

A. Specific Aims

Hemoglobinopathies are the most common monogenic disorders in the world. Despite the high prevalence of SCD in West Africa, relatively little research has been conducted in this region utilizing modern genomic technology. In addition, life expectancy of patients with SCD has been greatly extended in industrialized countries, in part as a result of improved medical management. However management of SCD and development of comprehensive life-course care has lagged behind in Africa. Recent findings indicate that genetic variation associated with fetal hemoglobin (HbF) levels strongly influences severity in patients with sickle cell disease (SCD) and β -thalassemia, offering potential insights into novel therapies.

Specific Aims:

1. Establish a research consortium between investigators at three African university hospitals (Ghana, Nigeria, Tanzania), the UK (King's College London, Univ. Oxford), and Loyola University, Chicago

To accomplish these aims, we will:

1. Organize a comprehensive training program and protocol development.
2. Recruit a cohort of 2000 SCD patients in each of the 3 African sites.
3. Follow the patients for 2 years with standardized exams.
4. Collect and analyze DNA, HbF and clinical severity data from Nigeria.
5. Genotype

B. Background and Rationale

B.1 SCD is the signature event of recent evolutionary selection in humans, and a source of fundamental knowledge about genetics

New evidence suggests that the life-threatening form of malaria, caused by *P. falciparum*, was transferred to humans from gorillas years ago [2]. Human susceptibility was conferred by a mutation on the surface of red blood cells, resulting in the most important parasite disease in our species [2]. The virulence of malaria, and its impact on children, has in turn driven a series of intense selection processes which provide the best understood examples of evolutionary selection in humans. A point mutation in the β -globin gene found on 5 different haplotype backgrounds and reaching an allele frequency of 25% in endemic areas appears to be the most effective natural defense. The development of better genomic technology should provide us with the tools to further understand and manage SCD. Furthermore, the identification of modifier genes for SCD, and the characterization of their *SCD is characterized by significant clinical heterogeneity. While some patients suffer life-threatening complications at a young age, including stroke and pulmonary complications, others live into the 5th and 6th decade with few major vascular episodes. Understanding the genetic basis of variability in severity is of central importance in the process of predicting the clinical course and selecting treatments that have appropriate risk-benefit profiles for patients with SCD. Over the last 50 years, anecdotal reports have suggested that some patients with SCD have few clinical complications, and this variability cannot be accounted for by environmental or hematologic variables. Longitudinal data from the Sickle Cell Unit (SCU), UWI, Mona which has followed patients from birth, has identified an unbiased sub-set of patients with the mildest clinical course [3] Recent genetic evidence published by one of our collaborators now demonstrates that genetic variation associated with HbF levels also influences clinical severity in SCD patients [4]. This is consistent with the observation that a key mechanism of action for hydroxyurea – an agent now widely used to treat crises in SCD – is to raise HbF.*

B.2 Moving from observation to mechanisms and knowledge that can be applied

At birth, a developmentally regulated gene expression switch leads to replacement of HbF with adult hemoglobin (HbA). This developmental switch is not complete, however, such that residual amounts of HbF are produced throughout adulthood. Across healthy individuals, a 20-fold variation in HbF level can be observed [5]. In SCD patients, HbF interferes with polymerization of deoxygenated HbS decreasing sickling events and their consequences. HbF is consequently the strongest modifier of clinical severity in SCD: SCD patients with elevated production of HbF have less severe complications and live longer [6, 7]. Substantial

progress has recently been made toward the identification of genes which modify HbF expression [4, 8-13]. These include the β -globin locus itself, the HBS1L-MYB intergenic region on chromosome 6q23, and the BCL11A gene on chromosome 2p15 [9, 11, 14-16]. A recent association study by Lettre et al showed that the minor allele of the SNP rs4671393 in the BCL11A locus is additively associated with increased HbF levels among African-American and Brazilian SCD patients [4]. Importantly, Sankaran et al. also showed that BCL11A is a direct negative regulator of HbF production [17]. Together known variation at these loci can now account for 20-50% of phenotypic variation in HbF [5].

