Hydroxyurea Use and *Plasmodium falciparum* Prevalence Among Children With Sickle

Cell Anemia in Homa Bay, Kenya: A Cross-sectional Study

by

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Amy Herring

Thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in the Duke Global Health Institute
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Abstract

Hydroxyurea, a mainstay of sickle cell management in the developed world, offers a range of potential benefits to children with sickle cell disease. There is strong evidence that hydroxyurea induces production of fetal hemoglobin (HbF) in red blood cells, which is generally associated with reduced morbidity and fewer incidents of clinical events in sickle cell patients. Based on findings from in vitro investigations, it has also been suggested that hydroxyurea may confer some protection against malaria parasitemia by inhibiting proliferation of the malaria-causing parasite - *Plasmodium falciparum*.

The purpose of this study was to examine the effects hydroxyurea use on *P. falciparum* infection, parasite density, HbF and morbidity among children with sickle cell disease living in a malaria endemic setting. We analyzed baseline data of 95 children (aged 1 – 10 years) enrolled in the EPiTOMISE clinical trial (Enhancing Preventive Therapy of Malaria in children with Sickle cell anemia in East Africa) between January 2018 and September 2018.

Our analyses revealed no significant difference in the prevalence of *P. falciparum* infection between hydroxyurea users and hydroxyurea non-users, prevalence ratio [95% Confidence Interval] = 1.14 [0.76, 1.71]. Among infected children, median (IQR) log parasites densities were also similar between hydroxyurea users, -0.96 (-1.67, 0.41), and hydroxyurea non-users, -0.12 (-1.32, 3.48), p-value = 0.146.

We did observe substantial hematological benefits among hydroxyurea users including, an approximate 1 unit increase in median hemoglobin concentration and a 2.7-fold increase in median percentage HbF. However, this observation did not translate to any meaningful difference in the prevalence of morbidity events.

In agreement with the few studies on hydroxyurea use in malaria endemic settings, these results suggest that there may be no added risk of *P. falciparum* infection to sickle cell patients who routinely use hydroxyurea. Furthermore, our results reflect that hydroxyurea use is associated with increased HbF and a better hematological profile in this population. There is need for more research on hydroxyurea use in sub-Saharan Africa to help resolve any existing concerns and conflicting data in the current body of literature.

Dedication

To my beautiful grandmother, Ms. Erina Bikwasiroha (1938-2019). Thank you for showering me with your unconditional love, support, wisdom and prayers every step of my academic journey. I miss you dearly jaja.

Table of Contents

iv

List of Tables

List of Figures

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1. Introduction

1.1 Sickle Cell Disease and Malaria

Sickle cell disease is a hereditary blood disorder which distorts the ability of hemoglobin to bind and transport oxygen to tissues and organs. It is marked by chronic anemia, pain crises, jaundice, progressive organ damage, frequent episodes of acute illness and an increased risk of serious bacterial infections among other complications.[1] Individuals who inherit sickle cell disease often require lifelong interventions including blood transfusions and prophylactic therapy to manage their symptoms and help improve their quality of life.

A 2010 review on the global burden of sickle cell disease estimated that approximately 275,000 children with sickle cell disease are born each year, with 85% of these births occurring in sub-Saharan Africa.[2] Of note, the true prevalence of sickle cell disease in sub-Saharan Africa is likely to be much higher than reported in the available literature and registries, due to the widespread absence of newborn screening across the region. Moreover, the clinical course of sickle cell disease is often made worse in this population due to the limited access to quality health care and the high risk of exposure to infectious diseases which may potentially increase disease severity.[1,3] Among the latter, malaria is of great concern due to its tendency to augment hemolytic anemia and precipitate episodes of pain crises in sickle cell patients.[4]

Malaria is a mosquito-borne parasitic infection caused by five species of the genus *Plasmodium*; the most lethal and common being *Plasmodium falciparum*, which is prevalent across sub-Saharan Africa.[5] In 2017, the World Health Organization reported

1

219 million cases of malaria in 90 countries and an estimated 435,000 resulting deaths. Most deaths occurred among children under the age of five living in sub-Saharan Africa.[6]

In sickle cell disease, an individual inherits two copies (one from each parent) of the sickle cell hemoglobin allele (HbS) that is responsible for the defective polymerization and atypical properties of sickle cell hemoglobin.[7] Persons who inherit one sickle cell allele, along with one normal hemoglobin allele (HbA), acquire the sickle cell trait (HbAS). Most people with sickle cell trait do not experience any symptoms associated with sickle cell disease and typically live normal lives.[8] In addition, evidence from multiple studies has shown that individuals with sickle cell trait enjoy a significant degree of protection against malaria compared to individuals without the sickle cell trait. [4, 9-12] Owing to this evolutionary advantage, the HbS allele has been sustained over several generations among populations living in malaria endemic settings such as western Kenya. This has resulted in a disproportionately higher number of children in sub-Saharan Africa being born to two parents with the allele and subsequently inheriting sickle cell disease. Unfortunately, the protection against malaria conferred to those with sickle cell trait is lacking among those with sickle cell disease (HbSS). The latter group is the primary focus of this paper.

Individuals with sickle cell disease are more susceptible to complicated and life-threatening malaria requiring hospitalization than individuals without sickle cell disease.[4] It is worth noting however, that malaria's contribution to overall sickle cell morbidity and mortality is still poorly quantified. A number of studies have reported

finding either no significant difference[13] or a significantly lower risk[14, 15, 16] of malaria

infection in children with sickle cell disease compared to children without sickle cell

disease. Nevertheless, it is still widely agreed among researchers and health care

providers alike that malaria infection is considerably more fatal in sickle cell disease,[4, 13,]

[14] which warrants the use of more cautious prevention measures in this vulnerable

population. Many countries in sub-Saharan Africa have implemented guidelines for

lifelong malaria chemoprophylaxis[17] for people living with sickle cell disease. These

include Kenya[18] and Uganda[19], as well as Nigeria[20] which has both the highest burden of

sickle cell disease[21] and one of the highest incidences of malaria[6] in the world. Despite

this, agreement on the appropriateness of malaria chemoprophylaxis for all sickle cell

patients is not unanimous. For instance, in Tanzania, there is no specific guideline and

the decision to prescribe preventive treatment is left to the discretion of the clinician.

In addition to chemoprophylaxis, other malaria prevention measures that are

available to sickle cell patients living in malaria endemic settings include sleeping under

insecticide-treated bed-nets and residual house spraying.

1.2 Hydroxyurea Therapy and Fetal Hemoglobin

In most developed countries, where malaria has been eradicated, the clinical

management of sickle cell disease mostly entails supportive care to prevent severe

anemia, pain crises and pulmonary complications of sickle cell disease. Past and present

strategies include: hydroxyurea therapy, micronutrient supplementation and blood

transfusions. More recent advances such as bone marrow transplants and gene therapy[22]

do offer curative treatments for sickle cell disease, but these are extremely rare, even in the developed world.

The expansion of sustainable and effective interventions for patients with sickle cell disease is a major necessity in sub-Saharan Africa, where an estimated 50-80% of children with sickle cell disease die before they reach their fifth birthday.[23-25] However, the majority of clinical trials and observational studies on the management of sickle cell disease have been conducted in Europe and North America, leaving several unanswered questions about the potential benefit of these interventions to African populations.

Among the most notable sickle cell therapies is hydroxyurea, a potent disease-modifying agent which has been a hallmark of sickle cell management in the developed world since 1998.[23] This antineoplastic drug has multiple positive effects on the bone marrow, vasculature and composition of blood. In sickle cell therapy, its primary mechanism of action involves the induction of fetal hemoglobin (HbF) in red blood cells.[22, 23] HbF plays a critical role in ameliorating the clinical and hematological features of sickle cell disease by replacing the defective HbS in red blood cells. Because of this, sickle cell patients with high percentage HbF are able to transport oxygen with more efficiency, and consequently suffer fewer incidences of blood transfusions, hospitalizations, pain crises, acute chest syndrome and sickle-related organ damage.[27]

HbF differs from normal adult hemoglobin (HbA) in the ability of the former to bind oxygen with a higher affinity. This physicochemical property is integral to enabling the prenatal transfer of oxygen from mother to fetus during pregnancy,[28] and similarly, it helps compensate for the low binding ability of HbS in individuals with sickle cell

disease. At birth, HbF constitutes approximately 60-80% of total hemoglobin in the full-term infant. For infants without sickle cell disease, this percentage decreases gradually; being completely replaced by HbA over the first six months of life.[29] In sickle cell disease, HbF is gradually replaced by HbS and early symptoms of the blood disorder typically begin to occur at around six months.[30] However, some individuals with sickle cell disease may also inherit other hemoglobin-related polymorphisms, distinct from the HbS allele, which enable them to continue synthesizing low to moderate levels of HbF well beyond infancy.[31, 32] These individuals tend to experience lower morbidity, a milder or asymptomatic disease course, and longer lifespans than individuals who lack this intrinsic ability.[33]

1.3 Hydroxyurea Use in Malaria-Endemic Settings

In contrast to the developed world, the use of hydroxyurea in sub-Saharan Africa remains limited due to considerable challenges such as resource constraints, absence of adequate safety and efficacy data for this population, as well as unanswered questions about the potential interaction between hydroxyurea and malaria. [34-36] One hypothesis suggesting that hydroxyurea may play a key role in promoting the progression of malaria notes that hydroxyurea increases the adherence of red blood cells to endothelial tissues, which could potentially delay the elimination of infected red blood cells from circulation.[37] Although this claim raises a critical question regarding the risks associated with hydroxyurea therapy in malaria endemic settings, it is founded primarily on in vitro experiments performed on cultured cells and does not correlate with the larger clinical evidence. Human studies over the past thirty years have demonstrated that the

beneficial effects of hydroxyurea include a lower risk of vascular adhesion[22] and vaso-occlusion.[27] Equally important, a conflicting hypothesis posits that hydroxyurea may confer protection against malaria through the induction of HbF, owing to the observed suppression of *P. falciparum* proliferation in HbF-rich cells.[38, 39]

In a 2017 randomized placebo-controlled trial of hydroxyurea in 207 Ugandan children who were followed for 12 months, hydroxyurea use did not increase the incidence or severity of malaria.[35] More recently, a three-year non-randomized clinical trial carried out in Uganda, Kenya, Angola and Democratic Republic of Congo observed that the benefits of hydroxyurea use in relation to malaria infection were significant, with a rate reduction of more than 50% between pre-treatment and post-treatment periods.[36] While findings from both these studies seem to dispute the potential adverse interaction between hydroxyurea and malaria, more evidence is needed to resolve the conflicting arguments and existing doubts.

Additional studies would provide a large and more diverse evidence base to reassure clinicians that there would be no unexpected adverse events resulting from hydroxyurea use, even among populations not represented in the most recent clinical studies. There is also an important need to examine data on the effects of hydroxyurea use in real-life settings, where sickle cell patients are subjected to less rigorous monitoring than that received in a clinical trial.

1.4 Study Rationale and Objectives

To expand on the limited literature examining the association between hydroxyurea use and malaria, we performed a cross-sectional study to explore the

association between *P. falciparum* infection and hydroxyurea use among children with sickle cell disease in western Kenya. In addition, we examined the effect of hydroxyurea use on parasite density, percentage HbF and sickle cell morbidity, with the overall aim of increasing our understanding of the potential benefits or risks of hydroxyurea therapy to children with sickle cell disease living in malaria endemic settings.

