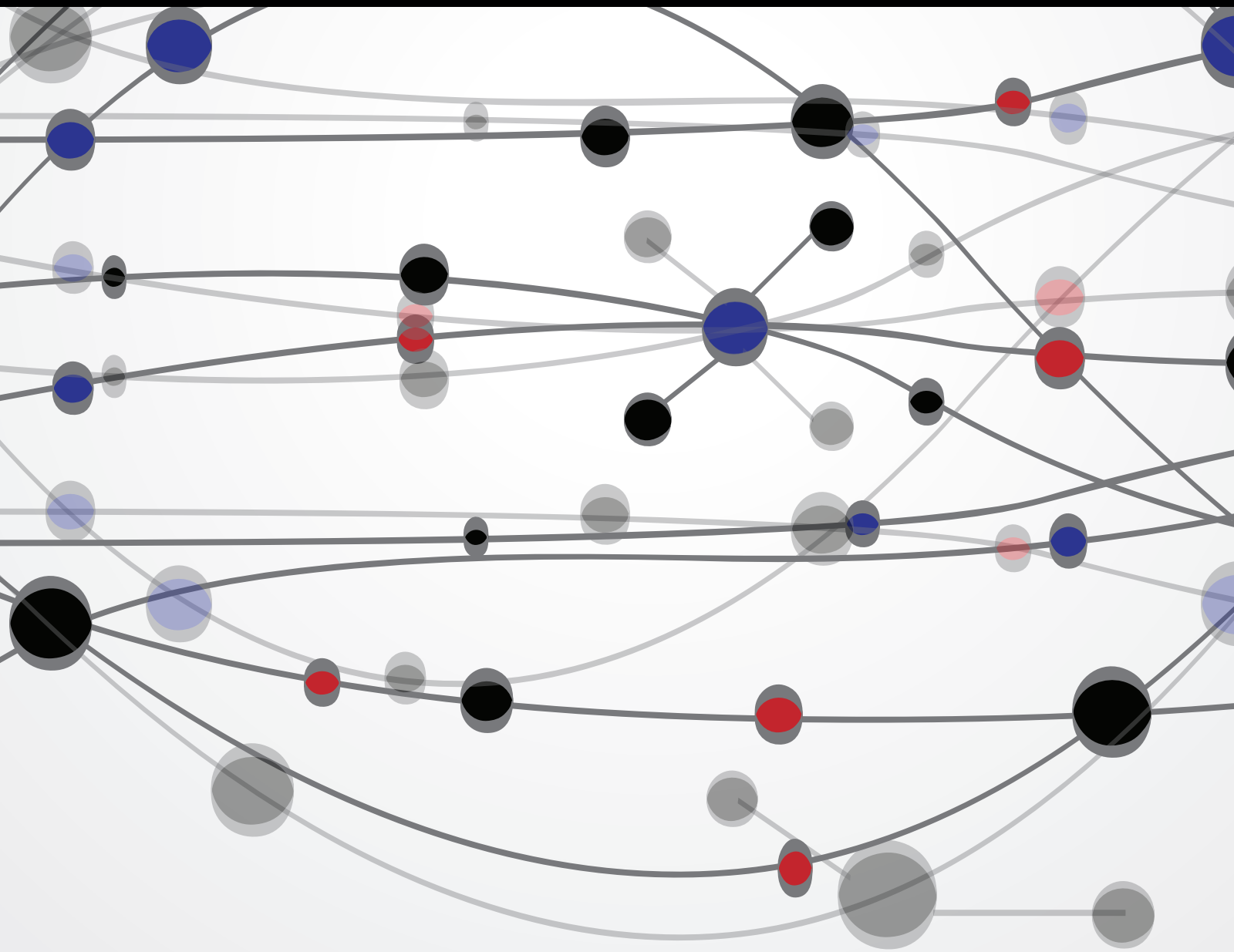
The Scientific World Journal

Updates in Hemoglobinopathies

Guest Editors: Youssef Al-Tonbary, Fernando Tricta, Amal El-Beshlawy, Mohamed Ahmed Badr, and Ahmed Mansour



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Editorial

Updates in Hemoglobinopathies

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I am honored to invite you to enjoy reading this special issue of The Scientific World Journal.

One review article is "*Ineffective erythropoiesis in β-thalassemia*." The authors concluded that this ineffective erythropoiesis could be the conjunction of several mechanisms of which the final consequence is the arrest of maturation and increased apoptosis of erythroblasts during their terminal differentiation stage.

Another review article discusses the biologic complexity in sickle cell disease. This complexity is likely to be one of the major barriers to the development of successful new treatments which, to date, has largely concentrated on individual mechanistic pathways. Future development of therapeutics needs to continue.

Another review article titled "*Phytomedicines and nutraceuticals: alternative therapeutics for sickle cell anemia*" highlights the feasibility of botanicals, mainly antisickling phytomedicines and nutraceuticals, as attractive potential candidates for sickle cell anemia therapy and strongly collaborates the ethnomedical usage of the plants.

A peer-reviewed original article was carefully selected from many articles submitted to the journal. It studied the impact of migrations on the health services for hemoglobin disorders in Europe. Its results show that countries with traditional strong prevention and treatment programs are well prepared to face these challenges, while others are urgently needed to address these problems in a systematic way.

More articles will be published in this special issue. On behalf of the guest editors of this special issue of The Scientific World Journal, I look forward to receiving your comments and any suggestions you may have.

Youssef Al-Tonbary
Fernando Tricta
Amal El-Beshlawy
Mohamed Ahmed Badr
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Review Article

Sickle Cell Disease: New Opportunities and Challenges in Africa

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Sickle cell disease (SCD) is one of the most common genetic causes of illness and death in the world. This is a review of SCD in Africa, which bears the highest burden of disease. The first section provides an introduction to the molecular basis of SCD and the pathophysiological mechanism of selected clinical events. The second section discusses the epidemiology of the disease (prevalence, morbidity, and mortality), at global level and within Africa. The third section discusses the laboratory diagnosis and management of SCD, emphasizing strategies that been have proven to be effective in areas with limited resources. Throughout the review, specific activities that require evidence to guide healthcare in Africa, as well as strategic areas for further research, will be highlighted.

1. Introduction

Sickle cell disease (SCD) consists of a group of disorders characterised by the presence of sickle haemoglobin. Although over 700 structural hemoglobin (Hb) variants have been identified, only two (Hb S, Hb C) reach high frequencies in Africa. The common SCD syndromes in this region include homozygous HbSS disease (HbSS) commonly known as sickle cell anaemia (SCA) and Hb SC disease. SCD was known in some parts of Africa before the twentieth century: inhabitants of western Africa gave the disease-specific names that evoke acute, painful episodes or death or refer to children destined to die and to be reborn as their own siblings [1, 2]. Africa is the major origin of the sickle (β^S) mutations [3]. There are four chromosomal haplotypes that are associated with the β^S mutation. They are named after the regions where they have the highest frequency: Benin, Senegal, Bantu (Central African Region (CAR)), and Arab-Indian. The haplotypes are defined by restriction fragment length polymorphisms (RFLPs) in the β-globin locus. Due to the population specificity of the haplotypes, it is believed that the sickle cell mutation arose independently in these populations and remained to this day [4].

1.1. Normal Human Hemoglobin. Human Hb is encoded by a cluster of genes located on chromosomes 11 and 16 that are expressed in a developmentally regulated manner. They are tetramers of two pairs of α-like and β-like globin chains. Adult and fetal hemoglobin have $\alpha\beta$(Hb A, $\alpha_2\beta_2$), δ(Hb A2, $\alpha_2\delta_2$), or γ chains (Hb F, $\alpha_2\gamma_2$), whereas in the embryo, α-like chains—termed ζ_γ (Hb Portland, $\zeta_2\gamma_2$) or $\varepsilon\,\zeta_2\varepsilon_2$—and α and ε chains form Hb Gower 2 ($\alpha_2\varepsilon_2$) (Figure 1) [5].

Embryonic hemoglobin production is confined to the yolk sac. Thereafter the major site of synthesis is the fetal liver. HbF is the predominant type of hemoglobin in fetal life, but around birth there is a switch from fetal to adult globin gene expression, when HbF is gradually replaced by adult hemoglobin, such that by 6 months of age the major Hb is HbA ($\alpha_2\beta_2$). Residual amounts of HbF, however, continue to be synthesized throughout adult life, and the amounts vary

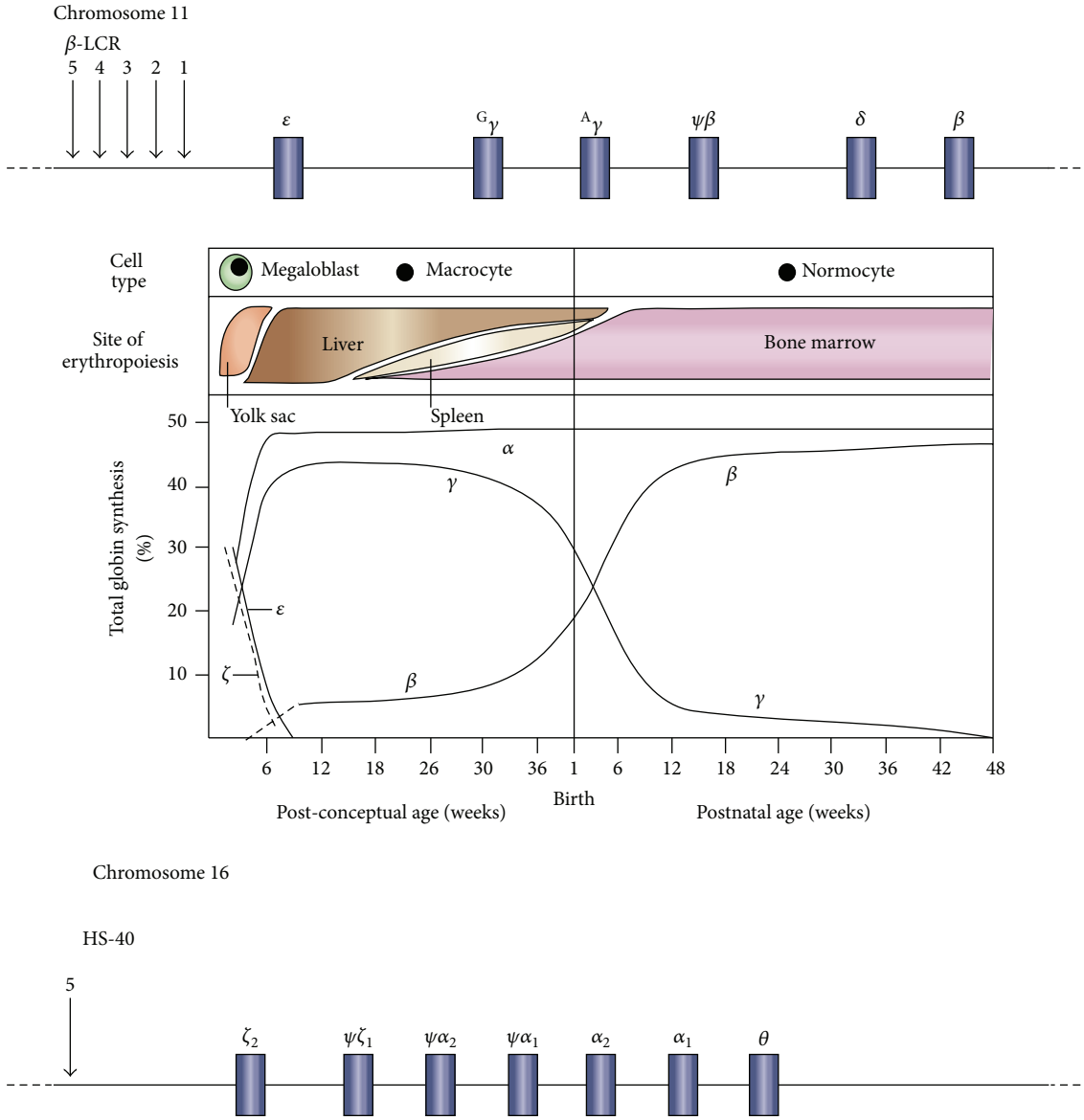

Figure 1: Developmental control of human haemoglobin (Hb) expression [6].

considerably, with the majority of adults having less than 1% HbF.

1.2. Pathophysiology of Clinical Events. Sickle haemoglobin (HbS) results from a substitution of one amino acid (Valine) for another amino acid (Glutamic acid) at position six of the β-globin polypeptide chain. This substitution is caused by a single-base mutation in codon 6 within the β-globin gene on chromosome 11, where the sequence GAG occurs instead of GTG.

Due to the abnormal amino acid in the β-globin chain, HbS forms long, insoluble polymers when deoxygenated, and the red blood cells (RBCs) containing HbS become less deformable and form a "sickle" shape. It was previously thought that the clinical consequences were simply due to this abnormal, rigid sickle red blood cell occluding small blood vessels. However, there is increasing evidence that the pathogenesis of the various clinical events, both acute and chronic, results from a series of complex mechanisms which are not limited to the RBC [7]. These relate to concentration of HbS and other haemoglobin variants such as HbF within the cell which reduces its ability to polymerise [8], disturbances in the red cell membrane making the cell less responsive to oxidant stress, and altered membrane lipids resulting in increased rigidity [9–11]. Additionally, adhesion molecules such as integrins ($\alpha_4\beta_1$), ($\alpha_v\beta_3$), their receptors (VCAM-1, ICAM-4), selectins interact with endothelial cells, RBC, and a variety of soluble proteins within the plasma, such as thrombospondin (from platelets) and von Willebrand factor from endothelial cells to mediate vasoocclusion within the macro- and microvasculature [12–16]. Finally there is compelling evidence of the role of nitric oxide (NO) in SCD [17]. NO is a potent regulator of basal vasodilator tone. It also inhibits the expression of cellular adhesion

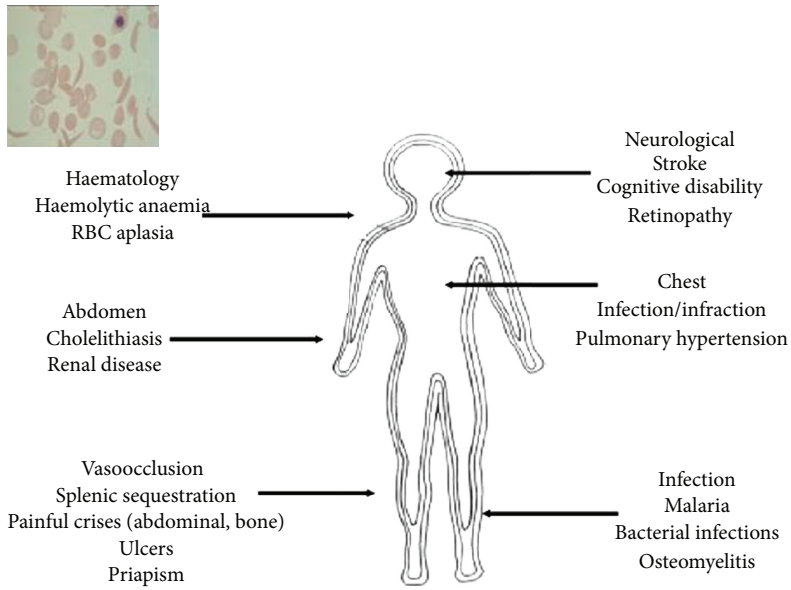

Figure 2: Selected clinical consequences of SCD.

molecules [18]. The increase in haemolysis in SCD results in an excess of haemoglobin in the plasma, which exceeds the scavenging capacity of haptoglobin. The result is that there is abnormal "cell-free" haemoglobin, which circulates in plasma, binding to and consuming NO, so causing a reduction in the concentration of NO [19]. This results in vasoconstriction, increased adhesiveness of erythrocytes, leukocytes and endothelial cells, and platelet aggregation.

1.3. Clinical Events in SCD. Although SCD stems from an abnormality of the RBC, it is essentially a multisystem disorder, affecting almost every organ system of the body, as shown in Figure 2. The clinical consequences can be divided into 4 groups: haemolysis and haematological complications, vasoocclusion, infection, and organ dysfunction.

2. Haemolysis and Haematological Complications

At birth, individuals with SCD do not have anaemia, but with the synthesis of adult Hb, they develop chronic haemolytic anaemia that is present throughout life. This may be interspersed with acute episodes of reduction in haemoglobin "anaemic crises". Hyperhemolysis crises are defined by a sudden fall in steady state haemoglobin accompanied by increased reticulocytosis and exaggerated hyperbilirubinemia. The chronic haemolysis in SCD may result in gall bladder disease due to high levels of bilirubin. Although the main cause of anaemia in SCD is chronic haemolysis, there are other types of anaemia that may occur. Acute splenic sequestration, when there is rapid onset of trapping of red blood cells in the spleen, is characterised clinically by a sudden increase in splenic size, at least 2 cm below the left coastal margin, accompanied by a reduction in haemoglobin or haematocrit by 20% of baseline level. This has been described in SCD and is a significant cause of mortality [20]. Anaemia may be secondary to infections such as malaria, bacterial and viral diseases. Of the latter, RBC aplasia in the bone marrow has been notably described and has been associated with infection with parvovirus serotype B19 [21].

3. Vasoocclusion

Vasoocclusion (VOC) is thought to be the underlying cause of painful crises, acute splenic sequestration, and priapism (painful and prolonged penile erection). Painful crises, considered the hallmark of SCD, are defined as severe pain lasting for 2 or more hours that is attributable to SCD. The sites that are normally affected include the arms, legs, back, abdomen, chest, and head. Painful crises do not include other causes/types of pain in SCD such as dactylitis, acute chest syndrome, right upper quadrant syndrome, osteomyelitis, and appendicitis. It is the most common cause of hospitalisation and frequent pain (defined as 2 or more painful events a year for three years) is associated with poor quality of life and increased risk of death [22].

4. Infection

Individuals with SCD are reported to be susceptible to infections with encapsulated organisms such as *Streptococcus pneumoniae* [23–25]. The use of oral penicillin in the USA had a significant impact on reduction in mortality [26], and it is now policy in many high-income countries to give penicillin prophylaxis and antipneumococcal vaccination to SCD patients [27]. It was previously thought that the situation in Africa may be different. Aside from the fact that the data regarding the clinical spectrum of SCD are limited, there was controversy regarding the role and significance of pneumococcal disease in causing morbidity and mortality in SCD in this setting [28]. However, there is

emerging evidence to confirm that pneumococcal disease is a significant cause of bacteraemia in SCD [29], with calls to introduce interventions for preventing infections as a critical factor in improving survival [30, 31]. The various factors that are associated with increased infections in SCD may be directly related or unrelated to the immune system. Some infections may be the result of a complication or treatment of SCD itself. SCD patients are at high risk of transfusion-transmissible infections particularly with human immunodeficiency virus and viral hepatitis since they receive frequent, often unplanned emergency blood transfusion (BT) [32–35]. This is particularly important in Africa, given the high prevalence of HIV infection and the operational problems in providing adequate blood-transfusion services. Long-term BT may result in iron overload, which in itself is associated with infections due to *Yersinia Enterocolitica* [36]. SCD causes end-organ damage to the lung, liver, kidney, and skin, making these sites susceptible to infection by unusual organisms. In addition, skeletal complications, poor perfusion, and blood supply to bone tissue are also thought to contribute to increased susceptibility to infections of the bone, osteomyelitis, which is often due to salmonella infections [37]. Other factors include high bone marrow turnover due to chronic haemolysis which results in increased susceptibility to viral infection. Parvovirus B19 infections are one of the viral infections that predispose to poor outcome with erythrocytic aplasia that may lead to life-threatening anaemia [21, 38, 39]. However, the epidemiology of this virus in Africa is poorly defined [40–42]. Individuals with SCD may have impairment of the immune system, involving both cellular immunity and humoral immunity. The most well-described immune defect is caused by reduced function of the spleen. Patients with SCD have repeated splenic infarction due to vasoocclusion which causes loss of the splenic vasculature leading to hyposplenism [43]. Reports have suggested that 14% patients with SS-SCD are functionally asplenic at 6 months of age, with this number gradually increasing: 28% at 1 year, 58% at 2 years, 78% at 3 years, and by 5 years, 94% are affected [44]. This is from an area without malaria. One of the roles of the spleen is filtration of unopsonised bacteria and remnants of red blood cells from intravascular space as well as opsonised bacteria [45]. Furthermore, the spleen is involved in the synthesis of soluble mediators of immunity. Therefore patients with SCD, with a functional asplenia, have been reported to have impaired antibody responses as well as lacking specific antibodies, particularly against *Salmonella* species and *Streptococcus pneumonia* [46]. This is thought to be due to deficiency of a complement factor involved in the activation of the immune system. The classic pathway is activated by antigen-antibody interaction which causes fixation of complement components C1, C2, and C4 which then activate C3, whereas in the alternate pathway the antigen directly activates C3. Activation of C3, which is an opsonin, results in fixing of antigens on the microorganism [47] making them susceptible to enhanced phagocytosis by neutrophils and monocytes/macrophage. Johnston et al. illustrated that patients with SCD have an abnormality in the activation of this pathway with failure of full activation and fixing of C3 to encapsulated bacteria [48]. This results in failure of SCD patients to phagocytose invading organisms, particularly *Streptococcus pneumoniae*. The distinction between factors directly related to the immune system or not is somewhat arbitrary as there is a lot of overlap between the various factors. Although there have been reports of different patterns of infections in patients with SCD, summarised in Table 1, this review focuses on invasive bacterial infections as detected by blood culture. In the absence of prophylaxis, infections are thought to be the leading cause precipitating clinical events and associated with increased mortality [23, 49].

Table 1: Clinical syndromes and common causative organisms reported in SCD.

Syndrome	Organisms	Reference
Septicaemia	*S. pneumoniae, H. influenza, Salmonella* spp, *E. Coli, S. Aureus*, and *M. Pneumoniae*	[28, 29, 50]
Pneumonia	*S. pneumoniae*, *M. Pneumoniae*, *Chlamydiae pneumonia*	
Meningitis	*S. pneumoniae*	
Osteomyelitis	*Salmonella* spp., *E. Coli*, Gram negative organisms, and *S. Aureus*	[37, 51, 52]
Aplastic anaemia	Parvovirus	[21, 38, 39]
AIDS and Hepatitis	HIV Viral hepatitis B,C	[32, 33, 53]
Abdominal pain	*Helicobacter pylori, Yersinia enterocolitica*	[36]

5. End-Organ Dysfunction

With increase in survival, major organs in individuals with SCD are eventually damaged. The brain and lungs are particularly affected, with stroke, defined as an acute neurological syndrome due to vascular occlusion or haemorrhage in which symptoms and signs last for more than 24 hours, being a well-described event. Acute chest syndrome (ACS) is an acute respiratory illness characterised by new pulmonary infiltrates on chest X-ray and falling arterial oxygen saturation [54, 55]. Both these events have been reported to occur with high prevalence in SCD and are also risk factors for death [23, 55, 56].

6. Heterogeneity of Clinical Events in SCD

The clinical expression of SCD is heterogeneous (Table 2). There is interindividual variability ranging from near complete asymptomatic to severe debilitating illness. There is also variability within an individual, with changes in the type and frequency of clinical events with age. Finally, there is variability in clinical events depending on the geographical location. This is due to the differences in environmental factors such as nutrition, socioeconomic status, and climate that will influence the natural history of disease. The general pattern of clinical disease is characterised by quiescent

Table 2: The prevalence of selected clinical consequences of SCD.

Clinical event	Prevalence	References
Haemolysis		
Anaemia	Chronic	[57–59]
Cholelithiasis	Prevalence is 40% by adolescence	[60, 61]
Aplastic anaemia	Associated with parvovirus B19 infection	[61–63]
Hyperhemolysis	Limited reports from Africa	[64–67]
Vasoocclusion		
Pain	More than 60% patients Most common cause of admission Frequent pain is a risk factor for mortality	[22, 23, 68, 69]
Acute splenic sequestration (ASS)	Frequently occurs before the age of 3 yrs	[23, 70, 71]
Leg ulcers	Prevalence is 10–25% adults	[72, 73]
Priapism	Prevalence is 10–40% males Occurs frequently in 5–14 years age group	[74]
Organ dysfunction		
Neurological events		
Stroke	Prevalence is 10% in children risk factor for mortality High rate of recurrence Leads to poor quality of life	[75]
Cognitive/silent	Prevalence is 20% Risk factor for overt stroke Leads to impairment of executive function	[76–79]
Retinopathy	Prevalence is >30% in HbSC	[80]
Chest		
Acute chest syndrome (ACS)	Prevalence is 40% Occurs frequently in children Has severe consequences in adults 12.8 per 100-patient years 59	[54–56]
Pulmonary hypertension	Prevalence is 30% Risk factor for mortality	[79, 81–84]
Avascular necrosis of femoral head	Prevalence is 10–50% in adults	[85–87]
Renal disease	Prevalence of chronic renal failure is 5%–20%	[88]
Infections		
Malaria	There is low prevalence of malaria in SCD. However, when malaria occurs in SCD it is associated with increased risk of morbidity due to severe anaemia and mortality	[89, 90]
Bacterial infections	10% children under 5 years	[91]

Modified from [92, 93].

periods interspersed with acute events, which are referred to as crises.

The reasons for this heterogeneity are not fully understood [94]. Interindividual variation in fetal hemoglobin (HbF) levels is one of the main modifiers that contribute to the clinical heterogeneity observed in SCD patients. Higher expression of HbF in adulthood ameliorates morbidity and mortality in SCD [56, 95].

It is now clear that common HbF variation is a quantitative genetic trait shaped by common polymorphisms. Multiple genes, together with an environmental component, determine the measured value of HbF in any given individual. Genetic variation at three major loci accounts for a relatively large proportion (20%–50%) of the phenotypic variation in HbF levels: (1) a single-base substitution (T/C) at position −158 of the $^{G}\gamma$ globin gene, termed *Xmn*I $^{G}\gamma$ site [96]; (2) the *HMIP* locus (*HBS1L-MYB intergenic polymorphism*) on chromosome 6q [97]; and (3) the oncogene *BCL11A* on chromosome 2 [98]. These variants have been well reported in nonanemic Northern Europeans and Sardinians, a β-thalassemia cohort, in SCD patients from Brazil, and in the African-American Cooperative Study of Sickle Cell Disease (CSSCD) [99–101]. There is very little description of the three main genetic polymorphisms explaining phenotypic variation in HbF levels and clinical phenotype in native African SCD patients [97, 102].

