank you very much for taking time to read/listen to this information.

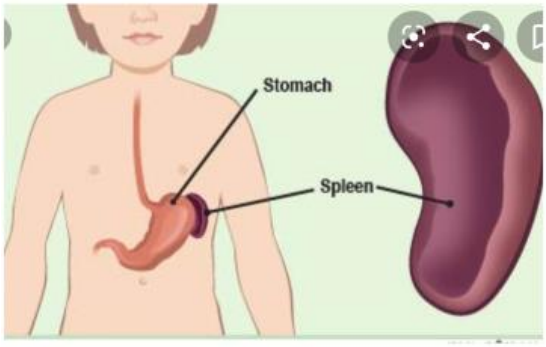
Appendix 17: Patient information sheet for controls (>18 years)

Title: 'A study to assess the spleen size and performance in sickle cell disease and the link with disease-causing agents'

My name is Dr Adama Isah Ladu. I am a student at the Liverpool School of Tropical Medicine working with Prof. Imelda Bates. We would like to invite you to take part in our research study. Before you decide, we would like you to understand why the research is being done and what it would involve for you. I will go through the information sheet with you and answer any questions you have. If you decide to take part, you will be given this information sheet to keep and will be asked to sign a consent form.

What is the research for?

We would like to find out information regarding one of the organs in the tummy known as the spleen. This is located on the left upper part of the belly as shown in the picture below. The spleen helps clear the body from old and damaged cells, and germs like malaria.



However, in individuals with sickle cell disease (SCD), the spleen size can become altered by the disease process and this may interfere with its ability to filter the blood from old cells and germs. In this study, we would like to look at the size and performance of the spleen. This will help us identify SCD patients who have problem with their spleen and can help us plan on how to look after them properly in the future.

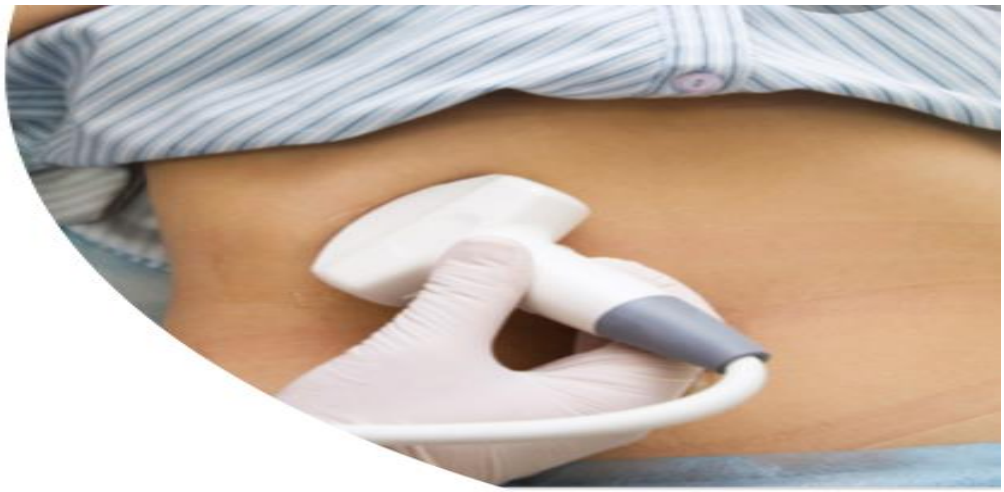
Why am I asking you to help?

We are inviting you to participate because we want to compare the spleen size in persons with sickle cell disease and those without the disease.

What happens if I want to help?

I will collect information about your age, weight and height. I will take a very small amount of blood sample (1ml).

You will also have an ultrasound scan of your tummy. The test is safe and painless and will help us take pictures of your spleen.Your parents can also be around during the procedure if you want them to. You will be lying down during the procedure on a couch with part of your tummy exposed for the test. A special gel will be applied to your tummy, this prevents discomfort when the ultrasound instrument is applied to your tummy. The instrument will enable us obtain live picture of your spleen. After the procedure, the gel will be wiped off your tummy and you can leave for home immediately.



Do I have to help?

You can choose if you want to take part. If you do not want to take part that is also okay. We have discussed this with your parents, and they know we are asking for your agreement.

What are the benefits of taking part in the study?

If you agree to take part, the information obtained from your blood test and ultrasound scan will provide us important information about the size of the spleen in normal individuals. This will be compared with findings from individuals with sickle cell disease in this environment, which will help us look after them in a better way.

Are there any risks to me taking part in the study?

We do not expect anything bad or dangerous to happen to you. You may feel some discomfort during the blood collection, we will apply a little cream over the area to minimize this. The tummy scan will not hurt too because of the special gel we will use during the test.

Will anyone else know about this?

We will not tell anyone else that you are in this research and information collected about you from the research will be put away safely so that only team members involve in the study can see them. When the study is finished, we may share the results with other doctors during meetings and presentations, but no one will be able to identify you from this.

What if a problem comes up during the study?

If you are not satisfied or any problem arise, you can tell you mum or dad and they can inform any member of the study team, or you can tell us yourself.

What happens now?

If you feel you have understood what the study is all about and what will happen, and want to take part, I will provide you with a form to either write your name or use a thumbprint at the end. You will be given a copy to keep in addition to this information leaflet. You mum or dad or guardian will need to sign a similar form to agree to you taking part.

Thank you very much for taking time to read/listen to this information.

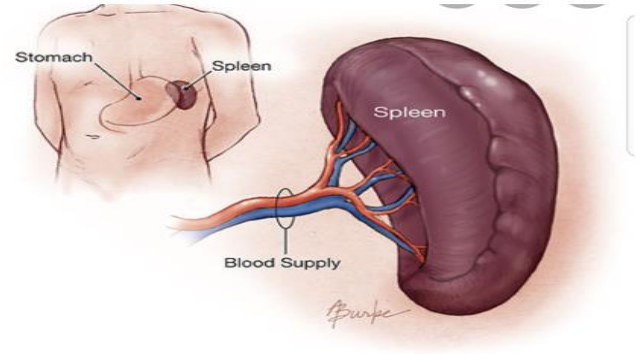
Appendix 18: Patient information sheet for parents of minors (controls)

Title: 'A study to assess the spleen size and performance in sickle cell disease and the link with disease-causing agents'

My name is Dr Adama Isah Ladu. I am a student at the Liverpool School of Tropical Medicine working with Prof. Imelda Bates. You are being invited to allow your child take part in a research study. Before you decide, we would like you to understand why the research is being done and what it would involve for your child. I will go through the information sheet with you and answer any questions you have. If you decide to take part, you will be given this information sheet to keep and will be asked to sign a consent form.