These important new developments in SCD need to be pursued in Africa. A recent large-scale genome-wide association study demonstrates both the challenges and the promise of genomic research in West Africa [18]. In a study from The Gambia only the HbS locus was found to be in association with malaria, apparently as a result of low tagging efficiency from weak linkage disequilibrium (LD) characteristic of African populations. In addition, LD varied to such a degree between The Gambian population and the Yorubas that none of the signals from this study would have replicated in Ibadan. Our project will yield further dissection of the genetics of HbF and other modifying factors in SCD in the well-studied Yoruba population.

B.3 Clinical management for SCD: current practice in Nigeria and Jamaica

Prevention, screening, early detection and strict follow up are the key components of the management of SCD in Nigeria. Premarital counseling should be offered and family pedigree constructed; at the present, however, there are no formal counseling resources at UCH Ibadan, although nurses and doctors perform this function. Newborn screening is also crucial to improving survival in the early post-partum period and some limited efforts have been made in this direction at UCH. Intensive immunization and malaria prophylaxis are implemented when possible and folic acid is recommended. As noted, HbF screening can be used to predict severity, and it is also of value during treatment with hydroxyurea, however routine measurement of HbF is not carried out. More aggressive measures include use of transfusions and non-invasive imaging to identify patients at high risk for vascular accidents, and these are also rarely available.

Further progress has been made in Jamaica. The SCU has over 40 years of clinical experience and clinical research. The first detailed clinical registry established for the Jamaica Study of Sickle Cell Disease (JSSCD) led to the recognition of pneumococcal sepsis and acute splenic sequestration as the two major causes of mortality in the under-5 age group [19]. In response to this, a program of penicillin prophylaxis and parental education, where parents/guardians are taught to palpate for splenic enlargement, was established. These simple and low-cost interventions have resulted in a decrease in the mortality rate in the under-10 year age group from >18% to <2% in 20 years [20]. Additionally, clinical research and studies on interventions done at the Unit have informed the publication of a clinical care manual, which is now used in Jamaica and also in the wider Caribbean region and which acts as a guide to cost-effective diagnosis, health maintenance and management of complications of the disease [21]. Sited in a middle income country, the philosophy of the SCU is guided by the need to minimize the expense of clinical care while not compromising on outcomes. Outpatient management of most complications is encouraged and the day-care facility at the Unit has acted as a model for the management of complications such as severe painful crises, limiting the need and associated expense of hospitalization [22].

It is important to recognize, however, that it may be necessary to modify the clinical management of SCD in Africa beyond the model developed in Jamaica. A crucial aspect of SCD in the Caribbean and the US is prevention and treatment of S. pneumoniae infections. As pointed out by Serjeant, however, recent studies of children in Uganda and Nigeria show that S. pneumoniae is found in less than 10% of septicemia and the offending organism was more likely to be Staphylococci, E. coli, Salmonella and Klebsiella [23]. Likewise, S. pneumoniae did not occur in any of 19 patients with bacteremia in Lagos, Nigeria [23]. It has been suggested that this might reflect a number of factors, including widespread use of over-the-counter antibiotics, early death before reaching medical attention, or retained splenic function as a result of endemic malarial infection [23]. Interventions used to reduce mortality in non-malarial areas may therefore be inappropriate. Critical implications of these observations for our proposed study include the fact that much remains to be learned about the course of SCD in Africa, results of methods universally used to study SCD could vary from one social context to another, and clinical research must be an integral part of any research project on SCD [24-27]. Given the Jamaican experience, we argue that our project could lead to improvement in clinical care.