7. Epidemiology of Sickle Cell Disease

7.1. Prevalence. The prevalence of SCD can be objectively determined by calculating the birth prevalence of affected children, which requires accurate diagnosis and registration

at birth. Since this is not done in most African countries, an alternative method is to use the prevalence of the carrier or heterozygous states (HbAS) to calculate the expected birth rate of SCA based on the gene frequency and Hardy-Weinberg equation. Approximately 300,000 children are born every year with SCD in the world, and countries such as the United States of America, United Kingdom, and Jamaica have well-documented SCD population. However, this SCD population constitutes only 1% of the global population of SCD, as over 75% are in Sub-Saharan Africa [103, 104]. It has been estimated that SCD results in the annual loss of several millions of disability-adjusted life years, particularly in the developing world [105]. Hemoglobinopathies alone represent a health burden comparable to that of communicable and other major diseases [106].

7.2. Population Genetics and Dynamics: SCD, Malaria, and Migration. Compared to noncarriers, healthy carriers of recessive genes for SCD have a well-documented survival advantage against the lethal effects of malaria. As a result, carriers are more likely to reach reproductive age. Consequently, the birth prevalence of SCD is high in Africa [107–109]. The resurgence of malaria in many parts of the world will serve to maintain these polymorphisms, but even if this selective force were removed it would take many generations for the gene frequencies of these conditions to fall significantly [110]. Any changes resulting from variation in selection or population dynamics will, however, be very small compared with the effect of the demographic transition that many countries have undergone over recent years [110]. Specifically, there is a high prevalence of hemoglobin S (HbS) in Africa and hemoglobin C (HbC) in parts of West Africa [111]. Since subjects that are homozygous for HbC do not present with severe disease like HbSS, it is anticipated that the frequency of HbC will progressively increase even if malaria is not controlled [112]. Internal migration in Africa has led to SCD, which was previously rare, being introduced in South Africa through an influx of migrants from West and Central Africa [113]. The high birth prevalence of SCD has highlighted the burden of SCD, such that in 2006, the World Health Organization (WHO) recognized SCD as a public health priority [114]. There is limited information about the burden of SCD to the health system and the impact that it has on individuals.

7.3. Mortality. There is a higher rate of mortality among individuals with SCD, with reports suggesting that if untreated most children with SCD die in early childhood. Studies done in Nigeria, reported mortality of up to 90% [115] but recent estimates suggest that mortality rate has decreased and is more likely to be up to 50% by 20 years. This mortality rate in Africa is similar to those reported in the early 1960s in the United States of America and United Kingdom. However, with early diagnosis and comprehensive treatment, significant reductions in mortality have been achieved, with recent reports of improved survival; 85.6% survive to 18 years in the USA [116], 84% in Jamaica, and 99.0% to 16 years in the UK [117]. The common causes of death in the USA, UK, and Jamaica are infections, acute splenic sequestration, and acute chest syndrome [23, 49, 118, 119] with the highest incidence between 1 and 3 years of age.

8. Laboratory Diagnosis of Sickle Cell Disease

The laboratory diagnosis of SCD is based on the demonstration of HbS and the absence or significant reductions in HbA, with variation in the percentage of two other hemoglobins—HbF, HbA_2—in RBCs. Commonly available screening tests in Africa include sodium metabisulphite sickling test and sickle solubility tests and confirmatory tests using electrophoresis and chromatography to confirm the sickle phenotype (SS/AS/SC/Sβ^-thalassaemia). The three tests widely used are haemoglobin electrophoresis, isoelectric focusing (IEF), and high performance liquid chromatography (HPLC). DNA-based assays precisely describe the genotype; however, for clinical purposes, diagnosis usually involves screening (sickling or solubility test) followed by confirmation of the sickle phenotype using gel electrophoresis, IEF or HPLC.

8.1. Screening Tests. In most African hospitals, screening is done, using the "sickling test", which involves making a thin blood film which is then put under hypoxic conditions by the addition of sodium metabisulphite. This will result in RBCs containing HbS becoming deformed (i.e., forming sickle cells) as detected by light microscopy. A "positive" sickling test identifies the presence of sickled RBCs, which occurs in both homo- (SS) and heterozygous (AS) states. The sickle solubility test is another method used for screening which is based on the principle that HbS becomes insoluble when it is deoxygenated. Additional confirmatory tests are required to confirm SS-SCD or SCD involving other Hb types, when these screening assays are used.

8.2. Confirmatory Tests. These tests are based on the principle that different haemoglobin isoforms have different overall ionic charge, which makes them migrate with different velocities in an electric field. HBE can be done under alkaline or acidic conditions. HbA, HA_2, HbF, and HbS migrate towards the anode under an electric field with different rate of mobility. During alkaline Hb electrophoresis the resolution between HbS and HbF can be poor, particularly in individuals with high HbF levels, for example, neonates. Under acidic conditions, HbF migrates relatively more rapidly and is therefore distinguishable from both HbA and HbS. Isoelectric focusing uses the same principles but is slightly more expensive than HBE. However, it is able to identify more Hb variants that would not be detected by HBE. It also has the advantage that it does not require commercial reagents. HPLC uses cation exchange chromatography to identify the various hemoglobins in an individual. It has the advantage in that it can also accurately quantify the Hb levels. In resource-rich countries, screening has largely been replaced by HPLC and confirmation is then done by IEF or HBE. This is mainly because HBE and IEF are labour intensive, time consuming and would not identify abnormal bands or quantify Hb. Furthermore, the quantification of Hb fractions by HPLC is used to monitor patients who are on Hydroxyurea therapy or exchange blood transfusion.

8.3. Molecular Diagnosis of SCA. The most popular molecular diagnosis of β^S mutation, based on restriction enzyme digestion, is performed on HBB PCR products. The point mutation, which results in SCD, abolishes the restriction site for the restriction enzyme *DdeI*. Digestion of DNA of individuals homozygous for HbAA would result in two fragments 188 bp and 192 bp. Analysis of heterozygous HbAS samples would result in three fragments one of 380 bp and the two digested fragments of 180 bp and 192 bp. Homozygous HbSS samples would result in 380 bp fragments being produced (Figure 3). This method is simple and cost effective and could be used for prenatal genetic diagnosis in African settings [120].

9. Management of Sickle Cell Disease

As a chronic disease, the natural history of SCD is characterised by quiescent periods interspersed by acute events, known as crises, leading to patients seeking health care and frequent hospitalisation. The "crises" range from defined syndromes such as acute chest syndrome (ACS), acute splenic sequestration (ASS), to less well-defined symptoms that include pain, fever, anaemia, worsening of jaundice, and leg ulcers. Other circumstances include pregnancy, dehydration, and extreme cold weather. With the increased life span of individuals with SCD, there has been an increasing awareness of the importance of improving the quality of life as well as preventing damage to major organs. SCD is associated with increased mortality. The causes of mortality in the USA, UK, and Jamaica included infections, ACS, ASS, and aplastic crises [23, 49, 118, 119]. The management of patients with SCD involves interventions that improve survival, prevent complications, treat acute events, and reduce end-organ damage. Specific conditions or circumstances when SCD patients require extra care include surgery requiring general anaesthesia, due to increased risk of developing acute sickling complications and sudden death. Over the past 3 decades there has been an improvement in the understanding of the different pathogenic mechanisms responsible for sickle cell events and organ dysfunction. Through a series of clinical trials, effective interventional strategies have been established.

9.1. Newborn Screening (NBS). The highest incidence of death occurs in the first 3 years of life [23, 49, 118, 121]. Identification of children at birth by newborn screening (NBS), and institution of preventative care has improved survival [116, 122, 123]. Patients who are identified at birth can be given counselling and advice about the course of illness. They can then be enrolled in comprehensive care programmes that provide prompt and effective care of acute events and prophylaxis against complications, resulting in overall positive impact on survival and quality of life. Countries with large SCD populations and adequate resources have started NBS programmes.

9.2. Comprehensive Care Including Dedicated Day Care Facilities. The identification of SCD at birth has to be accompanied by enrolment into programmes that provide comprehensive care by multidisciplinary teams comprising nurses, genetic counsellors, social workers, paediatricians, haematologists, orthopaedic surgeons, ophthalmologists, and internists. These programmes provide appropriate advice, counseling, and support to parents and affected individuals. This includes advice such as drinking adequate quantities of fluid to avoid dehydration and wearing warm clothing in cold weather. Specific health education that will enable them to recognise acute events and seek medical care is also essential. Teaching mothers to recognise enlargement of the spleen and anaemia was effective in diagnosing and treating anaemia due to ASS [71, 124]. Patients are also seen on a regular basis and provided with folic acid supplements. The evidence for the burden of folate deficiency in SCD is limited. Prompt treatment of crises (fever and pain), particularly at outpatient or in day-care facilities, has been found to be effective and reduces the burden of hospitalization to the individual and the health system [125–128]. Long-term care should be provided by a multidisciplinary team including professionals who have specialized in haematology and blood transfusion for adults and paediatric haematologists in children. In settings where there is a low prevalence of SCD or limited number of health care professionals, SCD patients can receive care from general health care workers. In such a setting, guidelines for management can be provided to general health care workers with a system of referral to specialised centres.

9.3. Prevention and Treatment of Infections. In the absence of intervention, bacterial infection is the leading cause of mortality in individuals with SCD, and the age group that is most affected is 1 to 3 years [37, 49, 118]. Bacterial infection in SCD is mainly due to *Streptococcus pneumoniae,* resulting in pneumonia, sepsis, and meningitis. The highest incidence of invasive pneumococcal disease is in children less than 6 years of age [91, 118]. In a landmark study in the USA, Gaston and colleagues demonstrated an 84% reduction in incidence of pneumococcal infection with the use of oral penicillin [26]. Interventions with daily oral penicillin and vaccination against pneumococcal infections have successfully reduced mortality in developed countries [26, 116, 129]. In Africa, these interventions have not been implemented as the evidence to demonstrate a similar role of bacterial infections was lacking. This made it difficult for hospitals and governments in developing countries to implement these interventions. Furthermore, published reports have actually questioned the role of prophylaxis against *Streptococcus pneumoniae* (SPN), in Africa [28]. However, there has been increasing evidence of the role of bacterial infections, particularly due to SPN in causing high childhood mortality [130, 131]. Since SCD patients are highly susceptible to SPN infections due to impaired immunity, this makes it even more likely that SPN infections will have a more significant role in SCD mortality. Therefore, there has been an increase in the appeal to implement these interventions [30, 132].

Malaria is widely considered to be one of the major causes of illness and death in patients living with SCD in SSA [90, 104]. Although, SCD individuals have an element

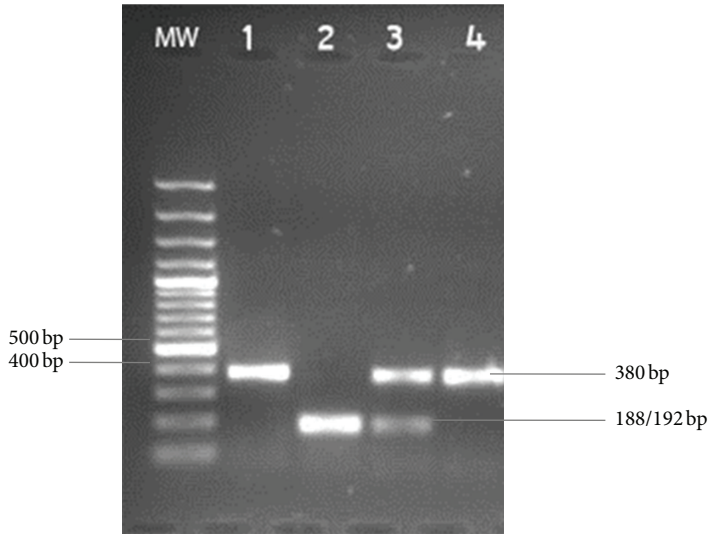

Figure 3: RFLP of HBB fragment with DdeI. Lane 1: undigested control, Lane 2: HbAA control, Lane 3: HbAS control, and Lane 4: HbSS MW: molecular weight marker.

of protection against malaria; with a lower prevalence of malaria infection [133–135] and a lower parasite density [136], the risk of mortality when SCD patients get malaria is significantly higher [137]. It is recommended that individuals with SCD who live in a malaria endemic area should receive prophylaxis against malaria [138]. There is ongoing debate as to what is the most appropriate agent that can be used for chemoprophylaxis. The increasing resistance by *Plasmodium falciparum* parasites to chloroquine has meant that most countries have had to stop using chloroquine. Sulphadoxinepyrimethamine has antifolate properties and is not recommended for prophylaxis in patients with SCD who are considered to be folate deficient. Most malaria-endemic countries have therefore been unable to decide which drug to use for prophylaxis in SCD, with options limited to proguanil (paludrine), mefloquine (Lariam), Malarone, or Doxycycline. Current practice in malaria-endemic countries involves use of insecticide-treated nets and prompt diagnosis and treatment of malaria.

9.4. Blood Transfusion (BT). SCD is contributing to the anaemia in under fives and pregnant women in areas of high prevalence. Patients with SCD have a compensated chronic haemolytic anaemia which allows them to carry on with normal activities at steady-state haemoglobin with narrow reserve capacity to accommodate strenuous physical activities. The steady state haemoglobin varies from person to person and is related to the level of HbF, co-inheritance of alpha thalassaemia, or heterozygosity for another haemoglobin type such as HbC. Although individuals with SCD have chronic anaemia which is tolerated, rapidly worsening anaemia can occur, and this presents as an emergency. It can be caused by ASS, aplastic crises, and hyperhemolysis or associated with other events such as bacterial infections and malaria. Under these circumstances, anaemia is life threatening and requires prompt treatment with blood transfusion. The products that are used (whole blood or packed RBCs) and the method of transfusion (simple or exchange) are determined by the clinical situation, availability of resources, and the capacity to provide the blood product and establish venous access [139]. Blood transfusion is also effective in other situations, such as acute stroke [140], ACS [141], and perioperatively [142]. Blood transfusion works by increasing the level of Hb, thus improving oxygen delivery. It also reduces the proportion of sickle RBCs in the circulation. Exchange or red cell transfusion has also been shown to be effective in reducing the level of HbS to less than 30% [143–146]. This is thought to reduce the deleterious effects of HbS and improve outcome. Long-term blood transfusion therapy (LTBT) has been found to be effective in the prevention of brain injury due to cerebrovascular disease [140]. Blood transfusion is associated with risks which have to be weighed against the benefits when considering implementing this as an intervention. These will be reviewed in the section on stroke.

9.5. Pain. Pain, the defining feature of SCD and its commonest symptom, starts early in life and persists throughout life. It is the commonest symptom of SCD and is related to disease severity. Studies in children in developed countries suggest that painful episodes and acute chest syndrome were the most frequent complications of sickle cell disease and that the pain crises are a major predictor of adverse outcome in children along with anaemia and leucocytosis. In adults, large proportion of patients die during an acute episode of pain, making it a risk factor for early death along with acute chest syndrome and stroke. However due to its subjective nature, patients with SCD may not be having appropriate assessment and adequate pain management necessary to prevent complications relating to the pain such as the development of a chronic pain syndrome resulting in worsening of the sickle cell condition. Training is essential for adequate assessment of pain intensity, reporting, documentation by patients, care giver, and health workers. Prompt management

of pain requires attention to the precipitating causes (stress, infection, dehydration, acidosis, and allodynia). Adequate oral analgesic should be administered for mild pain and parenteral for moderate to severe pain according to WHO step ladder for analgesia in patients. When the expected relief is not obtained in response to adequate doses of analgesics, this should alert to the condition of opioid-induced hyperaesthesia, allodynia, or the progression of acute pain to chronic pain [147–149]. However, many health facilities in Africa do not have access to opioids.

9.6. Hydroxyurea. Hydroxyurea (HU) (also known as hydroxycarbamide) has been reported to be effective in improving survival and reducing morbidity in some SCD patients (Table 3). The clinical outcomes include reduction in frequency of painful episodes and hospital admissions [150]. Hydroxyurea therapy is also monitored by a number of laboratory parameters which include increased HbF levels, mean corpuscular volume (MCV), and reduction in WBC count. Hydroxyurea has been found to be effective in the prevention of brain injury due to cerebrovascular disease [151].

9.7. Nitric Oxide. Lung dysfunction results from a combination of repeated pulmonary infections and infarctions as well as increased vasoconstriction leading to pulmonary hypertension [54, 55]. The latter has recently been shown to be associated with reduced bioavailability of nitric oxide [19], which has resulted in the development of potential therapies such as L-arginine, citrulline, and inhaled nitric oxide which is aimed at increasing NO levels through different pathways [153–157].

9.8. Stem Cell Transplant. The only cure that is available for SCD is stem cell transplantation (SCT), which replaces the host's bone marrow with stem cells containing normal β-globin genotype. Since the first successful transplant reported in 1984 [158], there has been significant reduction in risks due to SCT and increasing success, with the best results, of up to 85% event free survival, occurring with HLA-matched sibling donors and transplantation early in the course of the disease before end-organ damage occurs [159]. One limitation of SCT is the availability of sibling donors [160], and therefore there have been attempts to improve survival for unrelated stem-cell donors [161, 162]. The second limitation of SCT is that this line of treatment requires tremendous resources, and it becomes increasingly difficult for transplant physicians practicing in the developing world to reconcile the difference between what is possible and what is available. Moreover, it is more difficult to address because the clinical course of SCD is extremely heterogeneous. Despite the knowledge of various genetic and environmental factors known to alter disease severity, it is still difficult to accurately identify children with risk of severe disease before extensive damage has occurred. Until such time that a low-risk, definitive cure is available, the cornerstone of management of SCD is the prevention of early mortality, prevention of end organ damage, and improvement of the quality of life.

Table 3: Summary of study outcomes for hydroxyurea use in adults and children.

Outcome	Impact in adults	Impact in adolescents
Clinical outcomes		
Pain crises	↓↓↓	↓↓
Hospitalisations	↓↓↓	↓↓↓
Blood transfusion therapy	↓↓↓	↔ (insufficient data)
Acute chest syndrome	↓↓↓	↔ (insufficient data)
Laboratory markers		
Foetal haemoglobin	↑↑↑	↑↑↑
Haemoglobin	↑↑↑	↔ (not significantly significant)
Mean corpuscular haemoglobin	↑↑↑	↑↑↑
White blood cell count	↓↓↓	↓↓↓
Prevention of end organ damage		
Brain	↔	↔
Spleen	↔	↔
Kidney	↔	↔
Mortality	↓	↔=

↓↓↓: high-grade evidence for decrease; ↓: low-grade evidence for a decrease; ↑↑↑: high-grade evidence for increase; ↑: low-grade evidence for an increase; ↔: not evaluated/not significantly different/insufficient data. Source [152].

9.9. Gene Therapy. Since SCD is caused by a defective gene, definitive treatment would involve replacement of this gene with a normal gene. This has been done successfully in the sickle transgenic mouse [163], but progress in humans has been limited by identification of appropriate vectors and efficacy for gene transfer and low level expression of globin genes.

9.10. Role of Programmes for Control and Management of SCD. From a public health perspective, the policy for approaching the control of SCD in national health programmes needs to work in the context of countries with limited resources in health. Although, there is ongoing debate whether care of SCD should be integrated into existing health care services or whether there should be separate disease-specific programmes for SCD, the WHO recommends [164] that, for countries where the birth rate of affected infants is above 0.5 per 1,000 births, they should develop separate programmes for these conditions. It is recommended that counties with a high prevalence of SCD start planning effective control measures. In this context, control of SCD encompasses two elements: providing best possible care for affected individuals and preventing the birth of affected individuals.

With regard to providing best possible care, the following are options, depending on available resources, that have been recommended by Weatherall et al. in 2006 [105].

Option one: best possible patient care with the use of prophylactic penicillin following diagnosis, together with retrospective genetic counselling.

Option two: best possible patient care, together with a newborn screening program and the use of penicillin

for all homozygous babies, together with retrospective screening and counselling.

Option three: best possible patient care, together with newborn screening and the use of prophylactic penicillin from birth for homozygotes, together with population screening and prospective genetic counselling.

Option four: option three, plus the availability of prenatal diagnosis, bone marrow transplantation, or both.

The management of SCD involves early diagnosis of affected people, the provision of the most appropriate basic, cost-effective treatment, and genetic counselling and psychosocial support. The long-term goal is to ensure appropriate management at different levels of health care with development of referral centres for specialised diagnosis and treatment. This approach ensures a cost-effective way of effectively dealing with a highly prevalent condition in areas where the resources are limited. However, it is important that these centres are not limited to urban areas or centred on academic or research oriented health facilities. In order to avoid this, there must be active strategies to ensure that appropriate management is built into services at all levels of health care with adequate support from these specialised centres. Management of SCD needs to be accompanied by strategies that aimed at two levels of prevention: tertiary prevention which involves early diagnosis of SCD and prevention of complications and more ambitiously secondary prevention which tries to reduce the number of children that are born with SCD. (Note that primary prevention aims to ensure that individuals are born free of SCD). Preventative services involve community education, population screening, and genetic counselling that would encourage people to undergo screening before conception, during the antenatal or postnatal period. There are several issues that need to be addressed with regard to prevention of SCD. The aim of screening is to detect SCD in the foetus, discuss the consequences of a diagnosis of SCD, and provide options for treatment and prognosis. Since SCD is a recessive disorder, during pre-conception screening, the chances of getting an affected child are variable. There is difficulty in advising a couple not to have children as the risk of getting an affected child may be relatively low (1 in 4) and does not increase with each pregnancy. The highest risk would be for two individuals who are SS who wish to have children. This is different from thalassaemia, where children with the most severe form, thalassaemia major, will inevitably have severe disease. Therefore, one could argue that this therefore justifies the use of prenatal diagnosis as this would identify pregnancies with SCD children, and then parents would be given the appropriate information regarding the consequences and prognosis of SCD and allow more reproductive options to families. Prenatal genetic diagnosis represents one type of reproductive option as it provides parents with the option to test at-risk pregnancies and make decisions regarding affected pregnancies. The availability and acceptability of prenatal diagnosis and termination of an affected pregnancy are of particular importance in low-resource countries where neither health services nor families can afford to pay for long-term treatment of SCA [165]. Close to two-thirds of a sample of 130 Cameroonian parents with affected children reported they would accept termination of an affected pregnancy for SCA [120], a considerably higher proportion when compared to the Cameroonian preclinical, clinical medical student, and physicians in a previous study (22.4, 10.8 and 36.1%, resp.) [166]. Trends reported in Nigerian parents were slightly different where 92% of a sample of 53 SCA heterozygous carrier mothers favored prenatal diagnosis and 63% indicated they would opt for termination of an affected pregnancy [167]. However, in a survey of 403 health workers in a tertiary health care centre in Nigeria, only one-third of the respondents accept termination of pregnancy as an option if prenatal screening is positive for SCA, whereas close to half of the respondents (42%) were against the idea. Another study reported that 21.4% of Nigerian doctors would accept termination of an affected pregnancy for SCA [168]. Experience of the effective practice of prenatal genetic diagnosis for SCD (amniocentesis and fetal DNA analysis) was reported in Nigeria and Cameroon [169, 170]. The views of parents towards prenatal diagnosis and in some cases medical termination of pregnancy may be associated with their experience of affected patients and the psychosocial and/or economic impact of SCA on families. Nevertheless the discrepancy between perception of a professional and parents underscores the necessity for more studies to unravel the ethical dilemma around prenatal genetic diagnosis to offer a service that does not conflict with social and cultural values of the affected population. Preimplantation genetic diagnosis is a mechanism for accurate genetic diagnosis, careful selection of unaffected embryo and implantation to allow fertile or infertile couples to have offspring without SCA. It is an expensive procedure using assisted conception by in vitro fertilization or intracytoplasmic sperm injection. It requires close collaboration between fertility specialists, molecular biologists, geneticists, and genetic and fertility counselors and may be an option to individuals who may object to prenatal diagnosis followed by termination.

Although SCA is the most severe form of the disease (compared to SC/Sβ thalassaemia, etc.), there is still wide variability in disease severity. Therefore, even with the correct identification and diagnosis of SS with screening, it would be difficult to predict those who would develop severe disease and have a poor outcome.

10. Conclusion and Future Challenges

Because of their uneven distribution in high-frequency populations, reflecting their complex population genetics, the true magnitude of burden of SCD is still unknown. In many African countries there are few or virtually no facilities for appropriate diagnosis and management of SCD. There is limited data about frequency, clinical course, or mortality. Without this information it will be impossible to persuade African governments about the burden of this disease. The WHO Africa has recommended a set of public health interventions to reduce the burden of SCD in African region, namely, improving awareness, preventing the disease,

early detection, improving the provision of health care for affected individuals by providing effective clinical, laboratory, diagnostic, and imaging facilities adapted to different levels of the health system, screening of newborns, training of health care workers, developing protocols for treatment, providing genetic counseling, patient support groups, advocacy, and research [171]. The situation will be improved by commitment by member states to integrate SCD prevention and control in national health plans and provide conducive environment for various stakeholders to contribute to the reduction of SCD prevalence, morbidity, and mortality. It will also require concerted action on the part of the international community of the richer countries, together with input from other major international health organizations and funding agencies [172, 173].

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Review Article

Ineffective Erythropoiesis in β-Thalassemia

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In humans, β-thalassemia dyserythropoiesis is characterized by expansion of early erythroid precursors and erythroid progenitors and then ineffective erythropoiesis. This ineffective erythropoiesis is defined as a suboptimal production of mature erythrocytes originating from a proliferating pool of immature erythroblasts. It is characterized by (1) accelerated erythroid differentiation, (2) maturation blockade at the polychromatophilic stage, and (3) death of erythroid precursors. Despite extensive knowledge of molecular defects causing β-thalassemia, less is known about the mechanisms responsible for ineffective erythropoiesis. In this paper, we will focus on the underlying mechanisms leading to premature death of thalassemic erythroid precursors in the bone marrow.

1. Introduction

Normal human adult hemoglobin (Hb) A (HbA) consists of two pairs of globin chains, $\alpha_2\beta_2$, of which synthesis is normally tightly coordinated to ensure equal production. β-thalassemia, one of the most common inherited hemoglobinopathy in the world, is due to autosomal mutations in the gene encoding β-globin which induce an absence or low-level synthesis of this protein in erythropoietic cells [1]. The consequence of these mutations is an imbalance of α/β-globin chain synthesis, mostly evident in the homozygous forms, leading to the accumulation of free α-globin chains forming highly toxic aggregates [2]. Thalassemic patients suffer from anemia resulting from shortened red blood cell (RBC) survival, by hemolysis, and erythroid precursors premature death in bone marrow (ineffective erythropoiesis).