1.4.1 Specific Aims

There were three specific aims for this study:

1. To compare prevalence of *P. falciparum* infection among children with sickle cell disease who do and do not routinely use hydroxyurea

   o **Hypothesis:** Children who routinely use hydroxyurea will have a lower prevalence of *P. falciparum* infection than those who do not use hydroxyurea

2. To correlate percentage HbF in red blood cells with *P. falciparum* density among children with sickle cell disease who are infected with *P. falciparum* parasites

   o **Hypothesis:** Children with high levels of HbF will have a lower density of malaria parasites than children with low levels of HbF

3. To compare self-reported hematologic morbidity among children with sickle cell disease who do and do not routinely use hydroxyurea

   o **Hypothesis:** Children who routinely use hydroxyurea will have a lower prevalence of blood transfusions, hospitalizations, pain crises, dactylitis, splenomegaly and school absenteeism compared to those who do not use hydroxyurea

2. Methods

2.1 Overview

This was a cross-sectional study nested within the EPiTOMISE clinical trial – a randomized, three-arm, open-label clinical trial of malaria chemoprevention in children with sickle cell disease in Homa Bay, Kenya. Our study examined data captured from baseline interviews, therefore constituting a cross-sectional survey. For each study participant, we obtained a blood sample at enrollment, which we analyzed for presence and quantity of *P. falciparum* parasites, as well as measurement of hematological correlates. We compared the prevalence of *P. falciparum* infection and distribution of log parasite densities among 95 children with sickle cell disease who were reported to be using or not using hydroxyurea. We also evaluated differences in hematological factors and sickle cell morbidity between the two groups. Cross-sectional surveys, blood draws and physical examinations were conducted by local study personnel.

2.2 Setting

Our study location, Homa Bay County, is a high malaria transmission area with a small peri-urban center bordering the south shore of Lake Victoria. The total land area of the predominantly rural county is approximately 1,173 square miles and it holds an estimated population of 1,177,181 people.[40]

According to the Malaria Atlas Project, the mean estimate of HbS allele frequency in this region is approximately 0.15 and the *P. falciparum* infection rate in children aged 2 to 10 years is predicted to be greater than 40%.[41] Between 2012 and 2013,

a study by Onchiri *et al.* also estimated a 45.8% prevalence of laboratory-confirmed malaria parasites among 677 children aged 6 months to 15 years who sought care for febrile illness at the government facility, Homa Bay County Hospital (HBCH).[42]

In 2013, a sickle cell anemia clinic operated by Academic Model Providing Access to Health Care (AMPATH) HematoOncology and Moi Teaching and Referral Hospital (MTRH) was established at HBCH. More than 1,400 children with sickle cell disease receive regular treatment at this clinic; making it an ideal location to recruit participants for research on sickle cell disease.

For the ongoing EPiTOMISE clinical trial, majority of study participants have been recruited from the sickle cell anemia clinic. All cross-sectional interviews, physical examinations and collection of samples have been conducted at the EPiTOMISE study site which is also housed at HBCH.

2.3 Study Population

Our study population was comprised of 95 children enrolled in the EPiTOMISE clinical trial in Homa Bay, Kenya between January 2018 and September 2018. All study participants identified as members of the Luo ethnic group.

The following inclusion criteria were met by all study participants:

- Aged between 12 months to 10 years
- Confirmed HbSS status by hemoglobin electrophoresis
- Permanent residence in either Homa Bay County or Rongo or Awendo sub-counties of Migori County for the next two years

- Ability of participant to adhere to study medication regimen, and willingness of parent or legal guardian of child to provide informed consent

Participants were excluded if they met any of the following exclusion criteria:

- Had known allergies or sensitivities to any of the study medications

- Had a chronic medical condition other than sickle cell disease such as HIV or malignancy requiring regular treatment

- Were currently enrolled in another clinical trial

- Were living in the same household as a previously enrolled EPiTOMISE study participant

- Had QTcF intervals > 450 msec on repeated ECG

2.4 Procedures

2.4.1 Ethical Considerations

The study protocol was written by the primary authors and covered under the EPiTOMISE clinical trial (Pro00077428) which was approved by the Duke University Institutional Review Board and Moi University Institutional Research and Ethics Committee. All participants were screened for sickle cell disease prior to enrollment, and a parent or legal guardian provided written informed consent for each child to enroll in the EPiTOMISE study. This consent extended to the use of the collected data for secondary analyses related to relevant epidemiological and clinical research questions. Consent forms were translated from English to Kiswahili and Dholuo. Before data collection, each participant was assigned a unique ID to protect their identity.

2.4.2 Data Collection

2.4.2.1 Cross-sectional Interviews

Trained study staff conducted structured interviews with a parent or legal guardian for each participant. Questions were asked in the respondents preferred language: English, Kiswahili or Dholuo. The information gathered was captured on paper-based case report forms (Appendix A) and uploaded to the REDCap database on the same day. It included: socio-demographic data, comprehensive medical history data and detailed information on the use of any prophylactic medications such as hydroxyurea, proguanil or penicillin.

Each respondent was queried about the number of times a participant had experienced a given morbidity event in the previous 12 months, and also asked to list all of the medications used routinely by the study participant. Respondents were also asked to provide information about the dose, dosing schedule and initiation date for each therapy. The data we obtained from cross-sectional interviews were primarily self-reported.

2.4.2.2 Physical Examination

Participants underwent comprehensive physical exams which included measurement of spleen size using a handheld ultrasound device. During these examinations, participants were also evaluated for potential symptoms of malaria. Any participants suspected of having malaria received a rapid diagnostic test (RDT) and subsequent treatment with artemether/lumefantrine if they tested positive for *P. falciparum* infection.

2.4.2.3 Collection and Handling of Blood Samples

During the screening visit, a blood sample (1mL) for each participant was obtained by venipuncture and collected in a purple-topped vacutainer (EDTA) labelled with the participants unique ID. Samples were stored under refrigeration for up to five days before being transported in a cooler box to the AMPATH Oncology and Hematology lab in Eldoret, Kenya, where complete blood count and hemoglobin analysis and quantitation were performed.

For detection and quantification of *P. falciparum* parasites, we collected dried blood spots (DBS) on chromatography filter cards for each study participant, and shipped the samples to Duke Clinical Research Institute (DCRI) for analysis by real-time quantitative polymerase chain reaction (qPCR).

2.4.2.4 Hemoglobin Electrophoresis and Quantitation

To obtain a diagnosis for HbSS, prepared blood samples were run on hemoglobin gels (Quickgel) and visually inspected against Helena hemo controls to identify markers for abnormal hemoglobin bands. The relative percent of each hemoglobin band was determined by scanning the dried gels in the Quickscan using acid blue filter. Detailed procedures for hemoglobin electrophoresis and HbF quantitation used at the AMPATH HematoOncology laboratory are described in Appendix B (SOP for Hemoglobin Electrophoresis Testing).

2.4.2.5 Detection and Quantification of *P. falciparum* Parasites

DBS were punched into 96-well plates and extracted with Chelex-100. These plates of genomic DNA were then tested for *P. falciparum* using a duplex real-time PCR assay targeting both *P. falciparum pfr364* and human β-tubulin as described below.

We prepared a 384-well template-free reaction plate consisting of our probes and Taqman Environmental Master Mix. We added 1 µL of each extracted genomic DNA sample to a well containing master mix. Samples were added in duplicates, placed in adjacent columns. A series of standards prepared from 3D7 culture of known parasite densities were included in the experiment as positive controls. We also included a negative control using the same nuclease-free water that was used to prepare the master mix. Like our samples, all our controls were added in duplicates.

Following a 3-minute spin in a plate spinner, we ran our reaction plate on a QuantStudio 6 real-time PCR system, selecting a standard curve as the type of experiment. At the end of the experiment, we exported our results to an excel file which we aggregated with the rest of our participant data for further analysis and interpretation. A detailed procedure of our gDNA testing is described in Appendix C (SOP for gDNA Testing for *P. falciparum*).

2.5 Measures

2.5.1 Socio-demographic Data

We gathered information related to age, sex, ethnicity, residence, school enrollment status and whether participants had any living or deceased siblings with sickle cell disease.

2.5.2 Hydroxyurea and Concomitant Medications

The main exposure of interest was hydroxyurea use. This was a dichotomous variable defined as "Using HU" or "Not Using HU." Participants were categorized as "Using HU" if they were reported to be taking hydroxyurea for any indication at the time they were enrolled; they were otherwise categorized as "Not Using HU." Data on hydroxyurea dose, administration schedule and duration of use was also obtained during the interviews. Similarly, we collected detailed information on use of folate, proguanil and penicillin.

2.5.3 Hematological Factors

Fetal hemoglobin, which we hypothesized to be a mediator of the association between hydroxyurea use and *P. falciparum* infection and parasite density, was a continuous variable measured quantitatively by hemoglobin electrophoresis. It was reported as a percentage of total hemoglobin. Other hematological correlates measured were: total hemoglobin concentration in grams per deciliter (g/dL), mean corpuscular volume in femtoliters (fl) and platelet, neutrophil and white blood cell counts ($*10^9$/L).

2.5.4 *P. falciparum* Infection and Parasite Density

The primary outcomes of interest were *P. falciparum* infection and parasite density, both of which were ascertained by qPCR analysis of dried blood spot samples. *P. falciparum* infection was defined as having any detectable parasites; while participants were considered uninfected if they had no detectable parasites. Using a relative quantitation method, we generated a standard curve from a series of *P. falciparum* 3D7

reference samples to estimate parasite density from cycle threshold (CT) values. Our samples were tested in duplicates, and the average number of parasites/μL from each set of duplicates was reported as the parasite density for that sample. For discordant duplicates, where one result was positive for *P. falciparum* and the other was negative, we recorded a positive result for *P. falciparum* infection and reported the parasite density from the single positive result as the overall parasite density of that sample.

2.5.5 Sickle Cell Morbidity

Our secondary outcomes of interest included self-reported morbidity events which were defined in case report forms (Appendix A) as follows:

- Pain crisis - *any severe and uncontrolled pain, without obvious cause, lasting for more than two hours*

- Dactylitis - *any pain or tenderness experienced in the hands or feet, with or without inflammation*

- Unconfirmed malaria - *receipt of antimalarial drugs for suspected malaria episodes that were not confirmed by any objective diagnostic test*

- Hospitalization - *hospitalization at HBCH or other inpatient facility with any admitting diagnosis*

- Blood transfusion - *receipt of red blood cells from any caregiver for any indication*

- Absence from school - *missing school due to any illness-related event over the past 30 days*

Respondents were asked to report the number of times they had experienced each event in the past 12 months (absence from school was reported

15

for the past 30 days). Pain crisis, dactylitis, unconfirmed malaria and hospitalization were described as ordinal variables; with three levels indicating the frequency of events: "None", "1 – 2 times" and "3 or more times". Blood transfusions and absence from school were captured as discrete measures; with the actual number of blood transfusions and days of school missed reported.

An additional morbidity event, spleen size, was measured at enrollment by ultrasound imaging and defined according to the published literature[43] as follows:

- Normal - *spleen length within the 90th percentile of normal spleen lengths for healthy children of that age*

- Enlarged - *spleen length > 90th percentile of normal spleen lengths for healthy children of that age*

- Autosplenectomy - *no identifiable spleen on ultrasound*

2.6 Statistical Analyses

All statistical analyses were performed using RStudio software version 3.5.1. All statistical tests were two-sided and set at a significance level of 0.05. Missing data were handled separately for each outcome by dropping observations with missing values. The number and percentage of participants excluded from each analysis have been reported under the results section. The full reproducible code and data set used in our study is available on request.

2.6.1 Categorical Variables

Categorical variables were described by frequencies, with the use of tables, Venn diagrams and bar graphs to summarize our results. To evaluate the effects of HbF on sickle cell morbidity, we created three HbF categories: low HbF (< 10%), moderate HbF (10 - 19%) and high HbF (≥ 20%).

For our estimation of prevalence of *P. falciparum* infection among hydroxyurea users versus hydroxyurea non-users, we used the *epi.2by2* function to compute crude and adjusted prevalence ratios (PR) and confidence intervals [95% CI]. The *epi.2by2* function is built under the R package 'epiR' version 0.9-99 and uses the exact test approach and maximum likelihood to calculate point estimates, confidence intervals and test for homogeneity.[44]

Using Fisher's exact test, we also ran analyses on the prevalence of unconfirmed malaria, hospitalizations, blood transfusions, pain crises and dactylitis over the past 12 months; absence from school in the past 30 days; and spleen size at enrollment among hydroxyurea users versus hydroxyurea non-users.