C. Preliminary Studies

C.1 The Loyola program of research in Africa

The PI began the parent project with NIH support of the International Collaborative Study of Hypertension in Blacks in 1991 [28]. Over 11,000 participants were recruited in the first phase of this epidemiologic description of the evolution of hypertension risk in West Africa, the Caribbean and the US [28]. This project opened a new phase of research on populations of African origin, demonstrating how phenotypic expression of cardiovascular traits reflects the interplay of common genetic factors and contrasting environmental impacts. Subsequently this collaboration was extended to include large scale family linkage and case control studies of blood pressure, obesity and diabetes [29-34]. Our data were used to provide the first clear description of haplotype diversity in West Africa relative to other regions, laying the basis for the HapMap project [35], and we subsequently provided the sampling frame for the Yoruba population included in the HapMap. The research group at Loyola has also made contributions to statistical genetics [36-39]. He holds an adjunct university appointment in Ibadan.

C.2 The experience of the Sickle Cell Unit at the University of the West Indies, Kingston

The SCU began as an out-patient clinic of the Department of Medicine, UWI in 1966. It became a Medical Research Council (UK) funded unit in 1972 and remained so until 1999, when the UWI assumed control of its operation and positioned it within the Tropical Medicine Research Institute. This unit is the only comprehensive clinical care facility for the management of SCD in the English-speaking Caribbean. Housed in a separate facility, the SCU operates an ambulatory clinic and a day-care ward where complications such as acute painful crises, acute febrile illness and acute anemia are managed. Clinical data are captured in an electronic database system. Over 90% of patients with acute painful crises are managed as outpatients and aggressive acute day-care has acted as a model for SCD centers elsewhere. There are now over 5000 patients in the database; over two-thirds have used the services of the SCU during the past year. In addition to clinical care, the SCU performs neonatal screening on behalf of the Ministry of Health. Between 1995 and 2006, the SCU screened ~150,000 births; 889 persons with SCD were detected, of which 550 had homozygous β^S disease (HbSS). Of these, 395 have been enrolled in our clinic for clinical care and are being followed. In 1972, the SCU started the Jamaica Sickle Cell Cohort Study (JSCCS) and data from this study has facilitated the description of the evolution of SCD from birth and has led to improvements in the care of individuals with SCD in Jamaica and worldwide. Based on over 40 years of clinical experience and observational studies of the JSCCS, in October 2008, the SCU published "The Clinical Care Guidelines of the Sickle Cell Unit". These guidelines for health maintenance and management of acute complications in both outpatient and inpatient facilities are essential to ensure a standard of care that is informed by best practice and takes into account resource limitations in Jamaica and the wider English-speaking Caribbean region.

C.3 Studies of SCD in Ibadan, Nigeria

The University of Ibadan is the oldest medical school in West Africa and continues to be one of the premier training institutions; the Department of Hematology was established in 1972. The Hematology Day Care Unit (HDCU) runs a daily emergency care unit Monday to Friday. The HDCU is manned by the Consultant hematologist on duty for the month, hematology residents and the nursing staff. The hematology day care unit at the UCH serves as a referral center to most parts of South Western Nigeria. About 50% of the patients seen in the unit are SCD patients and the unit has a record of over 2000 patients with SCD. Patients consist of children and adults – some of whom are in their 7th decade. Registration is done manually. Follow up cases and newly referred cases (consultative clinics) are seen on Thursday afternoon in the Medical Outpatient clinic. The Hematology Department is actively involved in both clinical and epidemiologic studies of SCD [44-47]. Dr. Akingbola is currently conducting a research project entitled "Differentiation between acute bone infarction and acute bone infection in sickle cell anemia using serum monocyte chemoattractant protein – 1 (MCP-1) assay as a marker" through the sponsorship by the University Advancement Fund. The aim of this study is to evaluate the effectiveness of an MCP-1 assay in predicting bone infection in patients with SCD presenting with acute bone pain. Dr. Akingbola has worked on a variety of other clinical topics in SCD, including contraception, microalbuminuria as a predictive factor for renal failure and abdominal pain crisis.