The first description of thalassemia was reported by Dr. Thomas Cooley in 1925. There are a multiplicity of different genetic mutations in β-thalassemia that give rise to a clinically heterogeneous spectrum ranging from asymptomatic expression (thalassemia minor) and mild clinical anemia (thalassemia intermedia) to classical, fatal Cooley's anemia. The term "Cooley's anemia," now termed β^0-thalassemia major (TM), has been used synonymously with clinically severe forms of β-thalassemia, characterized by a very high

α/non-α chains ratio, severe ineffective erythropoiesis, and dependence on RBC transfusions to sustain live. Regular transfusions (average every month) expose these patients to iron overload and its live threatening systemic consequences, which require iron chelation [1].

It is well established that the α/non-α ratio correlates with the severity of disease [1, 3]. However, genotypic variability impairing globin chain synthesis at known loci is often insufficient to explain the heterogeneity in clinical phenotypes of individual patients with the same genotype, suggesting that other genetic modulations might exist [1, 4]. The molecular mechanisms underlying the heterogeneity and occasional severity of the syndrome remain obscure and are not the object of this paper.

The pathophysiology of β-thalassemia has been the subject of several extensive reviews, particularly on its molecular and genetics basis [5]. In this paper, we will focus on the mechanisms leading to end-stage maturation blockade and thalassemic erythroid precursors premature destruction in the bone marrow. Their understanding will probably be the key for developing novel therapeutic approaches improving anemia in β-thalassemia. In order to describe mechanisms underlying ineffective erythropoiesis, we will first summarize the current knowledge on normal hemoglobin synthesis and normal erythropoiesis.

2. Hemoglobin Synthesis

Two distinct globin chains α and β (each carrying an individual heme molecule) interact to form hemoglobin dimers α/β, and two dimers combine to form a hemoglobin tetramer $\alpha_2\beta_2$: the functional form of hemoglobin carrying oxygen.

Excepted the very first weeks of embryogenesis, in which zeta chains are produced, one of the globin chains is α and the second chain is called "non-α." The main (98%) hemoglobin type in the normal human adult consists of two α and two β chains ($\alpha_2\beta_2$ HbA); the minor type (2%) consists of 2α and 2δ chains ($\alpha_2\delta_2$ HbA2). Usually, globin chains synthesis is relatively balanced, even if some studies report a slight excess of α chains as a soluble pool [3, 6, 7]. In contrast, fetal erythrocytes contain another type of hemoglobin consisting of 2α and 2γ chains ($\alpha_2\gamma_2$ HbF), which can attract more oxygen effectively from the maternal blood.

Globin chains originating from a common ancestral type display a varying degree of homology. Two clusters of globins genes are known: the first one on chromosome 16 for α-like genes (two α-globin genes, α_1 and α_2, and two zeta genes) and the second one on chromosome 11, for β-like genes. The $5'$ to $3'$ order of β-like globin genes on chromosome 11 (ε-$^{G}\gamma$-$^{A}\gamma$-δ-β, two γ genes) reflects their sequential activation and silencing, during the transition from embryonic to fetal and from fetal to adult "hemoglobinopoiesis," called "hemoglobin ontogeny" or "hemoglobin chain switch."

3. Erythropoiesis

Erythropoiesis is defined as the pathway producing mature RBC from hematopoietic stem cells. This process includes several steps restricting differentiation and proliferation of cells which undergo this erythroid program, depending on sequential and specific erythroid gene expression. Erythropoiesis is regulated by combined effects of microenvironment and growth factors that promote survival, proliferation, and/or differentiation of erythroid progenitors and nuclear factors that regulate transcription of genes involved in survival and establishment of the erythroid phenotype. RBC production is orchestrated by a complex network of transcription factors, among which GATA-1, the master gene of erythropoiesis, positively regulates specific erythroid genes such as erythropoietin receptor (EpoR), glycophorin (GpA), and globin chains. Moreover, together with the transcription factor STAT5 (activated through EpoR activation by erythropoietin (Epo)), GATA-1 induces the expression of the anti-apoptotic protein Bcl-xL [8].

Committed erythroid progenitors differentiate into the first morphologically identified cell of the erythrocyte lineage: the proerythroblast. Next steps of erythroid differentiation are accompanied by temporally regulated changes in cell surface protein expression, reduction in cell size, progressive hemoglobinization, and nuclear condensation (successively called basophilic erythroblast, polychromatophilic erythroblast, and acidophilic erythroblast, the last nucleated cell of the mammalian erythrocyte lineage), which culminate in reticulocytes cells by nucleus, RNA, and mitochondria extrusion. In addition, erythroid maturation requires a transient activation of caspase-3 at the basophilic stage and translocation into the nucleus of the inducible heat shock protein 70 (Hsp70) to protect GATA-1 from caspase-3 cleavage [9, 10].

This process occurs within the erythroblastic island, in which a macrophage is surrounded by erythroblasts at all stages of maturation [11, 12]. The production of erythrocytes is the largest quantitative output of the hematopoietic system with estimated production rates of 2×10^{11} erythrocytes per day. The program of erythroid proliferation and differentiation must be positively and negatively regulated to ensure a continuous but tightly controlled production of RBC.

3.1. Positive Regulation of Erythropoiesis. Erythropoiesis is controlled by the combined effect of two major cytokines, stem cell factor (SCF) and Epo. SCF induces proliferation and survival and slows down differentiation of early erythroid progenitors and precursors towards the basophilic erythroblast stage. Epo is responsible of the finely tuned homeostatic control of erythrocyte numbers by tissue oxygenation. Interaction of Epo with the EpoR induces, through JAK2 activation, multiple signalling pathways involving PI3 kinase, Akt, and STAT5, which prevent apoptosis, supporting erythroid progenitors proliferation and allowing erythroid program to occur [13–15].

3.2. Negative Regulation of Erythropoiesis by Apoptosis. The negative regulation of erythropoiesis is mainly due to apoptosis, a fundamental cellular mechanism allowing clearance of unneeded or potentially dangerous cells. Apoptotic programs require the action of a family of cysteine-dependent and aspartate-specific proteases called caspases. Two classes of

caspases are described: initiators (caspase-8 and -9) and effectors (caspase-3 and -7) [16, 17]. Caspase-8 is activated by the death receptor pathway after cell surface receptor-ligand interaction [18]. In contrast, caspase-9 is activated by events causing intracellular damages and alterations in mitochondrial membrane potential (i.e., the mitochondrial pathway) [19, 20]. Activated caspase-8 and caspase-9 then activate effectors such as caspase-3 that cleaves GATA-1, Tal-1 [21, 22], and proteins involved in cytoplasm, nucleus, and DNA integrity, which allow the cell death program to occur.

3.3. Role of Cell Death Receptors and Epo. Death receptors of the TNF receptor (TNF-R) superfamilies (Fas-L, TNF-α, TRAIL) activate the extrinsic apoptotic pathway. Fas and Fas-L are expressed in cultured erythroblasts, but controversies regarding the level and differentiation stage at which they are expressed have been reported. Some studies suggest the existence of a negative regulatory feedback operating at low Epo level in a paracrine pathway. In this system, Fas-L expressing mature erythroblasts displays cytotoxicity against immature erythroblasts expressing Fas [23, 24]. Epo is able to partially protect immature erythroid cells from Fas-mediated apoptosis. Fas and Fas-L are therefore major regulators of erythropoiesis. In addition Fas/Fas-L interaction results, through caspase-8 activation, in GATA-1 cleavage which blocks erythroid differentiation and maturation [25].

The control of mature RBC production may be summarized as follow: at low doses of Epo, cells die by apoptosis; at intermediary doses, cells are arrested in their differentiation and maturation (through GATA-1 cleavage) or enter a program of apoptosis depending on the number of mature erythroblasts in the bone marrow; at high dose, erythroid progenitors and precursors pursue their maturation independently of the number of mature erythroid precursors.

3.4. Role of Caspases and Hsp70 in Differentiation and Maturation of Erythroid Cells. The terminal differentiation of erythroid cells exhibits some similarities with apoptosis, such as reduction in cell size, chromatin condensation, and degradation of nuclear components. A transient activation of caspases by the mitochondrial pathway has been shown, by our group and others, to be required for erythroid cells differentiation but to not induce neither GATA-1 cleavage nor apoptosis. We have more recently reported that Hsp70, an ubiquitous chaperone constitutively expressed during erythroid differentiation, protects GATA-1 in the nucleus from caspase-3-mediated proteolysis during caspase activation. These results strongly indicate that Hsp70 is another key erythroid antiapoptotic protein protecting GATA-1 from caspase-3-mediated cleavage and consequently allowing Bcl-x_L expression [9, 10].

4. β-Thalassemia Ineffective Erythropoiesis

4.1. Evidences for an Ineffective Erythropoiesis in β-Thalassemia. Dyserythropoiesis in β-thalassemic patients was suspected for a long time since it is largely recognized that many patients with an inadequate transfusional regimen have a dramatic expansion of the hematopoietic marrow and extramedullary hematopoiesis, which can lead to extensive bone deformity and/or bone marrow mass and splenomegaly. Erythrokinetic assays, done in the 50′s, showed that the rate of peripheral RBC destruction in β-thalassemia was insufficient to explain severe anemia [26, 27]. Then, ferrokinetic studies done in the 70′s, studying incorporation of ^{59}Fe into newly formed RBC, suggested that probably 60%–80% of erythroid progenitors were arrested in proliferation and/or underwent death [28].

The bone marrow of patients suffering from β-thalassemia contains five to six times the number of erythroid precursors observed in healthy controls [29], with increased basophilic and polychromatophilic erythroblasts and decreased orthochromatic erythroblasts [29–32]. Moreover, it has been shown that β-thalassemic bone marrow erythroblasts contain electron-dense alpha-globin inclusion (aggregates) beginning at early polychromatophilic stages, which increase in size and frequency during subsequent maturation [33].

Taken together, these results resume the findings of β-thalassemia dyserythropoiesis in human: expansion of very early erythroid precursors (proerythroblasts and earlier stages) and then ineffective erythropoiesis. Ineffective erythropoiesis defines the suboptimal production of mature erythrocytes from a proliferating pool of immature erythroblasts. It is thus characterized by (1) accelerated erythroid differentiation, (2) maturation blockade at the polychromatophilic stage, and (3) death of erythroid precursors [29, 30, 32, 34, 35] (Figure 1).

Although early erythroid progenitors expansion is believed to be due to a dramatic increased in Epo level as a result of the anemic state feedback [36], other mechanisms, yet not known, might be involved. In addition, precise pathophysiological mechanisms of accelerated erythroid differentiation and maturation arrest are still unknown. However, mechanisms underlying cell death were more studied and we intend at that point to review the different players of this death "game."

4.2. Enhanced Apoptosis Is a Key Feature of Ineffective Erythropoiesis in Human β-Thalassemia. It was evidenced in the 90s that β-TM erythroid precursors, but neither lymphoid and nor myeloid precursors underwent increased apoptosis (among 3- to 4-fold increased compared to healthy controls) as detected in human and mice bone marrow by an increase in DNA laddering (a sign of enhanced nucleosomal DNA cleavage, occurring specifically during apoptosis) and then confirmed by TUNEL labelling and exposure of phosphatidyl serine assessed by Annexin V labelling by FACS analysis [29, 30, 32]. *In vitro* findings corroborate the reduced cell expansion in β-TM erythroid cultures and enhanced apoptosis at the polychromatophilic stage of differentiation [32].

In spite of the markedly increased rate of apoptosis of β-thalassemic erythroid precursors, BM smears of these patients do not show high increased number of dying erythroblasts. Indeed, 15% to 20% of bone marrow erythroid precursors ($CD45^-/CD71^+$) present apoptotic features in

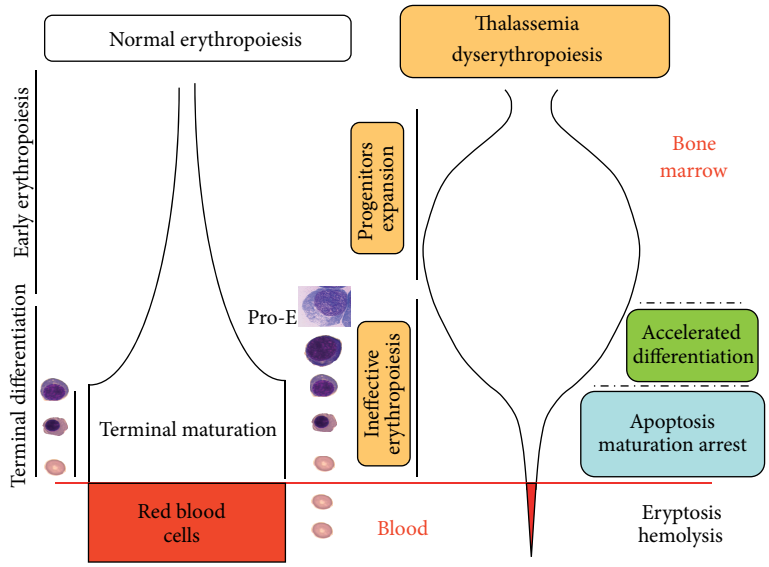

Figure 1: Difference between normal and β-thalassemia ineffective erythropoiesis. Erythropoiesis is the pathway producing mature RBCs from hematopoietic stem cells, including several proliferation and differentiation steps. Erythroid differentiation is accompanied by temporally regulated changes in cell surface protein expression, reduction in cell size, progressive hemoglobinization, and nuclear condensation and extrusion. β-thalassemia dyserythropoiesis in human is characterized by expansion of very early erythroid precursors (proerythroblasts and earlier stages) and then ineffective erythropoiesis. Ineffective erythropoiesis defines the suboptimal production of mature erythrocytes from a proliferating pool of immature erythroblasts characterized by (1) accelerated erythroid differentiation, (2) maturation blockade at the polychromatophilic stage, and (3) death of erythroid precursors.

aspirates [29, 30, 32]. This paradox might be explained by increased phagocytosis of abnormal precursors erythroblasts expressing phosphatidyl serine by bone marrow macrophages whose number and activation are enhanced, respectively, by about 2-fold in TM [29, 37, 38]. As a consequence, the delivery of thalassemic RBCs to the peripheral blood in the β-TM major patients is much reduced.

4.3. Apoptotic Pathways Involved in β-Thalassemia Ineffective Erythropoiesis. Studies of apoptotic death receptor pathways have shown that Fas and FasL are coexpressed early and at all stages of terminal differentiation. Both proteins are downregulated in bone marrow or spleen in proerythroblast and basophilic cells in β-thalassemic mice compared to control mice *in vivo*. No statistically difference was found in more mature cells. This down regulation in Fas/FasL expression might be a marker of erythropoietic stress [39] and might explain at least in part erythroid expansion.

Regarding the intrinsic apoptotic pathway, it was expected that it would have been also involved because it could be induced by cellular oxidant injury. Nevertheless, the involvement of this mitochondrial pathway has not been evidenced to date in β-thalassemia [40].

4.4. Role of Oxidant Injury in β-Thalassemia Ineffective Erythropoiesis

4.4.1. Excess of Unmatched Globin Chains Generates Reactive Oxygen Species (ROS). Occurrence of increased death at the polychromatophil stage of differentiation in TM [32] coincides with the stage of intense hemoglobinization [4, 27, 41, 42].

It could partially be explained by accumulation and precipitation of the unmatched α-globin chains at this stage, forming aggregates [33]. Indeed, there is several evidence that membrane components oxidation might play an important role in β-thalassemia pathophysiology [5, 43]. Free α-globin which is highly unstable and bound to heme and iron could generate reactive oxygen species (ROS) that damage cellular proteins, lipids, and nucleic acids [44, 45]. Data describing ROS production during erythroid differentiation in thalassemia are scarce [46]. A significant increase in ROS production both in early and late erythroid precursors compared to normal erythroblasts was evidenced in β-thalassemia intermedia [35] or β-thalassemia/HbE [34]. Thus it was speculated that the excess of ROS, by damaging components of RBC, might reduce lifespan of these cells and cause a premature RBC clearance by hemolysis (a passive process) [45]. However there is no robust evidence that ROS production is the direct cause of increased apoptosis in β-thalassemic erythroid cell precursors [5]. The observation that there are no differences [35] or a dramatic decrease [34] in ROS levels during thalassemic erythroid differentiation pleads against a direct mechanism leading to high ROS levels and apoptosis, since significant increased apoptosis was only observed in late differentiation stage (polychromatophilic stage and after) [32]. Actually, the direct link between increase of ROS and apoptosis has never been demonstrated in normal human erythroid cells. However in other cell models, ROS activate apoptosis signal regulating kinase 1 (ASK1) and Jun-kinase which can induce apoptosis (extrinsic or intrinsic

signaling pathway) [47]. ROS are also known to trigger the intrinsic apoptotic cascade via interactions with proteins of the mitochondrial permeability transition complex [48].

In conclusion, even if increased ROS levels might be an actor of erythroid cell precursors death by inducing damages of erythroid cell components, the pathophysiological relationship between apoptosis and accumulation of the unmatched α-globin chain in erythroblasts needs to be clarified. To date, no data provide clear evidences that the apoptotic mechanism involves ROS. On the other hand, the role of ROS in accelerated cell differentiation, another characteristic of ineffective erythropoiesis, is questionable [34] since antioxidants inhibit the *in vitro* erythroid progenitors differentiation from mice fetal liver [49].

4.4.2. Damaging Membrane Structures: A Cause of Ineffective Erythropoiesis? Unmatched α-globin chains and ROS production could induce alterations in membrane deformability, stability, and cellular hydration in addition to damages to cytoskeleton, explaining peripheral hemolysis [50]. Protein 4.1, a major component of the skeleton controlling its crosslinking, is partially oxidized in β-thalassemia's mature erythroblasts [51]. α-globins accumulation can occur even from the proerythroblast stage and such deposits were shown to colocalize with areas of defective assembly of the membrane skeletal proteins spectrin and protein 4.1 [31, 52]. Furthermore, very early in erythropoiesis, a defective assembly of the transmembrane band 3 protein was reported, which was not spatially related to α-globin deposits. This band 3 defect seems to disappear at the intermediate or late normoblast stage, suggesting either that the defect was temporary or that it severely affected band-3-deficient erythroid precursors which died and were removed. Furthermore, oxidant injury led to clustering of band 3, which in turn produced a neoantigen that bound IgG and complement [5, 53]. Extramembranous IgG/complement complex provided signals for macrophages to remove such affected erythroid precursors and RBC.

All these studies showed that β-thalassemic RBC membranes exhibited abnormalities in membrane skeletal proteins. Thus, it could also be postulated that the accumulation of α chains destabilizes the membrane and could participate in ineffective erythropoiesis, but it cannot be the unique explanation of the major increase of apoptosis [30, 31].

4.4.3. The α-Hb-Stabilizing Protein (AHSP) Is an Erythroid-Specific Private Chaperone Protein That Specifically Binds α-Hb. Recently, an α-hemoglobin-stabilizing protein (*AHSP*) was identified. This protein specifically binds to and stabilizes free α chains. *AHSP* is a 102 amino acid erythroid-specific protein induced by the essential erythroid transcription factor GATA-1 [54]. *AHSP* is abundant in late-stage erythroid precursors, in which its expression kinetics match with α-globin ones [55]. Stabilization of native folded α-globin by *AHSP* might be particularly important when heme quantity is limited, for example, during iron deficiency. Indeed, the presence of functional iron response element (IRE) in the 3′ untranslated region of human *AHSP* mRNA stabilizes this transcript in low iron conditions [56, 57].

A second *AHSP* function is to detoxify the excess of α-globin chains. Protein interaction screening has shown that *AHSP* binds free α globin and α-hemoglobin (unmatched α -globin chains bound to heme) (α-Hb). Subsequent investigations provide evidence that *AHSP* acts as a protein-specific molecular chaperone to fold and stabilize α-globin for HbA synthesis [58] and to protect erythroid cells against the deleterious effects of excess free α-Hb [45]. *AHSP* stabilizes the structure of α-Hb and detoxifies it by inhibiting the ability of heme iron to participate in chemical reactions that generate damaging ROS in RBC [45, 54, 59, 60]. In β-thalassemia mice model, loss of *AHSP* deficiency exacerbates anemia [45]. $AHSP^{-/-}$ mice present mild hemolytic anemia and hemoglobin precipitation in RBCs [45]. $AHSP^{-/-}$ mice exhibited also an elevated proportion of immature erythroid precursors, a maturation arrest, and excess of apoptosis [45]. This effect could also reflect ineffective erythropoiesis as a consequence of excess of free α chains.

The role of *AHSP* in human disease remains an open question. Naturally occurring mutations that ablate *AHSP* expression or alter the protein structure are rare [61, 62]. However, in human studies it was found that quantitative variation in *AHSP* expression between different individuals is extremely common [61]. Causal relationships between decreased *AHSP* expression and severity of thalassemic syndromes have not been established unequivocally [57].

4.5. Role of Heme and Heme Inhibitors in β-Thalassemia Ineffective Erythropoiesis. Excess of α-globin chains are associated with reduced heme production in late erythroid progenitors [35]. How the globin chain imbalance might affect the rate of heme synthesis is still a matter of investigation. The reduction of heme biosynthesis in β-thalassemic erythropoiesis has nevertheless a positive action to prevent the cytotoxic effect of free heme excess. Other cytoprotective mechanisms in response to oxidative stress in β-thalassemic erythroid cells also probably involve PRDX2 protein. PRDX2 is abundantly expressed during β-thalassemic erythropoiesis and binds heme in erythroid precursors, possibly playing an additional role to protect maturing cells by free heme from apoptosis [35].

Another protective factor in β-thalassemic erythropoiesis involves the heme-regulated inhibitor of protein translation, which represses globin translation in heme-deficient erythroid precursors. Heme-regulated inhibitor of protein translation plays a role in murine β-thalassemia, since anemia is more severe in β-thalassemic mice genetically lacking this protein [63, 64]. Roles of heme or heme inhibitors in ineffective erythropoiesis are still not known.

4.6. Role of Inflammatory Cytokines in Ineffective Erythropoiesis in β-Thalassemia Patients. Increased level of several inflammatory cytokines has been reported in β-TM and might contribute to ineffective erythropoiesis, through the well-known mechanism of "anemia associated with a chronic disease."

Further studies have shown an increased of TNF-α concentration in β-TM patients, unrelated to splenectomy [65, 66] or only in the splenectomised patients group [67–69]. In these studies, the authors suggested that the main cause for TNF-α rise was macrophage activation due to iron overload and the antigenic stimulation induced by chronic transfusion therapy.

TNF-α inhibits erythropoiesis *in vivo* and *in vitro* [70–80]. However, the mechanism by which TNF-α inhibits erythroid progenitor cells remains unclear. TNF-α induces an increase of apoptosis within the compartment of immature erythroblasts and a decrease in mature erythroblasts. TNF-α inhibits directly the BFU-E colony growth [70, 79, 80] whereas inhibition of CFU-E colony growth by the TNF-α is indirect via stimulation of β-interferon production from accessory cells [81, 82]. TNF-α might act directly on the TNF receptor expressed on immature erythroblasts or by inducing ceramide synthesis, lipids component of the cell membrane which can act as a signaling molecule involved in TNF-induced apoptosis. It was also reported that the inhibitory effect of TNF-α on erythroid maturation might be involved in NF-κB induction [70]. TNF-α cannot only directly inhibit erythroid differentiation but also facilitate proliferation of nonerythroid precursor cells (such as dendritics cells) in chronic disease with inflammatory syndrome [80].

Furthermore, it was described that transforming growth factor-β (TGF-β) plasma level was higher in β-TM splenectomized patients as compared to control group as well as other cytokines from TGF-β superfamily [83]. Therefore, TGF-β is a paradoxical inhibitor of normal erythropoiesis that acts by blocking proliferation and accelerating differentiation of erythroid progenitors [84]. Its potential role in pathophysiological mechanisms of β-thalassemia ineffective erythropoiesis has not been studied to date.

4.7. Other Pathophysiological Pathways Involved in β-Thalassemia Ineffective Erythropoiesis

4.7.1. Macrophages Number and Activation Are Enhanced. As described above, macrophages number and level of activation are enhanced in bone marrow of β-thalassemic patients [29, 37]. It was suggested that macrophages activation was due to iron overload and antigenic stimulation related to chronic transfusion therapy [83]. Those activated macrophages selectively phagocyte apoptotic erythroblasts exhibiting "eat me signal" [37, 38, 85], thereby contributing to the ineffective erythropoiesis.

It has been demonstrated that thalassemic erythrocytes are phagocytosed by activated macrophages *in vitro* [85], and the mean number of β-thalassemic cells ingested by monocytes was found to be approximatively 30% higher than that for normal monocytes [86]. Mechanisms involved in the recognition of apoptotic erythroblasts by macrophages are not fully understood, but CD36 at the surface of macrophages and phosphatidyl serine residues exposed on apoptotic erythroblasts membrane appear to be involved [37]. Furthermore, oxidant injury that produced a IgG-bound neoantigen band 3, associated to complement, provided signals for macrophages to remove such affected erythroblasts [53].

4.7.2. Epo Response to Anemia in β-Thalassemia Major. In β-thalassemia, Epo is dramatically increased in response to anemia and hypoxia [87]. Nevertheless, Epo response is blunted as compared with Epo response in aplastic anemia or iron deficiency anemia [88, 89]. Underlying mechanisms for the blunted Epo response in patients with β-TM are not well understood. Three hypotheses are suggested:

(i) increased capture and faster clearance of Epo by erythroid cells hyperplasia [90],

(ii) inhibition of Epo renal synthesis by inflammatory cytokines (IL-1α, IL-1β, TGF-β, or TNF-α) [91] as showed in other anemias of chronic disease [92–96] or by impaired renal function [89, 97–101]; very high serum Epo levels were found in young patients with β-TM and β-thalassemia intermedia, comparable to the levels found in patients with aplastic anemia, which were different from the levels found in older thalassemia patients with the same degree of anemia; it is suggested, therefore, that a decrease in serum Epo levels could develop during the course of the disease [102, 103],

(iii) The low oxygen-Hb affinity and subsequent right shifting of oxygen-Hb dissociation curve would facilitate tissue oxygen availability, decreasing the hypoxic burden to anemia. Both low oxygen-Hb affinity and increased 2,3-diphosphoglycerate levels [104] present in β-TM might induce an inadequate Epo response to a given degree of anemia.