2.6.2 Continuous and Discrete Variables

For our continuous and discrete data: age, hematological values, parasite density, blood transfusions and absence from school, we computed the median (IQR) values and performed Kruskal Wallis tests to compare the distribution among hydroxyurea users versus hydroxyurea non-users. Due to the heavily skewed distribution of parasite density, we performed a log10 transformation of parasites/μL and used values for log (parasite density) in our graphical and statistical analyses.

2.6.3 Correlation Analysis

Ordering parasite density as low *P. falciparum* [log (parasite density) < 0] and high *P. falciparum* [log (parasite density) ≥ 0], we performed a correlation analysis to measure the association between parasite density level and HbF level. We computed Spearman's rank-order correlation statistics using the *spearman.test* function built under the R package 'pspearman' version 0.3-0. The 'pspearman' package uses Ryser's formula to calculate the exact null distribution.[45]

3. Results

3.1 Missing Data

Our study population consisted of 95 participants. Overall, our data was relatively complete with only a few missing observations. There were 5 participants (5.3%) with missing complete blood count results due to hemolysis of their blood samples; 2 participants (2.1%) had missing values for *P. falciparum* infection and parasite density because their DBS samples were not included in the inventory shipped to DCRI for analysis; 27 participants (28.4%) had missing spleen size data, the majority (n=20) of whom were recruited before the ultrasound device was installed at the study site.

3.2 Demographic and Social Characteristics

The overall median (IQR) age of study participants was 5.2 (3.0 - 7.7) years. Our sample consisted of 60.0% boys (n=57). Regarding management of sickle cell disease, 87.4% of our sample reported seeking regular care at either a government hospital (n=79) or private health facility (n=4). Nearly all (n=93) study participants reported having access to an insecticide-treated bed net.

At the time of our surveys, 70.5% of the children (n=67) were currently enrolled in school. Among those 53.7% (n=36) were reported to have missed at least one day of school due to illness in the past 30 days. Family history revealed that 18.9% of our sample (n=18) had at least one full sibling living with sickle cell disease, while another 9.5% (n=9) reported having at least one full sibling with sickle cell disease that was deceased.

3.3 Hydroxyurea and Concomitant Medications

The prevalence of hydroxyurea use in our sample population was 60.0% (n=57), and majority of users (n=47) reported taking at least 1500mg of hydroxyurea per week. There was no association between gender and hydroxyurea use, however, hydroxyurea users were generally older, with a median (IQR) age = 6.2 (4.6 - 8.4) years compared to hydroxyurea non-users with a median (IQR) age = 3.2 (2.3 – 6.3) years, p-value < 0.001.

Only three other medications were reported to be routinely used for sickle cell management in our study population: folate, proguanil and penicillin. The prevalence of use for folate was 96.8% (n=92), for proguanil 93.7% (n=89) and for penicillin 75.8% (n=72). Figure 1 is an illustration of medication use in our study population.
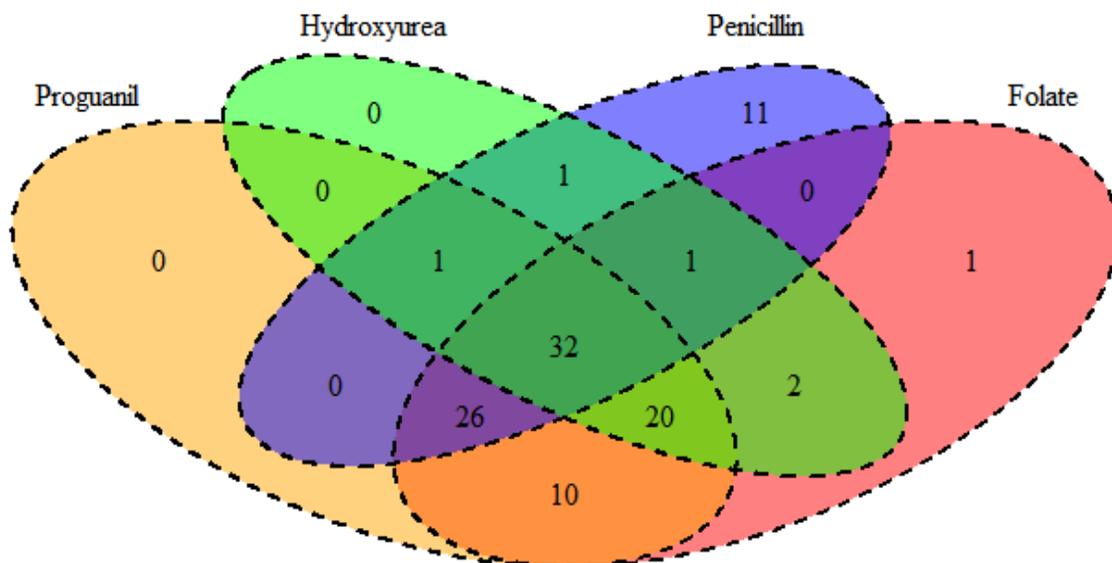

Figure 1: A Venn diagram of Medications Used by Study Participants

3.4 Hematological Factors

The overall median (IQR) percentage HbF in our sample population was 6.0 (0.0, 17.8). The difference in percentage HbF among hydroxyurea users, 11.4 (0.0, 19.2), versus hydroxyurea non-users, 4.3 (0.0, 13.6), was not statistically significant (p-value=0.072). However, based on the firmly established evidence linking elevated HbF to reduced sickle cell morbidity[27], we believe that the 2.7-fold increase observed among hydroxyurea users in our study population is clinically relevant.

Hemoglobin concentration and MCV values were significantly more favorable among hydroxyurea users compared to hydroxyurea non-users. We also observed significantly lower neutrophil and WBC counts among hydroxyurea users, which may be indicative of mild bone marrow suppression, a known side effect of hydroxyurea.[26, 27] Results from our analyses of hematological correlates are summarized in Table 1 and Figures 2 - 7.

Table 1: Median (IQR) Values of Hematological Correlates

	All participants	Using HU	Not Using HU	p-value [a]
Fetal Hemoglobin (%)	6.0 (0.0, 17.8)	11.4 (0.0, 19.2)	4.3 (0.0, 13.6)	0.072
Hb concentration (g/dL)	7.6 (7.0, 8.4)	8.0 (5.6, 8.5)	7.2 (6.6, 7.7)	0.007
MCV (fl)	90.6 (83.6, 100.5)	95.4 (89.2, 102.2)	85.6 (79.0, 89.5)	<0.001
Platelet count ($*10^9$/L)	429 (298, 552)	394 (306, 512)	466 (300, 572)	0.224
WBC count ($*10^9$/L)	15.1 (12.1, 20.1)	13.2 (11.9, 17.1)	18.6 (13.1, 23.3)	0.006
Neutrophil count ($*10^9$/L)	4.9 (3.5, 6.8)	4.2 (3.3, 5.9)	5.6 (4.2, 7.3)	0.037

[a] Kruskal-Wallis Test

FIG. 2A

FIG 2B.

FIG. 2C

FIG. 2D

FIG. 2E

FIG. 2F

Figure 2 shows the distribution of hematological correlates in the study population, stratified by hydroxyurea use. The line cutting through each group indicates the group median. The plots displayed are for: Fig. 2A. Fetal Hemoglobin, Fig. 2B. Hemoglobin Concentration, Fig. 2C. Mean Corpuscular Volume, Fig. 2D. Platelet Count, Fig. 2E. Neutrophil Count and Fig. 2F. White Blood Cell Count.

Figure 2: Scatter Plots of Hematological Correlates

3.5 P. falciparum Infection and Parasite Density

The overall prevalence of *P. falciparum* infection within our sample population was 52.7% (49/93). The prevalence of *P. falciparum* infection among hydroxyurea users (55.4%, 31/56) and hydroxyurea non-users (48.6%, 18/37) was similar (Prevalence Ratio [PR] = 1.14, 95% Confidence Interval [CI] 0.76, 1.71) (Figure 3A).

Among the infected children, the overall median (IQR) log (parasite density) was -0.79 (-1.53, 0.54). There was no significant difference in median (IQR) log (parasite density) among hydroxyurea users, -0.96 (-1.67, 0.41), and hydroxyurea non-users, -0.12 (-1.32, 3.48), p-value = 0.146 (Figure 3B-3C).

3.6 Association between Fetal Hemoglobin and Parasite Density

Initially, we constructed a plot of log (parasite density) against fetal hemoglobin to determine if there was a discernable relationship. There was no apparent trend between the two variables (Figure 4A). Categorizing HbF as low (< 10%), moderate (10 - 19%) and high (≥ 20%), we examined the distribution of log (parasite density) across the three strata. We observed similar median (IQR) values, p-value = 0.415 (Figure 4B).

Ordering parasite density as low *P. falciparum* [log (parasite density) < 0] and high *P. falciparum* [log (parasite density) ≥ 0], we performed a Spearman's rank-order correlation test of parasite density level versus HbF level. There was no significant correlation (Spearman's correlation coefficient (rho) = 0.15, p-value = 0.303).

FIG. 3A

FIG. 3B

FIG. 3C

Figure 3A shows a bar graph of prevalence of *P. falciparum* infection among hydroxyurea users versus non-hydroxyurea users.
Figure 3B shows overlaid histograms of log (parasite density) among hydroxyurea users versus non-hydroxyurea users.
Figure 3C shows a scatter plot of log (parasite density) among hydroxyurea users versus non-hydroxyurea users.

Figure 3: Plots of *P. falciparum* Infection and log (parasite density)
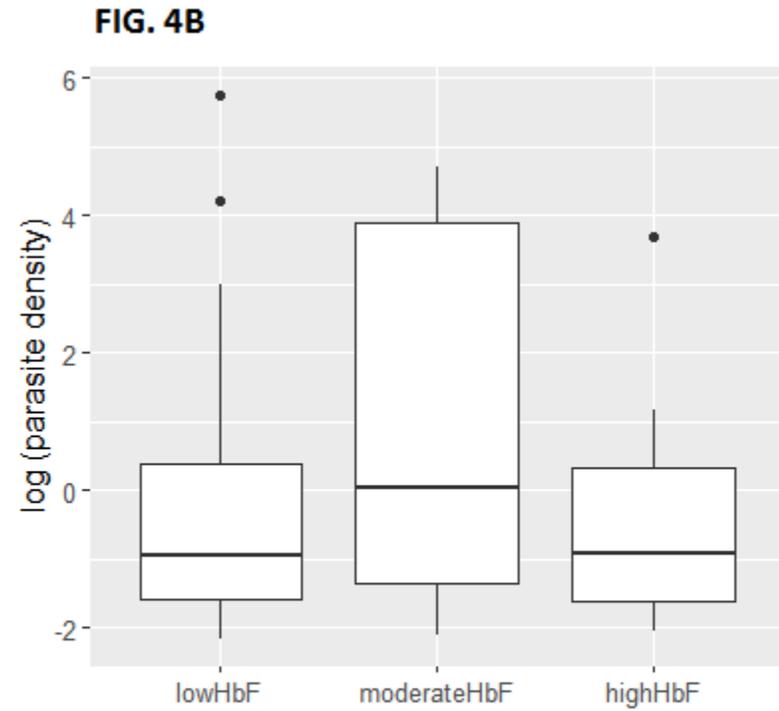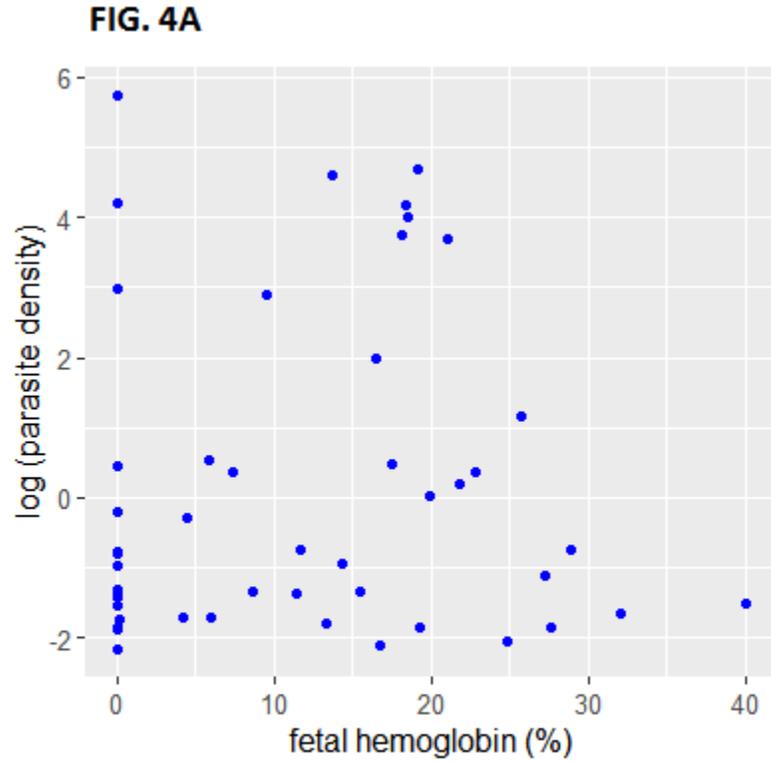
FIG. 4A

FIG. 4B

Figure 4A shows a scatter plot of log (parasite density) versus percentage fetal hemoglobin.
Figure 4B shows box plots of log (parasite density) stratified by low HbF (<10%), moderate HbF (10-19%) and high HbF (>=20%).