C.4 Research on the genetics of SCD by Dr. G. Lettre

Before assuming his current position at the Montreal Heart Institute, Dr. Lettre was a post-doctoral fellow with Dr. Joel Hirschhorn at the Broad Institute in Cambridge. Dr. Lettre is a co-investigator on the parent grant for this FIRCA. He has worked extensively in gene mapping of complex diseases [48-50] and has an

active research program focused on the identification of genes that modify clinical heterogeneity in SCD. As part of the group led by Dr. Stuart Orkin at Children's Hospital, Boston, he co-authored papers demonstrating the role of BCL11A in the regulation of HbF expression and the association of HbF loci with measures of SCD severity [4]. He will provide expert advice about on-going developments in the genetics of SCD, genotype candidate loci using the Sequenom iPLEX platform available to his laboratory, and receive aliquots of DNA from the collected samples for re-sequencing and more intensive study when funds become available.

D. Experimental Methods and Design

D.1 Exchange of investigators among the study sites

The primary goal of this project will be to establish an effective collaborative relationship between investigators at UCH Ibadan, UWI Kingston, and the two participating medical institutions in North America. Investment in research on SCD is not distributed proportionally to the geographic burden of the disease. Jamaica, as a middle income country, has sufficient resources to develop a high quality medical care program, and participates in a range of important research, including genomics. In West Africa, however, many obstacles prevent the implementation of effective research on SCD, despite the enormous burden. At the same time, there are crucial research opportunities in West Africa that could advance our understanding of SCD, in a fashion similar to what has been accomplished, through our group and others, in hypertension, diabetes, and the HapMap.

We propose to support a 4 week training period in Chicago for Dr. Akingbola, followed by a 4 week education and training visit to Kingston. In Chicago she will participate in seminars on clinical research and genomics. In addition she will be enrolled in the Masters in Public Health program offered on-line by the Department. Course work will include biostatistics, epidemiology, health policy, ethical research, international health and outcomes research. In Chicago she will start the program and receive appropriate support and feedback, and subsequently finish upon return to Nigeria. While the cost of tuition makes it prohibitive to consider awarding an official degree, she will be able to receive guidance and instruction from faculty in biostatistics, epidemiology, clinical trials, health policy and ethics. Under the supervision of faculty in Biostatistics she will participate in the development of the computer database for the clinical aspects of the study in Nigeria. While we do not anticipate that she will function as a data manager, a working knowledge of the system and the ability to generate summary data will be important to her advancement as a researcher. Finally, we will develop a plan to accumulate an up-to-date reference library on clinical and basic science research on SCD for use by Dr. Akingbola and other members of the Hematology Department. An assistant in the Department will work with her to accumulate textbooks, journal articles and web-based resources.

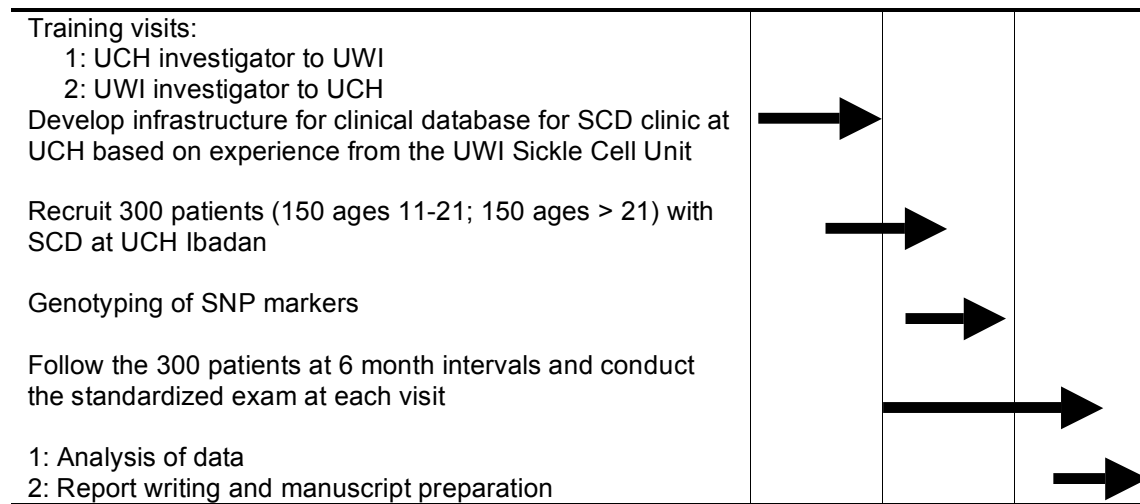
In Kingston Dr. Akingbola will study the organization of the SCU, observe the state-of-the-art management and treatment of SCD patients, learn the structure of the database at the Sickie Cell Unit used to track the patient cohorts, and participate in academic seminars. She will also have the opportunity to deepen her understanding of the genetics of SCD through mentoring from Dr. McKenzie.

