Organ damage in sickle cell disease study (ORDISS): protocol for a longitudinal cohort study based in Ghana

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ABSTRACT

Introduction Sickle cell disease is highly prevalent in Africa with a significant public health burden. Nonetheless, morbidity and mortality in sickle cell disease that result from the progression of organ damage is not well understood. The Organ Damage in Sickle Cell Disease Study (ORDISS) is designed as a longitudinal cohort study to provide critical insight into cellular and molecular pathogenesis of chronic organ damage for the development of future innovative treatment.

Methods and analysis ORDISS aims to recruit children aged 0–15 years who attend the Kumasi Centre for Sickle Cell Disease based at the Komfo Anokye Teaching Hospital in Kumasi, Ghana. Consent is obtained to collect blood and urine samples from the children during specified clinic visits and hospitalisations for acute events, to identify candidate and genetic markers of specific organ dysfunction and end-organ damage, over a 3 year period. In addition, data concerning clinical history and complications associated with sickle cell disease are collected. Samples are stored in biorepositories and analysed at the Kumasi Centre for Collaborative Research in Tropical Medicine, Ghana and the Centre for Translational and International Haematology, University of Pittsburgh, USA. Appropriate statistical analyses will be performed on the data acquired.

Ethics and dissemination Research ethics approval was obtained at all participating sites. Results of the study will be submitted for publication in peer-reviewed journals, and the key findings presented at national and international conferences.

INTRODUCTION

Sickle cell disease (SCD) comprises a group of inherited red blood cell conditions that result from the abnormal production of haemoglobin. Over 305 000 babies are born worldwide annually with SCD mostly in low and middle income countries, and about 75% or more of these births occur in sub-Saharan Africa, posing an increasing health burden1 and contributing to early childhood mortality.2 SCD affects approximately 2% of newborns in Ghana.3

Clinical syndromes of SCD include anaemia, infection, and the consequences of blood vessel blockage (vaso-occlusion). The latter deprives tissues of oxygen and is indicated as the cause of acute painful episodes, the hallmark of SCD, and other complications such as stroke, acute chest syndrome, priapism, leg ulceration and chronic organ failure. Stem cell transplantation offers curative possibilities although this is not universal, and other treatment options are generally limited in Africa.3 Improved knowledge and successful primary public health prevention strategies have positively impacted childhood survival transforming SCD into
a chronic disease. Nonetheless, progressive deterioration of organ function and end-organ damage is inevitable and appears to be irreversible.4–6 The mechanisms that lead to these complications, studied mostly outside sub-Saharan Africa, are not fully understood. Further understanding through a longitudinal cohort study of patients with SCD may provide novel insights into cellular and molecular pathogenesis of chronic organ damage, and opportunities for the development of innovative treatment and precisely timed interventions to prevent onset of organ damage.

In Ghana, a pilot Newborn Screening (NBS) project for SCD was established in Kumasi (the second largest city) and Tikrom (a nearby rural community) from 1993 as an international collaborative study.7 Newborns identified with SCD are registered in the Kumasi Centre for Sickle Cell Disease (K-CSCD) at the Komfo Anokye Teaching Hospital (KATH), and followed up until 15 years of age through the Child Health Directorate. This NBS project was subsequently adopted by the Government of Ghana in 2010 to scale it up as a national public health programme.

All patients enrolled in the K-CSCD have their haemoglobin (Hb) genotype confirmed with isoelectric focusing (IEF) in the neonatal period, and alkaline Hb electrophoresis beyond the neonatal period. K-CSCD provides comprehensive care for patients with available facilities and services including blood transfusion, radiology, laboratory, pharmacy, orthopaedics, and ophthalmology. There is a team of two consultant paediatricians, two specialist paediatricians, three residents (registrars), three house officers, a nurse in charge, eight other nursing staff, and three auxiliary personnel who help with data recording and retrieval of medical records on clinic days. Clinics are held every day.

STUDY OBJECTIVES
There are currently no data on the spectrum of organ dysfunction and end-organ damage in the SCD patient cohort attending K-CSCD. The Organ Damage in Sickle Cell Disease Study (ORDISS) was designed as a longitudinal cohort study of children with SCD attending K-CSCD to document acute events and the progressive deterioration in organ function with age, and to identify candidate and genetic markers of specific organ dysfunction and end-organ damage. Specific objectives are:

1. To determine the proportion of children with SCD attending K-CSCD who develop specific organ dysfunction.
2. To determine levels of biomarkers of organ dysfunction (heart, kidney, liver, lung, brain and skeletal muscle) from multiple candidate plasma and urine samples.
3. To determine haematologic and haemolytic markers in the recruited children attending clinic for routine evaluations or acute illness management.
4. To compare clinical evidence of organ dysfunction with biochemical and genetic markers.