Nevertheless, increased Epo level, secondary to profound anemia, is believed to be the cause of early erythroid progenitors and precursors expansion in β-TM [87]. In β-TM mice model, it was shown that persistent activation of JAK2, as a consequence of high levels of Epo, drives erythroid expansion and extramedullary hematopoiesis, thus might constitute a target, by using JAK2 inhibitors, to treat this complication and decrease transfusion burden [105].

4.7.3. Iron Overload in Thalassemia. Tissue iron overload is the most important complication of β-thalassemia and is a major focus of therapeutic management [42, 106].

Blood transfusion is a comprehensive source of iron loading for β-thalassemia patients. Nevertheless, iron overload occurs also in patients who have not received transfusions such as patients suffering from thalassemia intermedia [42, 107, 108]. Decreased levels of hepcidin in these patients explain this paradoxical feature. Hepcidin is a key regulator of iron homeostasis: it blocks iron release from macrophages and hepatocytes and inhibits intestinal iron absorption. Its liver expression increases in response to iron overload and inflammatory stimuli [4, 109]. If hepcidin expression would be correctly regulated, it should be increased in β-thalassemia patients in order to decrease intestinal iron absorption. However, the opposite effect is observed [110, 111]. Indeed, two hepcidin erythroid regulators have been reported: the growth differentiation factor 15 (GDF15) and the twisted gastrulation protein homolog 1 (TWSG1) [112, 113]. High concentrations of both proteins, members of the

TGF-β superfamily, were evidenced in β-thalassemia serum compared to normal human serum. These proteins down-regulate hepcidin secretion by hepatocytes [112, 114]. GDF15 expression is associated with cellular stress and apoptosis and is expressed at low level during normal erythropoiesis. While β-thalassemic erythroid differentiation, GDF15 has been shown to be secreted by apoptotic erythroid cells at final stages. In contrast, the highest levels of TWSG1 were detected at early stages of erythroblast differentiation, before hemoglobinization [113].

Red blood cell membranes from thalassemic patients carry abnormal deposits of iron, presumed to mediate a variety of oxidative induced membrane dysfunctions. The combination of iron overload and increased outpouring of catabolic iron from the reticuloendothelial system overwhelms the iron-carrying reticuloendothelial system and the iron capacity of transferrin, the main transport protein, to bind and detoxify iron. Non-transferrin-bound fraction of plasma iron might promote the generation of malonyldialdehyde [115] and free hydroxyl radicals, propagators of oxygen-related damage [42, 116, 117]. In addition, several pathobiochemical consequences in thalassemic RBC membranes, such as increased lipid peroxidation and protein thiol oxidation, have been linked to the deposition of generic iron on the cytosolic leaflet of plasma membrane. This mechanism could also contribute to erythroblast apoptosis [118].

It has been shown in mouse model of β-thalassemia intermedia that decreasing iron availability of erythroid cells limits the formation of toxic alpha-chain/heme aggregates and improves ineffective erythropoiesis and anemia [119].

Moreover, it was hypothesized that oral iron chelators, which have an enhanced capacity to penetrate through cell membrane, might be useful in chelating these pathologic iron deposits responsible for ROS generation [118]. This suggestion receives further support from *in vitro* and some *in vivo* studies using these treatments. It was shown that membrane free-iron content decreased as did heme content of RBC membranes from deferiprone-treated thalassemic patients [118]. It has been recently described that deferasirox therapy in β-TM patients is associated with higher levels of circulating erythroid burst-forming unit than controls and other iron chelators [120].

Furthermore, β-TM patients with severe hemochromatosis may develop severe endocrine complications due to iron overload. Hypogonadism, hypothyroidism, and hypoadrenalism may also contribute to anemia. Thus endocrinopathy must be monitored regularly and treated with hormone replacement [121].

4.7.4. Masked Deficit of Folic Acid in Thalassemia. Folic acid deficiency has been reported in both thalassemia major and minor [122–124], as a consequence of increased folate use caused by increased erythropoiesis. It can lead to overestimation of RBC deficiency. Daily folate supplementation is currently advised for patients with hemoglobinopathy [124].

5. Conclusion

The pathophysiological mechanisms of ineffective erythropoiesis in β-thalassemia could be the conjunction of several mechanisms of which the final consequence is the arrest of maturation and increased apoptosis of erythroblasts during their terminal differentiation stage.

Putative actors of ineffective erythropoiesis are suggested to be (1) oxidative stress induced by the excess of α-globin secondary to the α/β globin imbalance, (2) iron overload, and (3) endocrines and cytokine and environmental factors. Key questions still remain to be addressed: how deposition of α-globin chains and/or ROS production in erythroid precursors induce apoptosis and if antiapoptotic processes (involving heat shock protein) in erythroid cells are deficient or overwhelm. In the future it will be critical to decipher the precise role and mechanisms of these components in order to understand the ineffective erythropoiesis in β-thalassemia and to develop new therapeutic strategies based on these potential targets.

Authors' Contribution

J.-A. Ribeil, J.-B. Arlet, G. Courtois, and O. Hermine contributed equally to this work.

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Review Article

Biologic Complexity in Sickle Cell Disease: Implications for Developing Targeted Therapeutics

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Current therapy for sickle cell disease (SCD) is limited to supportive treatment of complications, red blood cell transfusions, hydroxyurea, and stem cell transplantation. Difficulty in the translation of mechanistically based therapies may be the result of a reductionist approach focused on individual pathways, without having demonstrated their relative contribution to SCD complications. Many pathophysiologic processes in SCD are likely to interact simultaneously to contribute to acute vaso-occlusion or chronic vasculopathy. Applying concepts of systems biology and network medicine, models were developed to show relationships between the primary defect of sickle hemoglobin (Hb S) polymerization and the outcomes of acute pain and chronic vasculopathy. Pathophysiologic processes such as inflammation and oxidative stress are downstream by-products of Hb S polymerization, transduced through secondary pathways of hemolysis and vaso-occlusion. Pain, a common clinical trials endpoint, is also complex and may be influenced by factors outside of sickle cell polymerization and vascular occlusion. Future sickle cell research needs to better address the biologic complexity of both sickle cell disease and pain. The relevance of individual pathways to important sickle cell outcomes needs to be demonstrated *in vivo* before investing in expensive and labor-intensive clinical trials.

1. Introduction

Sickle cell disease (SCD) is a group of disorders caused by a mutation in the sequence of beta globin, leading to polymerized hemoglobin (sickle hemoglobin, hemoglobin S), hemolytic anemia, painful vaso-occlusive events, vascular remodeling, acute and chronic organ injury, and shortened lifespan. Sickle cell disease affects over 70,000 individuals in the United States, and there are at least 75,000 hospitalizations costing over $500 million annually for treatment of SCD complications [1]. While survival has greatly improved, the average lifespan for people with hemoglobin SS was estimated in 1994 to be in the midforties [2], significantly less than the average American. Despite well-described genetic and biochemical properties of sickle hemoglobin and many basic science discoveries about sickle cell pathophysiology, modern-day therapy continues to be limited to symptomatic treatment of pain, oxygen supplementation, antibiotics, red blood cell transfusions, and hydroxyurea. Hydroxyurea is an agent that induces fetal hemoglobin production and is the only drug approved for adults by the United States Food and Drug Administration that directly affects sickle cell outcomes. Stem cell transplantation from a histocompatible donor has a high cure rate, but many patients do not have a suitable donor.

Since the passing of the National Sickle Cell Control Act in 1972, over one billion dollars have been allocated from the National Heart, Lung and Blood Institute of the National Institutes of Health (NIH) for SCD research [3]. This funding has resulted in a significant body of research on SCD. The United States National Library of Medicine website lists over 7000 articles since 1950 meeting the search terms of "sickle cell research;" 482 are human clinical trials. As of December 1, 2012, the website http://www.clinicaltrials.gov/ showed 96 open intervention trials in sickle cell disease. Table 1 shows the most common types of studies. Some of these emerging therapies have been recently reviewed [4].

Director of the Division of Blood Diseases and Resources at the NIH, W. Keith Hoots, recently wrote, "Research over the decades indicates that the primary defect in hemoglobin

that results in polymerization of the protein under low oxygen conditions and resultant cellular deformity of the red blood cell initiates a complex downstream pathogenesis associated with vascular injury, and organ ischemia. Deciphering this in a manner that informs successful therapies that improve all target organs continues to challenge hematologists" [5]. It is likely that this complexity is a barrier to successful translation of basic science discoveries in SCD to effective therapeutics. Sickle hemoglobin polymerization is associated with many abnormal downstream processes, but no single pathway has been shown to play a *primary or critical* role in complications occurring in people with SCD. Most mechanistically based clinical interventions have been designed to target individual pathways, but there is likely to be ongoing interaction within the body between different processes, so that even if one pathway is successfully blocked, others may still be active and continue to promote vaso-occlusion or other complications. To address biologic complexity in SCD, this paper will analyze examples of promising clinical trials that did not yield expected benefits, contrast reductionism with systems biology, present models that facilitate the visualization of interactions of mechanisms in SCD complications, and then discuss implications for future research.

Table 1: Major types of sickle cell intervention studies registered on the website http://www.clinicaltrials.gov/, as of December 1, 2012, of a total of 96 trials.

Pathway/mechanism	Number of studies
Bone marrow transplantation	21
Hemoglobin F induction	9
Nitric oxide related	8
Analgesic regimens	6
Nutritional supplements	4
Adhesion inhibition	3
Transfusion therapy	3
Red cell hydration	3
Noninvasive ventilation	3
Statins	2
Renin-angiotensin pathway in nephropathy	2
Iron chelation	2
Educational tools	2
Antiinflammation	2
Gene transfer	1
Carbon monoxide donation	1
Anti-coagulation	1

2. Unexpected Outcomes of Promising Clinical Trials

Preclinical studies and clinical trials targeting three different sickle cell pathways will be reviewed, including inhibition of adhesion by poloxamer 188, inhibition of Gardos channel-induced erythrocyte dehydration by senicapoc (ICA-17043), and treatment of acute pain episodes with inhaled nitric oxide. Comprehensive reviews of approaches to sickle cell treatment have been published elsewhere [4, 6, 7].

2.1. Antiadhesion Therapy with Poloxamer 188. Fluorocarbon emulsions, including identical but variously named compounds Pluronic F-68, Flocor, RheoThRx, and poloxamer 188, have been studied in SCD since 1975 [8]. Pluronic F-68 was demonstrated to reduce *in vitro* sickle red cell static rigidity (stiffness), filterability through a 5 micron filter, and abolish adherence to endothelial monolayers [9]. It is believed to bind nonspecifically to the red cell membrane, "lubricating" the cells and "providing a hydrated, poorly compressible barrier that appears to block hydrophobic adhesive interactions (cell-cell, cell-protein, and protein-protein) in the blood [10]." Preclinical studies demonstrated "a reduction in blood viscosity, erythrocyte aggregation, adhesion to vascular endothelium, and an improvement in microvascular blood flow."

A phase II randomized, double-blinded placebo-controlled trial (RCT) testing poloxamer 188 was conducted with fifty subjects with SCD who presented within 4–18 hours of onset of acute pain. They were treated with either placebo or poloxamer 188 infusion of 300 mg/kg for 60 minutes, followed by 47-hour maintenance infusion of 30 mg/kg/hr. In the 31 subjects who completed the 48 hour infusion, there was a statistically significant reduction in pain duration by 36 hours and a 3–5-fold reduction in analgesic use.

This was followed by a phase III RCT using poloxamer 188, with 255 individuals enrolled at 40 study sites [11]. In the poloxamer 188-treated group, there was a "modest" 9-hour reduction in pain duration, with more pronounced effects in children under 15 years old (21-hour reduction) and subjects receiving hydroxyurea (16-hour reduction). There were no significant differences between the poloxamer 188- versus placebo-treated groups in time to discharge, pain severity ratings, or total analgesic use. Pharmacokinetics showed mean steady state drug concentration "within the therapeutic range of rheological and antiadhesive effects." The differences in responses between the phase II and III trials were attributed to the more stringent definition of pain duration used in the Phase III trial, and the authors concluded that poloxamer 188 may most benefit children with SCD under 15 years of age or those being treated with hydroxyurea.

Close comparison shows differences between the Phase II and III trials. There was a shorter duration of pain prior to beginning study drug in the Phase II trial. The inclusion criteria for the Phase II trial included 4–18 hours of moderate pain on presentation, and there was a median of 17 hours between onset of pain and start of study infusion. In contrast, subjects in the phase III trial had 1.87–2.25 days of pain from onset of crisis to randomization, with an additional 2.3 hours between randomization to start of study infusion, or almost 3 times longer pain duration before study drug infusion was begun. In addition, the loading dose of poloxamer 188 was 3-fold higher in the phase II trial compared to phase III. Lastly, the phase II trial allowed nonsteroidal anti-inflammatory drugs (NSAIDs) for analgesia, but they were not allowed during the study drug infusion and for 12 hours following

discontinuation in the phase III trial. The absence of opioid-sparing effect of NSAIDs may have contributed to the lack of difference in opioid usage.

2.2. Gardos Channel Inhibition. Erythrocyte water content is an important determinant of sickle hemoglobin concentration and polymer formation. Erythrocyte hydration status is controlled by KCl and water loss through two transport systems, K-Cl cotransport and the calcium-dependent potassium, or Gardos channel. Gardos channel inhibition in sickle cells was found to improve erythrocyte hydration status [12]. The orally available antifungal agent, clotrimazole, could completely inhibit deoxygenation-induced Gardos channel-mediated K^+ loss. Oral clotrimazole was found to reduce K^+ loss and improve erythrocyte volume in transgenic mice expressing hemoglobin S, Antilles, and D Punjab (SAD mice) [13] and in a phase I-II trial in five adults with hemoglobin SS [14]. However, clotrimazole as a potential red cell hydrating agent was felt to be limited by its poor oral absorption, short half-life, and the development of elevated hepatic transaminases at high doses.

Based on the structure of clotrimazole, alternative agents were developed to more potently and specifically inhibit the erythrocyte Gardos channel. Senicapoc (ICA-17043) was found to inhibit SAD mouse red cell dehydration *in vitro* and *in vivo* and an increase in hematocrit of 7% [15]. A phase II RCT in humans showed favorable hematologic responses to senicapoc 10 mg daily compared to placebo, with 0.68 gm/dL increase in hemoglobin concentration and reduction in dense erythrocytes, reticulocytes, lactose dehydrogenase (LDH), and indirect bilirubin [16].

This was followed by a phase III RCT with 297 adult subjects randomized at 75 study centers to receive senicapoc for 52 weeks [17]. The dose was 20 mg twice daily for four days, followed by 10 mg daily. The primary endpoint was the frequency of sickle cell-related painful crises requiring medical facility treatment. The study was discontinued early due to lack of efficacy. Despite improvement in hematologic parameters, there was no difference in painful crisis rates between subjects receiving senicapoc compared to placebo. The authors proposed that a possible reason for the discrepancy between hematologic responses and painful crisis rates was that reduction in hemolysis rate increases bioavailability of NO, which in turn enhances nocioceptive pain signaling.

Of note, the Gardos channel, also known as the intermediate conductance Ca^{2+}-activated K^+ channel, $K_{Ca}3.1$, KCNN4, or SK4, is expressed in other cell types, such as T- and B-lymphocytes, macrophages, endothelial cells, fibroblasts, vascular smooth muscle cells, and neurons. In blood vessels, the $K_{Ca}3.1$ channel plays a role in endothelium-derived hyperpolarizing factor- (EDHF-) induced vasodilation, which may play a major role in the microcirculation [18]. Chemical inhibition of the channel or KCNN4−/− knockout in mice resulted in hyperresponsiveness to stress due to enhanced adrenocorticotropic hormone (ACTH) secretion by the anterior pituitary [19]. The $K_{Ca}3.1$ channel has approximately 50% homology with $K_{Ca}2$ family channels, which are small conductance channels located in neurons and involved in afterhyperpolarization. These channels are expressed in high concentrations in the brain and are critical for learning and memory [20]. Therefore, it is possible that the unexpected results of the senicapoc trial may be related to the drug's effect on cell types other than sickle erythrocytes, particularly on EDHF-dependent vasodilation or in the nervous system.

2.3. Inhaled Nitric Oxide for Acute Painful Episodes. Nitric oxide (NO) is an important vasoactive molecule, with effects on vascular smooth muscle dilation and modulating leukocyte and platelet activation. The use of nitric oxide in treatment sickle cell disease acute chest syndrome (ACS) was first reported in 1997 [21], followed by additional critical care medicine reports of clinical benefit in ACS, stroke, and multiorgan failure syndrome [22–24]. Cell-free hemoglobin has been found to reduce NO concentrations [25]. Individuals with hemoglobin SS had 20-fold higher plasma heme concentrations (4.2 μM), and NO consumption was greater in sickle cell plasma and linearly correlated with plasma heme concentration. Forearm blood flow was lower in patients with hemoglobin SS, which improved after infusion of sodium nitroprusside (SNP), a NO donor. Giving inhaled NO at 80 ppm reduced NO consumption of sickle cell plasma by oxidizing and nitrosylating hemoglobin. Inhaled NO at 80 ppm was found to increase skin oxygenation in people with SCD but had no effect on forearm blood flow [26].

In SAD mice, inhaled NO at 20 ppm improved survival rates during exposure to hypoxia when it was given 30 minutes prior to hypoxic exposure and continued during the entire exposure [27]. However, there was no benefit if a lower concentration of NO was used, or if it was given only before or only during hypoxia. The benefits of NO preinhalation and treatment during hypoxia-induced vaso-occlusion were proposed to include reduction of platelet and erythrocyte adhesion, but these mechanisms were not tested.

There have been three published studies of inhaled NO therapy for acute pain episodes in SCD. In the first study, 20 pediatric subjects with SCD were enrolled, 10 who were treated with inhaled NO 80 ppm for 4 hours and 10 with placebo [28]. The group who received inhaled NO had lower pain ratings and less morphine used at 6 hours, but no significant difference in duration of hospitalization. In the second study, nine adults with SCD were treated with inhaled NO 80 ppm for 4 hours and nine with placebo [29]. The group who received inhaled NO had lower pain ratings at six hours, but no difference in morphine utilization. A multicenter RCT of inhaled NO was conducted with 150 children and adults age 10 years and older with SCD presenting with acute pain crisis [30]. Inhaled NO was given for up to 72 hours, with an initial concentration of 80 ppm for the first four hours, 40 ppm for the next four hours, and then pulsed delivery at 5 ppm for the remainder of the 72 hour period. There was no difference between groups for the primary outcome measure, time to resolution of pain, or duration of hospitalization, pain scores at 24 hours, or total opioid use.

3. Lost in Translation?

With the strength of the preclinical findings, why were these therapeutic strategies not effective in reducing the impact

of acute pain, the most common complication of sickle cell disease? Sickle cell clinical research probably suffers from the same challenges as other clinical trials in the United States. In 2012, the Institute of Medicine published a report that discusses the major challenges with current clinical trials, including high costs due to elaborate administrative procedures, failure to enroll sufficient numbers of participants, regulatory issues, and failure to publish negative results [31]. For sickle cell disease research, it has been recommended that clinical trials design be improved, especially to ensure sufficient enrollment, redefine study endpoints, and account for different clinical subphenotypes [4].

In the studies reviewed in this paper, the outcome measures for acute pain were different in many of the studies conducted. The need to use multiple study sites to accommodate the number of subjects necessary for large clinical trials makes consistency of study treatments and outcome measures extremely important. For example, in the phase III inhaled NO RCT, there were institutional differences between study sites, such that two sites had significantly different outcomes than the others. In the poloxamer 188 studies, there were several methodologic differences between the phase II and III trials which may account for the disparity in efficacy results.

3.1. Lack of Models for Acute Pain in Sickle Cell Disease. Beyond challenges inherent to clinical trials, there are some recurring themes in these studies. First, preclinical studies were surrogates, but not sufficiently good model systems, for actual pain episodes. In general, preclinical *in vivo* evidence of the relevance of individual pathways on important sickle cell outcomes has been lacking. For antiadhesion therapy with poloxamer 188, the general "masking" effect was a good approach to address the redundancy of multiple red cell, leukocyte, platelet, and endothelial cell adhesion molecules, but the nearly complete endothelial adhesion blockade demonstrated *in vitro* had not been reported in published animal studies prior to human clinical trials. In the case of Gardos channel inihibition, there was strong evidence for improvement in red cell volume, reduced hemolysis, and anemia, but no testing in a model mimicking painful crisis prior to the phase III clinical trial. Preclinical data for inhaled NO showed beneficial effects on survival in hypoxic conditions in transgenic sickle cell mice, but it was only helpful when given before hypoxia was initiated. Inhaled NO had no effect on forearm blood flow, but somewhat improved skin hemoglobin oxygen saturation. In healthy adults, it has been shown that inhaled NO 40 ppm corrects hypoxia-induced pulmonary hypertension without an effect on systemic vasodilation [32]. While it would be predicted that inhaled NO would promote vasodilation, reduce inflammation, and improve red cell hydration, none of these outcomes were reported in the three inhaled NO clinical trials, making it difficult to ascertain whether there was any improvement to these proposed mechanisms resulting from treatment.

At the heart of the matter, it remains an article of faith that sickle cell polymerization and microvascular occlusion is the actual cause of acute pain in SCD. There is currently no way to visualize vaso-occlusion to corroborate that sites of sickle erythrocyte microvascular occlusion correspond to locations where patients feel pain or to show that therapies that restore or maintain normal blood flow will relieve vaso-occlusion and pain. Intravital microscopy of various vascular beds in rodents has been used to study agents that block adhesion of sickle erythrocytes and leukocytes, including *ex vivo* rat mesenteric vessels, transgenic mouse cremaster, and brain window [33–35]. While vascular occlusions can be visualized, animals are anesthetized during the procedures and cannot be evaluated for pain. In humans, conjunctival and skin blood flow have been shown to be abnormal in people with SCD [26, 36], but these are not typically sites of pain, and blood flow has not been specifically measured at these sites during acute pain episodes. Bulbar conjunctival blood flow was found to improve after erythrocytapheresis [37] and in a small number of subjects treated with poloxamer 188 [38].

3.2. Drug Delivery and Timing. Even if a therapy is effective in the person with SCD, there may be difficulty in maintaining effective pharmacokinetics, timing of therapy, and/or drug delivery to the site of vaso-occlusion. A treatment may work well at certain concentrations *in vitro* or in transgenic mice, but these same concentrations may not be achievable in the setting of a person with a larger volume of distribution for the necessary duration of the acute painful crisis. In early nitric oxide inhalation studies, acute treatment with inhaled NO 80 ppm was for 4 hours, and followup was for 4–24 hours. It is possible that these time courses were too short to be effective, since vaso-occlusion commonly does not resolve in 6 hours. In the case of established tissue injury from ischemia, pain may not necessarily resolve within a day. In the larger randomized controlled trial of inhaled NO, the equivalent of NO 5 ppm was given for up to 72 hours, an overall lower concentration, not previously reported to have an effect on vasodilation or inflammatory markers, and the authors state that this lower rate of administration may have been insufficient to generate systemic nitrite, an NO metabolite which may mediate its tissue effects.

As in stroke therapy with neuroprotectants and thrombolytics [39], timing of therapy in acute painful crisis may be an important factor in effectiveness. Sickle cell pain may be similar to myocardial and cerebral infarction, in that the benefits of therapies to reverse vessel occlusion and restore perfusion need to be initiated very early in the ischemic process to be effective, and otherwise the ischemic injury is established and cannot be readily reversed. We currently have no way of knowing whether there is still ongoing vaso-occlusion when a person with SCD presents for treatment of pain, if the pain is instead associated with reperfusion or due to persistent transmission from afferent sensory neurons that have been activated in ischemic tissues. If pain is a postocclusive event, then treatments that reduce sickle cell polymerization, adhesion, thrombosis, or vaso-constriction may not have much effect on the acute pain experience, though they may prevent worsening or progressive vaso-occlusion.

In the hemoglobin SAD mouse hypoxia studies, inhaled NO was only effective in preventing mortality when the animals were pretreated for 30 minutes prior to hypoxic exposure, which would not be feasible in people. Nitric oxide may be effective if therapy is begun at the very outset of vaso-occlusion *and* if vaso-constriction and inflammation are important inciting factors. Individuals in the clinical trials may present for treatment to the clinical research site several days after the initiation of the vaso-occlusive event, after unsuccessful home therapy. A similar effect of timing was seen in poloxamer 188 studies, where the drug was more effective in the Phase II trial when it was given earlier in the onset of the painful episode.

3.3. Complexity of Sickle Cell Disease. Lastly, it is likely that therapies which target specific components of sickle cell pathophysiology do not sufficiently inhibit the entire process that makes up acute pain episodes. Poloxamer 188 has the potential to inhibit multiple cell-cell interactions, but adhesion has not yet been demonstrated to be a major mechanism contributing to acute painful episodes. While the effects of senicapoc on sickle erythrocyte hydration and total hemoglobin concentration has been consistent between *in vitro* experiments, transgenic mice, and humans, it may also have effects on the $K_{Ca}3.1$ channels of vascular cells and/or neurons that could adversely affect acute painful episodes. Inhaled NO could potentially improve blood flow by vasodilating vessels and reduce inflammation and platelet adhesion, but it may also promote cyclic GMP-mediated nociceptive pain transmission. Even if each of these therapies had fully beneficial effects on vaso-occlusion, alone they may not be adequate to reverse a painful crisis in progress if vessels are completely occluded with irreversibly sickled erythrocytes or if the pain is due to reperfusion response rather than vaso-occlusion.

Early reports discussing complexity in SCD date back to 1974 and 1983 [40, 41]. Frenette in 2002 described sickle cell vaso-occlusion is as a "multistep and multicellular paradigm," involving sickle erythrocyte and leukocyte adherence in addition to sickle hemoglobin polymerization [42]. In 2009, HebbeI provided a detailed review of the many "subbiologies" that are involved in sickle cell vaso-occlusion and recommended multimodality chemoprophylaxis to target them simultaneously [43], a recommendation reiterated by others [4, 44]. Complexity in SCD has been recognized for several decades, but for practical considerations and the mandate for feasible hypothesis-driven research, most SCD research has focused on understanding and intervening in individual pathways.