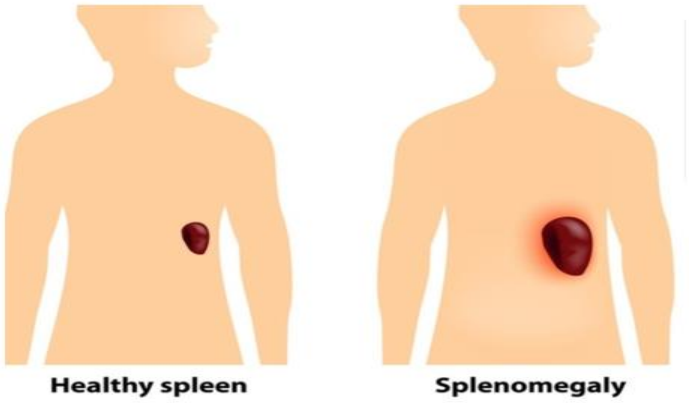
Purpose of the study

We would like to find out information regarding an organ in your child's tummy known as the spleen. This is located on the left upper part of the belly as shown in the picture below. The spleen helps clear the body from old and damaged cells, and germs like malaria.



However, in individuals with sickle cell disease (SCD), the spleen size can become altered by the disease process and this may interfere with its ability to filter the blood from old cells and germs. In some, it may become enlarged, while in others, it may become small or even absent. In this study, we would like to look at the size and performance of the spleen in SCD patients. This will

help us identify those who have problem with their spleen and can help us plan on how to look after them properly in the future.



Why are we asking your child to take part?

We are inviting your child to participate because we want to compare the spleen size in persons with sickle cell disease and those without.

What will be involved if I agree for my child to participate?

Information about your child's age, haemoglobin genotype, weight and height will be collected. They will be offered ultrasound scan of their tummy in order to determine their spleen size. The procedure is safe and painless. During the scan, your child will be lying on a couch and a probe will be placed on their skin over the part of the tummy to be examined. A gel will be applied on to the skin so that the probe makes good contact with their body. The probe takes live pictures of the spleen, which will enable us to determine the size.

You**r** child will also be required to provide blood sample for a test call HPLC. The test will help us measure the level of a blood protein known as haemoglobin F (HbF), which is important in maintaining the size and function of the spleen. The test will require about 1 **ml** of blood to be taken.

Does my child have to participate?

You can choose if you want your child to take part. If you want more time to decide, you can take up to 24 hours to do so. If you do not want your child to take part, you do not have to give reason.

Are there any costs to the study?

The blood test will be performed free of charge, and if you are happy for your child to have the ultrasound scan, we will book you an appointment for the procedure. If you need help with transport costs for you and your child to attend for the appointment, this will be taken care of too.

What are the benefits and risks of my child taking part in the study?

If you agree for your child to take part, the information obtained from the blood test and ultrasound scan will provide us important information about the size and function of the spleen in normal individuals in this environment, which can be used to compare with individuals with sickle cell disease.

We do not think there is any major risk to your child from participating in the study. They may feel some discomfort during the blood collection, but we will apply a little cream over the area to minimize this. The tummy scan will not hurt too because of the special gel we will use during the test.

Right to anonymity & confidentiality

Your child's name, answers and test results will be treated with privacy and confidentiality and will not be given to people not involved in the study or their medical care. Information will be kept on forms which will be kept securely locked.

With your consent, information from this study may sometimes be shared with other research teams if it will answer important questions. We will also tell other doctors about the results using reports and presentation, but no one will be able to identify your child from this.

Who is (are) responsible for the study and funding?

The study is sponsored by Liverpool School of Tropical Medicine (LSTM), United Kingdom (UK). The principal investigator is Dr. Adama Ladu, who is currently a PhD student at LSTM under the Commonwealth scholarship. She is also a registered doctor and haematologist with the Medical and Dental Council of Nigeria.

Who has approved the study?

The Health and Research Ethics Committee (REC) of the University of Maiduguri Teaching Hospital, Nigeria, and the REC committee of Liverpool School of Tropical Medicine (LSTM), UK have given approval for the conduct of the study.

Who to contact for further information?

You are free to ask any question regarding the study. If you need any further information, please contact the PI using the details below:

Dr Adama Isah Ladu: Department of haematology and blood transfusion, University of Maiduguri Teaching Hospital. Borno state. Telephone number: +234 80 37720130

What if I am unhappy or if there is problem?

Should you wish further information about your **child / relation** rights, safety and well-being in the research, or if you have a problem, you feel you cannot come to any of the study team members, please contact the UMTH REC secretariat using the following details:

Health and Research Ethics Committee (HREC) Secretariat,

University of Maiduguri Teaching Hospital

Or

You can also contact the chairperson of the Liverpool School of Tropical Medicine Research and Ethics committee:

Email lstmrec@lstmed.ac.uk.

Postal address: Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK.

Thank you very much for taking time to read/listen to this information.

Appendix 19: Consent form for controls (>18 years)

Title: 'A study to assess the spleen size and function in sickle cell disease and the link with disease-causing agents'

Participant ID number: ----------

Please initial box

1. I confirm that I have read and understood the information sheet dated ……. for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered to my satisfaction.

2. I understand that participation in this study is voluntary, and I am free to withdraw my consent at any time, without giving any reason, without any penalty.

3. I understand that data collected during this study may be looked at by individuals from LSTM, UK and other regulatory authorities. I give permission for these individuals to have access to my records.

4. I understand that some of my samples will be analysed in laboratories in AKTH, Kano Nigeria and LSTM, UK for those test that cannot be performed here in UMTH. I consent for my samples to be analysed in these facilities.

5. I hereby declare that I have not been subjected to any form of coercion in giving consent to the above.

6. I agree / do NOT agree for blood samples and data about me collected in this study being stored for further use in the future.

7. I agree / do NOT agree to get feedback of any incidental finding detected during the ultrasound scan

8. I agree to take part in this study.

Signing this declaration does not affect your right to decline to take part in any future study

.…….	……	……..
Name of participant	Date	Signature
……	…….	……
Name of Witness	Date	Signature
……	…….	……
Name of Researcher	Date	Signature

Thank you

Appendix 20: Consent form for parents of minors (controls)

Title: 'A study to assess the spleen size and function in sickle cell disease and the link with disease-causing agents'

Participant ID number: ----------

Please initial box

1. I confirm that I have read and understood the information sheet dated ……. for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered to my satisfaction.

2. I understand that participation of my **Child** in this study is voluntary, and I am free to withdraw consent at any time, without giving any reason and without any penalty.

3. I understand that data collected during this study may be looked at by individuals from LSTM, UK and other regulatory authorities. I give permission for these individuals to have access to my **Child** records.

4. I understand that some samples will be analysed in laboratories in AKTH, Kano Nigeria and LSTM, UK for those test that cannot be performed here in UMTH. I consent for my **Child** samples to be analysed in these facilities.

5. I hereby declare that I have not been subjected to any form of coercion in giving consent to the above.

6. I understand that only study approved staff will have access to information that can identify me or my **Child.**

7. I agree / do NOT agree for blood samples and data about my **Child** collected in this study being stored for further use in the future

8. I agree / do NOT agree to get feedback of any incidental finding detected during the ultrasound scan of my child.

9. I agree for my **Child** to take part in this study.

Signing this declaration does not affect your right to decline to take part in any future study.

…….
Name of participating child

……	…….	……
Name of Parent/Guardian	Date	Signature

If no signature from parent or guardian, a witness is required.

……..	…….	……
Name of Witness	Date	Signature

……	…….	……
Name of Researcher	Date	Signature

Thank you.