METHODS AND ANALYSES
ORDISS is an international collaborative study conducted at three institutions: Department of Child Health, KATH/Kwame Nkrumah University of Science and Technology (KNUST), Ghana; Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR), Ghana; and Centre for Translational and International Haematology at the Heart, Lung, and Blood Vascular Medicine Institute (VMI), University of Pittsburgh, USA.

Participants and recruitment
A consecutive purposive sampling method of all individuals willing to participate is being employed to recruit and follow-up participants for the next 3 years in a longitudinal design.

Eligibility criteria
Eligible participants are families of children with SCD comprising all genotypes, confirmed with both IEF and alkaline electrophoresis with cellulose acetate membrane, who are registered at K-CSCD, aged 0 to 15 years and younger at recruitment, and receive outpatient or inpatient care at KATH. Patients not known to KATH and older than 12 months of age must be registered at K-CSCD for at least 12 months prior to becoming eligible for enrolment.

Exclusion criteria
Exclusions are children with SCD and co-morbid chronic conditions including malignancies, seizure disorders, and history or clinical signs and symptoms of HIV infection. In addition, patients who cannot be followed-up for a minimum of 12 months during the study, and families who decline informed consent or assent are excluded.

Recruitment and enrolment procedures
ORDISS was initially introduced to the families of children with SCD at the monthly Sickle Cell Disease Association meeting, a national support group for parents and patients with SCD, which is held at KATH premises.

Enrolment (Entry) Visit
Consecutive clinic attending families are opportunistically approached during routine clinic visits, and the study introduced to them prior to phlebotomy. Signed or thumb-printed informed consent is obtained from parents/caregivers and assent from children with SCD aged 7 years and over. Consenting families (participants) are enrolled into the study, and the child (subject) is assigned a unique study identification number that will be used as the subject identifier throughout the study. Participants’ demographics, clinical information, and past medical history are recorded. These include the subject’s age, gender, standing height, weight, head circumference, heart rate, respiratory rate, blood pressure, oxygen saturation (SpO2), and SCD complications relating to eyes, ears, head, nose, and mouth. Examinations of the throat, lymph nodes, chest with auscultation, heart with auscultation, abdomen, liver, spleen, genitalia,
extremities, joints, and neurological examinations are performed by specialist paediatricians or residents, and recorded. In addition, data on parental ethnicity, religion, marital status, and educational level are collected. All information gathered is written in the subject’s medical records, and entered into an electronic Case Report Form (e-CRF).

Using standard practice of phlebotomy, blood is collected from each subject into di-potassium ethylenediaminetetraacetic acid (K₉EDTA) tube and serum separator (SS) tube with gel, each 3–4 mL; 10–20 mL of midstream urine is also collected from each subject at specific visits. Blood and urine samples are collected from 8am to 12pm on the clinic day. Urine samples are collected from children aged 3 years and over using sterile urine containers, while infant urine collection bags are used for younger children. The latter urine samples are subsequently decanted into sterile urine containers. The blood in the K₉EDTA tube is inverted 8–10 times to ensure adequate mixing of the blood with the EDTA anticoagulant; the blood in the SS tube is allowed to adequately clot.

The samples from each subject are duly labelled with the specific study identification number. The K₉EDTA-anticoagulated blood samples are sent, in a cryobox at room temperature, to the KATH Laboratory where aliquots are taken and immediately used for haematologic analyses; these include full blood count (FBC) with white blood cell (WBC) differential, performed electronically, and reticulocyte count, performed manually. The remainder of the blood sample and the urine sample are then placed on wet ice in a cold box and transported to KCCR for further processing, storage and analyses.

Demographic and clinical information, as well as FBC results, of the subjects are entered into a tablet adapted specifically for ORDISS with CommCare software, which allows creation and management of mobile applications through a website. The study database was developed at KCCR where the server is also held. Data are transmitted via a mobile phone network, subject to strength of connectivity, at the end of each clinic day from KATH to the KCCR server for cloud storage and management. This is subsequently extracted into Microsoft Excel spreadsheet format for statistical analyses.