To date, systems biology high-throughput and large dataset methodologies, or "omics" studies, of SCD have included transcriptome analysis of blood outgrowth endothelial cells, monocytes and reticulocytes [45] in humans, and kidneys in transgenic sickle cell mice [46]. Blood outgrowth endothelial cell transcriptome analysis showed that individuals with SCD and arterial occlusion in the Circle of Willis had higher expression of genes regulated by NFK-B and RelA, regulators of inflammation [47]. Sickle cell monocytes demonstrated differential expression of genes involved in heme metabolism, cell-cycle regulation, antioxidant and stress responses, inflammation, and angiogenesis [48]. Proteome analysis of sickle cell erythrocytes and plasma have shown upregulation of antioxidant proteins, an increase in cytoskeletal defects, an increase in protein repair and turnover components, a decrease in lipid raft proteins, and apolipoprotein dysregulation [49]. Genome-wide association studies (GWAS) were performed using DNA from over 1000 subjects in the Comprehensive Study of Sickle Cell Disease cohort [50]. Several genes that were identified were associated with severity of SCD symptoms and certain complications. Epigenetic analysis of reticulocytes before and after hydroxyurea demonstrated increased expression of microRNAs (miR)-26b and miR-151-3p to be associated with fetal hemoglobin response [51].

4. A Systems and Network Approach to Sickle Cell Disease

Reductionism in science dates back to René Descartes in the 1600s [52]. His approach was to "divide all the difficulties under examination into as many parts as possible… beginning with simplest and most easily understood objects, and gradually ascending… to the knowledge of the most complex."

Systems theory was first coined by Ludwig von Bertalanffy in the 1940s, defining it as the transdisciplinary study of the abstract organization of phenomena, independent of their substance, type, or spatial or temporal scale of existence. It investigates both the principles common to all complex entities and the (usually mathematical) models which can be used to describe them [53].

Donella Meadows defines a system as "a set of things interconnected in such a way that they produce their own pattern of behavior over time. The system may be buffeted, constricted, triggered, or driven by outside forces. But the system's response to these forces is characteristic of itself, and that response is seldom simple in the real world… Our own bodies are magnificent examples of integrated, interconnected, self-maintaining complexity [54]." Key definitions used in systems models include *stocks*, which are measurable materials or information; *flows*, or the movement of stocks, including inflow and outflow; and *feedback loops*, closed chains of causal connections from a stock that regulate the behavior of stocks. The concept of stabilizing or balancing feedback loops is analogous to its physiologic definition, where balancing feedback is required for homeostasis. In sickle cell disease, an example would be the interaction between free heme released by hemolysis and natural heme scavengers, such as haptoglobin and hemopexin. Health reflects a state of balanced equilibrium, whereas illness results from disequilibrium or loss of homeostasis.

Systems biology is the application of systems thinking to the study of biologic processes. Kitano wrote that systems biology is a ""holistic" approach to interconnect different cellular processes, such as metabolism and genetic regulation, instead of traditional reductionist methods [55]." Breitling

states that it is "based on the comprehensive study of the molecular diversity of living systems, both natural and synthetic, the identification of simplifying general principles and patterns that are recurring features in living and engineered systems, and the integration of our biological knowledge in complex models of the regulatory networks that characterize life [56]." Machado defined that "systems biology is used to model complex biological processes using computational tools and high throughput experimental data [57]." However, Joyner warns that systems biology methods need to be used in the context of physiologic principles, such as homeostasis, feedback, redundancy, and adaptation, rather than applied generically and without guiding hypotheses [58].

Network thinking was defined by Mitchell as "focusing on relationships between entities rather than the entities themselves [52]." A network is a collection of *nodes* connected by *links*. Nodes to connected to many others are *hubs*. Hubs are highly vulnerable to failure or can be targeted for attack. Examples of nodes are cells or molecules, and links can be relationships or physical connections, such as synapses, fibers, or routes. Alon has applied engineering principles to model biologic functions [59]. He wrote that, "Simplicity occurs in biologic networks. There are only a few types of recurring interactions, or *network motifs*. Each motif can perform defined information processing functions... Most biologic functions are carried out by specific groups of genes and proteins which form *functional modules*. For example, proteins work in coregulated groups such as pathways and complexes. This is analogous to modules in engineering, subroutines in software, or replaceable parts in machines. The working definition of a module *is a set of nodes that have strong interactions and a common function*."

These concepts of systems thinking and network analysis were used to develop diagrammatic models that incorporate and which show potential interactions between multiple mechanisms putatively involved in acute pain episodes (Figure 1) and chronic vasculopathy (Figure 2) in SCD. These are not classic biologic network models with specific proteins or genes as nodes but include as modules major mechanistic pathways in SCD for which there is existing evidence. In these models, the primary defect of sickle hemoglobin polymerization causes secondary processes of hemolysis and vaso-occlusion, which further transduce their effects to blood vessels and organs through oxidative, inflammatory, vasomotor, coagulation, and angiogenic intermediaries. The roles of balancing feedback loops in homeostasis and the effects on the vascular bed are central features of the models.

4.1. Acute Pain Model. Figure 1 shows potential relationships between mechanistic pathways in SCD that lead to acute pain episodes. The most commonly considered pathway in this process begins with sickle hemoglobin polymerization, leading to formation of rigid sickle erythrocytes and microvascular occlusion when these cells become trapped in small vessels. In the presence of inflammation (infection or ischemia and reperfusion), vascular endothelial cells express a number of adhesion molecules that facilitate adherence of mature erythrocytes, reticulocytes, activated leukocytes, and/or platelets. Endothelial tissue factor expression can activate coagulation factors and thrombosis.

Hemolysis can also contribute to vaso-occlusion, through the release of free heme, reactive iron species, and membrane microparticles. Free heme can bind NO and reduce its bioavailability, which promotes vaso-constriction, inflammation, and platelet aggregation. Heme and reactive iron species can directly cause injury and oxidative damage to endothelium. Erythrocyte membrane microparticles with exposed phosphatidylserine may active platelets and promote coagulation. Hemolytic anemia reduces oxygen delivery to tissues and contributes to reduced tissue and organ perfusion chronically, likely leaving them vulnerable to the effects of acute vaso-occlusion.

After vaso-occlusion has occurred, there is local tissue ischemia and reperfusion, which includes inflammatory and oxidant responses. Our group has demonstrated elevated levels of hypoxia-inducible factor- (HIF-) associated angiogenic growth factors in children with hemoglobin SS during steady state [60], suggesting a chronic baseline state of tissue ischemia even in the absence of symptomatic pain or acute complications. In particular, levels of stromal-derived factor- (SDF-) 1α, produced by ischemic endothelium and other cells, were associated with the number of bone marrow-derived circulating progenitors with angiogenic potential ($CD34^+$/$VEGFR2^+$) and total white blood cell (WBC) count. Total WBC has been found to be associated with SCD severity in some series, and the relationship between WBC and SDF-1α suggests that those people with the most ongoing tissue ischemia may have the most vaso-occlusive organ injury. Tissue ischemia has a reinforcing effect on inflammation, through HIF-mediated angiogenic stimulation of WBC release from the bone marrow.

Pain is itself a complex process and is not diagrammed in detail in this model. The pain experience is the product of the nociceptive input from injured tissue but also involves cognitive, contextual, mood, and individual differences, such as sex, age, and genetics. Stress related to acute pain can induce neuro-endocrine responses, such as stress steroids, catecholamines, and pain peptides (substance P, neurokinins). Catecholamines promote vaso-constriction, and the pain peptides can be proinflammatory. The current model also does not attempt to delineate all of the external factors that may also influence a pain episode, such as environment (ambient temperature, second-hand smoke) or psychosocial factors. Neuropathic pain resulting from ischemic injury directly to nerves may account for some of the pain experienced in SCD and would not necessarily respond to antisickling therapies or those targeting inhibition of vaso-occlusion. Chronic pain may involve "imprinting" of the nervous system by epigenetic modifications (DNA methylation, histone modification) that regulate gene expression [61]. Chronic pain and opioids can alter the structure of the brain [62, 63]. To summarize, there are many pathways that can interact in acute pain episodes in SCD, some positively reinforce others, and some factors are outside of the body.

4.2. Chronic Vasculopathy Model. A model with similar features describes the development of chronic vasculopathy in

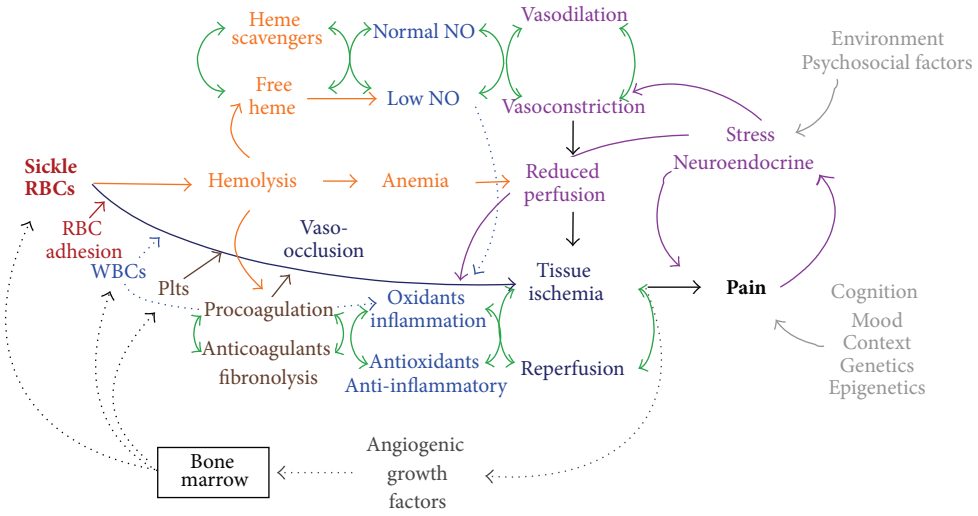

FIGURE 1: Acute pain model. This diagram shows proposed interactions between pathophysiologic mechanisms in sickle cell disease that lead to acute painful episodes. Key to mechanisms: green: balancing feedback loops, red: erythrocytes, orange: hemolysis, light blue: inflammation and oxidant stress, dark blue: ischemia and reperfusion, lavender: vasomotor, brown: coagulation, gray: angiogenesis, and light gray: pain modifiers. See text for details.

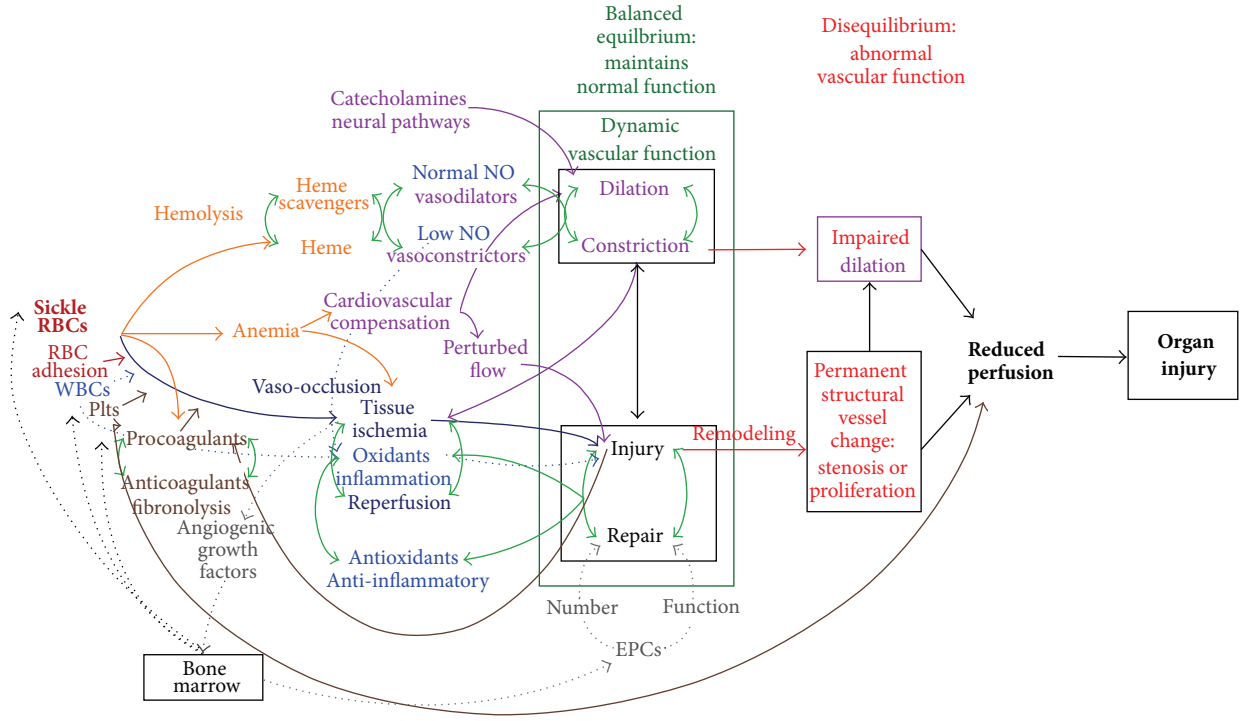

FIGURE 2: Chronic vasculopathy model. This diagram shows proposed interactions between pathophysiologic mechanisms in sickle cell disease that lead to chronic vasculopathy and organ injury. Key to mechanisms: Green arrows: balancing feedback loops, Red: abnormal vascular structure and function, orange: hemolysis, light blue: inflammation and oxidant stress, dark blue: ischemia and reperfusion, lavender: vasomotor, brown: coagulation, and gray: angiogenesis. See text for details.

SCD (Figure 2). In the vasculopathy model, dynamic vascular function is the central process affected by SCD derangements. In the healthy state, there is balanced equilibrium between vasodilation and constriction, and between endothelial injury and repair.

Hemolysis will cause disequilibrium in favor of vasoconstriction, and longterm exposure can eventually cause sustained impairment in vasodilation and reduced vessel compliance (stiffness). Early stage remodeling may cause arterial wall stiffness and reduced compliance before arterial stenosis is apparent in imaging studies. This may be the case in those children with elevated transcranial doppler (TCD) velocities in the cerebral arteries that is associated with high stroke risk, but whose brain magnetic resonance arteriography (MRA) shows no arterial stenoses.

Anemia reduces oxygen delivery and organ perfusion. Ischemia and reperfusion due to the combination of anemia and repetitive vaso-occlusion stimulate HIF-associated angiogenic growth factors. Anemia-associated compensatory circulatory changes are likely to cause disturbed blood flow, which can promote endothelial injury, and potentially contribute to vessel wall remodeling (intimal proliferation and arterial stenosis). Our group has demonstrated elevated serum concentrations of platelet-derived growth factor-(PDGF-) AA, a mediator of vascular remodeling, and brain derived neurotrophic factor (BDNF), a biomarker of cerebral ischemia, in children with hemoglobin SS and high TCD velocities [64], consistent with the model. High frequency of red cell transfusion therapy reduced PDGF-AA, soluble VCAM-1, and RANTES in children with hemoglobin SS and high TCD velocities, suggesting that correction of anemia and reduction in sickle cells by transfusion reduces vascular injury, inflammation, and vascular remodeling responses [65].

Considering sickle cell disease through the lenses of these models helps explain the potential limitations of therapies that are targeted to single pathways. While the therapies discussed earlier may have some multimodal effects, there are many additional contributors to acute pain that were not modulated by individual therapy. These models contrast conceptually to the bimodal model proposed by Kato, in which certain SCD complications are primarily related to either hemolysis or vaso-occlusion, and that individuals exhibit one or the other subphenotype [66]. In these models, hemolysis and vaso-occlusion occur simultaneously in both acute pain and chronic vasculopathy, although not necessarily equally in magnitude at any given time.

Complexity in SCD does not necessarily mean that the disease is hopelessly complicated and cannot be successfully treated. In these models, sickle hemoglobin polymerization is the network hub, and therefore most vulnerable to attack (correction). This is consistent with the clinical observation that red cell transfusions and hydroxyurea, which correct hemoglobin S polymerization and/or sickled erythrocytes, are often effective SCD treatments and also reduce downstream mediators. However, while hydroxyurea is an alternative to transfusions for certain SCD indications, it is not an equivalent therapy. In the setting of established structural vascular disease, such as significant cerebral vasculopathy in individuals with stroke, hydroxyurea therapy in combination with phlebotomy to relieve iron overload was not as effective as transfusions in preventing stroke [67]. Even chronic red blood cell transfusions are not effective in preventing recurrent stroke in up to 20% of individuals [68], suggesting additional unidentified stroke mediators in a subset of individuals with the most severe disease.

Viewed from this perspective, replacement of sickle erythrocytes by stem cell transplantation, gene therapy correction of the hemoglobin S mutation, or very effective fetal hemoglobin induction are likely to be the most effective SCD treatments in the long run. However, until these are widely available, should severe sickle cell disease be treated like thalassemia, with lifelong chronic red cell transfusions? How do we identify those at highest risk who would benefit from life-long transfusions begun early in life?

5. Future Directions

This paper of selected clinical trials and discussion of complexity in SCD has identified some challenges in the search for alternative effective therapies. There are logistical and methodological issues related to clinical trials, such as consistent study endpoint definition and effective timing and delivery of therapeutics; lack of good model systems in which to test the effect of therapeutics; and the larger problem posed by complexity—it is difficult to shut down all aspects of the system at once without using transfusions or stem cell transplantation. Acknowledgment of complexity does not imply that therapies targeting individual intermediary mechanisms in SCD should be abandoned but necessitates that their effectiveness needs to be tested in highly predictive model systems prior to embarking on large-scale clinical trials. In this section, some possible approaches to these challenges are suggested.

5.1. Strategic Mechanistic Testing of Therapeutics in the Transgenic Sickle Cell Mouse Model. There is a need to develop preclinical model systems for complications such as acute pain or chronic vasculopathy that are highly predictive of those processes in people with SCD. There are currently transgenic sickle cell mouse models of acute chest syndrome and pulmonary hypertension, but none yet for SCD-associated stroke or acute painful crisis. The most extreme model used in transgenic sickle cell mice is hypoxia-induced death, which presumably occurs from sickle cell-induced vaso-occlusion in the entire animal. While not similar to human acute pain episodes in severity, drugs that are potent enough to prevent this degree of sickling or vaso-occlusion should be effective in less extreme situations. For example, the lack of beneficial effect of inhaled NO in the phase III RCT was accurately predicted by the lack of rescue in the hypoxia-exposed SAD mice who were treated with only posthypoxia inhaled NO. If there was a way to image sites of pain while simultaneously measuring pain behaviors in the sickle cell mouse, it might be useful to titrate the severity of the hypoxic exposure to a sublethal dose that might simulate acute painful events.

Transplantation of sickle cell mouse bone marrow or injection of sickle mouse erythrocytes into a transgenic strain with a desired gene knockout may be used to prove the essential role of an individual molecule in sickle cell pathogenesis. These approaches have been used to demonstrate the role of P-selectin in sickle cell microvascular occlusion [69] and superoxide produced by NADPH oxidase in cerebral artery microvascular occlusions [35]. Tissue-specific conditional knockouts in a transgenic sickle cell mouse would be an elegant but technically challenging experimental approach to test the role of individual molecules in the "native" environment of the transgenic sickle cell mouse. However, such an approach should only be used in pathways in which there is no physiologic redundancy. At least ten different sickle erythrocyte membrane proteins or components have been identified as potentially involved in adhesion or interaction with endothelial cells [70], so that knocking down only one is unlikely to have major impact on vaso-occlusion, unless it is a *critically* important molecule that has greater impact than all of the others in combination.

5.2. Develop Imaging Systems for Vaso-Occlusion. One of the most basic tenets of the field is that acute pain episodes are caused by the occlusion of microvessels by sickled erythrocytes. While this makes theoretic sense on the basis of hemoglobin polymerization, rheologic and microvascular studies, this has never been proven in people with SCD. When does acute pain begin relative to vaso-occlusion? Do individuals with full body pain really have sickling everywhere? Does vaso-occlusion in one part of the body induce pain at distant sites through crosstalk between neurons in the sensory pathway or central nervous system? The field needs ways to visualize both sites of vaso-occlusion and pain pathways to demonstrate that they are actually related and to have an outcome measure for testing pain treatments. Vaso-occlusive sites could potentially be visualized by radionuclide-tagged particles that home to ischemic tissue markers, such as SDF-1α.

5.3. Interdisciplinary Pain Research. The pain research field apparently has similar challenges in developing chronic pain treatments as has been described here for SCD. Borsook wrote "Drug development for pain often fails, paralleling many other CNS areas, because preclinical and experimental clinical proof-of-concept (POC) studies do not translate well to clinical conditions and patient populations [71]." Proposed biomarkers for pain include functional brain imaging with analysis of focal brain regions and chemical biomarkers, such as CNS neurotransmitters (e.g., glutamate, GABA, and glycine) and brain metabolites (e.g., NAA, choline), which can be measured *in vivo* using magnetic resonance spectroscopy (MRS) [72]. Functional MRI (fMRI) with support vector machine learning analysis of the whole brain is able distinguish pain without the need for verbal communication [73]. Since pain is such a large part of SCD symptomatology, future research collaborations with established pain neurobiologists equipped to use these state-of-art approaches are warranted as suggested by the NIH Blueprint for Neuroscience "Grand Challenge on Pain [74]."

5.4. Develop a Consensus Set of Meaningful Study Endpoints and Test for Mechanistic Markers during Clinical Trials. For agents that prevent pain, the number and duration of acute painful episodes, including both facility- and home-treated events, and quality of life measures related to pain may be a more accurate assessment of the true effectiveness of a pain prevention therapy. In the treatment of acute pain, there needs to be agreement on the definition of who should be hospitalized for therapy to assure comparability of groups and on the definition of what constitutes "resolution of pain." It has also been recommended that study endpoints better match the mechanism of action of the therapeutic [4, 44]. For example, pain outcomes may not be applicable to agents that reduce hemolysis (Gardos channel inhibitors) or target NO signaling, since they may be more likely to be beneficial in the vasculopathy subphenotype (pulmonary hypertension, priapism). Analogously, there needs to be consensus on vasculopathy endpoints. For example, is echocardiographic measurement of tricuspid regurgitant velocity acceptable as a noninvasive surrogate of pulmonary hypertension? In the case of any endpoint, does it respond rapidly enough to therapy to be feasibly measured as a study outcome?

To better understand the role of therapeutic mechanisms, biomarkers or other functional outcome measures should be included as part of early phase clinical trials. As mentioned in the earlier discussion of inhaled NO, none of the published clinical trials reported on vasodilation, platelet aggregation, or inflammatory biomarkers, such as sVCAM. Senicapoc's red cell effects were consistently measured in each clinical trial, so that lack of efficacy in reducing pain frequency appears to be unrelated to improvement in cell hydration status. In this situation, the data helps guide investigators in the analysis of therapeutic and unwanted side effects. Measurement of biomarkers makes clinical trials more labor intensive and is unlikely to be feasible at every study site. However, it provides important evidence to explain drug efficacy or lack thereof.

5.5. Directed Delivery of Therapeutics to Ischemic Sites. In combination with biomarker and functional assays, early phase clinical trials should include well-described pharmacokinetics to establish that the administered doses result in plasma concentrations that are comparable to those used in preclinical studies. However, systemic concentrations of a drug may not be the same as the amount delivered to the affected tissues, especially in areas of reduced perfusion. There is an opportunity to apply nanotechnology to selectively deliver analgesics, antisickling, other vaso-occlusion disrupting agents, or drugs that improve tissue oxygenation to ischemic regions (presumably corresponding to vaso-occlusion) by targeting ischemia markers, such as SDF-1α.

5.6. Systems Biology, "Omics," and Computational Modeling. Systems biology and "omics" technologies may be useful in understanding complex biologic systems. This approach is in its infancy in SCD and could be applied to dissecting specific problems, such as identifying gene variants or microRNAs that predispose to higher risk of well-defined complications

such as stroke, chronic pain, changes in gene expression, or epigenetic modifications that occur with therapies. Another goal for the application of "omics" to SCD would be to identify master regulators that control multiple pathophysiologic mechanisms, so that these could be targeted for inhibition.

Predictive computational models can potentially be used to integrate multiple types of patient data, such as laboratory values, radiographic findings, circulating biomarkers, and genetic and epigenetic data, with disease phenotype to define risk categories. The ability to identify individuals at highest risk for severe complications would allow the option of early treatment with high-risk therapies, currently red blood cell transfusion or stem cell transplantation, well before the onset of complications. The predictive strength of such modeling approaches would be enhanced by including as many individuals with SCD as possible, perhaps through a collaborative national data registry and biorepository system.

5.7. Improve Understanding of the Effects of Psychosocial Determinants and the Environment on SCD Complications. It has been well described that stresses related to poverty and racism affect cardiovascular risk and disease and disproportionately affect African Americans. It is very likely that such gene-environment interactions are additional factors influencing SCD complexity and outcomes and are currently not adequately understood. For example, how do chronic undernutrition, lack of utilities, poverty, or personal or familial mental illness affect the frequency and severity of acute illness or the response to therapy in a person with SCD? Epidemiologic methodologies including geocoding could be applied to studying some of these factors in SCD. Such variables can be added to computational predictive models to help us begin to understand the relative contributions of factors in SCD complications.

6. Conclusions

SCD is caused by a single mutation in beta globin but triggers several pathophysiologic pathways and results in a highly complex disease. This complexity is likely to be one of the major barriers to the development of successful new treatments which, to date, has largely concentrated on individual mechanistic pathways. Future development of therapeutics needs to continue to focus on correcting the underlying problem of sickle hemoglobin polymerization but should also include development of better model systems for acute and chronic SCD complications, methods for visualizing and measuring vaso-occlusion and associated pain, directed delivery of therapies to sites of vaso-occlusion, systems biology approaches to identify master regulators of the multiple downstream effectors of hemolysis and vaso-occlusion, and better understanding of the contribution of gene-environment interactions on sickle cell disease complications. Considering the number of pathophysiologic processes caused by SCD, it is astonishing how well the body maintains homeostasis sufficient for growth, development, and general health for periods between acute illnesses. The approach to this disease should also include an effort to identify mechanisms that are crucial to maintaining homeostasis and wellness. While there have been many life-saving advances in the treatment of SCD, much work remains to achieve the goal of curing the disease and developing safe and effective therapies to improve health and well-being.