Appendix 21: Assent for Controls (< 18 years)

Assent for non-sickle cell disease minor		
Title of Project: **A study to assess the spleen size and performance in sickle cell disease and the link with disease-causing agents.** **Participant ID number**: ---------- Date……		
If you would like to part in the study, please fill and sign the form below.		
Has somebody explained the study to you?	Yes	No
Do you understand what this study is about?	Yes	No
Have you asked all the questions you want to ?	Yes	No
Have your questions been answered in a way you understand?	Yes	No
Do you understand its ok to stop taking part at any time?	Yes	No
Are you happy to join in?	Yes	No
I have read the information sheet for the above study dated …	Yes	No
I agree to take part in the spleen study	Yes	No
Name of child	Date	Signature or thumbprint
If no signature from the child, a witness is required		
Name of Witness	Date	Signature or thumbprint
Name of Researcher	Date	Signature or thumbprint
Thank you		

Appendix 22: Standard operating procedure for argyrophilic inclusion red cells

Purpose

To describe the process of staining peripheral blood film using a silver stain for the identification of argyrophilic inclusion (AI) containing red cells.

Principle

This is based on the detection of red cells containing argyrophilic inclusion demonstrated by silver stain. The silver stain was originally used to demonstrate the nucleolar organizer regions (NORs) of chromosomes, to evaluate their function, and to identify chromosomes in cytogenetic preparations (Bukhari *et al.*, 2007; Lindner, 1993; Ogunsola and Antia, 2018). The technique was first applied by Tham and colleagues to red blood cells (Tham *et al.*, 1996). The silver stain demonstrates all intracellular argyrophilic particles. The number of erythrocytes containing argyrophilic inclusions was directly related to the splenic reticuloendothelial function. The argyrophilic inclusions were shown to be Howell–Jolly bodies, Pappenheimer bodies and other inclusions visible in patients with a decreased or absent splenic function.

If the PE count is used as a gold standard and 3% as the upper limit of normal for both PE and AE counts, then the AE count has a sensitivity of 88.9% and a specificity of 97.1%. The AE counts are reproducible (intraobserver rs = 0.736,P <.001; interobserver rs = 0.578, P = .002). They correlate well with PE counts when all the counts are compared (rs = 0.647, P <.001). The study indicated that the AE count is possibly as sensitive as PE count and has the advantage that no special equipment is

required other than an ordinary light microscope. Therefore, it may be done in any haematology laboratory.

Materials/ Reagents

- Gelatin
- Silver nitrate
- Formic acid
- Potassium iodide
- Filter paper
- Formalin
- Ethanol
- Distilled water
- Beaker
- Eosin
- Glass slides
- Pipette
- Amber-glass bottles
- Microscope

3.1 Staining reagents

(i) Solution A (2%), a colloidal developer solution:

- Dissolve 2 g of Gelatin in 100 ml of distilled water and 1 ml of formic acid (1% v/v) at room temperature; requires at least 30 minutes at room temperature to dissolve.
- Or continuous shaking at room temperature for 10 minutes
- Filter through Whatman filter paper.

(ii) Solution B (50%), an aqueous silver nitrate solution:

- Dissolve 50 g silver nitrate in distilled water in a proportion of 1:2 (w/v).

- Silver nitrate solution should be prepared just before use.
- Or can be prepared beforehand and stored in refrigerator and protected from light. Store in capped amber-glass bottles or by other light-protective means.

  NB: Small-capped bottles should be used in which different quantities of silver nitrate like 1g, 2g, 3g or 4 g can be kept in small-capped bottle and stored away from light before use

(iii) Working solution:

- Prepare fresh in acid-clean glassware just before use.
- Take one part of solution A and mix with two parts solution B.
- Filter through Whatman filter paper into plastic bottle
- Use immediately because it degrades immediately (approximately 3 minutes).

Method

Collection of blood sample

Aliquot of blood from an EDTA sample can be used for making the blood smear.

Preparation of blood smears

- Place 5 microliter of blood near one end of the glass slide.
- Using a second slide to spread, draw the spreader backwards into the drop of blood at 45 degree or more depending on the haematocrit,
- Push forward in one quick movement. This should produce a film that is tongue shape, with a base, body, and tip.
- Air dry the film.
- Label the slides with the subject ID, initials and date one side of the slide.

Staining of blood smears

- Fix air dried smears in 3:1 ratio of 95% ethanol to formalin mixture for 3min (solution made by diluting 150 mL of formalin (37% formaldehyde) to 500 mL with 95% ethanol)
- Wash the smear with distilled water.
- Shake off water as much as possible- blot to dry with towel
- Cover the smear with three drops of freshly prepared working solution - silver staining solution.
- Drop a 40 X 24 mm coverslip on the slide to ensure even spread of the staining solution.
- Incubate in dark at 38^0C for 20 min.
- Remove cover slip and wash thoroughly in distilled or deionized water.
- Counterstain with 1% eosin for 30 seconds. Wash in water.
- Dehydrate in increasing concentration of ethanol.
- Clear in xylene
- Mount with DPX

Estimation of argyrophilic inclusion positive red cells (AI)

- Using the oil immersion objective, identify a field that contains red cells in monolayer.
- Using a multiparameter counter tally, count the total number of red cells with one or more distinct black inclusions and normal red cells per field simultaneously.
- Count a minimum of 500 red cells and express the number of AIs as a percentage.
- Repeat the process in another area of the film and take the mean of the two results.

- Only red cells with distinct black granules are counted as positive. Red cells with a diffuse or a fine reticular or punctate pattern of brown staining are not regarded as positive for argyrophilic inclusions (may be due to the presence of RNA as indicated by polychromasia and punctate basophilia or retics count)

Limitations

Slightly higher counts in patients with haemolytic anaemias may be due to the hematologic disturbance and not indicative of abnormal splenic function. Kent et al. found an increase in the percentage of red cells with autophagic vacuoles in patients with hematologic disturbance and reticulocytosis but intact spleens. A high proportion of reticulocytes contained vacuoles. Therefore, the impact of reticulocytes count will need to be corrected for/accounted for; this is in order to get the correct AI count.

Quality control: Pre test runs to check effect of the several parameters so as to identify optimal staining parameters

- Conduct test run to check the effect of reduction with potassium iodide on the quality of slides; test with one batch of slides pre-treated with potassium iodide and another batch from the same sets of people not pre-treated (reduction with potassium iodide is supposed to reduce background staining).
- Conduct test run to check effect of temperature: stain different bacthes using different time interval to obtain the optimum staining time. Standard is 30 minutes at room temperature, but with every one-degree increase rise in baseline room temperature, the duration of staining can be reduced .
- Try different brands of gelatin; brands made from bovine - type b gives the least black precipitate.

Risk assessment

Aim

Silver nitrate reagents is hazardous; the aim is to describe safety precautions to be observed during the procedure in order to minimize this risk.

Hazard statement

Silver nitrate solution may intensify fire, protect the work area with paper towels, and keep away from combustible materials, heat, hot surfaces, sparks, open flames, and other ignition sources. No smoking.

- May be corrosive to metals, keep only in original container.
- It is very toxic to aquatic life with long lasting effects and should not be released to the environment. It should be disposed of in accordance with national regulations.

Precautions

Causes severe skin burns and eye damage, general safety procedure should include use of relevant personal protective equipment such as gloves, safety glasses and a laboratory coat or gown when handling.