Figure 4: Plots of log (parasite density) versus Fetal Hemoglobin

3.7 Sickle Cell Morbidity

We examined the prevalence and frequency of several morbidity indicators in our study population. Overall, the most prevalent sickle cell-related events experienced over the past 12 months were: unconfirmed malaria (77.9%, 74/95) and pain crises (83.2%, 79/95). Among the 67 children who were currently enrolled in school, there was a relatively high prevalence of school absenteeism due to illness over the past 30 days (53.7%, 36/67). We observed no significant difference in morbidity events among hydroxyurea users versus hydroxyurea non-users (Table 2). Prevalence of morbidity across levels of HbF was also similar (Table 3).

Table 2: Prevalence and Frequency of Morbidity Events by Hydroxyurea Use

	Using HU (n=57)	Not Using HU (n=38)	p-value
Hospitalizations in the past 12 months - n (%)			
None	30 (52.6%)	19 (50.0%)	
1 – 2	22 (38.6%)	17 (44.7%)	0.725[b]
3 or more	5 (8.8%)	2 (5.3%)	
Episodes of unconfirmed malaria in the past 12 months - n (%)			
None	14 (24.6%)	7 (18.4%)	
1 – 2	28 (49.1%)	19 (50.0%)	0.891[b]
3 or more	15 (26.3%)	12 (31.6%)	
Episodes of pain crisis in the past 12 months - n (%)			
None	11 (19.3%)	5 (13.1%)	
1 – 2	35 (61.4%)	21 (55.3%)	0.397[b]
3 or more	11 (19.3%)	12 (31.6%)	
Episodes of dactylitis in the past 12 months - n (%)			
None	28 (49.1%)	12 (31.6%)	
1 – 2	18 (31.6%)	13 (34.2%)	0.161[b]
3 or more	11 (19.3%)	13 (34.2%)	
Received any blood transfusions in the past 12 months - n (%)			
	12 (21.1%)	6 (15.8%)	0.600[b]
Missed any days of school due to illness in the past 30 days - n/N$_s$* (%)			
	22/48 (45.8%)	14/19 (73.7%)	0.057[b]
Number of blood transfusions received in the past 12 months - median (IQR)			
	1 (1 – 1)	1 (1 – 2)	0.192[a]
Number of absences from school due to illness in the past 30 days - median (IQR)			
	5 (2 – 7)	5 (3 – 6)	0.947[a]

[a] Kruskal-Wallis Test, [b] Fisher's Exact Test, *N$_s$ is the number of participants enrolled in school

Table 3: Prevalence of Morbidity Events by HbF Level

Overall (n=95)	Low HbF (n=53)	Moderate HbF (n=28)	High HbF (n=14)	p-value [b]
Hospitalizations in the past 12 months - n (%)				
46 (48.4 %)	27 (50.9%)	13 (46.4%)	6 (42.9%)	0.809
Episodes of unconfirmed malaria in the past 12 months - n (%)				
74 (77.9%)	42 (79.2%)	21 (75.0%)	11 (78.6%)	0.940
Episodes of pain crisis in the past 12 months - n (%)				
79 (83.2%)	44 (83.0%)	26 (92.9%)	9 (64.3%)	0.075
Episodes of dactylitis in the past 12 months - n (%)				
55 (57.9%)	31 (58.5%)	18 (64.3%)	6 (42.9%)	0.387
Received any blood transfusions in the past 12 months - n (%)				
18 (18.9%)	10 (18.9%)	5 (17.9%)	3 (21.4%)	0.962
Missed any days of school due to illness in the past 30 days - n/N_s* (%)				
36/67 (53.7%)	22/37 (59.5%)	13/21 (61.9 %)	1/9 (11.1%)	0.025

[b] Fisher's Exact Test, *N_s is the number of participants enrolled in school

3.8 Spleen Size

At enrollment, we obtained spleen length measurements by ultrasound imaging for a subset of our study population. The demographic characteristics of participants included in our spleen analyses (n=68) versus participants excluded due to missing spleen data (n=27) were similar: mean age (5.3 vs 5.4 years, p-value = 0.880); proportion of boys (35.3% vs. 51.8%, p-value=0.167). The overall prevalence of splenomegaly (11.8%, 8/68) and autosplenectomy (16.2%,11/68) in our study population was low. We observed no significant differences in the prevalence of either event among hydroxyurea users

versus hydroxyurea non-users (Table 4). Spleen measurements were also similar across

levels of HbF (Table 5).

Table 4: Spleen Size at Enrollment by Hydroxyurea Use

	All participants (n=68)	Using HU (n=39)	Not Using HU (n=29)	p-value [b]
Normal	49 (72.0%)	26 (66.7%)	23 (79.3%)	
Splenomegaly	8 (11.8%)	4 (10.2%)	4 (13.8%)	0.220
Autosplenectomy	11 (16.2%)	9 (23.1%)	2 (6.9%)	

[b] Fisher's Exact Test

Table 5: Spleen Size at Enrollment by HbF Level

	Low HbF (n=38)	Moderate HbF (n=20)	High HbF (n=10)	p-value [b]
Normal	28 (73.6%)	14 (70.0%)	7 (70.0%)	
Splenomegaly	5 (13.2%)	2 (10.0%)	1 (10.0%)	0.943
Autosplenectomy	5 (13.2%)	4 (20.0%)	2 (20.0%)	

[b] Fisher's Exact Test

4. Discussion

The main objective of this study was to examine the effect of hydroxyurea use on the prevalence of *P. falciparum* infection and parasite burden among children with sickle cell disease living in a malaria endemic setting. We found that hydroxyurea use was not associated with the prevalence of *P. falciparum* infection, nor, among infected children, with the density of parasites. Three other studies on hydroxyurea use in sub-Saharan Africa have observed either no significant harm [34, 35] or a significant benefit [36] to using hydroxyurea in relation to its effects on malaria incidence. [34, 35, 36] Our results lend further credence to the current body of literature regarding the potential interaction between hydroxyurea use and malaria.

In relation to the broader benefits of hydroxyurea on sickle cell morbidity, evidence from clinical trials conducted by the REACH[35] and NOHARM[36] studies has suggested that hydroxyurea use in sub-Saharan Africa is safe, effective and feasible. In this study, we examined the effects of hydroxyurea use on a rural population outside of the confines of a clinical trial, and therefore, we offer some insight on potential outcomes in a real-life setting.

The hematological benefits we observed among hydroxyurea users in our study population are congruent with the findings from recent clinical trials. Most notably, the hemoglobin concentration was 0.8 g/dL higher among self-reported hydroxyurea users (p-value = 0.007). This is comparable to the mean 1.3 g/dL difference (p-value < 0.001) between the treatment group and placebo arm of the NOHARM randomized clinical trial; as well as the 1.0 [95% CI, 0.8 – 1.0] g/dL increase from baseline after 1 year of

treatment reported by the nonrandomized REACH trial. While our own study failed to

detect a statistically significant difference in percentage HbF between hydroxyurea users

and hydroxyurea non-users (p-value = 0.072), the firmly-established evidence[27] on the

clinical benefits associated with an elevated HbF leads us to suggest that a 2.7-fold

difference between our study groups is clinically relevant and noteworthy.

Consistent with the known side effects of hydroxyurea therapy, we did observe a

marginally lower neutrophil count among hydroxyurea users compared to hydroxyurea

non-users (4.2 *10^9/L vs 5.6 *10^9/L, p-value = 0.037). Although this may be indicative of

mild bone marrow suppression, there was no evidence of severe neutropenia, and

neutrophil counts in both groups were within the acceptable range for patients with

sickle cell disease.[46, 47] Based on neutrophil count, our findings support the recent claims

that hydroxyurea use may be safe and well-tolerated in sub-Saharan populations.[35, 36]

Concerning the expected improvement in clinical course of sickle cell disease

among hydroxyurea users versus hydroxyurea non-users, our analyses showed no

significant difference in the prevalence or frequency of any of the seven morbidity

indicators that we examined. This may be partly attributed to limitations in our study

design, which are discussed later on in this chapter.

The primary goal of hydroxyurea therapy in sickle cell management is to induce

production of HbF. While studies have demonstrated that sickle cell patients with high

percentage HbF generally experience lower morbidity, [27, 31-36, 48] our findings did not

reflect any meaningful differences in sickle cell-related events across groups with low

HbF, moderate HbF and high HbF. It is worth noting however, that despite the high

prevalence of hydroxyurea use (60.0%) in our study population, only 14.7% (n=14) of our sample had HbF values at or above the suggested protective threshold of 20%.[33] This discrepancy may be ascribed to a variety of factors including: ineffective dosing, poor compliance and short duration of therapy. Studies have shown that the HbF response to hydroxyurea therapy is dose-dependent, and that patients achieve the maximum benefits from hydroxyurea therapy when their treatment is escalated to the maximum tolerated dose (MTD). [48, 49] However, dose escalation requires close therapeutic monitoring, routine lab tests and coordinated efforts between patients and care providers to minimize the risk of toxicity. This capacity is lacking in low resource settings like our study location, making it difficult for prescribers to attempt to achieve MTD in their patients.

One study challenging the literature on MTD reported no significant difference in response to hydroxyurea among 161 Omani children receiving low dose therapy (10–15.9 mg/kg/day) and high dose therapy (16–26 mg/kg/day).[50] The severity of disease and clinical history of patients across the two groups was comparable at baseline. While this finding does not discount the documented efficacy achieved at higher doses, it suggests that there is still significant potential for clinical improvement even in the absence of MTD. In agreement with this assertion, the majority of hydroxyurea users (82.5%, 47/57) in our study population were using low doses of hydroxyurea (1500 - 2500 mg per week), but were able to attain significantly more favorable hematological values compared to hydroxyurea non-users.

Another key consideration concerning hydroxyurea therapy in sub-Saharan Africa is sustainability. Percentage HbF among compliant hydroxyurea users has been observed to increase significantly for each year of use.[49] Approximately 75.0% of our study participants reported being on hydroxyurea therapy for less than a year at the time they were interviewed. It is therefore possible that even at an optimal dose, a longer duration may be required to realize a sufficient increase in HbF and a positive change in clinical events. It is therefore important that any national policies directing the roll out of hydroxyurea therapy, must commit fully to facilitating the long-term provision of this intervention to sickle cell patients, so as not to interrupt any gains that recipients might attain.

There were several limitations to our study including: the large amount of self-reported data and low ability to ascertain medical history and medication compliance; temporal ambiguity regarding the initiation of hydroxyurea therapy and the occurrence of clinical events; potential confounding by indication/severity given that hydroxyurea is typically prescribed to sickle cell patients who are at high risk of morbidity and would therefore be more prone to the clinical events we examined than participants who were not prescribed hydroxyurea.