Interim (Follow-up) Visit

On subsequent annual visits after recruitment (ie, interim visits) over 3 years, clinical procedures and data collection will be replicated, with emphasis on each subject’s current ailments, episodes of acute illness not treated at KATH, episodes of enuresis, and current medications. In addition, educational performance is assessed and documented from preceding school-term reports to determine whether this is maintained during the study. Furthermore, blood and urine samples will be collected and identical procedures applied. Additionally, the process of data transmission with tablets via internet to KCCR will be repeated for each interim visit.

Acute illness visit and hospitalisation

During acute illness of subjects, blood and urine samples will be collected together with samples for acute illness blood tests requested by attending clinicians. These blood tests will also help to rule out illness due to other infections such as malaria, and allow comparison with steady-state laboratory values. Identical volumes of blood and urine will be collected from each subject during an acute illness; the samples will be processed using equivalent outlined procedures before these are transported on wet ice to KCCR.

Biorepository sample collection and analyses

At KCCR, the blood samples collected at each ORDISS visit are centrifuged for segregation of the major blood components. Each of plasma (from K₉EDTA tube) and serum (from SS tube) is harvested and aliquoted into two (2) tubes for storage. Buffy coat is then collected from each sample into single tubes. Genomic DNA is manually extracted from aspirated buffy coat samples using the QIAamp DNA blood mini kit (QIAGEN, # 51106). DNA extracts are stored in double eluates/ aliquots. Each sediment of red cells is stored in a single tube after washing three times with 1X phosphate buffered saline. A 4.5 mL single aliquot of each urine sample is also stored. All samples at each stage (ie, recruitment, interim and acute illness) of the study will be processed with a consistent approach at KCCR, and stored at −80°C.

A duplicate biorepository (ie, single aliquots) of DNA extracts, plasma and serum samples is maintained at the VMI. The samples are transferred carefully into intact stockings, and organised into bundles in the stockings; the mouth of each stocking is tied with a string and labelled with a sticker that bears the stocking number, sample type (ie, serum, plasma or DNA), and stage (visit) collected. The stockings are then placed in a tank containing liquid nitrogen (at −196°C), the tank stoppered tightly, labelled and shipped via air flight to VMI, observing all protocols. An electronic file in Microsoft Office Excel format showing the samples in the various stockings and stocking bundles being shipped are also sent electronically to VMI. The first shipment of duplicate biorepository to VMI has already been completed, and will subsequently take place once every year.

A sub-aliquot of each deposit of red cells will be used for haemolysate preparation. The haemolysates will be analysed to ascertain the haemoglobin (Hb) phenotype and determine the percentage of foetal Hb (HbF) of subjects at KCCR. DNA extracts will also be analysed for genetic markers of organ injury at VMI. Plasma, serum and urine samples will be batch assayed for chemical biomarkers of haemolysis, organ dysfunction and end organ damage both at KCCR and VMI. Assays will be performed using standardised validated enzyme-linked immunosorbent assays and colorimetric techniques. Laboratory investigations are presented in the table 1, and concise definitions of organ damage with diagnostic criteria are shown in table 2.
**Table 1** Organ Damage in Sickle Cell Disease Study (ORDISS): Laboratory Investigations

| Classification       | Parameters                      | Marker of Interest                                      | References |
|----------------------|---------------------------------|---------------------------------------------------------|------------|
| Fundamentals         | Basic/general organ function     | FBC with WBC differentials, reticulocyte count and percentage, Hb phenotype, percentage HbF | (10-12)    |
| Index of Injury      | Haemolysis                       | Plasma Hb, haem, haptoglobin, haemopexin, haem oxygenase-1, total and fractionated Hb, soluble C91, soluble CD163, arginase | (13-15)    |
|                      | Vascular and Systemic Inflammation | IL-1β, IL-2, IL-3, IL-4, IL-6, IL-8, IL-10, soluble VCAM-1, soluble ICAM-1, P-selectin, E-selectin, nitric oxide metabolites, TNF-α, IFN-α, Methaemoglobin, oxidised phospholipids, alpha-1 microglobulin, isoprostanes | (13, 15-17) |
| Organs of Interest   | Kidney and Liver, Lung and Brain Heart and Skeletal Muscle | Creatinine, blood urea nitrogen, alanine transaminase, aspartate transaminase, total and fractionated protein CPK-1, brain-derived neurotrophic factor Total CPK, CPK-2, troponin T and I, CPK-3 | (21-23) (21, 24) (21, 25-27) |

WBC=white blood cells, , VCAM=vascular cell adhesion molecule, , TNF=tumour necrosis factor.
CPK, creatine phosphokinase; FBC, full blood count; Hb, haemoglobin; ICAM, intravascular cell adhesion molecule; IL, interleukin; IFN, interferon; TFN, tumour necrosis factor; VCAM, vascular cell adhesion molecule, WBC, white blood cells.