Subsequently Dr. Reid will travel to Ibadan to meet with UCH faculty and staff and familiarize himself with the clinical environment in Nigeria. He will present technical assistance in the development of the clinical assessment procedures and the database, provide a seminar explaining how the SCD guidelines were developed in the Caribbean and demonstrate their impact. During this visit he will set in place the framework for a clinical research program that can be used to investigate the course of SCD and define key infectious threats in Ibadan. We anticipate that this will be accomplished in a two week period.

We have budgeted a further 2 week visit to Chicago for Dr. Akingbola in years 2 and 3 to review the data that have been collected, prepare manuscripts, and plan future directions of the project. Dr. Tayo, who travels to Nigeria twice a year as part of other on-going NIH-supported projects, will also devote 2 weeks of all 3 years to providing technical assistance to the UCH Ibadan team. Finally, Drs. Cooper, Tayo and Lettre will also visit Jamaica and Nigeria with funds provided by other research projects. During these visits they will work closely with Dr. Akingbola to analyze data, improve the recruitment protocol and explore new opportunities for research collaboration. During the visit, they will also evaluate the use of the SCD patient database and identify possible areas of improvement for its use by other clinicians and researchers at UCH. Dr. Cooper will also conduct a short course of prevention, as developed through the CDC prevention seminars in Africa which he directed.

Time Line

Activity	Year 1	Year 2	Year 3
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D.2 Develop a cohort of 300 SCD patients at UCH, Ibadan

A nurse, a phlebotomist, a laboratory technician and a data manager will be hired at each site to establish the patient cohort and electronic database. The Department of Hematology at UCH takes over the care of patients with SCD at the age of 11. In order to capture the full spectrum of information on these patients we will recruit 150 established clinic patients ages 11-21 and 150 ages > 21 from the existing rolls. We will restrict enrollment to patients from the Ibadan metropolitan area to increase the likelihood of complete follow up. In the first 3 months of the project we will adapt, pilot test, and refine the forms and electronic database used to record clinical information and track the longitudinal course of the patients. The clinical nurse will identify and enroll patients, and assist in the standardized exam. These patients will subsequently be seen at 6 month intervals, on a minimum of 3 follow up visits. We anticipate that regular follow up will present a significant challenge; therefore we propose to provide funds to cover travel and a means of contacting patients in the community who are lost to follow up.

Loss to follow up will not be random (e.g., patients with a fatal event are more likely to be unaccounted for). A full time nurse, who obtains detailed contact information, is the most effective approach to this challenge. It should be noted that cell phone use is now very widespread in Nigeria so through the extended family networks shared by most patients we should at least be able to establish vital status. An additional resource for patient recruitment and follow-up will be the SCD “club” in Ibadan. Organized as a patient support group, similar clubs exist for a variety of diseases. In addition to psychological support, these organizations pool resources to provide medications that may be in intermittent supply at the hospital.

The intake history and physical examination will be based on that recommended by the SCU, Jamaica in its clinical care guidelines [21]. Standardized forms will be developed to capture acute events such as severe acute painful episode, acute chest syndrome and stroke. These forms may be completed either 1) at presentation - if the patient seeks medical attention at the UCH for the acute event, 2) during telephone contact in defaulted patients or 3) at the routine 6 monthly follow-up visits to document events that occurred since the patient was last seen, if medical attention was not sought at the UCH.