Finally, our study population consisted of participants from a clinical trial whose characteristics may differ significantly from children with sickle cell anemia in the general population. This limits the generalizability of our findings.

5. Conclusion

While bone marrow transplants and gene therapy offer definitive cures for sickle cell disease, these interventions are not free of risk and require huge resources and sophisticated care to implement successfully. Management of clinical manifestations through hydroxyurea therapy is the best and most feasible intervention currently available for children in developing countries. Hydroxyurea use is simple, cheap, cost-effective and more sustainable compared to frequent blood transfusions. The emerging body of evidence suggests that it might also be equally as safe and effective in reducing pain crises, acute chest syndrome, hospitalizations and bacterial infections in sub-Saharan populations, as documented among populations in Europe and North America where most sickle cell studies over the past 30 years have been conducted.

While additional research may help elucidate the association between hydroxyurea and malaria, recent findings, including this one, suggest that hydroxyurea presents no added risk for *P. falciparum* parasitemia, malaria incidence or disease severity in individuals with sickle cell disease. Moreover, there is compelling evidence that hydroxyurea therapy can significantly improve the quality of life for children with sickle cell disease living in sub-Saharan Africa and help curb the high under five mortality rate in this population.

Future studies should focus on obtaining additional safety and efficacy data from diverse populations; quantifying the mediating effects of HbF to establish a more precise threshold; and evaluating the feasibility of adopting, sustaining and maximizing the benefits of long-term hydroxyurea therapy within national control programs.

Appendix A

CASE REPORT FORM

Inclusion/Exclusion

Record the date of consent	___/___/_____ DD MM YYYY
Was consent obtained to collect blood in a PAXgene tube for future research?	○ Yes ○ No

In order to be eligible to participle in this study, an individual must meet all of the following Inclusion criteria:

Inclusion

Is the patient's age greater than 12 months and less than 10 years at enrollment?	○ Yes ○ No
Is the patient currently attending or willing to attend the study sickle cell anemia clinic at HBCH?	○ Yes ○ No
Is the patient's residence in either Homa Bay County or the Rongo or Awendo sub-counties of Migori County?	○ Yes ○ No
Does the patient NOT have immediate, apparent, or reported plans to relocate residence away from Homa Bay County or the Rongo or Awendo sub-counties of Migori County in the next two years?	○ Yes ○ No

Is the patient able to take oral medication and willing to adhere to the medication regimen, or is the caregiver willing to give the medication regimen as prescribed?	○ Yes	○ No
Is the patient's parent or legally authorized representative (LAR) able and willing to give informed consent?	○ Yes	○ No
Has the patient's hemoglobin genotype been confirmed as HbSS by electrophoresis?	○ Yes	○ No

Comments on inclusion criteria

In order to be eligible to participle in this study, an individual must not meet any of the following Exclusion criteria:

Exclusion		
Is the patient taking routine antimalarial prophylaxis for an indication other than sickle cell anemia?	○ Yes	○ No
Does the patient have a known allergy or sensitivity to sulfadoxine, pyrimethamine, amodiaquine, proguanil, dihydroartemisinin, piperaquine, artemether, lumefantrine, pencillin, or derivatives of these compounds?	○ Yes	○ No
Does the patient have a known chronic medical condition other than sickle cell anemia requiring frequent medical attention (examples: malignancy, HIV)?	○ Yes	○ No
Is the patient currently participating in another clinical research study, or has he/she participated in one in the past 30 days?	○ Yes	○ No
Does the patient live in the same household as a previously-enrolled study participant?	○ Yes	○ No
Does the patient chronically use any medications known to prolong the QT interval in children (see Appendix J of the protocol for a list of the medications; also see below)? **Any azole antifungals** (including Fluconazole, Itraconazole, Ketoconazole, Posaconazole, Voriconazole)	○ Yes	○ No

Any Fluoroquinolone antibiotics (including Ciprofloxacin, Gatifloxacin, Levofloxacin, Moxifloxacin, Ofloxacin, Sparfloxacin)

Antiretrovirals (Lopinavir, Saquinavir, Efavirenz)

Other antibiotics (Azithromycin, Clarithromycin, Roxithromycin, Telithromycin, Metronidazole, Telavancin, Pentamidine, Foscarnet)

Psychotropics (Fluoxetine, Citalopram, Escitalopram, Haloperidol, Risperidone any Tricyclic antidepressants (including Amitriptyline, Nortriptylene, Imipramine, Doxepin))

Pulmonary (Albuterol, Levalbuterol, Formoterol, Salmeterol, Terbutaline)

Has the patient received a blood transfusion in the past 120 days?	○ Yes	○ No
Does the patient have a Fridericia's corrected QT interval (QTcF) interval >450 msec?	○ Yes	○ No

Comments on exclusion criteria

Demographics

Child Gender	○ Male ○ Female
Child Date of Birth	____/ ____/ _____ DD MM YYYY
County/Subcounty of residence	○ Homa Bay County - any subcounty ○ Migori County - Awendo subcounty ○ Migori County - Rongo subcounty
Location	
Sub Location	
Village	
Self-identified tribe/ethnic group	○ Luo ○ Kisii ○ Luhya ○ Other
Specify Other	

Medical History

Is the child currently being treated for any of these conditions?	❑ HIV (Please note that this chronic health condition is an exclusion criteria)
	❑ Tuberculosis (Please note that this chronic health condition is an exclusion criteria)
	❑ Malnutrition
	❑ Diabetes
	❑ Any other infections
	❑ Any other medical problem
	❑ None of the above

~~Specify other infections~~

Specify other medical problems

Is the child allergic to any medication(s)?	○ Yes ○ No
Medication allergies	
How many medications is the child allergic to? (enter 1-5)	
Medication #1: What is the name of the medication?	

Medical History

Medication #1: What is the reaction to this medication?	○ Rash ○ Anaphylaxis ○ Nausea/Vomiting ○ Other
Medication #1: Specify Other	
Medication #2: What is the name of the medication?	
Medication #2: What is the reaction to this medication?	○ Rash ○ Anaphylaxis ○ Nausea/Vomiting ○ Other
Medication #2: Specify Other	
Medication #3: What is the name of the medication?	
Medication #3: What is the reaction to this medication?	○ Rash ○ Anaphylaxis ○ Nausea/Vomiting ○ Other
Medication #3: Specify Other	
Medication #4: What is the name of the medication?	
Medication #4: What is the reaction to this medication?	○ Rash ○ Anaphylaxis

Medical History

	○ Nausea/Vomiting ○ Other
Medication #4: Specify Other	

Medication #5: What is the name of the medication?	
Medication #5: What is the reaction to this medication?	○ Rash ○ Anaphylaxis ○ Nausea/Vomiting ○ Other
Medication #5: Specify Other	

Current medications

Does the child take any of the following regular or daily medications?

Folate	○ Yes ○ No

Medical History

Folate dose in mg	
Folate frequency	○ Daily ○ Monthly ○ Weekly ○ Other

Specify if Other

Proguanil	○ Yes ○ No
Proguanil dose in mg	
Proguanil frequency	○ Daily ○ Monthly ○ Weekly ○ Other

Specify if Other

Hydroxyurea	○ Yes ○ No
Hydroxyurea dose in mg	
Hydroxyurea frequency	○ Daily ○ Monthly ○ Weekly ○ Other

Specify if Other

Medical History

Penicillin	○ Yes	○ No
Penicillin dose in mg		
Penicillin frequency	○ Daily ○ Weekly	○ Monthly ○ Other
Specify if Other		
Does the child take any other regular or daily medications?	○ Yes	○ No
How many other daily medications does the child take? (enter 1-5)		
Medication #1: What is the name of the medication?		
Medication #1: What is the dose?		
Medication #1: Unit?	○ mg	○ Other
Specify Other		
Medication #1: How often does the child take this medication?	○ Daily ○ Weekly	○ Monthly ○ Other

Medical History

Medication #1: Specify frequency taken if Other

_____

Medication #1: What is the indication for this medication?

Medication #2: What is the name of the medication?	
Medication #2: What is the dose?	
Medication #2: Unit?	○ mg ○ Other

Specify Other

Medication #2: How often does the child take this medication?	○ Daily ○ Monthly ○ Weekly ○ Other

Medication #2: Specify frequency taken if Other

_____

Medication #2: What is the indication for this medication?

Medication #3: What is the name of the medication?	
Medication #3: What is the dose?	
Medication #3: Unit?	○ mg ○ Other

Medical History

Specify Other

_____

Medication #3: How often does the child take this medication?	○ Daily ○ Monthly ○ Weekly ○ Other

Medication #3: Specify frequency taken if Other

_____

Medication #3: What is the indication for this medication?

_____

Medication #4: What is the name of the medication?	

Medication #4: What is the dose?	

Medication #4: Unit?	○ mg ○ Other

Specify Other

_____

Medication #4: How often is this medication taken?	○ Daily ○ Monthly ○ Weekly ○ Other

Medication #4: Specify frequency taken if Other

_____

Medication #4: What is the indication for this medication?

_____

Medication #5: What is the name of the medication?	

Medical History

Medication #5: What is the dose?	
Medication #5: Unit?	○ mg ○ Other
Specify Other	
Medication #5: How often does the child take this medication?	○ Daily ○ Monthly ○ Weekly ○ Other
Medication #5: Specify frequency taken if Other	
Medication #5: What is the indication for this medication?	
Prior vaccinations	
Has the child received the 23 valent pneumococcal vaccine? *Please ask if possible to see the immunization card to confirm*	○ Yes ○ Not Applicable ○ No
Specify date of the 23 valent pneumococcal vaccine	○ Full date known ○ Partial date known
If **full** date known:	

Medical History

Specify date of the 23 valent pneumococcal vaccine	____/____/_____ DD MM YYYY
If **partial** date known: Enter **Partial** date: MONTH *Select UNK if month is unknown*	
Enter **Partial** date: DAY *Select UNK if day is unknown*	
Enter **Partial** date: YEAR *Select UNK if year is unknown*	
Has the child received the quadravalent meningococcal vaccine? *Please ask if possible to see the immunization card to confirm*	○ Yes ○ Not Applicable ○ No
Specify date of the quadrivalent meningococcal vaccine	○ Full date known ○ Partial date known
If **full** date known: Enter **full** date	____/____/_____ DD MM YYYY
If **partial** date known:	

Medical History

Enter **Partial** date: DAY *Select UNK if day is unknown*	
Enter **Partial** date: MONTH *Select UNK if month is unknown*	
Enter **Partial** date: YEAR *Select UNK if year is unknown*	
Prior surgeries	
Has the child ever had surgery?	○ Yes ○ No
How many surgeries has the child had? *(Enter 1-3)*	

Surgery 1: What type of surgery?

Surgery 2: What type of surgery?

Surgery 3: What type of surgery?