Conflict of Interests

The author has no financial interests in any of the commercial products mentioned.

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Research Article

The Impact of Migrations on the Health Services for Rare Diseases in Europe: The Example of Haemoglobin Disorders

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Migration from different parts of the world to several European countries leads to the introduction of haemoglobinopathy genes into the population, which creates several demanding needs for prevention and treatment services for Hb disorders. In this paper we examined the degree to which European health services have responded to such challenges and in particular to health services necessary to address the needs of patients with thalassaemia and sickle cell disease (SCD). Information on available services was obtained from international organizations, collaborated European project, and the Thalassaemia International Federation (TIF) Databases, which include information from published surveys, registries, field trips, and delegation visits to countries and regions by expert advisors, local associations, and other collaborators' reports. Results show that countries with traditional strong prevention and treatment programs are well prepared to face the above challenges, while others are urgently needed to address these problems in a systematic way. The Thalassaemia International Federation (TIF) is committed to monitor the progress, raise awareness, and support the promotion of more immigrant-oriented health policies to ensure their integration in society and their access to appropriate, adequate, and timely health services.

1. Introduction

Throughout history, poverty, land pressures, climate change, famine, war, and persecution have forced people to move from their homeland and in this context migration is not at all new. Migrants, like all people, carry with them personal health prints made up of ethnic and family disease susceptibilities and reflect the ways in which people and cultures have adapted to their physical environment and the mechanisms they have developed to deal with illness. As such, free population movements have always been considered important challenges to global health. Today as the gap between rich and poor countries is growing, people are moving faster and further, crossing vast climate and "disease zones", being forced, in greater numbers, to seek work and better life elsewhere. At the same time richer countries are actively recruiting people to address their emerging labour needs while modern means of transportation and communication make it much easier for people to migrate while seeking better opportunities in life.

Population movements have had a major impact on disease epidemiology and public health. In the past, the main concern has been the spread of communicable diseases linked to poverty, suboptimal hygiene conditions, and lack

of contemporary prevention programs and public health services. The more recent migration process observed in the end of the twentieth and dawn of the twenty-first century continues to contribute to the spread of communicable diseases but have in addition resulted in dramatic changes in the epidemiology of chronic diseases previously unknown or of low prevalence in host populations. These new "imports" represent a significant additional challenge to health services on a global scale.

Migrants experience a unique journey linked to the classical four phases of migration: premigration preparation, arrival, integration, and return. Without underestimating the significance of all stages of migration, during the arrival and integration phase, poverty and social exclusion are considered to exert their greatest effect on individual and group health outcomes. This is the period when the health of migrants is influenced by the availability, accessibility, acceptability, and quality of services in the new host environment. Health services may not be accessible because of linguistic, cultural, religious, and social barriers and this situation may sadly persist for many years after their establishment in the new host country.

The above were clearly evidenced in countries which traditionally hosted immigrants from countries where Hb disorders have been highly prevalent including UK, where in 2000 it was shown that despite available quality health services for prevention, screening, and care, there was only 50% uptake of such services by immigrants [1] and only 50% chance of survival of patients with β-thalassaemia major at the age of 35 [2]. The UK presents an example of a country where, today, third- or fourth-generation immigrants, from haemoglobinopathy prevalent countries, live and work and despite this, it was not until such data were analyzed that national strategies for these diseases were put in place.

The global geographical distribution of the haemoglobinopathy genes is today well documented [3, 4] and it is well known that in Europe, such genes are endogenous mainly in the populations of the south, especially in the countries of the Mediterranean basin and to a lesser extent in some of the countries of Eastern Europe. In contrast, these genetic traits are quite rare in the western, central, and northern countries of the European Continent. Migrants from the Middle East (ME), South East Asia (SEA), and other mainly malaria-endemic (or previously endemic) countries of the world have been moving, over the 20th century, to the western part of Europe. In addition migrants from the South, previously less affluent countries of Europe, have also been migrating towards West and North Europe. Such populations have now reached their second, third, or even fourth generation in these countries. In more current years, however, the immigration statistics are dramatically changing. There has been a substantial increase of population movements from ME, Asia, and Africa, much less from the south of Europe, and more from the Eastern European countries who have recently gained accession to the European Union (EU). Host countries in the last decade include mainly the fifteen old states of the EU [5]. These changes bring about considerable challenges to all systems of the host countries including most importantly their health services system.

In this study we have evaluated the degree to which European health services have responded to such challenges and in particular to health services necessary to address the control of rare anaemias and more specifically of haemoglobin disorders (Hb disorders): thalassaemia and sickle cell disease (SCD). We have focused on the compilation and analysis of the current status of services in specific European countries that appear to receive the majority of migrants from high-prevalence areas.

2. Methods

2.1. Countries. In this study we have included 12 European countries: Austria, Belgium, Cyprus, Denmark, France, Germany, Greece, Italy, The Netherlands, Spain, Sweden and the United Kingdom (UK).

2.2. Criteria. In order to examine the impact of Hb disorders on the health services, we examined the specific control programs for such health issues, which have literally been introduced through long-term and recent migration in almost every European country. Such assessment required the gathering of information on the following aspects of migration:

(i) the size and origin of each migrant group or ethnic minority in each host country and whether these are permanent or temporary workers/residents;

(ii) the expected contribution of migrant groups to the haemoglobinopathy gene pool in the new host country;

(iii) the marriage and reproductive behaviour of the new population groups versus the second, third (or more) generation of immigrants.

In addition, the services were evaluated having in mind the needs of those affected by such disorders. The services to be provided and planned for are those that aim at the best possible survival and quality of life of patients. These include preventive measures which will allow informed choices by risk couples from these communities. In considering the needs for health care services, the rarity of the conditions in many countries was taken into account. Service needs according to published best practice guidelines [6] were built mainly on knowledge and experience derived from successful control programs implemented in some countries. The characteristics of such programs include

(i) spread of community awareness, education, screening, and counselling,

(ii) availability of possible solutions such as prenatal diagnosis or preimplantation diagnosis as available choices for informed couples,

(iii) neonatal screening programs for sickle cell disease,

(iv) system capacities to provide timely and accurate diagnosis,

(v) provision of medical and other care and monitoring,

(vi) provision of multidisciplinary care in expert centres with effective networking of centres within and between countries to support primary and secondary level services,

(vii) education of the medical and patients' communities.

In order to achieve the above objectives, appropriate public health and services planning were assessed regarding the existence of national policies to address the control of haemoglobin disorders, are regarded as essential. National policies may be disease specific as for example in a disease management programs (Cyprus, Italy, and Greece), under a general plan for rare diseases, or for chronic diseases in general (France).

2.3. Sources of Information. Since all Hb disorders and other rare anaemias are usually treated by the same clinical and public health services, it was possible for TIF, both through its own work and also through its partnership with national and international organizations and projects, to evaluate the current situation of services for Hb disorders and by extension for other rare congenital anaemias in European communities. In order to have a clear picture of the situation and to advocate for the development of services where they are most needed, TIF has developed, over the past few years, a global database for Hb disorders, which includes not only information on the burden of disease in each country, but also on available services and on the extent of access of patients to free and comprehensive care within national health systems. Having in mind the resources required for lifelong care, the existence of prevention measures as a tool to save and reallocate resources is also examined in this study. In order to estimate the magnitude of the problem the following parameters, numbers, and estimates were considered.

(1) The number of immigrants in each European country, whose origin is from countries or populations with a thalassaemia and SCD carrier rate of more than 1%. The influx from countries with a low prevalence is not included in our estimates and the major source of the data is the Eurostat Population Database [7].

(2) The carrier rate in each ethnic group which is derived from the carrier rate in the country of origin was used to estimate the number of carriers within each group. This was then added to the carrier rate of the indigenous population (for the low-prevalence countries the carrier rate is assumed to be 0.1% unless more accurate details are known) [5].

(3) Carrier rates and other figures were taken from the Thalassaemia International Federation (TIF) Databases, which include information from published surveys, registries, field trips, and delegation visits to countries and regions by expert advisors, local associations, and other collaborators' reports. On occasions, reports were used from industry and national/regional health authorities and from relevant scientific conferences. In this database most African and American continents data are incomplete and were thus supplemented from the modell's almanac [8].

(4) From the total carriers calculated as described above, the expected annual affected births were estimated according to the Hardy-Weinberg rule [9].

(5) The disease burden was completed by recording the number of known patients in each country. The number was again obtained from TIF member associations, published information, and national registries wherever possible.

3. Results

Assessment of the quality of services currently available in each country has been made possible through the work of ENERCA and the funded project from the Executive Agency for Health and Consumers. This funding was provided for the preparation of the White Book on the identification of the criteria for haemoglobinopathy reference centres and networks in the European Union. This White Book includes several components such as the existence of national and regional health policies supporting services for haemoglobin disorders, the assessment of a comprehensive disease management policy for chronic and/or rare diseases. The existence of a national policy for prevention of thalassaemia including education, screening, counselling, and prenatal diagnosis, the establishment of a neonatal screening program for sickle cell disease, and the institution of a health insurance policy that allows free coverage for chronic disease patients, as compared to partial coverage that requires out-of-pocket expenses.

In Table 1 we present several estimations for the number of Hb disorder carriers in different European countries. The results are presented to the nearest figures that were calculated on the available data on immigrant populations. It was assumed that Northern European populations have a thalassaemia carrier rate of 0.1% in their indigenous populations and no carriers of the sickle cell gene [3]. The importance is that in the countries where the prevalence is high in the indigenous population (Cyprus, Greece, and Italy) there are national policies to meet the needs of these disorders. In the rest of Europe, the proportion of immigrants is approximately similar, yet only the UK and France have disease-specific policies. The carrier frequency is rising most rapidly in Belgium and Spain where national planning is most urgently needed.

In Table 2 we present the disease burden from Hb disorders in each country under investigation in relation to the number of expected births and the number of known patients. In Table 3 we delineate the different health policies that constitute a prerequisite for the development and provision of diagnostic and clinical services to such patients, while in Table 4 we provide information regarding the most important prevention services available. In Table 5 we examine the different diagnostic and clinical services offered in each country such as designated treatment centres, reference laboratories, and the existence of specialized tests for treatment, including cardiac monitoring with magnetic resonance imaging (cardiac MRI $T2^*$) for the assessment of

Table 1: Estimations of the number of carriers in the countries studied.

Country	Total population	Total number of immigrants carriers of β-thalassaemia	Total number of carriers of b-thalassaemia in the indigenous population	Total number of immigrant carriers of HbE	Total number of immigrants carries of sickle cell	Total number of immigrant carriers of HbC	Carrier immigrants as a proportion of the total population	Carriers of Hb disorders as a proportion of the total population
(1) Austria	8210281	11842	8210	2453	4675	708	0.24%	0.34%
(2) Belgium	10438353	19403	10438	4073	39250	5169	0.65%	0.75%
(3) Cyprus	840407	3991	121019	354	583	20	0.58%	15%
(4) Denmark	5543453	6772	5543	4083	2277	330	0.24%	0.34%
(5) France	64057792	98219	64058	32607	172600	47884	0.54%	0.65%
(6) Germany	82329758	128419	82330	22955	53883	7135	0.25%	0.36%
(7) Greece	10737429	29289	837519	536	7626	183	0.35%	8.70%
(8) Italy	61261254	75748	2572972	9463	72870	21416	0.29%	6.50%
(9) The Netherlands	16715999	27656	16716	13751	30329	7703	0.47%	0.57%
(10) Spain	47042984	57257	715053	2434	92601	27796	0.38%	1.90%
(11) Sweden	9482855	21092	9483	12593	8720	912	0.46%	0.56%
(12) UK	63047162	107694	63047	27124	145038	25290	0.48%	0.58%

These results are the nearest figures that are calculated on the available data on immigrant populations. It was assumed that Northern European populations have a thalassaemia carrier rate of 0.1% in their indigenous populations and no carriers of the sickle cell gene. The importance is that in the countries where the prevalence is high in the indigenous population (Italy, Greece, and Cyprus), there are national policies to meet the needs of these disorders. In the rest of Europe the proportion of immigrants is approximately similar, yet only the UK and France have disease specific policies. The carrier frequency is rising most rapidly in Belgium and Spain where national planning is most urgently needed.

cardiac iron load in regularly transfused patients and the transcranial doppler (TCD) to assess stroke risk in sickle cell patients.

In Figure 1 we clearly see that thalassaemia and/or sickle cell disease genes are more common among the immigrant groups of European countries, and the gene predominance reflects the origin of the immigrant groups residing in each country. The increase in migrations from sub-Saharan Africa for example is seen in Belgium, France, Spain, Italy, and the UK, while other countries are influenced by migrations from Southern and Eastern Europe, West Pacific, and Asia such as Spain and France. The trend of accelerated influx of migrant populations from high-prevalence areas is presented in Figures 2 and 3, comparing the migrant populations between 2001 and 2011 in Belgium and Spain, respectively. Such trends are seen in most European countries and those of African descent seem to be increasing at a faster rate.

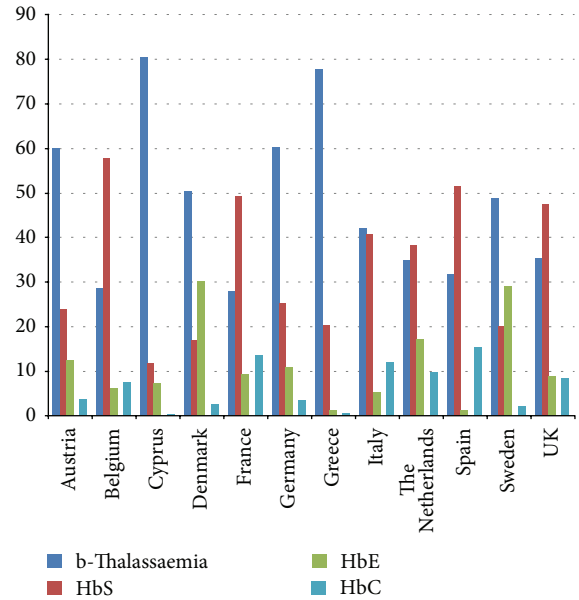

Figure 1: Relative proportion of carriers of Hb disorders among immigrant populations in selected European Countries. The denominator used included the total number of carriers of Hb disorders in each country.

4. Discussion

The Thalassaemia International Federation (TIF) constitutes an international federation of 110 national patient support organizations from 60 countries around the world. Its mission is to support the development and implementation of national control and prevention programs and promote optimal management for Hb disorders. The ultimate goal is to secure equal access to quality health care for all patients with Hb disorders around the world.

TIF works in close and official relations with WHO headquarters and regional offices, promotes the two WHO's specific resolutions on thalassaemia and SCD, and supports WHO's plan of action for Noncommunicable Diseases (NCDs) and the inclusion of these diseases into the national policies for NCDs. TIF is also active in Europe, supporting the

Table 2: Disease burden with the expected births and the number of known patients.

Countries	Expected thalassaemia births/1000 live births	Expected SCD births/1000 live births	Number of known or estimated patients with thalassaemia syndromes	Number of known or estimated patients with sickle cell syndromes
(1) Austria	0.0015	0.00008	NA	NA
(2) Belgium	0.002	0.0035	100	400
(3) Cyprus	5.5	0.0018	639	40
(4) Denmark	0.0018	0.00004	NA	NA
(5) France	0.0016	0.0018	571	10000
(6) Germany	0.0016	0.0001	1500	3000
(7) Greece	1.6	0.009	3241	1080
(8) Italy	0.46	0.11	7000	6000
(9) The Netherlands	0.0018	0.0008	250	750
(10) Spain	0.067	0.0011	113	>200
(11) Sweden	0.0025	0.00021	50	100
(12) UK	0.0018	0.0013	920	15000

Table 3: Health policies which support services.

Country	National register for Hb disorders	Hb disorders under national policy: chronic diseases	Hb disorders under national policy: rare diseases	Hb disorders under national policy: Blood diseases	National policy for prevention
(1) Austria	No				No
(2) Belgium	No	No	No	No	No
(3) Cyprus	Yes			Special policy	Yes
(4) Denmark	No	No	No	No	No
(5) France	Yes		Yes		Yes
(6) Germany	Initiated			Special policy	No
(7) Greece	Yes			Special policy	Yes
(8) Italy	Yes (regional)			Special policy	Yes
(9) The Netherlands	Initiated	No	No	No	Yes
(10) Spain	Regional	No	Yes	No	No
(11) Sweden	No	No	No	No	No
(12) UK	Yes			Special policy	Yes

inclusion and promotion of Hb disorders into the community action and Council Recommendations on Rare Diseases [10–12]. Many other relevant World Health Assembly resolutions have been supported by TIF including those on the control of birth defects, viral hepatitis, health inequalities, social determinants of health and the haemoglobin disorders [13, 14].

In the above context, TIF supports the transposition of the EU Directive on Patients' rights for Cross Border Health Care into national legislation. Most importantly, it is involved in the promotion and networking of Reference Centres for Rare Diseases, including Hb disorders, which are amongst the prerequisites of the above directive. In addition, TIF operates on its own plan of activities, including its educational program, and participates in relevant EU projects such as Ithanet and ENERCA. Awareness and education of patients and health professionals is also achieved through the above information and organization networks.

With regards to immigrant groups information, there are several reasons why much or part of it may be approximations under- or overestimated. The quality of available data on carriers for example is often based on outdated, inappropriately sampled studies and/or small local surveys referring to selected groups rather than representing total populations. However we have used the best possible estimates derived not only from published data but also from TIF's database as described before.

The effect of migrations cannot be assessed only on the numbers of migrants. The behaviour of these groups in terms of marriage, reproduction, use of health services, permanency in the new host country, and other sociological factors also needs to be studied and considered. For example,

Table 4: Policies and available prevention services.

Country	Carrier screening available	Carrier screening free	Neonatal screening	Prenatal diagnosis available
(1) Austria	No	No	No	No
(2) Belgium	Yes	No	Regional	Yes
(3) Cyprus	Yes	Yes	No	Yes
(4) Denmark				
(5) France	Targeted	Yes	Regional	Yes
(6) Germany	No	No	No	Yes
(7) Greece	Yes	Yes	No	Yes
(8) Italy	Yes	Yes	Regional	Yes
(9) The Netherlands	Yes		Yes	Yes
(10) Spain	Regional	Yes	Regional	No
(11) Sweden	No		No	No
(12) UK	Yes	Yes	Yes	Yes

Table 5: Diagnostic and screening services.

Country	Designated treatment centres serving all patients	Reference labs	Networks of labs	MRI T2*	TCD
(1) Austria	No	No	No	No	
(2) Belgium	Majority	Yes	No	No	Yes
(3) Cyprus	Yes	Yes	Yes	Yes	No
(4) Denmark	No	No	No		
(5) France	Yes	Yes	Yes	Yes	Yes
(6) Germany	Minority	Yes	Yes		Yes
(7) Greece	Yes	Yes	Yes	Yes	No
(8) Italy	Yes	Yes	Yes	Yes	Yes
(9) The Netherlands	No	Yes	Yes	No	
(10) Spain	Minority	Yes	Yes		
(11) Sweden	No	No	No		
(12) UK	Yes	Yes	Yes	Yes	Yes

intermarriage with the host population is certainly observed to occur as well as marriage with other ethnic minorities; however these parameters are not usually officially recorded in most countries. For instance, of the approximately 10.000 Filippino residents (potential carriers of both thalassaemia and HbE, which is nonexistent in the Cypriot population) in Cyprus in 2011, 7.702 are between the ages of 15 and 44 and 513 women were married in Cyprus in the last 4 years. These are females who come to Cyprus as domestic workers on a four-year contract. None was married to a countryman of theirs, 56% were married to a Cypriot, and 23% were married to a husband from another thalassaemia-prevalent country (the chances of a Cypriot-Filippino marriage producing an affected offspring are roughly 1 : 1.750). This is an indication that not all "temporary" migrants return to their home country. Such social variables cannot always be estimated and the example from Cyprus was chosen because of the readily available data from a small country.

Another variable whose effect can only be approximated is the contribution of nonregistered migrants who are estimated to be 1–4% of the population in Europe. This group will face all the problems of their "legal" counterparts and in addition will have to face even more difficulties in accessing the new host countries' health services because of fear of exposure. Where a chronic disease is concerned, the problems seem insurmountable and the help of NGOs which may step in to give assistance (http://www.nowhereland.info/) is usually very limited.

Another difficulty in assessing the importance of migration on the national and EU burden of these diseases is that the majority of European countries do not maintain comprehensive registries of patients, that include diagnosis, age distribution, location within a country, data on new cases, complication rates, and mortality data. One of the objectives of the current work is to highlight this problem and alert national health policy makers to the need for promoting national policies for Hb disorders and for the improvement of access and integration of immigrant patients to these services. Specific health services registries are considered essential to provide effective planning. In fact, it is quite difficult to understand how appropriate multidisciplinary services may be developed and provided in order to cover the needs of such patients in the absence of such information. Thus, one may argue that the lack or existence of a registry is indeed a major

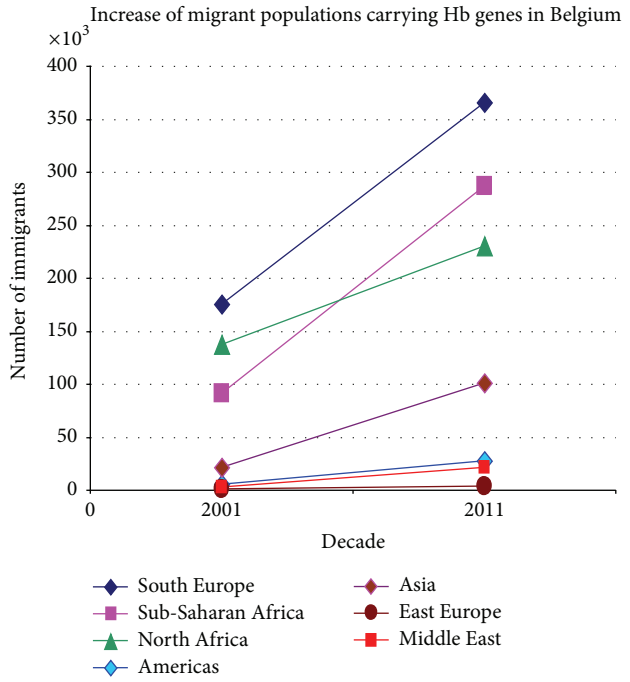

Figure 2: Increase in the number of immigrants carrying Hb disorders in Belgium between 2001 and 2011 according to the geographic region of origin.

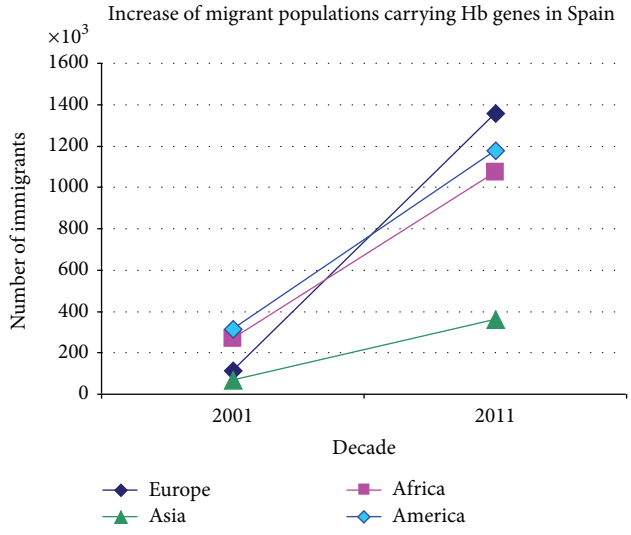

Figure 3: Increase in the number of immigrants carrying Hb disorders in Spain between 2001 and 2011 according to the geographic region of origin.

indicator of the quality of care in each country. Registries for Hb disorders exist currently only in France [15], Greece [16], Cyprus [17], Italy [18], and UK [19, 20]. Each of these referenced articles also demonstrates the usefulness of the derived information from national registries. The rest of the countries in this study provide estimates of the numbers of patients which, however, cannot be reliably confirmed.

In considering the services for chronic Hb disorders and their poor or limited uptake by the immigrant populations, one may elaborate on two major reasons. First there are constraints which originate from the social situation of the migrant groups in a host country, the low educational level especially of the new or first-generation migrants along with language and cultural barriers. Where hereditary disorders are concerned, there are also additional factors such as ignorance of the condition and its causes, including the possibility of carrier testing [21–24], social prejudice, and cultural and religious attitudes towards prevention and pregnancy termination. On many occasions, priority is rightly given to more acute problems related to family survival and occupational settlement in the new host country. The second factor is the response of health services in the host country to health problems which are initially and largely unknown and perhaps "foreign" to the indigenous population and not included in planning and policy making. The extent of the problem is usually only made known over time, since only a few such patients appear in hospital paediatric or adult departments and are considered as isolated cases.

Although, in more recent years, the importance of developing and implementing national programs in every EU member state by 2013 for rare diseases has been very much underscored, still to date surveys are generally lacking. Early diagnosis is often delayed and appropriate services for management are still largely absent or heterogeneously spread across, between and within European countries. In addition, strategic prevention of these disorders, in the vast majority of EU countries, is still absent at the national or regional level.

It is quite interesting that Mediterranean countries, which have a high prevalence of haemoglobinopathy genes in the indigenous population, are in recent years receiving immigrant groups from other high-prevalence countries. These countries, namely, Italy, Greece, and Cyprus, already have an infrastructure for the comprehensive management of these disorders with a long history of service development [25–27]. In these countries new affected births were drastically reduced by nationally controlled prevention programs mainly instituted by late 1980s. In recent years, however, the affected births are seen to be rising due to the poor response of the "new" immigrant groups as evidenced from the experience in Latio, Italy [28]. The question arises as to why these population groups do not take advantage of established prevention programs and health services. The most important reasons possibly relate to the lack of awareness of these immigrants for such programs due to the absence of previous experience from their countries of origin. In addition, the culture of carrier identification followed by possible prenatal diagnosis are not usually established in these population groups. Even when awareness is provided by the new host country's services, the decision of immigrants, at least in the first generation, is greatly influenced by their cultural and religious background linked to their country of origin [29, 30].