**Statistical analysis**

Subjects will be allocated to the two extreme quartiles of biochemical and clinical evidence of organ dysfunction in a case-control design. The primary analysis will be ANOVA with either the actual measurements of biomarkers or those normalised by appropriate transformations. Genotypes will be the independent variable and the dependent outcomes will be biomarkers. Analyses will be run using the most recent version of the STATA software. In the event that there is evidence for a significant interaction between single nucleotide polymorphism (SNP) and a clinical event, analyses will also be run on organ damage and non-organ damage subjects independently. As with other phenotypes, it is likely that multiple genetic variants operate to affect the risk of specific clinical events more than any single SNP or plasma biomarker, independently. Thus, SNPs will be tested in 2-way-ANOVA and if a significant interaction term is observed, the effect size will be compared with the linear model. The results will be interpreted in light of known pathways and feedback loops for specific biomarkers. An exploratory analysis will be performed examining the relationship among biomarkers belonging to the same pathways. Specifically, we will interrogate data to see if correlations among these factors differ by the status of a clinical phenotype, which would suggest differences in the overall network of factors; this will be performed using Spearman’s rank correlation and testing for heterogeneity among organ damage phenotypes using a t-test on the Fisher r-to-z transformations of the Spearman correlation coefficients performed. SNPs will be initially tested for association with the occurrence of acute organ damage as a dichotomous trait (eg, +ACS/-ACS). Statistical tests for differences in single locus allele and genotype frequencies will be calculated using PLINK. All loci will also be tested for Hardy-Weinberg equilibrium to assess the possibility of genotyping error. Genetic association will be concluded if the frequencies of either genotypes or alleles differ significantly between the extreme quartile classes (p<0.05). Odds ratios will be calculated using logistic regression.

**ETHICS AND DISSEMINATION**

**Ethical and safety considerations**

Research ethics approvals for ORDISS were obtained from both the Committee on Human Research, Publications and Ethics of KNUST (Approval No. CHRPE/AP/325/14) and subsequently renewed approvals (No. CHRPE/AP/104/16 and No. CHRPE/140/17), and University of Pittsburgh Institutional Review Board (Approval No. PRO14010452). The study is currently in an active phase which commenced in May 2015, and just began year three. Informed consent (and assent where applicable) is obtained from all participants. Blood samples are routinely collected from children with SCD attending the K-CSCD at KATH and collection of urine samples is a non-invasive procedure. Data transmission from the K-CSCD at KATH to KCCR is secure. Biorepository samples are transported from KATH to KCCR and from KCCR to VMI, and stored appropriately according to international standards. Samples sent to VMI are de-identified, and there is an ethics (institution review board) approved material transfer agreement between the collaborating institutions.

**Dissemination**

The results of ORDISS will be submitted for publication in peer-reviewed journals, and the key findings presented at national and international sickle cell disease and haematology conferences.