- Do not breathe dust.
- Wash hands thoroughly after handling.
- If contact with skin or hair, remove all contaminated clothing immediately, rinse skin with water.
- If contact with the eyes, rinse cautiously with water for several minutes.
- If swallowed, rinse mouth. Do not induce vomiting.
- If inhaled, remove person to fresh air and keep comfortable for breathing.
- Immediately call a poison centre/ doctor

Appendix 23: Standard operating procedure staining for HJB red cells.

Purpose

To describe the process of staining peripheral blood film using a May Grunwald Giemsa (MGG) stain for the identification of Howell-Jolly body containing red cells.

Principle

Howell–Jolly bodies are basophilic DNA remains from the nucleus of the erythrocyte precursor cell. Normally, the spleen clears the erythrocyte of these nuclear remnants or removes the erythrocytes from the circulation, but when the spleen is absent or has a decreased function, these Howell–Jolly body-containing erythrocytes remain in the circulation (William and Corazza, 2007; Corazza *et al.*, 1990). Therefore, the number of erythrocytes containing HJBs can used to assess the function of the spleen.

Materials/Reagents

- Giemsa
- May Grunwald stain
- Filter paper
- Methanol
- Distilled water
- Beaker
- Glass slides
- Pipette
- Microscope

Staining reagent

1. May-Grünwald stain diluted with an equal volume of distilled water
2. Giemsa stain diluted with 9 volumes of distilled water.

Method

Aliquot of blood from an EDTA sample can be used for making the blood smear.

Preparation of blood smears

See appendix 22 above.

- Air dry the film.
- Fix the thin film by dipping in absolute methanol for a few seconds and then letting the slide air dry.
- Label the slides with the subject ID, initials and date one side of the slide.

Staining procedure

- Add May-Gruenwald and stain for 5 minutes and wash in 2 to 3 changes of distilled water.
- Add Giemsa stain and allow to stain for 10 minutes before washing in 2 to 3 changes of distilled water.
- Wash slides with distilled water and allow to dry upright as this prevents residue from forming on the slides.
- Stored in a cool, dry place within a sealed box with ample desiccant.

Estimation of HJB containing red cells

- Using the oil immersion objective, identify a field that contains red cells in monolayer.
- Using a multiparameter counter tally, count the total number of red cells with distinct inclusions and normal red cells per field simultaneously.
- Count a minimum of 400 red cells and express the number of HJBs as a percentage.
- Repeat the process in another area of the film and take the mean of the two results.
- The range of HJBs count obtained from normal subjects will be used to determine the upper and lower limits and used subsequently to determine whether the level of HJBs counts in the study patients is normal, reduced or increased.

Appendix 24: Standard operating procedure for blood culture

Purpose

To describe the procedure for isolating and identifying pathogenic microorganism from the blood stream of febrile patients

Principle

Culture of blood specimen is the gold standard procedure for the identification of bacterial causing infection. Blood stream infection may be caused by pathogenic gram negative or gram-positive bacteria. Infrequently, contamination of the blood stream by normal commensals on the skin may occur during blood collection procedures. The type of organism is also influenced by the patients age, immune status, and presence of co-morbidities. Example of some gram-positive organisms causing bacteraemia include Staphylococcus aureus, Streptococcus pneumoniae, S. pyogenes (group A) and Enterococcus spp. While example of some gram-negative bacteria includes Escherichia coli, Klebsiella spp, Salmonella spp. (including S. Typhi), Pseudomonas aeruginosa, Neisseria meningitidis and Haemophilus influenzae.

Method

Collection of blood sample

Two sets of culture bottle are required for each patient.

a) Label the culture bottles with subject ID, subject initials, date, and time of blood collection.
b) Select an arm and apply a tourniquet to restrict the flow of venous blood. Select the most prominent vein for venepuncture.
c) Vigorously wipe the skin with 70% alcohol swab

d) Follow with iodine tincture or povidone-iodine swab over the selected area and allow to dry.

e) Insert the needle into the vein using a Bactec butterfly needle

f) Obtain an adequate amount of specimen: 3 ml for children and 10 ml for adults (NB: the minimum amount specified by the manufacturer will be used to guide the volume for both the paediatric and adult's patient in view of their low baseline haematocrit)

g) Disinfect the rubber septum of both blood culture bottles with 70% ethanol prior to inoculation.

h) The blood is drawn directly into the culture bottle.

i) Release the tourniquet and place a sterile cotton ball over the insertion site while holding the needle in place.

j) Withdraw the needle and have the patient hold the cotton ball firmly in place until the wound has stopped bleeding.

Specimen transportation

After inoculation, bottles should be transported to the microbiology laboratory without delay, in a sealed plastic bag or rigid specimen container. Aim to process the blood samples in a bacteriology laboratory as soon as possible after collection.

Blood culture procedure

The inoculated culture bottles are incubated at 37°C in air and inspected daily for 7 days for any colour change on the detector at the base of the bottle. If there is no colour change in the detector, blind sub-culture will be performed at day 5 and day 7 to detect early and late growth, respectively.

Blind sub-culture on day 5 and 7

- Remove the blood culture bottle (s) from the incubator.
- Mix the broth gently using a swirling motion.
- Sterilise the rubber top of the bottle with 70% alcohol.
- Using a sterile needle obtain culture broth.
- Allow a single drop of broth to inoculate the sub-culture media: at day 5, inoculate a blood (BA), chocolate (CA), and MacConkey (MAC) agar plates; at day 7, inoculate a CA agar plate only.
- Streak the inoculum out using a sterile microbiological loop to obtain single colonies.
- Incubate the plates for up to 48 hours at 37°C in air (BA / MAC) or 5-10% CO_2 (CA).

Processing of positive culture

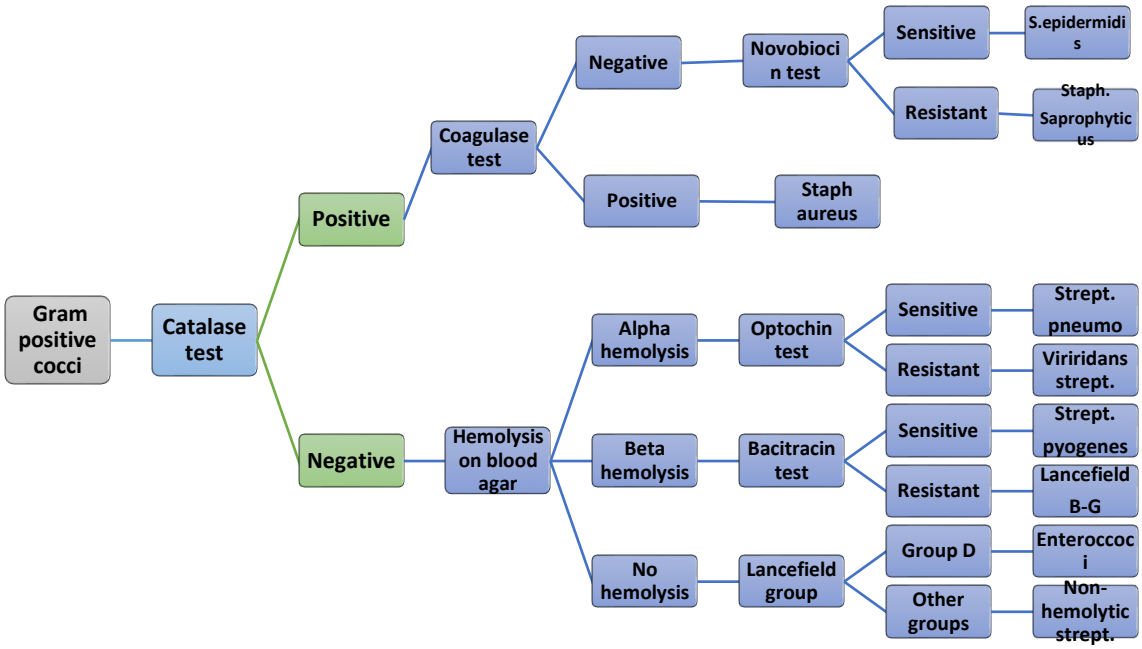
1. Perform a Gram stain.