Recent medical history	
In the past 12 months, has the child been hospitalized for any reason?	○ Yes ○ No
How many times has the child been hospitalized in the past 12 months?	○ Once ○ 6 to 10 times

Medical History

	○ Twice ○ More than 10 times ○ 3 to 5 times
In the past 12 months, has the child been treated for malaria?	○ Yes ○ No
How many times has the child been treated for malaria in the past 12 months?	○ Once ○ 6 to 10 times ○ Twice ○ More than 10 times ○ 3 to 5 times
In the past 12 months, has the child experienced pain crises, defined as pain lasting 2 hours or more that did not have an obvious cause?	○ Yes ○ No
How many times has the child experienced a pain crisis in the past 12 months? Pain crisis is defined as pain lasting 2 hours or more that did not have an obvious cause	○ Once ○ 6 to 10 times ○ Twice ○ More than 10 times ○ 3 to 5 times
How many times have pain crises required medical **evaluation** of any sort?	○ None ○ 3 to 5 times ○ Once ○ 6 to 10 times ○ Twice ○ More than 10 times

Medical History

How many times have pain crises required medical **treatment** of any sort?	○ None ○ Once ○ Twice	○ 3 to 5 times ○ 6 to 10 times ○ More than 10 times
How many times have the pain crises required hospitalization?	○ None ○ Once ○ Twice	○ 3 to 5 times ○ 6 to 10 times ○ More than 10 times
In the past 12 months, has the child experienced dactylitis, defined as pain or tenderness of the hands or feet without obvious cause?	○ Yes	○ No
How many times has the child experienced dactylitis, defined as pain or tenderness of the hands or feet without obvious cause?	○ Once ○ Twice ○ 3 to 5 times	○ 6 to 10 times ○ More than 10 times
In the past 12 months, has the child received any blood transfusions?	○ Yes	○ No
How many times has the child received blood transfusions?	○ Once	○ 6 to 10 times

Medical History

	○ Twice ○ More than 10 times ○ 3 to 5 times
Does the child have access to an insecticide-treated bed net to sleep under?	○ Yes ○ No
In the past week, how many nights did the child sleep under the bed net? *Enter a number 0-7. Enter 9999 if unknown*	
In the past 6 months, has the child's house been treated with residual spraying?	○ Yes ○ No
If yes, what was the house treated with?	○ Pyrethroid ○ Organo Phosphate ○ Unknown/Don't know
Does the child regularly receive medical care for sickle cell anemia?	○ Yes ○ No
Where does the child receive care?	○ Government Hospital ○ Government Health Center/Dispensary ○ Private Health Facility

Medical History

	○ Other

Specify Other

_____

How often does the child receive medical care for sickle cell anemia?	○ Once a month ○ Twice a year ○ Once every 3 months ○ Only when needed ○ Once a year ○ Other

Specify if Other

_____

Social History

Baseline data should be collected from person consenting on behalf of the child, namely a parent or guardian. Confirm that the person responding is the primary guardian/caregiver for the child

What is the relationship of the primary person accompanying the child?	○ Mother ○ Aunty ○ Father ○ Grandparent ○ Sibling ○ Other ○ Uncle
Specify if Other	
Does the child live with this person?	○ Yes ○ No
If no, who does the child stay with? *Check all that apply*	❑ Mother ❑ Aunty ❑ Father ❑ Grandparent ❑ Sibling ❑ Other ❑ Uncle
Specify if Other	
Does this person work outside the home?	○ Yes ○ No
If yes, what do they do?	○ Employed ○ Farmer

Social History

	○ Self employed ○ Other
	○ Casual

Specify if Other

In the past thirty days, how many days has this person missed work because of the child's medical problems?	

What is the highest level of education attained by this person?	○ No education
	○ Primary
	○ Some Secondary (not completed)
	○ Secondary completed
	○ Tertiary

How many people live in the household other than the child? *Include the respondent, Max 40*	

How many full siblings does the child have (living or deceased)? Full sibling is defined as a child with the same mother and same father *Max=40, Enter 0 if none*	

Social History	
How many full siblings have or had sickle cell anemia? *Living or deceased, Max 40*	
Have any of the full siblings died?	○ Yes ○ No
How many of the full siblings have died?	
How many of the deceased full siblings had sickle cell anemia?	
Does the child attend school?	○ Yes ○ No
If so, what class is the child in?	○ Nursery ○ Class 4 ○ Class 1 ○ Class 5 ○ Class 2 ○ Class 6 ○ Class 3
In the past thirty days, how many days of school has the child missed?	
How many of these days did the child miss because of medical problems?	

Review of Symptoms

Chief Complaint	○ Feeling well	○ Having symptoms
General *Check all that apply*	❏ Fever ❏ Pain ❏ Fatigue ❏ Jaundice	❏ Weight loss ❏ Night sweats ❏ No complaints
Pain Score *Enter 0-10 0=No Pain, 10=Worst pain ever experienced:* *Comment if unable to assess*		
Pain Comment		
HEENT: *Check all that apply*	❏ Hearing difficulties ❏ Swallowing difficulties	❏ Vision difficulties ❏ No complaints
Hearing difficulties duration	○ Days ○ Weeks	○ Months

Swallowing difficulties duration	❍ Days	❍ Months
	❍ Weeks	
Vision difficulties duration	❍ Days	❍ Months
	❍ Weeks	

HEENT Comments

Cardiopulmonary: *Check all that apply*	❑ Cough ❑ SOB	❑ Chest pain ❑ No complaints
Cough duration	❍ Days ❍ Weeks	❍ Months
Cough specifics	❍ Blood ❍ White	❍ Purulent ❍ None
SOB duration	❍ Days ❍ Weeks	❍ Months
SOB specifics	❍ At rest ❍ On exertion	❍ None

Chest pain duration	○ Days ○ Months ○ Weeks
Chest pain specifics	○ Sub sternal ○ Posterior ○ Right ○ None ○ Left

Cardiopulmonary comments

Gastrointestinal: *Check all that apply*	❏ Dysphagia ❏ Diarrhea ❏ Jaundice ❏ Melena ❏ Lack of appetite ❏ Bleeding per rectum ❏ Abdominal pain ❏ No complaints ❏ Constipation
Dysphagia duration	○ Days ○ Months ○ Weeks

Review of Symptoms

Jaundice duration	○ Days ○ Weeks	○ Months
Lack of appetite duration	○ Days ○ Weeks	○ Months
Abdominal pain duration	○ Days ○ Weeks	○ Months
Abdominal pain location *Check all that apply*	❑ RUQ ❑ LUQ ❑ RLQ	❑ LLQ ❑ Epigastric ❑ Suprapubic
Constipation duration	○ Days ○ Weeks	○ Months
Diarrhea duration	○ Days ○ Weeks	○ Months
Melena duration	○ Days ○ Weeks	○ Months

Bleeding per rectum duration	○ Days ○ Months ○ Weeks

Gastrointestinal comments

Genitourinary:
 	
Check all that apply
	❑ Dysuria ❑ Decreased
	
	        urine volume
	❑ Priapism
	
	        ❑ No complaints
	❑ Increased
	
	urine volume

Dysuria duration	○ Days ○ Months ○ Weeks
Priapism duration	○ Days ○ Months ○ Weeks
Increased urine volume duration	○ Days ○ Months ○ Weeks
Decreased urine volume duration	○ Days ○ Months ○ Weeks

Genitourinary comments

Musculoskeletal: *Check all that apply*	❑ Lower extremity edema ❑ Leg ulcer	❑ Joint pain ❑ No complaints
Lower extremity edema duration	○ Days ○ Weeks	○ Months
Leg ulcer duration	○ Days ○ Weeks	○ Months
Musculoskeletal joint pain duration	○ Days ○ Weeks	○ Months
Musculoskeletal joint pain location *Check all that apply*	❑ Neck ❑ RUE ❑ LUE ❑ LLE	❑ Back ❑ Chest ❑ Buttocks ❑ RLE

Review of Symptoms

Musculoskeletal Comments

Central Nervous System: Check all that apply	☐ Paresthesia ☐ Focal weakness ☐ Seizures	☐ Headache ☐ Confusion ☐ No complaints
Paresthesia duration	○ Days ○ Weeks	○ Months
Focal weakness duration	○ Days ○ Weeks	○ Months
Seizures duration	○ Days ○ Weeks	○ Months
Headache duration	○ Days ○ Weeks	○ Months
Confusion duration	○ Days ○ Weeks	○ Months

Central Nervous System Comments

Masses or skin lesions:	○ Yes	○ No

Masses or skin lesion duration	○ Days ○ Months ○ Weeks
Masses or skin lesion comments	

Physical Exam

History of fever >=37.5C or subjective fever in the last 24 hours?	○ Yes ○ No
Current temperature *Celsius*	

If febrile or subjective fever in last 24 hours, perform Rapid Diagnostic Test for malaria

Laboratory testing	
Was the child tested for malaria using an RDT at this visit?	○ Yes ○ No
Record lot # / identifier of RDT	
RDT control line	○ Present ○ Absent
RDT *P.falciparum* line	○ Present ○ Absent
pLDH line	○ Present ○ Absent
Other Finger prick blood blotted onto paper as dried blood spot?	○ Yes ○ No
ECG testing	

Physical Exam

Please note that the first 20 children allocated to the monthly DP regimen who live in Homa Bay town will require ECG's at each regular follow-up visit 4-6 hours following the 3rd monthly dose, at each visit (including final visit).

Is the child a participant in the ECG substudy?	○ Yes	○ No
Post-Treatment ECG		
Did the child return for a post-treatment ECG?	○ Yes	○ No
Date of the post-treatment ECG	___/___/_____ DD MM YYYY	
Are you the parent or legal guardian of the child?	○ Yes	○ No
What day was the child given the last dose of the medication?	___/___/_____ DD MM YYYY	
How many hours ago was the child given the last dose of the medication?	○ <4 ○ 4-6	○ >6
QTcF interval in msec		

Physical Exam

If QTcF interval >450 msec, repeat the ECG.	
Has the ECG been repeated?	○ Yes ○ No
What is the repeated QTcF interval in msec? *If QTcF interval > 450 msec in the repeat test, please complete the AE/SAE form*	
Vital signs	
Weight *kg*	
Height/Length *cm*	
Mid Upper Arm Circumference (only for children under 60 months of age) *cm*	
General Appearance:	○ No apparent distress ○ Not assessed ○ Abnormal
Comment if Abnormal	
Respiratory Rate *bpm*	

Physical Exam

Pulse Rate *bpm*	
Oxygen Saturation *%*	
HEENT:	○ Scleral icterus ○ No abnormalities ◉ ○ Conjunctival pallor ○ Not assessed ○ Other finding

Specify if other finding

HEART:	○ Systolic murmur ○ No abnormalities ◉ ○ Diastolic murmur ○ Not assessed ○ Other finding

Specify if other finding

LUNGS:	○ Abnormal breath sounds ○ Clear to auscultation ◉ ○ Other finding ○ Not assessed

Specify if other finding

Physical Exam

ABDOMEN:

○ Tenderness to palpation

○ Mass

○ Hepatomegaly

○ Splenomegaly

○ Other finding

○ No abnormalities

○ Not assessed

Specify if other finding

EXTREMITIES:

○ Joint tenderness to palpation

○ Joint swelling ⊚

○ Decreased shoulder ROM

○ Decreased hip ROM ⊚

○ Lower extremity edema

○ Other finding

○ No abnormalities

○ Not assessed

Specify if other finding

GAIT:

○ Normal

○ Abnormal

○ Not assessed

Lab

Was a venous blood sample drawn at today's visit? (conditional to enrollment and visits 3, 6, 9, 12)	○ Yes ○ No
Was a PAXGene blood sample drawn at today's visit? (conditional to enrollment visit only)	○ Yes ○ No
Was a dried blood spot card prepared at today's visit, with either fingerprick or venous blood? (all scheduled and acute care visits)	○ Yes ○ No
Results from MTRH lab	
Date lab tests were performed	___/___/_____ DD MM YYYY
Was the AMPATH lab able to provide test results?	○ Yes ○ No
What was the reason no lab results were available?	○ Not enough sample ○ Not specified ○ Other (e.g., vial dropped) ○ Sample hemolyzed
Please Specify	

CBC	
White Blood Cell count (*10^9/L) *normal range 4 - 10*	
Neutrophil count (*10^9/L) *normal range 2 - 7*	
Hemoglobin concentration (g/dl) *normal range 11.5 - 16.5*	
Platelet count (*10^9/L) *normal range 150 - 400*	
Mean corpuscular volume (MCV) (fl) *normal range 77 - 93*	
Chemistries	
Creatinine (mg/dL) *normal range 0 - 0.7*	
ALT/SGPT (units/L) *normal range < 30*	

Hb Electrophoresis	
HbF % (conditional to visits 6 and 12 only) *Number between 0-100%*	
HbS % (conditional to visits 6 and 12 only) *Number between 0-100%*	
Lab Adverse Events	
Did the child have any adverse events related to lab results? *If yes, fill in the Serious Adverse Event form(CRF)*	○ Not Assessed ○ Yes ○ No