**CONCLUSION**

It is envisaged that ORDISS will achieve its objectives and will substantially add to the modest amount of existing data on onset and progression of organ damage in children with SCD. ORDISS will also provide new insights into organ dysfunction and end-organ damage for appropriate and more precise timing of future therapeutic inventions.
| Classification of Complications | Clinical Manifestations of Organ Damage | Definition | Diagnostic Criteria |
|----------------------------------|----------------------------------------|------------|-------------------|
| Cardiac                          | Cardiomegaly                           | Enlargement of the heart and may involve the ventricles, the atria or both | Evidence of enlargement on CXR or ECG BP as measured sitting or supine in the steady state in a warm environment on three separate occasions separated by 15 min. the BP values greater than the 90th centile for age, sex and height. ECHO is the most commonly used technique used to measure cardiac function. MRI measurement of volume may be used. |
|                                  | Hypertension                           | BP exceeding the 90th centile for age | |
|                                  | Cardiomyopathy                         | Heart disease affecting the musculature of the heart leading to impairment of function. Chronic high cardiac output leads to cardiac hypertrophy and development of hypertrophic cardiomyopathy while iron overload causes dilated cardiomyopathy. |
| Pulmonary                        | Acute chest syndrome                   | Acute illness characterised by fever and/or respiratory symptoms accompanied by a new pulmonary infiltrate on CXR | Radiographic evidence of consolidation. A new segmental (involving at least one segment) radiographic pulmonary infiltrate. Temperature>38.50C,>2% decrease in SPO2, tachypnea, intercostal retractions, nasal flaring, use of accessory muscle, chest pains, cough, wheezing. |
| Musculoskeletal                  | Dactylitis                              | Inflammation caused by ischaemia/infarction of bone/bone marrow of the hands/feet resulting in swelling, redness and pain. It is seen primarily in children from 6 months to 3 years and generally does not occur beyond 5 years of age due to lack of haemopoietic marrow activity in the hands and feet. Condition resulting in dead bone tissue due to an interruption in blood supply most likely as a result of vaso-occlusion. | Soft tissue swelling of hands/feet and limited range of motion of extremities or pain and tenderness of hands and feet. Radiographic evidence of necrosis and subsequent bone changes. Plain films may be normal early in disease whereas MRI demonstrate early changes and provide more detail on the degree of bony involvement. |
|                                  | Avascular necrosis of joints           | Condition resulting in dead bone tissue due to an interruption in blood supply most likely as a result of vaso-occlusion. | |
| Neurological                     | Seizures                               | Acute onset of uncontrolled electrical activity in the brain which may produce a physical convulsion with minor physical signs, and thought disturbances. Circumscribed blood filled dilatation of a cerebral artery caused by weakening of arterial wall / intracranial haemorrhage / acute neurological syndrome resulting from impaired cerebral blood flow without evidence of haemorrhage. | EEG consistent with seizure, sustained abnormal electrical discharges that have a relatively discrete beginning and end or based on clinical history and neuroimaging(CT or MRI) Visualisation by MRA or angiogram of brain/ demonstration of haemorrhage on CT scan or MRI on brain/ MRI or CT Scan showing an infarctive CNS event consistent with symptoms and signs. |
|                                  | Stroke – aneurysm / haemorrhage / infarctive | | |
| Renal                            | Haematuria                             | Presence of red blood cells in the urine, due to acute papillary necrosis, UTI and less commonly glomerulonephritis, obstruction, analgesic toxicity, mycobacteria infection, tumours, arterio-venous malformation and vasculitis. | Greater than three red blood cells per high power field on urine microscopy. |
### Table 2

| Classification of Complications | Definition                                                                 | Clinical Manifestations |
|---------------------------------|---------------------------------------------------------------------------|-------------------------|
| Hepatobiliary                   | Inflammation of gallbladder lining, generally caused by infections, spasms of gall bladder in biliary tract, usually leading to liver enlargement and decreased haemoglobin concentration. | Cholecytitis                  |
| Cholelithiasis/Sludge           | Presence of stones or sludge in gallbladder or common bile duct.          | Splenic Acute splenic infarction |
| Hepatic sequestration           | Sequestration of red blood cells in hepatic sinusoids and decreased haemoglobin concentration. | Acute ischaemic necrosis of spleen |
| Ultrasound evidence of stones or sludge | Ultrasound evidence of stones or sludge in gallbladder or common bile duct. | Acute (L) upper quadrant pain which may be referred to the (L) shoulder and periumbilical region, upper quadrant pain-colicky and one or more of the following: |
| Sequestration of red blood cells in hepatic sinusoids | Sequestration of red blood cells in hepatic sinusoids and decreased haemoglobin concentration. | Imaging evidence of necrotic or ischaemic splenic parenchymal or surgical evidence of acute splenic parenchymal necrosis. |
| Decrease of>2 g/dL in haemoglobin concentration | Decrease of>2 g/dL in haemoglobin concentration from baseline leading to liver enlargement and decreased haemoglobin concentration. | Acute splenic infarction as a result of venous or arterial compromise |

**Organ dysfunction definitions adapted from:** Balsa et al., 2012

**Table 2 Continued**

| Organ Dysfunction | Definition |
|-------------------|------------|
| Carotid Artery     | Acute ischaemic necrosis of spleen as a result of venous or arterial compromise |
| Liver              | Acute ischaemic necrosis of spleen as a result of venous or arterial compromise |
| Splenic Artery     | Acute ischaemic necrosis of spleen as a result of venous or arterial compromise |
| Splenic Artery     | Acute ischaemic necrosis of spleen as a result of venous or arterial compromise |

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