Bacterial identification

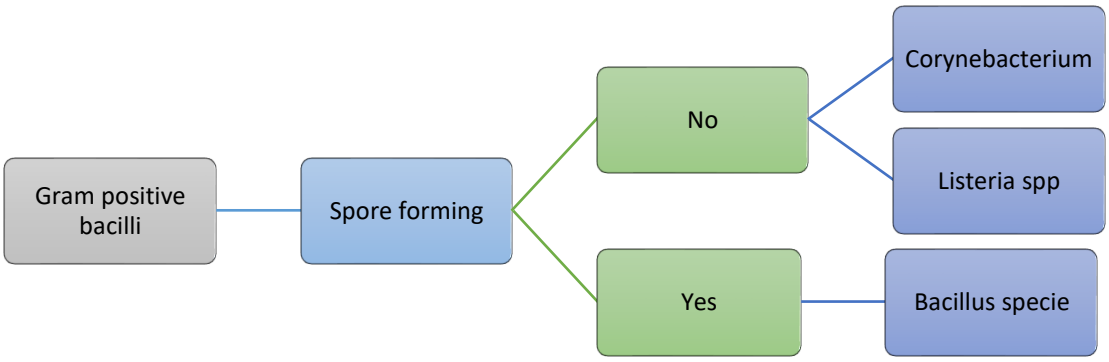
- Clinically significant isolates should be identified to species level using microbiological properties and biochemical test described below.
- Any organism considered to be a contaminant may not require further identification.
- Discard plates after seven days of incubation
- Store significant isolates at -80°C in a microbank bead containing glycerol and record the isolate details in the result logbook and database.

Gram positive bacteria

Gram positive cocci flow chart

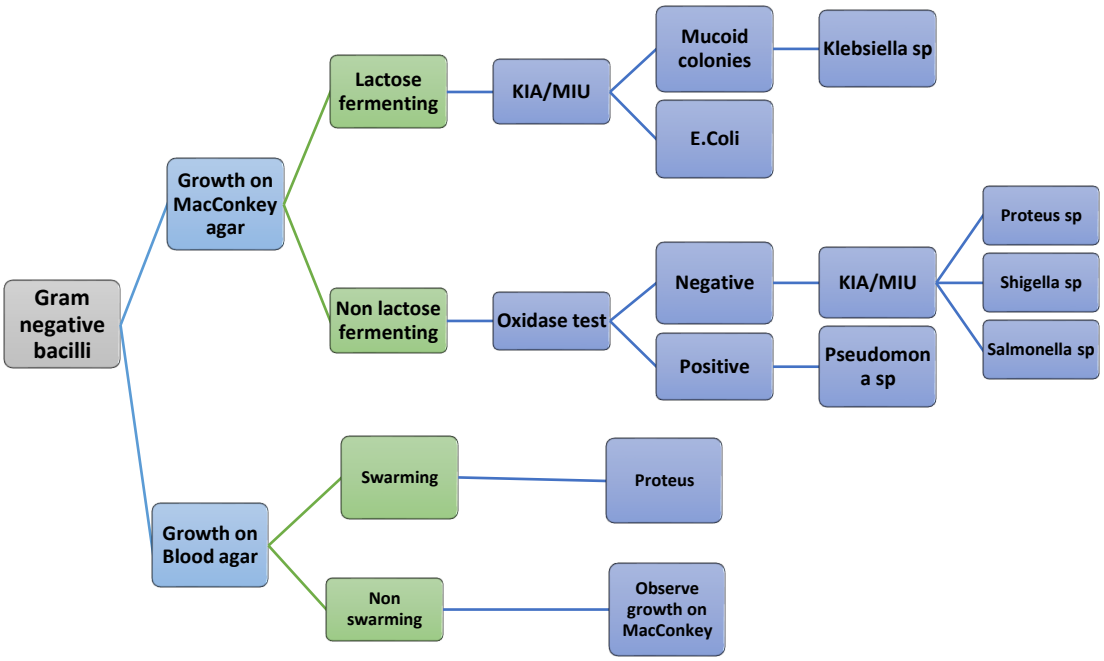


Gram positive bacilli flow chart



Gram negative bacteria

Gram negative bacilli flow chart



Gram negative Coccobacilli and Diplo-Cocci flow chart

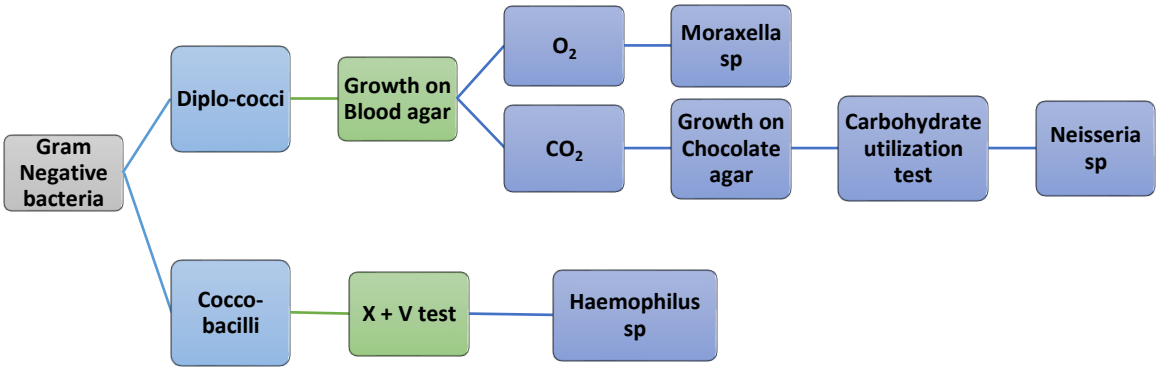


Table 1: Identification of Gram-negative organisms

Organism	Environment	Growth on BA	Growth on MAC	MIU			KIA				Oxidase
				Motility	Indole	Urea	H_2S	Glucose	Lactose	Gas	-
E.Coli	O_2	Grey moist	LF	+	+	-	-	+	+	+	-
S.Sonnei	O_2	Grey moist	NLF	-	-	-	-	+	-	-	-
Salmonella	O_2	Grey moist	NLF	+	-	-	+/-	+	-	+/-	-
Proteus	O_2	Swarm	NLF	+	-	+	+	+	-	-	-
Pseudomonas	O_2	.	NLF green	.	.	.	.		.	.	+
Klebsiella	O_2	Mucoid	LF Mucoid	-	-	+/-	-	+	+	+	-

LF: Lactose fermenters; NLF: Non Lactose fermenters; BA :Blood agar; MAC: MacConkey

Reporting

- Inform managing clinician of all positive results.