Baseline investigations would include: a complete blood count, Hb electrophoresis and a quantitation of %HbF, HbS and HbA2, iron studies, renal function tests (urea and creatinine), liver function tests (total protein, albumin, bilirubin – total and direct, AST, ALT), LDH and urine analysis. Samples for genetic testing will also be collected at baseline. Further investigations will be guided by the intake history and examination as well as review of the results of baseline investigations. These might include X-ray (chest if there is a history of recurrent acute chest, hips – if there is evidence of avascular necrosis of the femoral head, etc.), neuroimaging (CT, MRI if there is a history of cerebrovascular accident).

Patients will be ranked according to mildness/severity. Appropriate scales for use in Ibadan do not now exist. In general, more experience exists defining “severity”, which is taken to be the cumulative incidence of a set of major complications. “Mildness” is therefore defined as the absence of severe complications. We recognize that this approach may not be sufficiently robust to scale patients across the full range of conditions and additional pilot data will be required from the Nigerian site to assure that the data items can be collected accurately and that they occur with sufficient frequency. Clinical data points useful for this purpose include painful crisis events (bone and abdominal painful crisis), avascular necrosis, acute chest syndrome, septicemia, b19 infection, and leg ulceration. The average number of events per year adjusted for clinic entry

bias will be computed and will be used to assign a severity rank to each patient. Patients will be grouped by quartile of severity. In this exploratory analysis three different schemes will be used. Two of the schemes will be based on mildness rank within the study sample ("relative severity"). The first scheme will include pain events only and the second will use a combination of pain events and the other clinical events listed above. Patients who die or suffer a life-threatening event (e.g. stroke) during the period of follow-up will be excluded from the mild disease definition. The third scheme will identify patients having moderate to severe disease as those suffering from greater than or equal to three acute painful crisis or acute chest events per year ("absolute severity"). Patients who die or suffer a life-threatening event (e.g. stroke) are classified as having severe disease. This last scheme is based on clinical practice guidelines for the use of hydroxyurea therapy [51] which emerged from the outcomes of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia [52].

D.3 Genotype loci associated with variation in HbF

DNA will be extracted in Nigeria using established techniques and they will be stored and shipped in batches first to Chicago (this will insure safe passage). Quantity and quality will be verified and an aliquot shipped to Jamaica for genotyping in Dr. McKenzie's lab. An aliquot will also be shared with Dr. Lettre for additional future genotyping. The Jamaica lab has experience in SNP genotyping for candidate loci and recently examined susceptibility to oxidative stress in severe childhood malnutrition [53, 54]. A total of 9 SNPs will be genotyped in the three HbF-associated loci for association analysis. 3 SNPs (rs11886868; rs4671393; rs7557939) in BCL11A gene, 5 SNPs (rs28384513; rs7776054; rs9399137; rs9389268; rs4895441) in HBS1L-MYB region and rs7482144 in β -globin locus. HbF measurement will be performed in Ibadan using an ELISA assay [55]. The equipment for this assay is available at UCH and the grant will provide assay kits.

D.4 Statistical considerations

D.5

D.6 Limitations and future plans

Clinical research in much of Africa, including Nigeria, faces major practical challenges. Electrical power can be intermittent, strike actions by staff and faculty are a regular occurrence and physicians must cope with enormous patient volumes. Our team has coped successfully with these challenges over the last 20 years and our track record demonstrates that we can implement creative solutions. We also realize that standardization of the clinical measures, and, in particular, achieving high levels of follow-up, is also difficult. Cell phone use is very widespread in Africa and greatly facilitates contact. We note also that alternative clinical facilities in Ibadan are limited and many patients are intensely loyal to UCH. We further acknowledge that these cohorts lack some state-of-the-art clinical phenotype measures, which require sophisticated imaging (e.g. MRI, Doppler flow). These are not major obstacles however since the clinical outcomes are the most relevant measures, and that while of interest, more sensitive assays of HbF are not required in SCD since the levels are high.

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