Study Medication Dispense

Adherence	
Proguanil arm only: In the past two weeks, how many days of malaria prevention medication did the child miss?	○ 01 ○ 09 ○ 02 ○ 10 ○ 03 ○ 11 ○ 04 ○ 12 ○ 05 ○ 13 ○ 06 ○ 14 ○ 07 ○ Not ○ 08 Applicable
SP-AQ or DP arms only: For the last dose of malaria prevention medication, how many days of medication did the child miss?	○ Zero ○ 03 ○ 01 ○ Not ○ 02 Applicable
Are there difficulties getting the child to take the medication?	○ Yes ○ No

Please specify difficulties

Study Medication Dispense

How many times did you forget to give the child these malaria medications when you were busy?	○ Never ○ Often ○ Rarely ○ Unknown ○ Sometimes
Medication adverse event	
Based on the interval history and clinical evaluation, has a Serious Adverse Event occurred? (If yes fill out Serious Adverse Event form) *As a reminder, the following types of events qualify as serious: Death, Life Threatening, Inpatient hospitalization, Persistent or significant incapacity, and an important medical event*	○ Yes ○ No
Study medication dosage	
Proguanil arm only: Choose the weight in kilograms used to calculate today's Proguanil dosage	○ < =13 (1/4 tablet daily) ○ 14-21 (1/2 tablet daily) ○ 22-29 (3/4 tablet daily)
SP-AQ arm only: Choose the age in years used to calculate today's SP-AQ dosage	○ < 5 (1 tablet SP once, 1 tablet AQ for 3 days) ○ >= 5 (1 1/2 tablet SP once, 1 1/2 tablet AQ for 3 days)

Study Medication Dispense

DP arm only: Choose the weight in kilograms used to calculate today's DP dosage	○ < = 5 (1/4 tablet daily for 3 days) ○ 6-10 (1/2 tablet daily for 3 days) ○ 11-14 (3/4 tablet daily for 3 days) ○ 15-19 (1 tablet daily for 3 days) ○ 20-23 (1 1/4 tablet daily for 3 days) ○ 24-25 (1 1/2 tablet daily for 3 days)

After selecting the correct medication/dosage, write the child's Patient ID and the dispense date on the label

Enter the Batch Number of the medication package from the label:	
Enter the Expiry Date of the medication package from the label:	____/____/_____ _ DD MM YYYY
Clinical officer only: Was the correct medication and dosage dispensed to the child?	○ ○
Study nurse only: Was the correct medication and dosage dispensed to the child?	○ ○
Penicillin	

Study Medication Dispense

Choose the age in years used to calculate today's penicillin dosage:	○ 1-3 (1 tablet twice a day) ○ 3-5 (2 tablets twice a day) ○ > 5 (no penicillin)

After selecting the correct medication and dosage, write the child's Patient ID and the dispense

date on the label

Enter the Batch Number of the medication package from the label:	
Enter the Expiry Date of the medication package from the label:	___/___/_____ _ DD MM YYYY
Malaria treatment with Artemether-lumefantrine	
Did the child test positive for malaria at this visit using an RDT?	○ Yes ○ No
If yes, did the child require referral for admission to the hospital?	○ Yes ○ No
Artemether-lumefantrine 20/120mg tablets: Choose the weight in kilograms used to calculate the malaria treatment dosage	○ 5-14 (1 tablet twice a day for 3 days) ○ 15-24 (2 tablets twice a day for 3 days)

Study Medication Dispense

	○ 25-34 (3 tablets twice a day for 3 days)
	○ > 34 (4 tablets twice a day for 3 days)

After selecting the correct medication/dosage, write the child's Patient ID and the dispense date on the label

Enter the Batch Number of the medication package from the label:	
Enter the Expiry Date of the medication package from the label:	___/___/_____ _ DD MM YYYY
Immunizations	
Was the 23 valent pneumococcal vaccine administered today? (Conditional to enrollment visit only) *criteria: child must be over 24 months old and not received the vaccine previously or it has been more than 5 years since they have received the vaccine*	○ Yes ○ No
Was the quadravalent meningococcal vaccine administered today? (Conditional to enrollment visit only)	○ Yes ○ No

Lab Screening

Results from MTRH lab	
HbF % *number between 0-100%*	
HbS % *number between 0-100%*	
ECG Results	
ECG done today?	○ Yes ○ No
QTcF interval in msec *If QTcF interval >450 msec, repeat the ECG.*	
Has the ECG been repeated?	○ Yes ○ No
What is the repeated QTcF interval in msec? *If QTcF interval > 450 msec in the repeat test, please complete the Completion form*	

Malaria Testing

Date of test	___/___/_____ DD MM YYYY
Where was the test performed?	○ Study Clinic ○ Other
Please specify other location	
Can we have permission to access your records at this other facility	○ Yes ○ No
Type of test	○ Smear microscopy ○ RDT
Did the child have a fever before testing?	○ Yes ○ No
Was it a measured temperature greater than or equal to 37.5?	○ Yes ○ No
Test Result	○ Positive ○ Negative
Is this result by self-report, or confirmed at the visit?	○ Self reported ○ Confirmed
Was it confirmed by review of outside records (exercise book), or observed by study personnel?	○ Exercise book ○ Directly observed

Malaria Testing

Record lot # / identifier of RDT	
RDT control line	○ Absent ○ Present
RDT P.falciparum line *Children who test positive using an RDT in the study clinic should be treated with AL. Please complete the Studymeddispense form to ensure proper dosing of AL.*	○ Absent ○ Present
pLDH line	○ Absent ○ Present

Comments

Serious Adverse Events

Please note that only events which are Serious, Unexpected and Related are to be reported via the web form, per protocol. If you have questions about recording events, please contact the Clinical Officer immediately

Record Serious Adverse Event (e.g., Appendicitis)	
Is this a Serious Adverse Event requiring reporting?	○ Yes ○ No
Please select the criteria which best describes the seriousness of the event	○ Death ○ Life Threatening ○ Inpatient hospitalization ○ Persistent or significant incapacity ○ An important medical event
Was this event related to the study drug?	○ Related ○ Not Related
Was this event unexpected per product labeling?	○ Expected ○ Not Expected
Date the event began	○ Full date known ○ Partial date known

Serious Adverse Events

If **full** date is known: Enter **known** dat*e*	___/___/_____ _ DD MM YYYY
If **partial** date is known: Enter **Partial** Date: Day *Select UNK if day is unknown*	
Enter **Partial** Date: Month *Select UNK if month is unknown*	
Enter **Partial** Date: Year *Select UNK if year is unknown*	
Event Status	❍ Stopped ❍ Ongoing
Date the event ended	❍ Full date known ❍ Partial date known
If **full** date is known: Enter **known** date	___/___/_____ _ DD MM YYYY
If **partial** date is known: Enter **Partial** date: Day *Select UNK if day is unknown*	
Enter **Partial** date: Month *Select UNK if month is unknown*	

Serious Adverse Events

Enter **Partial** date: Year *Select UNK if year is unknown*	
Action taken with study drug	◯ Drug Withdrawn ◯ Drug Interrupted ◯ Dose Reduced ◯ Dose Not Changed ◯ Dose Increased
Outcome of the event	◯ Recovered/Resolved ◯ Recovered w/ Sequelae ◯ Not Recovered/Not Resolved ◯ Fatal ◯ Unknown
Date of Death	___/___/_____ DD MM YYYY

Describe the event in a narrative format include the signs and symptoms, evaluations by study personnel or other providers, treatments applied by study or non-study personnel, relevant lab data, and the outcome of the event.

Serious Adverse Events

SAE Narrative:

_____

_____

_____

_____

_____

_____

_____

_____

Interval History

Date	___/___/_____ DD MM YYYY
Are you the parent or legal guardian of the child?	○ Yes ○ No

If not the parent or legal guardian, please flag the chart to follow up on permission to obtain medical records with the child's parent or legal guardian

What is the relationship of the primary person accompanying the child?	○ Mother ○ Aunty ○ Father ○ Grandparent ○ Sibling ○ Other ○ Uncle

Specify if Other

Recent hospitalizations	
Since the last visit, has the child been hospitalized?	○ Yes ○ No
How many times were they hospitalized?	○ 1 ○ 4 ○ 2 ○ 5 ○ 3

Interval History

Hospitalization #1: Where were they hospitalized?	
Hospitalization #1: How many days were they hospitalized?	
Hospitalization #1: Did the child have any of these conditions? *Check all that apply*	○ Malaria ○ Dactylitis ○ Pain Crisis ○ Acute chest syndrome ○ Anemia ○ Pneumonia ○ None of the above
Hospitalization #1: Was the child treated with any of these therapies? *Check all that apply*	○ Antimalarials ○ Antibiotics ○ Pain Medications ○ Oxygen ○ Blood transfusion ○ None of the above
Hospitalization #1: Can we have permission to access the child's medical records at this other medical facility?	○ Yes ○ No
Hospitalization #2: Where were they hospitalized?	
Hospitalization #2: How many days were they hospitalized?	

Interval History

Hospitalization #2: Did the child have any of these conditions? *Check all that apply*	○ Malaria ○ Dactylitis ○ Pain Crisis ○ Acute chest syndrome ○ Anemia ○ Pneumonia ○ None of the above
Hospitalization #2: Was the child treated with any of these therapies? *Check all that apply*	○ Antimalarials ○ Antibiotics ○ Pain Medications ○ Oxygen ○ Blood transfusion ○ None of the above
Hospitalization #2: Can we have permission to access the child's medical records at this other medical facility?	○ Yes ○ No
Hospitalization #3: Where were they hospitalized?	
Hospitalization #3: How many days were they hospitalized?	
Hospitalization #3: Did the child have any of these conditions?	○ Malaria ○ Dactylitis

Interval History

Check all that apply	○ Pain Crisis ○ Acute chest syndrome ○ Anemia ○ Pneumonia ○ None of the above
Hospitalization #3: Was the child treated with any of these therapies? *Check all that apply*	○ Antimalarials ○ Antibiotics ○ Pain Medications ○ Oxygen ○ Blood transfusion ○ None of the above
Hospitalization #3: Can we have permission to access the child's medical records at this other medical facility?	○ Yes ○ No
Hospitalization #4: Where were they hospitalized?	
Hospitalization #4: How many days were they hospitalized?	
Hospitalization #4: Did the child have any of these conditions? *Check all that apply*	○ Malaria ○ Dactylitis ○ Pain Crisis ○ Acute chest syndrome ○ Anemia ○ None of the

Interval History

	○ Pneumonia above
Hospitalization #4: Was the child treated with any of these therapies? *Check all that apply*	○ Antimalarials ○ Antibiotics ○ Pain Medications ○ Oxygen ○ None of the ○ Blood transfusion above
Hospitalization #4: Can we have permission to access the child's medical records at this other medical facility?	○ Yes ○ No
Hospitalization #5: Where were they hospitalized?	
Hospitalization #5: How many days were they hospitalized?	
Hospitalization #5: Did the child have any of these conditions? *Check all that apply*	○ Malaria ○ Dactylitis ○ Pain Crisis ○ Acute chest syndrome ○ Anemia ○ None of the ○ Pneumonia above
Hospitalization #5: Was the child treated with any of these therapies?	○ Antimalarials ○ Antibiotics

89

Interval History

Check all that apply	○ Pain Medications ○ Oxygen ○ None of the ○ Blood above transfusion
Hospitalization #5: Can we have permission to access the child's medical records at this other medical facility?	○ Yes ○ No
Malaria testing or treatment	
Since the last study visit, has the child been tested for malaria using any type of test? *If yes, please complete the Malaria Testing Form **for each test** prior to the child leaving*	○ Yes ○ No

Interval History

Since the last visit, has the child been treated for malaria (including requiring hospitalization)?	○ Yes ○ No
When was the child treated for malaria?	___/ ___/ _____ DD MM YYYY
What medication was the child treated with?	❑ AL ❑ Amodiaquine ❑ DP ❑ Quinine ❑ SP/Fansidar ❑ Other ❑ Chloroquine
Please specify other medication	
Interval medical complications	
Since the last visit has the child received any blood transfusions?	○ Yes ○ No
How many blood transfusions did they receive? *Max=10*	
Since the last visit, has the child experienced a pain crisis, defined as pain lasting 2 hours or more without an obvious cause?	○ Yes ○ No