Quality control

- Media and identification tests should be quality controlled according to the relevant SOP

Limitations

Several factors may result in a false negative blood culture, including:

- Exposure to antimicrobial agents prior to blood sampling: a detailed drug hx should be obtained from all patients.
- Aim to collect blood culture sample prior to commencement of antibiotics in the hospital.
- Inoculation of an inadequate volume of blood: the minimum amount appropriate for each age group should be collected.
- Intermittent bacteraemia sampled at the incorrect time point; collect two blood culture specimens using two sites if feasible.

Materials

- Alcohol swab (70%), dry swab
- Tincture of iodine
- Syringe and needle: 10 ml (Children), 10 or 20 ml (adults)
- Tourniquets
- Gloves
- BACTEC aerobic blood culture bottles (Paediatric, Adult)

Appendix 25: Standard operating procedure for microscopy detection of malaria parasites

Purpose

To describe the procedure for preparing and staining blood smears for the detection and estimation of malaria parasites using microscopy.

Principle

Examination of thin and thick peripheral blood film is a standard method for detecting the malaria parasites. The thick film contains a large amount of de-haemoglobinase red cells and helps in quantification of parasite density. The thin film consists of evenly spread monolayer of red cells which helps in identifying the type of malaria specie.

Materials

1. Stock Giemsa solution
2. Buffered water pH 7.2,
3. Micropipette (1- 10 µL)
4. Slide staining rack
5. Grease free glass slides
6. Timer
7. Wash bottle
8. Slide drying rack
9. Spreader slide
10. Absolute methanol
11. Light microscope
12. Tally counter
13. Immersion oi

Method

Collection of blood sample

Aliquot of blood from an EDTA sample can be used for making the blood smear. A capillary blood sample can also be used.

Preparation of blood smears

- Place 2 to 3 microliter of blood near one end of the glass slide.
- Using a second slide to spread, draw the spreader backwards into the drop of blood at 45 degree or more depending on the haematocrit,
- Push forward in one quick movement. This should produce a film that is tongue shaped, with a base, body, and tip.
- Air dry the film.
- Label the slides with the subject ID, initials and date one side of the slide.

Staining procedures

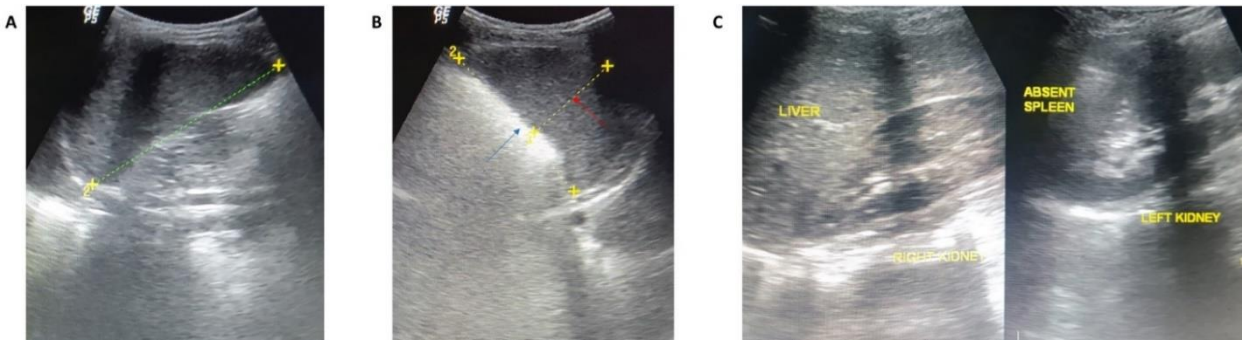
- Fix the thin film by dipping in absolute methanol for a few seconds and then letting the slide air dry.
- Cover blood films completely with freshly prepared 3% v/v Giemsa solution.
- Stain the slides for 15 minutes.
- Rinse each slide with buffered water.
- Allow the slides to dry completely.
- Stored in a cool, dry place within a sealed box with ample desiccant.

Malaria parasite quantification

Using the oil immersion objective, identify a field that contains red cells in monolayer.

- Using a multiparameter counter tally, count the total number of red cells containing asexual stages of the parasites and unparasitized red cells per field simultaneously.
- Count a minimum of 500 red cells and express the parasitemia as a percentage.
- Repeat the process in another area of the film and take the mean of the two results.

Appendix 26: Obtaining splenic dimensions on ultrasonography



A) Longitudinal view of the spleen; the spleen length is measured as the distance between the most superior margin and inferior margin (indicated by the callipers). B) Transverse view of the spleen. The spleen depth is measured as the maximal distance between the medial and lateral borders (red arrow). The spleen width is measured as maximal anterior to posterior diameter (blue arrow). C) Autosplenectomy is present when the spleen is not visible on scan in the absence of a surgical removal

Appendix 27: Ethics approval from LSTM

LSTM
LIVERPOOL SCHOOL OF TROPICAL MEDICINE
Since 1898

Pembroke Place,
Liverpool, L3 5QA, UK
Tel: +44(0)151 705 3100
Fax: +44(0)151 705 3370
www.lstmed.ac.uk

Dr Adama Isah Ladu
Liverpool School of Tropical Medicine
Pembroke Place
Liverpool
L3 5QA

Monday, 01 June 2020

Dear Dr Ladu,

Re. Research Protocol (20-050) 'Evaluation of spleen size and function: its relationship with malaria and bacterial infection among sickle cell anaemia patients in North-East Nigeria'

Thank you for your letter of 18 March 2020 providing your response to Action Point Letter and associated approvals for this project. I can confirm that the protocol now has formal ethical approval from the LSTM Research Ethics Committee, noting that the study should not commence until 1 July 2020, in line with Letter of Approval from University of Maiduguri Teaching Hospital dated 10 February 2020.

The approval is for a fixed period of three years and will therefore expire on 30 June 2023. The Committee may suspend or withdraw ethical approval at any time if appropriate.
Approval is conditional upon:

- Continued adherence to all in-country ethical requirements.
- Notification of all amendments to the protocol for approval before implementation.
- Notification of when the project actually starts.
- Provision of an annual update to the Committee.
  Failure to do so could result in suspension of the study without further notice.
- Reporting of new information relevant to patient safety to the Committee.
- Provision of Data Monitoring Committee reports (if applicable) to the Committee.