It is evident that, as a result of heavy past and continued migrations coupled with projections for future increase, the thalassaemias and sickle cell disease currently emerge as a

visible public health problem in most of Europe. The new host countries of Europe appeared initially unprepared for this new yet increasing problem, and health authorities were unaware and unwilling to invest in services for what may have been viewed as a temporary situation. With time, the problem grew and treatment services started to be provided in few centres. Eventually prevention had to also be considered as the numbers of immigrants and potentially the number of annual affected births were increasing. The United Kingdom was the first among the Northern European states to develop a specific program and appropriate services [1], albeit initially not at the national level, almost concurrently with the endemic south. Other countries in the north have since followed, but many still have a long way to go in order to develop optimal and comprehensive services.

Europe had not experienced the need for developing genetic programs, which involve the total population to the same extent as the thalassaemia-endemic areas. The concept of community genetics has indeed introduced many concerns including ethical and legal challenges, complicated by the fact that these hereditary disorders are more common to immigrant populations with different cultures affecting the practical application of such programs. Demographic and epidemiological data are prerequisites and constitute a priority for development of national programs and services. The carrier rate may be the same as that of the country of origin, which is the method to calculate the figures presented in this report, but it would have been better estimated by local surveys, which could provide figures on annual affected births and the total and at-risk pregnancies. Such information could offer better or more accurate estimates for the planning of services as for example the size of the clinical services, the needed screening program, the number of prenatal diagnoses to be carried out, and the needs for couples' counseling [4]. In very few parts of Europe this process has been completed while in others it has yet to be faced.

There has been a tendency for immigrants to gather in specific areas, often forming "ghettoes". For this reason, a second priority is micromapping through patient registries, with information on patient numbers and location so that needs for clinical and other services are rationally developed in geographically relevant locations. Clinical services should follow internationally accepted evidenced-based guidelines on which local, national, and regional standards of care could be integrated. It may be difficult for patients with rare anaemias in large countries to access expert centres of excellence as recommended by the comprehensive management programs of all currently available guidelines. This need can be met by networking and development of shared care with local centres as well as by physician training [31] within a comprehensive national policy for chronic and rare diseases. There is sufficient evidence, especially from survival data of various birth cohorts, that care in expert centres based on updated treatment protocols is associated with improved patient outcomes [17].

Designated treatment centres with access for all patients exist in Cyprus, Italy, Greece, and the UK (Table 4). In three other countries, only a portion of the patients have access to such centres. In addition, specialized laboratory and clinical tests necessary for addressing and preventing medical complications do not exist in the great majority of countries and even in reference or expert centres.

Acknowledging the limitation of the current paper, we would like to note that many of the research questions we posed in this study cannot be accurately answered because updates and/or reliable information may be unavailable. However, it was possible to build a picture of the current situation in Europe which may help raise awareness in the medical community and alert health authorities towards the particular needs in order to respond to the challenges that come with changes in the population demographics. Despite the rarity of the Hb diseases, their chronic nature and complexity result in a significant impact on health planning, which may lead to devastating and immense economic and other repercussions on individual patients and families. It must also be remembered that migrations are not constant but in fact are changing constantly in terms of size, direction, and impact, according to historical, environmental, political, and economic circumstances and therefore are difficult to follow. Because of this, the conclusions of this study reflect only on the very current situation and availability of health services for Hb disorders.

Financing services for Hb disorders is an important issue common to all chronic diseases' programs. Appropriate allocation of financial and human resources is essential for the sustainability of any such program. Some countries have adopted copayment systems, which may exert a difficult burden on migrants and low-income groups facing lifelong treatment and multidisciplinary care needs. In times of economic restraints, inequalities in access and health care services may be more likely to increase in an already global environment of severe inequalities in the health and social domains. Migrants with chronic diseases are particularly vulnerable to such situations.

The planning of programs for these genetic disorders is a public health exercise, which has not yet been adequately adopted in all European settings either as chronic disease policies or rare disease policies (Tables 3, 4, and 5). This is an unfortunate situation, since sufficient knowledge, experience, and expertise exist to adequately and effectively prevent and treat Hb disorders. In making plans, health authorities should also monitor the changes in the size and origins of incoming populations because changes may be significant over relatively short periods of time.

It is unquestionable that the prevention and treatment of thalassaemia and sickle cell disease patients have added to the health burden in many areas of the world by significantly contributing to budgetary constraints. However, governments around the world and particularly in Europe are already committed not only by having signed two important WHO specific resolutions [13, 14] but also by approving other EU directives on migrant health and recommendations on rare diseases. As a global NGO with responsibility and commitment for advocacy of patients' rights, TIF aims to continue its coordinated efforts to strengthen the support for the promotion of policies which will result in better outcomes for all patients with Hb disorders in the European and global arena. Each European country has a different approach to

funding and providing cost-effective solutions to the multiple needs of chronically sick patients, especially among ethnic minorities. TIF is committed to monitor the progress, raise awareness, and support the promotion of more immigrant-oriented health policies to ensure their integration in society and their access to appropriate, adequate, and timely health services.

Conflict of Interests

The authors declare that they have no conflict of interests with respect to the current paper.

Acknowledgments

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Review Article

Phytomedicines and Nutraceuticals: Alternative Therapeutics for Sickle Cell Anemia

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Sickle cell anemia is a genetically inherited disease in which the "SS" individual possesses an abnormal beta globin gene. A single base substitution in the gene encoding the human β-globin subunit results in replacement of β6 glutamic acid by valine, leading to the devastating clinical manifestations of sickle cell disease. This substitution causes drastic reduction in the solubility of sickle cell hemoglobin (HbS) when deoxygenated. Under these conditions, the HbS molecules polymerize to form long crystalline intracellular mass of fibers which are responsible for the deformation of the biconcave disc shaped erythrocyte into a sickle shape. First-line clinical management of sickle cell anemia include, use of hydroxyurea, folic acid, amino acids supplementation, penicillinprophylaxis, and antimalarial prophylaxis to manage the condition and blood transfusions to stabilize the patient's hemoglobin level. These are quite expensive and have attendant risk factors. However, a bright ray of hope involving research into antisickling properties of medicinal plants has been rewarding. This alternative therapy using phytomedicines has proven to not only reduce crisis but also reverse sickling (*in vitro*). The immense benefits of phytomedicines and nutraceuticals used in the management of sickle cell anemia are discussed in this paper.

1. Introduction

1.1. Hemoglobinopathies: Sickle Cell Anemia. Sickle cell disorder (SCD) is a group of hereditary illnesses affecting the red cell hemoglobin [1]. Various types of these disorders exist: including sickle thalassaemia and sickle cell anemia (HbSS), also known as drepanocytosis. The disease is most prevalent in the black race, but it is also known in other races surrounding the Mediterranean and in India [2].

Parents who possess heterozygous genotypes (HbAS) are sickle cell carriers and their offspring have a 1 in 4 chance of having a homozygous sickle genotype (HbSS) or a homozygous normal genotype (HbAA) as depicted in Figure 1.

Sickle cell disease was first recognized as a hematological disorder by Herrick in 1910 and its molecular pathology was established in 1949 by Linus Pauling. Molecular research traces its origin to the study of abnormal hemoglobin and the mechanisms by which a single base substitution in the gene encoding the human β-globin subunit, with the resulting replacement of β6 glutamic acid by valine, leads to the devastating clinical manifestations of sickle cell disease [1]. This substitution causes a drastic reduction in the solubility of sickle cell hemoglobin (HbS) when deoxygenated. Under these conditions, the HbS molecules polymerize to form intracellular fibers which are responsible for the deformation of the biconcave disc shaped erythrocyte into a sickle shape [3]. The normal and sickled red blood cells are shown in Figure 2.

The ailment is characterized by premature breakdown of the red blood cells causing constant anemia and occlusion of small blood vessels leading to excruciating body pains and other manifestations. The disease stems from inadequate oxygen transport by red blood cells. *In vivo*, sickled erythrocytes tend to block capillaries, causing stasis, and thereby starve organs of both nutrients and oxygen and eventually cause hypofunction or complete tissue destruction [1]. Sickle cell incidence has been closely linked to malaria incidence in tropical areas like Nigeria. These SS persons are least fit for

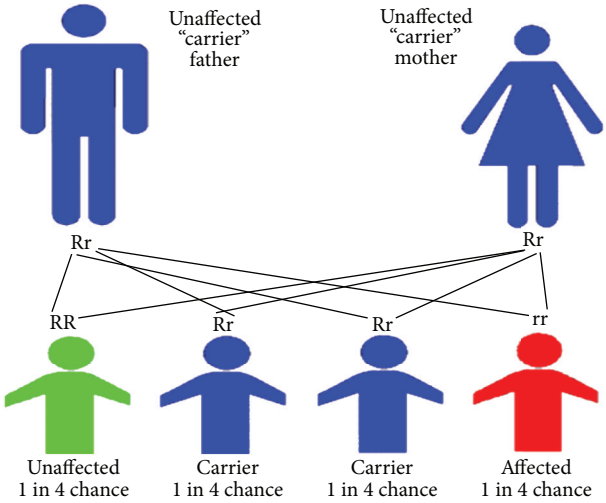


Figure 1: Sickle cell disorder inheritance pattern.

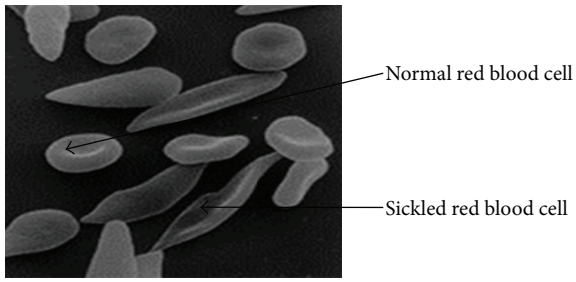


Figure 2: Normal and sickled red blood cells [1].

survival in a hostile malaria environment and survival rates are particularly low in childhood.

2. Approach to Therapy

There are several compounds such as amino acids, which prevent sickling by affecting the erythrocyte membrane, causing an increase in the cell volume of the erythrocyte and thus reducing the intracellular hemoglobin concentration below its minimum gelling concentration [4–8]. The most popular approach to prevent or reverse sickling *in vitro* and *in vivo* is to employ compounds or techniques which directly affect the hemoglobin (Hb) molecule.

A characteristic property of the gelation of deoxy-HbS is the existence of a delay time prior to polymerization of deoxy-HbS molecules [9]. A drug that prolongs the delay time prior to polymerization might be of therapeutic value in SCD, because a longer delay time decreases the probability of SS cell sickling. Reported antisickling/antidrepanocytary agents in this group include a formulated phytomedicine, Niprisan (Nix-0699), a chemical compound 5-hydroxymethyl-2-furfural [5HMF], and MX-1520 (a prodrug of a food additive, Vanillin) which modify intracellular sickled hemoglobin and inhibit sickling of red blood cells [10–13].

Presently, first-line clinical management of sickle cell anemia includes use of hydroxyurea, folic acid and amino acids supplementation (as nutritional supplements), penicillin prophylaxis (helps prevent infection), and antimalarial prophylaxis (helps prevent malaria attack), for example, paludrine in varying doses in childhood, adulthood, and pregnancy. The faulty "S" gene is not eradicated in treatment; rather the condition is managed and synthesis of red blood cells induced to stabilize the patient's hemoglobin level. Further management and treatment of this disorder with compounds or techniques which directly affect the hemoglobin [Hb] molecule (e.g., hydroxyurea, bone marrow transplantation, and blood transfusion) are very expensive and out of reach of the masses and besides expose the patient to mutagenicity, iron overload, and other fatal risks [14–17]. Monthly blood transfusion lowers the proportion of sickling cells to <30%, but it is stopped at 18 years of age. Others recommend transfusion of stroke patients (from cerebrovaso occlusion) for an indefinite period of time in view of the high recurrence risk (of the stroke). However, there is a predictable complication of long-term therapy because the anemia is not an iron deficiency condition, rather a hemolytic type. Therefore the patient already has the required iron concentration in the blood and may run the risk of iron-overload. Bone marrow transplantation is a more definitive treatment [15]. Another angle for drug relief adduces the reason for the stickiness of SS red blood cells to be due to the secretion of thrombospondin, a cell surface protein [14].

In summary these are the various approaches to sickle cell disease therapy

(a) Clinical/medical/pharmacological:

(i) blood transfusion, bone marrow transplantation.

(ii) chemotherapy: hydroxyurea, which increases HbF (an antagonist of HbS) stimulation, nitric oxide gas inhalation.

(iii) confers only symptomatic relief/maintenance of patient.

(iv) anti-inflammatory (for pain crisis).

(v) antimalarial and antibacterial drugs (paludrine and penicillin).

(b) Nutritional:

(i) multivitamin supplements, proper diet, calorie and protein intake, Vanillin.

(c) Phytomedicines/phytotherapy. Phytomedicines and naturally occurring antisickling agents:

(i) Niprisan with *Piper guineense*, *Pterocarpus osun*, *Eugenia caryophyllum*, and *Sorghum bicolor* as components, Ciklavit (*Cajanus cajan* as base), and hydroxybenzoic acids are used in SCD management [13, 18].

3. Antisickling Agents

Synthetic (otherwise called, orthodox) medicines developed so far for sickle cell management focus on symptomatic relief of pain and crisis alleviation. Examples of such drugs are zinc, piracetam (which aim to prevent sickle cell crisis by reducing red blood cell dehydration), PP-188 (Purified Poloxamer 188) blood drug (which reduces the viscosity of RBC's), and nitric oxide gas [19–21].

Research into antisickling properties of medicinal plants has been rewarding. This alternative therapy using phytomedicines has proven to not only reduce crisis but also reverse sickling (*in vitro*). Examples of these herbal drugs are Niprisan (renamed Nicosan) with *Piper guineense, Pterocapus osun, Eugenia caryophyllum,* and *Sorghum bicolor* as components; Ciklavit (*Cajanus cajan* seed extract as base), Aqueous extracts of *Zanthoxylum zanthoxyloides* roots, Ajawaron HF complex with *Cissus populnea* as main component, Aqueous and alcoholic extracts of *Terminalia catappa* leaves; *Carica papaya* unripe fruit and dried leaf extracts.

Zanthoxylum zanthoxyloides (otherwise called *Fagara*, orin-ata) roots have been analyzed for antiprotease and membrane stabilizing activity using a modified osmotic fragility technique to analyze membrane stabilization action [22]. It has been discovered that the antisickling (and anti-inflammatory) action of *Fagara* was due to its o-hydroxybenzoic acid constituent [23]. According to literature, these already documented herbs and compounds, for example, *Cajan*, *Fagara*, Niprisan, and Ciklavit are all still in the research stage and some have passed through clinical trials and health care safety standardizations and have been approved for use [4, 18, 24–29]. While ascertaining the efficacy of these drugs, their safety in humans is also important for survival. The mode of action of these herbal drugs is of particular interest. The possible mechanism of action of phenylalanine, an amino acid reported to have antisickling effect, has been adduced, indicating the role of several transport systems [3, 30, 31].

Aqueous and ethanolic extracts of several phytomedicines have been evaluated for significant *in vitro* antisickling activity. Recent studies support the claims of the traditional healers and suggest a possible correlation between the chemical composition of these plants and their uses in traditional medicine [32]. *Z. zanthoxyloides* has shown drepanocyte (sickling) reversibility, appreciable increase in hemoglobin gelling time, and improved rheological properties of drepanocytary blood [33]. Antisickling properties of amino acids have been recognized much earlier; of all the amino acids reported, phenylalanine was shown to be most active [6–8]. The mode of transport and possible mechanism of action of some amino acid benzyl esters, for example, L-phenylalanine benzyl ester (Phe-Bz), an aromatic compound, an antisickling agent was found to be effective at a low concentration and is therefore a potential therapeutic agent for the treatment of sickle cell disease [3].

The antisickling effect of cetiedil [alpha-cyclohexyl-3-thiopheneacetic acid 2-(hexahydro-1 Hazepin-1-yl) ethyl ester] has been reported [34]. Antisickling effect was achieved regardless of whether cetiedil was added before or after deoxygenation. The minimal gelling concentration of deoxy HbS was increased by less than 10% in the presence of cetiedil concentration and the oxygen equilibrium curves of HbS were not significantly affected. Erythrocytes treated with high concentrations of cetiedil were swollen and became spheroidal. It was concluded that the antisickling effect of cetiedil might be due to an effect on erythrocyte membranes. The hemoglobin (Hb) molecule has a high affinity for most substrates that reverse the sickling phenomenon [5]. The configuration of the region of Hb where antisickling agents bind has been determined, suggesting that the rate constant k is dependent upon the rate at which the substrate is transported across the membrane since the rate of combination with hemoglobin is very fast.

Another scope of work on antisickling agents with the focus on nutrition found that the concentrations of ascorbic acid and alpha-tocopherol were significantly depressed while that of retinol was slightly reduced in subjects tested. The depletion in the levels of the antioxidant vitamins A, C, and E may account for some of the observed manifestations of sickle cell anemia, such as increased susceptibility to infection and hemolysis [35]. Vitamin B_{12} levels have been observed to be diminished in patients with severe sickle cell disease. Patients with low vitamin B_{12} achieved a significant symptomatic improvement when treated with vitamin B_{12}, 1 mg intramuscularly weekly for 12 weeks. It was concluded that many patients with severe sickle cell disease may suffer from unrecognized vitamin B_{12} deficiency [36].

Research on antioxidant status and susceptibility of sickled erythrocytes to oxidative and osmotic stress has been reported using a range of diluted saline-phosphate buffer in a typical osmotic fragility test to determine osmotic stress/membrane integrity and AAPH (a peroxyl radical generator) to induce hemolysis with oxygenated and deoxygenated RBCs for oxidative stress analysis. It was discovered that though there are differences in antioxidant status between sickled and normal RBCs, these differences did not appear to be responsible for the observed difference in susceptibility to oxidative or osmotic stress-induced hemolysis [37].

The inhibition of erythrocyte membrane ATPases with antisickling and anesthetic substances and ionophoric antibiotics has been studied, in the light of the partition coefficient of these drugs in erythrocyte membranes, the changes they induce in the permeability properties of erythrocytes, and the subsequent effect of procaine on sickling of erythrocytes and their potential interaction with specific membrane components. In general, the drugs were found to inhibit both types of enzymic activities but with varying degrees of efficacy. $(Ca^{2+}\text{-}Mg^{2+})$-ATPase was more sensitive to the lipophilic anesthetics and $(Na^{+}\text{-}K^{+})$-ATPase to the ionophoric antibiotic, Amphotericin B [38].

Oral magnesium supplementation reduces the number of dense erythrocytes and improves the erythrocyte membrane transport abnormalities of patients with sickle cell disease. Children with SCD are reported to demonstrate or exhibit normal serum magnesium level with accompanying hyperphosphataemia and hypocalcaemia [39]. They have also

been observed to have decreased height and weight when compared with their peers. Although exact reasons for poor growth were not established, increased calorie and protein needs and deficiencies in zinc, folic acid, and vitamins A, C, and E were adduced to be the factors responsible [40, 41]. It has been suggested that the nutrient intake of patients with sickle cell disease is often inadequate and the study suggests that education of patients with SCD should focus on specific nutrient needs, with proper distribution of dietary intake among the food groups, ways to provide nutritious meals on a limited income, and methods for increasing calorie and protein intake. Patients with SCD that have adequate vitamin B_6 and B_{12} status, but elevated plasma homocysteine levels with indicated suboptimal folate status, especially pediatric sickle cell patients, may benefit from folate supplementation to reduce their high risk for endothelial damage [42].

A potential nutritional approach for the molecular disease SCD found that from both *in vitro* and pilot clinical trials, a "cocktail" of aged garlic extract, vitamin C, and vitamin E proved beneficial to patients. Ascorbic acid is important in SCA because significant oxidative stress occurs in the disease and its role as an antioxidant is very beneficial [43].

Ascorbate levels in red blood cells and urine in patients with sickle cell anemia have been analyzed [44] and it was reported that

(1) ascorbate is present in sickled red blood cell (SRBC), most likely due to ascorbate recycling, despite increased free-radical generation;

(2) there is increase in renal excretion, which may contribute to the low plasma levels of ascorbate;

(3) the presence of ample ascorbate in sickled red blood cells (SRBCs) and decreased plasma ascorbate suggests that ascorbate movement across the SRBC membrane may be different from normal red blood cell.

The effect of vitamin C on arterial blood pressure, irreversibly sickled cells (ISCs), and osmotic fragility in sickle cell anemic subjects also suggests a potential benefit of vitamin C supplementation to sickle cell anemia subjects because vitamins A, C, and E supplementation was shown to decrease arterial blood pressure, % ISCs (irreversibly sickled cells), and MCHC (mean corpuscular hemoglobin concentration) but increased Hb (hemoglobin) and PCV (packed cell volume) [45, 46].

The first widely accepted herbal formulation Niprisan (now Nicosan) produced by Wambebe of NIPRID, Abuja, Nigeria, was analyzed *in vivo* using transgenic mice under acute severe hypoxic conditions using a series of innovative analyses and tests and found to be very effective in reversing and preventing sickling [9, 12]. The kinetics of reversal of presickled erythrocytes by aqueous extract of *C. cajan* seeds was reported [47] as well as the antisickling properties of *Parquetina nigrescens* and a Nigerian herbal formula, Ajawaron HF, using the method of sodium metabisulphite-inhibition of sickling for the analysis [26, 48].

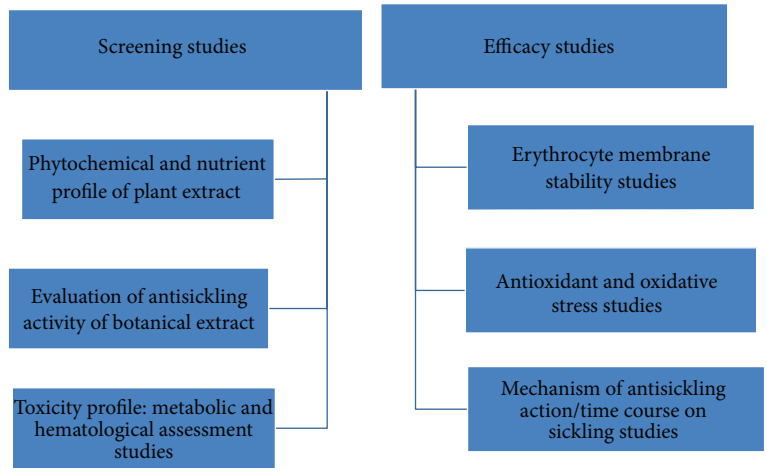
The antisickling effects of MX-1520, a prodrug of vanillin, have been analyzed using rodents *in vivo*. This prodrug was produced because vanillin rapidly decomposed in the upper digestive tract and so was ineffective when taken orally in its original form [11]. A naturally occurring aromatic aldehyde, 5-hydroxymethyl-2-furfural (5HMF), was found to modify intracellular sickled hemoglobin and to inhibit sickling of red blood cells. This aldehyde unlike previous ones was found to be bioavailable (i.e., did not get decomposed in the digestive tract, but was found in appreciable amounts in the blood stream) [10].

More research on antisickling agents have evolved since then especially in Nigerian universities, with the emphasis on antisickling action of extracts of phytomedicines and isolated antisickling agents contained in these phytomedicines. For example, the antisickling activity of *Carica papaya* unripe fruit extracts [49–52] and *Carica papaya* dried leaf extract [53, 54] have been reported.

4. Current Trends in Alternative Herbal Treatment of Sickle Cell Anemia

It is acknowledged worldwide that traditional medicine can be explored and exploited to be used alongside synthetic pharmaceutical products for enhanced health management. Due to the high mortality rate of sickle cell patients, especially in children, and since chemotherapy has its adverse effects, there is need for rational drug development that must embrace not only synthetic drugs but also natural products (phytomedicines/herbal drugs), naturally occurring antisickling agents which can be obtained from our vast forest resources and can be used to effectively manage the sickle cell patient and treat the anemic condition accompanying this disorder. Attempts to find alternative, cheaper, and less toxic therapies led to the scientific discovery of antisickling properties of some medicinal plants such as *Cajanus cajan* seeds, *Zanthoxylum zanthoxyloides (Fagara)* root, *Carica papaya* unripe fruit, and also *Parquetina nigrescens* whole plant extracts which boost blood volume—all these are locally used by traditional healers in Nigeria for diverse herbal remedies [23, 31, 48–52]. Medicinal plants are parts of a plant or the whole plant that possess healing properties and unlike orthodox (synthetic) medicines, which may have adverse side effects, medicinal plant formulations are considerably cheaper and safer to use. In a previous review, selected medicinal plants with antisickling properties which are currently in use for the management of sickle cell anemia were highlighted and their methods of extraction, the various methods of analyzing herbal extracts for antisickling activity via efficacy tests and analyses and research findings were also discussed [55]. Since then, more research studies have continued in our laboratories and some of the findings are summarized here (Scheme 1).

4.1. Carica papaya Dried Leaf Extract. *Carica papaya* is a member of the Caricaceae family, native to Nigeria and Central America, and is medicinal plant used as an alternative therapeutic agent for sickle cell anemia. The correlations between the chemistry and pharmacology of *Carica papaya* leaves have been reported. Phenolic compounds have been found in papaya leaves [56]. The presence of such compounds could partially explain the pharmacological properties of this



Scheme 1: A scheme of research showing a flowchart of methodologies and research phases used in our laboratories to screen and ascertain the efficacy of antisickling phytomedicines.

plant and demonstrate its importance in alimentation and daily intake. Phenolic compounds are important components in vegetable foods, infusions, and teas for their beneficial effects on human health.

Methanol extract of papaya leaves has been analyzed and important polar compounds (secondary metabolites) were identified and quantified using gas chromatography-mass spectrometry (GC-MS) in the selected ion-monitoring (SIM) mode. 5,7-Dimethoxycoumarin and polar molecules such as protocatechuic acid, *p*-coumaric acid, chlorogenic acid, kempferol, and quercetin were detected and identified in qualitative analysis. Quantitative analysis showed the presence of phenolic acids as the main compound, while chlorogenic acid was found in trace amounts, compared to the flavonoids and coumarin compounds [56].