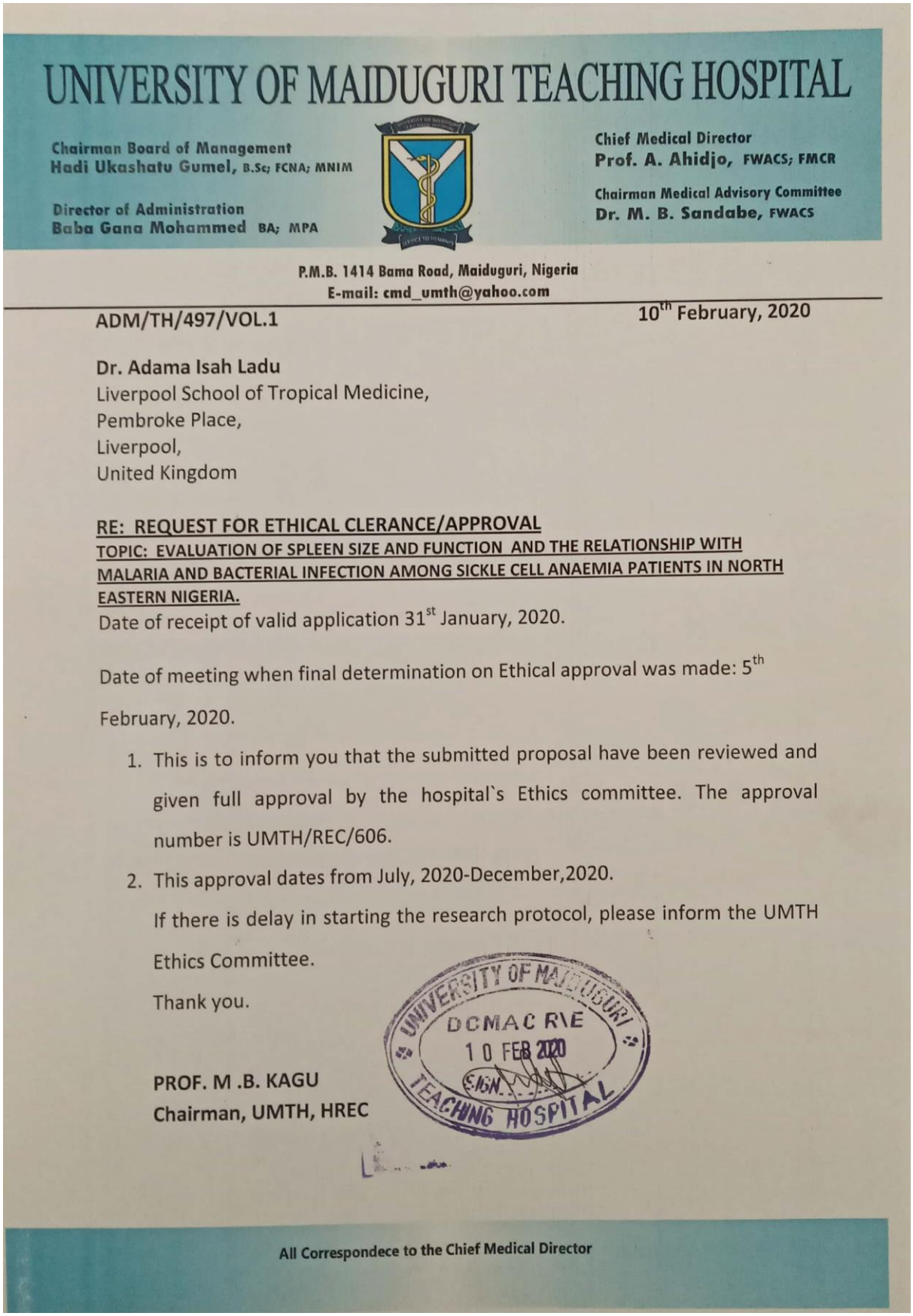
Failure to comply with these requirements is a breach of the LSTM Research Code of Conduct and will result in withdrawal of approval and may lead to disciplinary action.

The Committee would also like to receive copies of the final report once the study is completed. Please quote your Ethics Reference number with all correspondence.

Yours sincerely,

Professor Graham Devereux
Chair
Research Ethics Committee

Appendix 28: Ethics approval from UMTH



UNIVERSITY OF MAIDUGURI TEACHING HOSPITAL

Chairman Board of Management
Hadi Ukashatu Gumel, B.Sc; FCNA; MNIM

Director of Administration
Baba Gana Mohammed BA; MPA

Chief Medical Director
Prof. A. Ahidjo, FWACS; FMCR

Chairman Medical Advisory Committee
Dr. M. B. Sandabe, FWACS

P.M.B. 1414 Bama Road, Maiduguri, Nigeria
E-mail: cmd_umth@yahoo.com

ADM/TH/497/VOL.1

10th February, 2020

Dr. Adama Isah Ladu
Liverpool School of Tropical Medicine,
Pembroke Place,
Liverpool,
United Kingdom

RE: REQUEST FOR ETHICAL CLERANCE/APPROVAL
TOPIC: EVALUATION OF SPLEEN SIZE AND FUNCTION AND THE RELATIONSHIP WITH MALARIA AND BACTERIAL INFECTION AMONG SICKLE CELL ANAEMIA PATIENTS IN NORTH EASTERN NIGERIA.

Date of receipt of valid application 31st January, 2020.

Date of meeting when final determination on Ethical approval was made: 5th February, 2020.

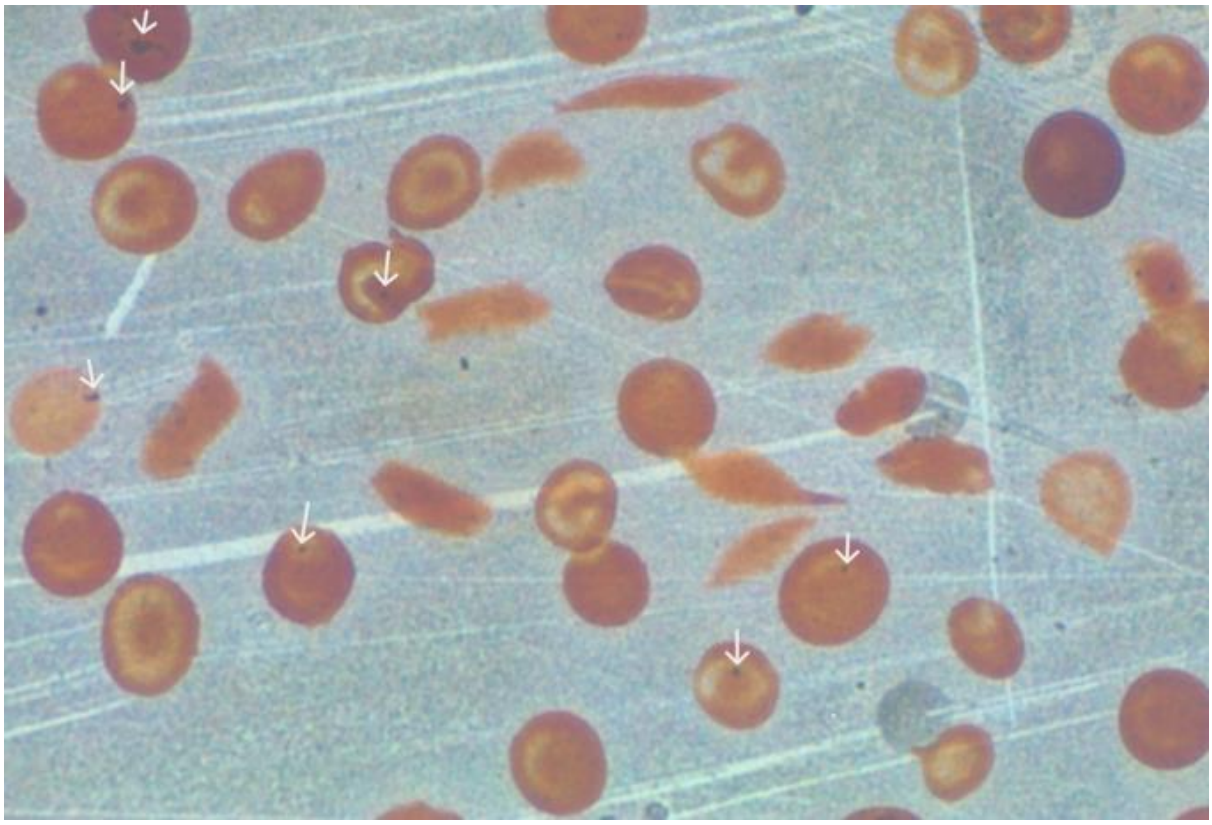
1. This is to inform you that the submitted proposal have been reviewed and given full approval by the hospital`s Ethics committee. The approval number is UMTH/REC/606.
2. This approval dates from July, 2020-December,2020.
   If there is delay in starting the research protocol, please inform the UMTH Ethics Committee.
   Thank you.

UNIVERSITY OF MAIDUGURI TEACHING HOSPITAL
DCMAC R\E
10 FEB 2020
SIGN

PROF. M .B. KAGU
Chairman, UMTH, HREC

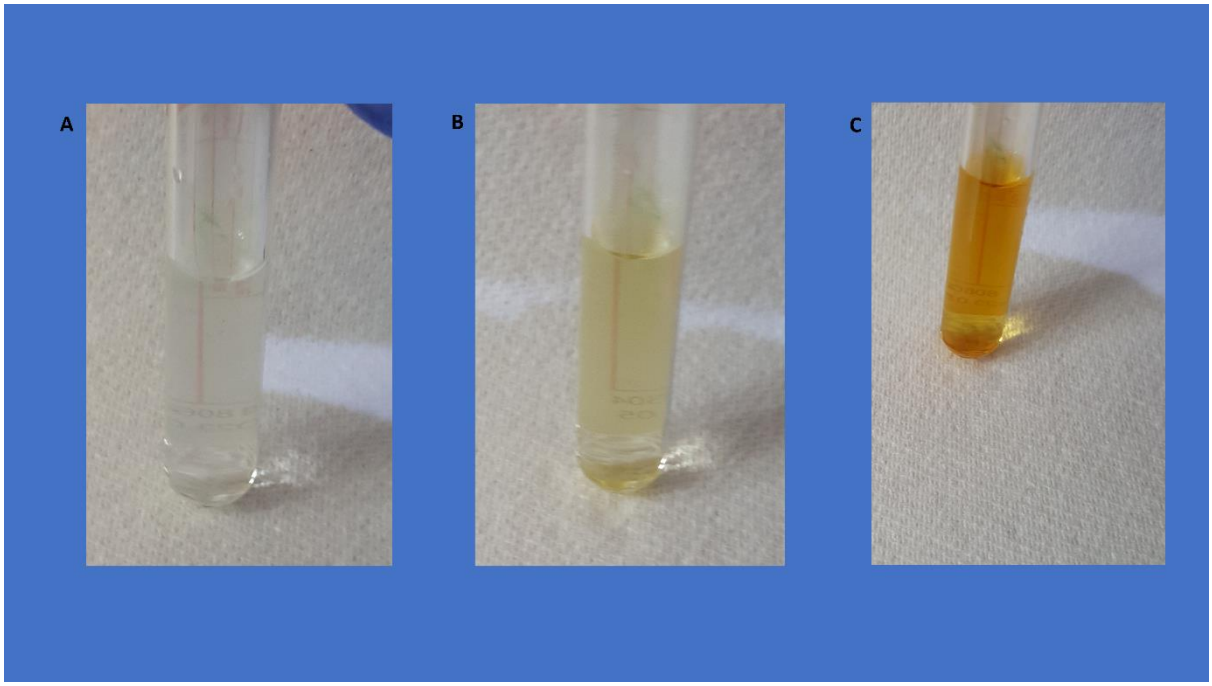
All Correspondece to the Chief Medical Director

Appendix 29: Overstained argyrophilic inclusion containing red cells smear



Effect of prolonged staining of slide. Silver-stained blood smear (x100/0.80) from a 22-year-old female HbSS patient showing deeply stained red cells (white arrows) making visualization of some of the argyrophilic inclusions difficult.

Appendix 30: Argyrophilic inclusion method staining time



Silver stain reaction time. A) Silver stain at 1 minute. The preparation appears clear and colourless. B) The preparation has begun to take a yellow colouration. C) The preparation has turned golden yellow and continues deteriorating on further standing

Appendix 31: List of publications / abstracts

A. List of publications during PhD

Published

a. The spectrum of splenic complications in patients with sickle cell disease in Africa: a systematic review

b. Evaluation of two red cell inclusion staining methods for assessing spleen function among sickle cell disease patients in North-East Nigeria

c. Determinants of Splenic Preservation among Patients with Sickle Cell Disease in North-Eastern Nigeria.

d. Clinical and Laboratory Factors Associated with Splenic Dysfunction Among Sickle Cell Disease Patients. Submitted to 'Transactions of the Royal Society of Tropical Medicine and Hygiene'. Revised version submitted. Awaiting Journal response.

e. Bacteraemia among Patients with Sickle Cell Disease in Nigeria: association with spleen size and function. Submitted to 'Mediterranean Journal of Haematology and Infectious Disease' . Revised version submitted. Awaiting Journal response

2. Under review

a. Preprint: Ultrasonographic assessment of spleen size Among Sickle Cell Disease patients and normal control in North Eastern in Nigeria. Submitted to Ultrasound; awaiting Journal response.

b. Preprint: Malaria Infection in Patients with Sickle Cell Disease in Nigeria: Association with Markers of Hyposplenism . Submitted to Hemoglobin. Awaiting Journal response.

B. Abstracts submitted for conference presentations during PhD

- Spectrum of splenic complications in sickle cell disease patients in Africa: A Systematic Review. Presented at the Liverpool School of Tropical Medicine Annual Postgraduate Research and Scientific conference. June 21 – 22, 2021

- Ultrasonographic splenic Indices and Age-Related Clinical and Laboratory Parameters in patients with sickle cell disease in North-Eastern Nigeria. Presented at the Annual Sickle cell disease and Thalassemia conference. Oct 20-22, 2022.
- Evaluation of Red cell Inclusions for Assessing Spleen Function Among Sickle Cell Disease Patients. Presented at the British Society of Haematology 63rd Annual Scientific meeting. April 23-25, 2023.
- Determinants of Splenic Preservation among Patients with Sickle Cell Disease in North-Eastern Nigeria. Presented at the British Society of Haematology 63rd Annual Scientific meeting. April 23-25, 2023
- Clinical and Laboratory Factors Associated with Splenic Dysfunction Among Sickle Cell Disease Patients. Presented at the British Society of Haematology 63rd Annual Scientific meeting. April 23-25, 2023