4.2. Effects of Papaya Leaf Extracts on Sickling. Many phytomedicines have been identified as potential antisickling agents, stemming from reported usage as ethnomedicines by the local folk. *Carica papaya* dried leaves have been indicated in sickle cell anemia management by local indigenous folk and in recent scientific research. A particular research examined methanolic leaf extracts of *Carica papaya* L. (Caricaceae) for possible *in vitro* antisickling and membrane-stabilizing activities involving the use of positive (p-hydroxybenzoic acid 5 μg/mL) and negative (normal saline) controls for the antisickling experiments and osmotic fragility test on Hb^{ss} red blood cells obtained from noncrisis state sickle cell patients. Fragiliograms indicated that the plant extract reduced hemolysis and protected erythrocyte membrane integrity under osmotic stress conditions. Pretreatment of SS cell suspensions with *Carica papaya* leaf extract inhibited formation of sickle cells under severe hypoxia, with only 0–5% sickle cells at 40 mins compared with untreated SS cell suspensions which had over 60% sickle cells. These results indicate the feasibility of *Carica papaya* as an attractive potential candidate for SCD therapy [53].

In another research, dried *C. papaya* leaves were extracted using the soxhlet extraction method with 5 different solvents to give five different fractions, namely, hexane, chloroform, ethyl acetate, butanol, and water. The research examined the crude extract and the various leaf extract fractions of *C. papaya* L. (Caricaceae) for possible *in vitro* antisickling activities on Hb^{ss} red blood cells obtained from noncrisis state sickle cell patients involving the use of positive (phydroxybenzoic acid 5 μg/mL) and negative (normal saline) controls for the antisickling experiments. Pretreatment of SS cell suspensions with *C. papaya* leaf extract and fractions all inhibited formation of sickle cells under severe hypoxia at varying degrees, with only 0–5% sickle cells in the crude extract at 60 min compared with untreated SS cell suspensions which had over 80% sickle cells. Analysis of two different concentrations of *C. papaya* crude extract (10 and 5 mg/mL) showed the 10 mg/mL extract as the concentration with highest antisickling effect. Butanol extract showed the highest antisickling activity at 10 mg/mL concentration, while the ethyl acetate extract had the highest antisickling activity at 5 mg/mL concentration. These results further indicate the possibility of *C. papaya* leaf extract as potential phytotherapy for sickle cell anemia [54].

4.3. Antioxidant Effects of Papaya Leaf Extracts, Cajanus cajan Seed Extract, Fagara zanthoxyloides Root Extract, and Parquetina nigrescens Plant Extract on the Erythrocyte. In demonstration of the ability of *Carica papaya* leaf extract to confer protective properties on the erythrocyte membrane, the effect of varied concentrations of the herbal extracts on erythrocyte membranes was analyzed using the osmotic fragility test, which revealed appreciable membrane-stabilizing (protective) effects of the herbs and their inhibitory action on hemolysis of red blood cells (Figures 3, 4, 5, and 6). The resistance of the erythrocytes can be measured by subjecting them to the action of various harmful agents. Red blood cells suspended in hypotonic salt (NaCl) solution take up water, swell, and become spheroidal and more fragile, and eventually burst. The increased fragility, which leads to lysis, is inversely proportional to the concentration of NaCl and directly proportional to the thickness of the red blood

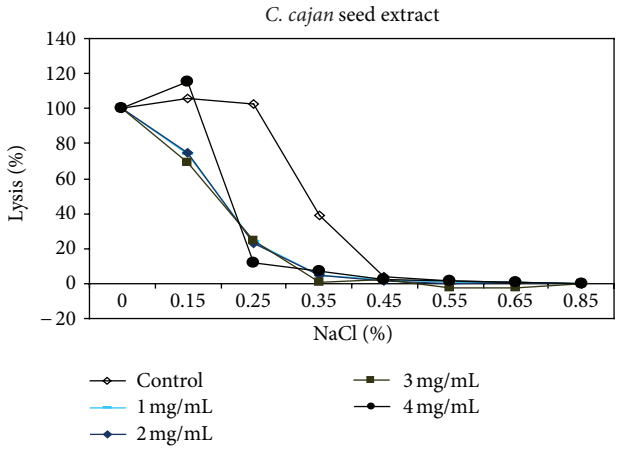


FIGURE 3: Osmotic fragiliograms after supplementation with various concentrations of *C. cajan* seed extract.

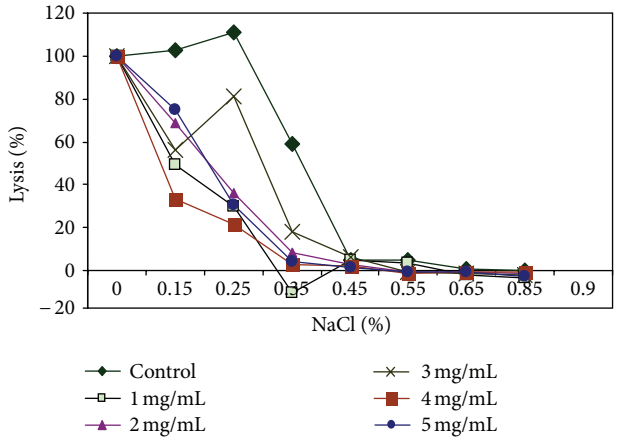


FIGURE 4: Osmotic fragiliograms after supplementation with various concentrations of *Fagara* root extract.

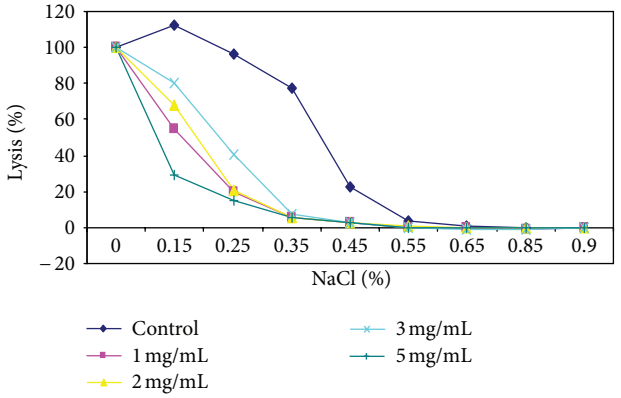


FIGURE 5: Osmotic fragiliograms after supplementation with various concentrations of *Parquetina nigrescens* plant extract.

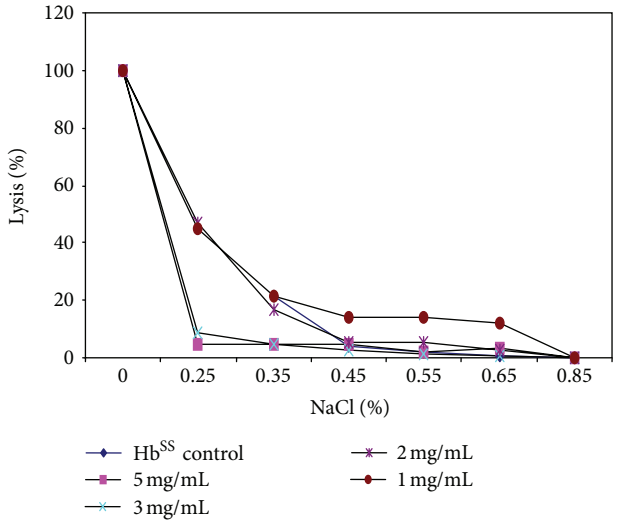


FIGURE 6: Osmotic fragiliogram after supplementation with various concentrations of *Carica papaya* extract.

cell [57]. An increase in osmotic fragility is equivalent to a decrease in osmotic resistance. Rounded cells lyse at relatively high salt concentrations. The osmotic fragility test measures accurately how nearly spherical red cells are. Increased osmotic fragility or decreased resistance means spherocytosis (found in hereditary spherocytosis, hemolytic anemia). Diminished osmotic fragility or increased resistance means excessive flatness of red cells (sickle cell anemia, jaundice, and thalassemia). In a study [53] most of the cells supplemented with papaya extract were still rounded after incubating in salt solutions. This observed inhibition/reduction in RBC lysis after treatment with the *C. papaya* extract is indicative of protective properties of the extracts on the RBC membrane, thus helping to maintain membrane integrity through membrane stabilization.

Sickle erythrocytes have been reported to have a distorted volume-to-surface ratio when compared to normal erythrocytes [45] and so a shift to the left in the osmotic fragiliograms suggests a higher osmotic resistance for most sickle cells. This shift was observed in the study, showing that the extract was able to protect the integrity of the erythrocyte membrane, increase its resistance to osmotic stress/lysis, and thus reduce membrane fragility. From these erythrocyte studies, one can infer that aqueous extract of *Carica papaya* reduced hemolysis and conferred some protective effect on erythrocyte membrane.

Active constituents of medicinal plants and naturally occurring compounds, known as antisickling agents, which improve the health of sickle cell individuals are rich in aromatic amino acids, phenolic compounds, and antioxidant nutrients [58] which are thought to be responsible for their observed antisickling action. A herbal preparation of *Cajanus cajan* was found to contain phenylalanine, carjaminose, and hydroxybenzoic acid as active constituents and are thought to be the reason for its antisickling effect [47]. Folk medicine reportedly uses *Parquetina nigrescens* L. (Asclepiadaceae) as a herbal remedy for the management of sickle cell anemia. A study was carried out to screen the leaves and stem of *Parquetina nigrescens* for antisickling activity, erythrocyte membrane-stabilizing effects, and any end organ toxicity. Percentage reversal and inhibition of sickling parameters were

analyzed on presickled Hb^{SS} blood cell suspensions using sodium metabisulphite solution as inducer and 5 mg/mL parahydroxybenzoic acid and normal saline as positive and negative controls, respectively. Effects of the plant extracts on the erythrocyte were assessed using osmotic fragility and the toxicity profile done via lethal dose LD_{50} and subacute toxicity studies on graded concentrations of extract. Results showed that *Parquetina nigrescens* has appreciable antisickling activity, has no toxic effect when administered at low concentrations, and protects the integrity of the erythrocyte membrane as evidenced in the fragiliogram by the reduction in hemolysis of the Hb^{ss} cells [59]. The presence of alkaloids and flavonoid glycosides could also act as an adjuvant that enhances the activity of the components actually responsible for the membrane protection effect noticed in the fragiliograms. From reported findings, one can appreciate the antioxidant properties of these phytomedicines and their role in maintaining the integrity of red blood cells and subsequently improving the quality of life in individuals with sickle cell anemia.

Various works have identified a number of herbal applications that have ameliorating effects on sickle cell disorders (Figures 7 and 8). The antisickling activities of dried *Carica papaya* leaves and roots of *Fagara zanthoxyloides* were investigated in a study to determine the antioxidant properties of the plant extracts and their effects on homozygous sickle cell (SS) erythrocytes *in vitro*. The antisickling activities of both extracts were determined as well as analyses of hematological parameters, hemolysis of SS cells, and formation of membrane-associated denatured hemoglobin (MADH) used to measure the effects of plant extracts on the erythrocyte. Folin-C total phenol and beta-carotene methods of assay were used to determine antioxidant activity, while the effect of plant extracts on oxidative stress was measured by assaying for superoxide dismutase, catalase, glutathione transferase levels, and lipid peroxidation. Results confirmed the potent antisickling activity of both plants. The levels of the oxidative stress enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione (GST) and lipid peroxidation were reduced after blood samples had been incubated with the extracts. The extracts therefore protected membrane integrity resulting in a reduction of red blood cells (RBCs) hemolysis without met-hemoglobin formation. It was concluded that both plant extracts possess potent antioxidant activity which may be responsible for their observed antisickling action [60].

Methanol extracts of herbs hitherto reported to have antisickling activity namely, *Carica papaya* leaf extract, *Fagara zanthoxyloides* root extract, *Cajanus cajan* seed extract, and *Parquetina nigrescens* leaf extract were evaluated in another study. An assessment of their antioxidation potential was determined by assaying for their phytochemical constituents, total phenol content, scavenging activity on DPPH, and total antioxidant status via the ferric thiocyanate method. The extracts had similar phytochemical constituents and exhibited high scavenging activity compared to gallic acid and ascorbic acid standards due to their relatively high total phenol content. These findings suggest that *Carica papaya* leaf extract, *Fagara zanthoxyloides* root extract, *Cajanus cajan* seed extract, and *Parquetina nigrescens* leaf extract are endowed with antioxidant phytochemicals which may act singly or synergistically to potentiate the antisickling action of the plants [61].

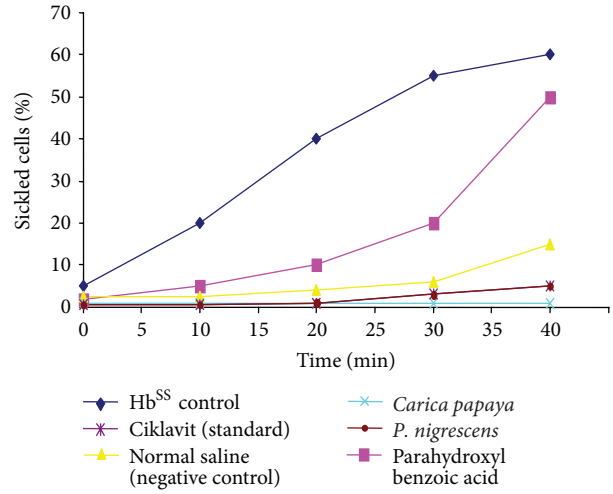


Figure 7: Comparison of antisickling activities of phytomedicines.

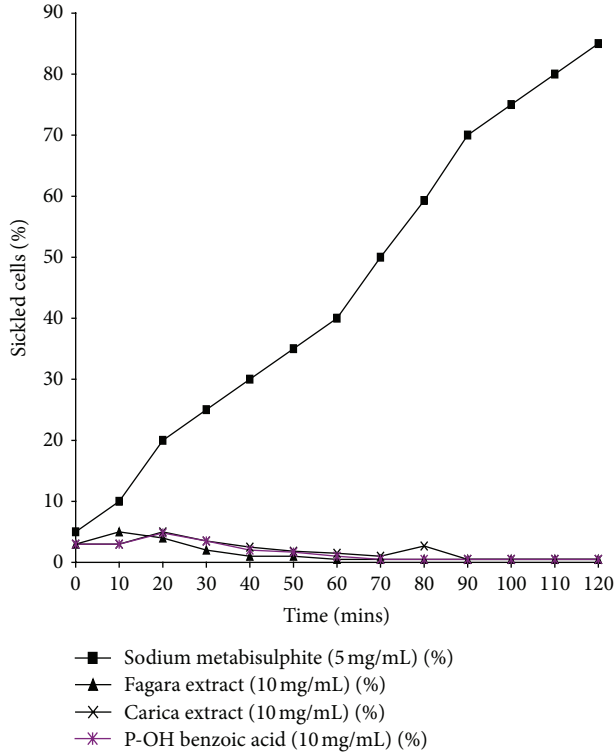


Figure 8: Time course of phytomedicines on Sickling.

5. Correlation between Oxidative Stress, Antioxidants, and Sickling

5.1. Oxidative Stress and Sickling. Oxidative stress is caused by an imbalance between the production of reactive oxygen species and a biological system's ability to readily detoxify the

reactive intermediates or easily repair the resulting damage. It arises when the cellular generation of reactive oxygen species (ROS) overwhelms the antioxidant defense system [62]. Oxidative stress is a large increase in the cellular reduction potential or a large decrease in the reducing capacity of cellular redox couples such as glutathione. Oxidative stress challenges often arise from sources such as radiation, metabolism of xenobiotics, and challenges to the immune system or abnormal functions [63].

Many health hazards such as atherosclerosis, cancer, Parkinson's disease, and Alzheimer's disease have been associated with oxidative stress. Amongst other associated diseases of oxidative stress are sickle cell diseases such as sickle cell anemia. Sickle cell crisis (the fall out of the sickling phenomenon) usually starts with inflammation of joints, which is mostly as a result of oxidative stress affected erythrocytes (RBCs). A very destructive aspect of oxidative stress is its production of reactive oxygen species, which include free radicals and peroxides [63].

However, ROS can sometimes be beneficial as they can be used by the immune system as a way to attack and kill pathogens and also as a form of cell signaling. Most of these oxygen-derived species are produced at low levels by normal aerobic metabolism and the damage they cause to cells is constantly repaired. However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to fall apart [63].

5.2. Antioxidants: Vitamins and Enzymes. Antioxidants are literarily known as "scavengers" or "moppers" of free radicals in an organic entity. They scavenge for free radicals and, consequently, are a very special group of nutritional supplements [64]. The term antioxidant (also "antioxygen") was originally used to refer specifically to a chemical that prevented the consumption of oxygen. In the late 19th and early 20th centuries, extensive study was devoted to the uses of antioxidants in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fueling of internal combustion engines. However, early research on the role of antioxidants focused on their use in preventing the oxidation of unsaturated fats, which causes rancidity. Then, antioxidant activity could be measured simply, by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption. Yet, it was the identification of β-carotene (precursor of vitamin A), vitamin C (ascorbic acid), and vitamin E (α-tocopherol) as antioxidants that revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms.

Antioxidants and their mechanisms of action were first explored when it was recognized that a substance with antioxidative activity is likely to be one that is itself readily oxidized. Further research into how vitamin E prevents the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevent oxidative reactions, often by scavenging reactive oxygen species (ROS) before they can damage cells.

Among the numerous antioxidants available, flavonoids are naturally occurring phenolic compounds in plants. In fact, the majority of antioxidants, both natural and synthetic, are phenolic compounds [65]. Vitamin C, beta-carotene, and vitamin E are all powerful natural antioxidants. Other natural antioxidants, which have been less well characterized, are the flavonoids and anthocyanins. These are all phenols and several thousand different structural variants are found in nature. It has been estimated that the total intake of these compounds in the typical diet is close to 1000 mg per day [65], dwarfing the antioxidant content of antioxidant vitamins in the typical diet. Due to their remarkable importance, antioxidants have been the focus of considerable research and the antioxidant properties of several plants *in vivo* and *in vitro* have been shown to be of great advantage to nutrition today.

For sickle cell disease (SCD), the study of antioxidants especially in various antisickling agents is of great importance because different antisickling agents have different degrees of effect. Antioxidants (scavengers of free radicals) are believed to be major components of these antisickling agents that add to their potential [37]. Thus, it is believed that the higher the antioxidant property of an antisickling agent, the higher its possible antisickling effect, as this enables it to reduce oxidative stress that contributes to sickle cell crisis.

In a reported research [60], aqueous extracts of *Carica papaya* and *Zanthoxylum zanthoxyloides* showed high total antioxidant properties (via β-carotene bleaching assay) and higher phenolic properties than garlic acid. This might explain why decoctions of these plants (used locally) over the years give relief to various oxidative stress associated diseases. The levels of the oxidative stress enzymes (SOD, CAT, and GST) were reduced after blood samples had been incubated with papaya extracts. These enzymes are excreted from the cell during cell damage. Lipid peroxidation, measured indirectly by the percentage of malonaldehyde (MDA) inhibited by plant extract, was also reduced by papaya extract. These findings further confirm the antioxidant activity inherent in the plant extract. Hb^{SS} individuals already in distress during oxidative stress-induced RBC membrane lysis do not need this situation aggravated by a plant extract that causes more oxidative stress to the erythrocyte membrane. Low levels of the oxidative stress enzymes were observed and indicate that papaya extracts can quickly mop up free radicals produced during sickle cell crisis and thus help preserve the integrity of the membrane, along with its inherent nutrients and the glutathione synergistic effect.

6. Herbal Preparations Already in Use as Government-Approved Phytomedicines and Nutraceuticals for Sickle Cell Anemia Management

6.1. Nicosan. Nicosan (formerly known as Niprisan), an antisickling phytomedicine, is reported to inhibit the polymerization of the hemoglobin S. As reported earlier, it is a cocktail of four medicinal plants, *Piper guineense, Pterocapus osun, Eugenia caryophyllum,* and *Sorghum bicolor*, as components and is currently being marketed in Nigeria in encapsulated

250 mg/350 mg doses for a once-daily administration. In a research, the biochemical effects of drug-drug interaction of Ciprofloxacin, a wide spectrum antibiotic, coadministered with Nicosan were examined using standard methods for biochemical, hematological, and antioxidant assays. Findings showed that the presence of Nicosan had a palliative effect on the oxidative free radicals produced as a result of the antibiotic administration [66].

6.2. Ciklavit. Ciklavit is a plant extract preparation available for the management of the sickle cell anemia condition. It contains primarily extracts of the plant *Cajanus Cajan*, proteins (essential amino acids), vitamins such as vitamin C (ascorbic acid), and minerals such as zinc. Ciklavit (*Cajanus cajan* extract) has been reported to have antisickling properties and to improve well being of sicklers. The study also revealed that the antisickling effect of Ciklavit may not probably be through nitric oxide generation or arginase inhibition, since there were no appreciable changes in these parameters. Earlier reports of the antisickling constituent of *Cajanus cajan* suggested cajaminose [67], phenylalanine, and hydroxybenzoic acid [30]. Phytochemical studies on the aqueous extract confirm the presence of phenylalanine and several other amino acids and phenolic compounds and tannins. The antisickling properties of amino acids in *in vitro* studies have been recognized much earlier. Of all the amino acids reported, L-phenylalanine, found to have antigelling effects, was shown to be most active [7]. The role played by other components in Ciklavit (besides *Cajanus cajan*) is basically nutritional. Blood levels of several vitamins and minerals are often low in individuals with sickle cell disease, including vitamin A and carotenoids, vitamin B6, vitamin C, vitamin E, magnesium, and zinc [44, 68–73]. These deficiencies cause a significant depreciation in blood-antioxidant status in these patients [74] and the resulting oxidative stress may precipitate vasoclusion-related acute chest syndrome [75]. Studies indicate that vitamin-mineral supplements of certain nutrients (vitamins C and E, zinc, and magnesium) or treatment with a combination of high-dose antioxidants can reduce the percentage of irreversibly sickled cells [40, 43, 71, 76, 77]. Zinc sulphate appears to help reduce red blood cell dehydration. Important studies indicate that it helps prevent sickle cell crises and reduce pain and life-threatening complications. A study on children with sickle cell suggested that supplements may help improve growth and weight gain. It may also boost the immune system and help protect against bacterial infections. Zinc deficiency is a common nutritional problem in sickle cell disease, so supplements may be important. Magnesium protects against potassium and water loss in sickle cells [19, 72, 73]. In view of these findings, it can be concluded that Ciklavit may cause a reduction in bone pains (painful crises) and may ameliorate the adverse effect of sickle cell anemia on the liver. It is also suggested that one of the antisickling effects or mechanisms of action of Ciklavit may involve the induction of fetal hemoglobin production. Ciklavit may therefore be a promising option for the treatment and management of sickle cell anemia.

7. Current Research Methodologies on Antisickling Phytomedicines

7.1. In Vitro Screening of Plant Samples for Phytochemical, Nutrient, and Antioxidant Composition. A study was carried out to screen the leaf extracts of *Parquetina nigrescens* and *Carica papaya* L. (Caricaceae) for possible antioxidant phytochemicals, proximate nutrient constituents, amino acid composition, and mineral content present in the samples using standard chemical and chromatographic procedures. Phytochemical screening confirmed the presence of folic acid, vitamin B_{12}, alkaloids, spooning, glycosides, tannins, and anthraquinones [78]. The study also showed that each of these plant extracts contained flavonoids and the antioxidant vitamins A and C. Some of the previously established antisickling amino acids were also present in the plants. Cyanogenic glycosides were absent from both plant extracts, indicative of the nontoxic effects of these plants when taken orally. These results indicate that the previously reported antisickling properties of these herbs may be due to their inherent antioxidant nutrient composition, thus supporting the claims of the traditional healers and suggesting a possible correlation between the chemical composition of these plants and their uses in traditional medicine [78].

7.2. Bioassay Studies on Therapeutic Efficacy and Safety Profile of Selected Plants in Rat Models

7.2.1. Antisickling Potency of Selected Phytomedicines. In a study [53], antisickling data was obtained from three typical independent experiments performed in duplicate using blood samples from twenty SS patients. Sickle cell suspensions were preincubated with extracts prior to exposure to 2% sodium metabisulphite solution. Results showed that the time course for 60% sickling was 40 minutes for the control (SS blood without extract). However, at the same time, PHBA (parahydroxy benzoic acid), saline, Ciklavit, *P. nigrescens,* and *C. papaya* (5 mg/mL) all reduced sickling to 50%, 15%, 5%, 2%, and 0% respectively (Figure 7). *C. papaya* showed the highest antisickling activity after 40 minutes of incubation compared to the other phytomedicines and chemical standards used.

7.2.2. Hb^{SS} Polymerization and Time Course for Sickling. Data from *in vitro* studies on the Time course for antisickling activity of the herbal extracts carried out on blood samples collected from confirmed noncrisis sickle cell individuals showed that all the extracts reflected the same delay time for Hb^{SS} polymerization.

Carica papaya did not prolong the delay time of Hb polymerization but greatly affected (appreciably) the time course for sickling (the most effective dose range being 5–10 mg/mL aqueous extract) compared with *Fagara*, parahydroxybenzoic acid, *Parquetina nigrescens,* and Ciklavit (Figure 7). The aqueous papaya extracts reduced the degree of sickle cell formation in a dose-dependent manner (1, 3, 5 mg/mL–10 mg/mL), with the highest dose exhibiting a more effective antisickling activity compared to the methanol extract concentrations and the Hb^{SS}-sodium metabisulphite

control. After 2 hours, in the presence of 5 mg/mL and 10 mg/mL extract concentrations and under the view of the microscope, the original discoidal shape was retained in nearly all cells, unlike the control where over 80% of the SS cells had assumed a sickle shape.

In conclusion, research into phytotherapy of diseases is a current trend in the management of tropical diseases and genetic disorders like sickle cell anemia, with a view to finding cheaper, alternative medicines that the wide populace can have immediate access to. The results outlined in this paper, indicate the feasibility of botanicals, mainly antisickling *phytomedicines* and *nutraceuticals,* as attractive potential candidates for sickle cell anemia therapy and strongly collaborate the ethnomedical usage of the plants.

Further in-depth *in vivo* studies using transgenic mice models and cell lines will provide the mechanism of action and subsequently a deeper appreciation of these phytomedicines and nutraceuticals currently used to improve the quality of life of individuals with sickle cell anemia.

Abbreviations

SS or Hb^{SS}: Hemoglobin S
RBC: Red blood cells.

Conflict of Interests

The author does not have a direct financial relation with the commercial identities, Ciklavit and Nicosan, mentioned in this paper that might lead to a conflict of interest. The author certifies that the products are not hers and that she was not approached to market these products. This review was done purely for scientific research reasons.

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