Interval History

How many pain crises did they have? *Max=15*	
Since the last visit, has the child experienced dactylitis, defined as pain or tenderness of the hands or feet without obvious cause?	⭘ Yes ⭘ No
How many times did they experience dactylitis?	
Interval medication allergies	
Since the last visit, has the child developed any new medication allergies?	⭘ Yes ⭘ No
What is the medication name?	
What is the reaction?	⭘ Rash ⭘ Anaphylaxis ⭘ Nausea/vomiting ⭘ Other
Please specify other reaction	
Interval surgeries	
Since the last visit, has the child had any surgeries?	⭘ Yes ⭘ No

Interval History

How many surgeries has the child had? *Enter 0 if none*	

Surgery #1: Specify the surgery

Surgery #2: Specify the surgery

Interval social history	
Does the child attend school?	○ Yes ○ No
In the past 30 days, how many days of school has the child missed?	
How many of these days did the child miss because of medical problems?	
Does the child's guardian work outside the home?	○ Yes ○ No
What type of work?	○ Employed ○ Farmer ○ Self employed ○ Other ○ Casual

Specify if Other

Interval History

In the past 30 days, how many days have you missed work because of the child's medical problems?	
Does the child have access to an insecticide-treated bed net to sleep under?	○ Yes ○ No
In the past week, how many nights week did the child sleep under the bed net? *Enter a number 0-7. Enter 9999 if unknown*	
In the past 30 days, has the child's house been treated with residual spraying?	○ Yes ○ No
If yes, what was the house treated with?	○ Pyrethroid ○ Organo Phosphate ○ Unknown/Don't know

Acute Care

Date	___/___/_____ DD MM YYYY
Since the last study visit, has the child been tested for malaria using any type of test? *If yes, please complete the Malaria Testing Form **for each test** prior to the child leaving*	○ Yes ○ No
Since the last visit, has the child received any antimalarials, antibiotics, or other medications other than those provided by the study?	○ Yes ○ No
What was the name of the medication?	
What was the indication for the medication?	○ Malaria ○ Pain/Pain Crisis ○ Bacterial Infection ○ Other ○ Pneumonia
Specify if Other	
When was this medication started?	___/___/_____ _ DD MM YYYY

Has the child visited any other medical providers for this condition?	○ Yes ○ No
What was the name of provider?	
What facility was visited?	
What medications were given at this facility?	
Can we have permission to access the child's medical records at this other medical facility? *Please complete permission form*	○ Yes ○ No

Completion

Date	___/___/_____ _ DD MM YYYY
Has the child completed the study?	◯ Yes ◯ No
Has the child withdrawn from the study?	◯ Yes ◯ No
Reason child withdrew from the study	◯ Failed Screening ◯ Parent withdrew informed consent or child withdrew assent ◯ Moved out of study area ◯ Clinical Officer decision ◯ Was unable to comply with study schedule ◯ Lost to follow up ◯ Met exclusion criteria after enrollment ◯ Died

97

	(Please complete the Serious Adverse Event form) ○ Other

Specify if Other

_____

Additional Comments

_____

_____

_____

CIOMS FORM

Please complete any adverse event reporting via the CIOMS form, available on the Internet.

A copy of the CIOMS form can be printed and completed as a paper source document from the below URL

https://cioms.ch/wp-content/uploads/2017/05/cioms-form1.pdf

Appendix B

Standard Operating Procedure for Hemoglobin Electrophoresis and HbF Quantitation

SOP #: Ep-L-003	**Version**: 1.0	**Effective date**: 03Jan2018	**Page** 1 of 7

Title: Hemoglobin Electrophoresis Testing

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Revision History:	**Version**	**Effective date**	**Note**
	1.0	01Jan2018	New SOP

EPiTOMISE

1

Title: Hemoglobin Electrophoresis Testing

I. Purpose
To describe the procedure for performing hemoglobin electrophoresis as a technique used to distinguish between individuals with normal hemoglobin and those with sickle cell disease and sickle cell trait.

II. Background
Electrophoretic technique encompasses separation of charged macromolecules such as proteins, resolved on the basis of their mobility in an electric field. In HbElecrophoresis, very small samples of hemolysate prepared from packed cells are applied to the QuikGel Alkaline Hemoglobin gel. The hemoglobin in the sample are separated by electrophoresis using an alkaline buffer and stained with Acid Blue Stain. The patterns are scanned on a densitometer, and the relative percentage of each band is determined.

In this study, Hb electrophoresis will be used to screen potential participants; participants must have a diagnosis of sickle cell disease confirmed by Hb electrophoresis to be eligible to enroll. Amounts of HbF and HbS will also be measured with Hb electrophoresis at months 6 and 12.

Reagents, Materials and Equipment
Equipment
- SPIFE 3000 Analyzer
- SPIFE 2000 Analyzer
- QuickScan 2000
- SPIFE QuickGel Holder
- SPIFE QuickGel Electrode
- SPIFE QuickBlock Remover
- SPIFE QuickGel Chamber Alignment Guide
- QuickGel Applicator Blade (10)
- QuickGel Applicator weights
- QuickGel applicator base
- QuickGelDispoable Sample Cups (10)
- QuickGelDispo Cup Tray
- Chamber Cover
- Vortex Mixer
- Power supply capable of providing at least 550volts

Materials and Reagents
- QuickGel Alkaline Hemoglobin Gels (10)
- Acid Blue Stain (1 Vial)
- Hemolysate Reagent (25ML)
- Citric Acid Destain (1pkg)
- QuickGel Blotter C (10)
- QuickGel Blotter X (20)
- 5% Acetic Acid
- 0.85% NaCl

REQUIREMENTS

2

EPiT◉MISE

Title: Hemoglobin Electrophoresis Testing

Specimen Collection and Handling
Specimen of choice: Whole blood collected in EDTA tubes.
Specimen Storage: If storage is necessary, whole blood and packed cells may be stored up to 1 week at 2-8ºC.

QUALITYCONTROL
Two controls for hemoglobin electrophoresis are available from Helena Laboratories: AA2 Hemo Control (Cat. No. 5328) and AFSC Hemo Control (Cat no 5331). The controls must be used as markers for the location of particular hemoglobin bands. They may be quantitated for verification of the accuracy of the procedure. At least one of these controls must be included on each gel run.

III. Responsibilities
Laboratory Technician
Research Nurse
Clinical Officer
Site Coordinator
Project Manager
Principal Investigators

IV. Definitions

Term	Definition
SOP	Standard Operating Procedure
RN	Research Nurse
CO	Clinical Officer
SC	Site Coordinator

3

EPiT◑MISE

Title: Hemoglobin Electrophoresis Testing

V. Procedures

1. **Specimen preparation:**
   1.1. Washed packed cell hemolysates must be prepared for each patient specimen.
   1.2. Whole blood sample
      1.2.1. Centrifuge anti-coagulated blood for 10min to separate cells from plasma.
      1.2.2. Remove plasma
      1.2.3. Wash packed cells 3 times by re-suspending in 5 to 10 volumes of normal saline solution (0.85% NaCl), centrifuging and aspirating supernatant as before.
      1.2.4. After washing the samples, prepare the hemolysates by mixing 10μL of the sample to 100μl hemolysate Reagent. Vortex or shake vigorously for 15 seconds.
   1.3. Control
      1.3.1. AA2 (Cat No.5328) no dilution is necessary
      1.3.2. AFSC (Cat No.5331) 1:2 (1 part control + 1 part Hemolysate Reagent)

2. **Chamber Preparation**
   2.1. The QuickGel chamber must be plugged into power supply.
   2.2. Set the timer 14minutes and the power at 550 volts. An electrophoresis time of 14 to 15 minutes is acceptable.
   2.3. Snap the electrophoresis lid into place on the chamber.
   2.4. Ensure that the chamber floor is cool (room temperature) before starting the test.

3. **Sample Preparation**
   3.1. Prepare hemolysates of patients' specimen and controls as per standard lab handling of specimen at AMPATH
   3.2. Slide the QuickGel disposable samples cups into row A of the Dispo cup Tray. Pipette 17μL of patient sample or control hemolysate into the sample cups and cover tray until ready to use. When ready, place the dispo cup tray into the applicator base. Proceed to Step 4.

4. **Gel Preparation**
   4.1. Carefully open one end of the pouch and remove one gel from the protective packaging. Reseal the pouch with the pouch tape to prevent drying of the gel. Remove the gel from the plastic mold and discard the mold.
   4.2. Dispense approximately 1ml of REP Prep onto the left side of the electrophoresis chamber.
   4.3. Hold the gel so that the samples numbered 1 to 10 are turned to the left side of the chamber. Place the left notch of the gel so that it fits the left pin of the chamber floor and gently roll the gel to the right side fitting the right notch to the right pin of the chamber floor. Use a lint-free tissue to wipe around the edges of the gel to remove excess REP Prep. Make sure that no bubbles remain under the gel.
   4.4. Using QuickGel blotter C, gently blot the entire gel using slight fingertip pressure on the blotter. Remove the blotter.

4

EPiTOMISE

Title: Hemoglobin Electrophoresis Testing

5. **Sample Application**
   5.1. Remove one QuickGel applicator blade from packaging.
   5.2. Place an applicator weight on top of the applicator blade.
   5.3. Place the blade into the vertical slot A of the applicator.
   5.4. While holding the white applicator knob, place the applicator into the designated slot on the applicator base aligning the small red dots on the applicator with those on the base.
   5.5. Then slowly lower the applicator knob allowing the blade to enter the sample cups and immediately start the timer for 30 seconds.
   5.6. After 30 seconds, lift the applicator knob and immediately place the applicator into the slots on the chamber floor, aligning the red dots.
   5.7. Slowly lower the knob to apply sample to the gel, set timer for 30 seconds.
   5.8. After 30 seconds, lift the applicator knob and remove the applicator from the chamber.
   5.9. Place two blotter X's horizontally along the top and the bottom sides of the gel backing. They should be positioned along the edges (not touching the gel) so that, when the lid closes the blotter X's do not interfere with the electrodes.
   5.10.   Close the lid, press the power switch to turn on the chamber and power supply.

6. **Electrophoresis/Staining**
   6.1. Electrophorese the gel for 14 minutes at 550volts.
   6.2. Turn off the power supply and the QuickGel chamber.
   6.3. Remove the blotter X's using the QuickGel block remover, remove the two gel blocks from the gels. Again use a lint free tissue to wipe around the edges of the gel backing to remove excess moisture. Remove the gel blocks from the gel. Again use lint free tissue to wipe around the edges of the gel backing to remove any excess moisture. Remove the gel from the chamber.
   6.4. Replace the electrophoresis lid with drying lid. Clean the two electrodes on the electrophoresis lid with deionised water after each use. Wipe with a lint free tissue.
   6.5. Fill a staining dish with prepared stain. Fill another container with destain solution.
   6.6. Place the gel into the stain for 4 minutes, remove the gel from the stain and allow draining on a blotter.
   6.7. Then place the gel into the destain solution for 30 seconds, remove it and allow it to dry on a blotter.
   6.8. Carefully place the gel in the chamber and close the drying lid.
   6.9. Turn on only the gel in the chamber. Dry the gel for 25 minutes or until dry. After drying, turn the chamber off and remove the gel.
   6.10.   Destain the gel in two consecutive washes of destain solution. Use a gentle alternately rocking and swirling technique. Allow the gel to remain in each wash 1:30 Minutes. The gel background should be completely clear. Tap the gel to remove the excess destain solution.
   6.11.   Ensure that the chamber floor is clean. Replace the gel onto the QuickGel chamber floor. Close the drying lid, turn on the QuickGel chamber and dry for 10 minutes or until dry. When drying is complete, turn off the QuickGel chamber and remove the gel.

5

EPiTOMISE

Title: Hemoglobin Electrophoresis Testing

7. **Evaluation of the hemoglobin bands**
    7.1. Qualitative evaluation:
        7.1.1. The hemoglobin gels may be inspected visually for the presence of abnormal hemoglobin bands. The Helena hemo controls provide a marker for band identification.
    7.2. Quantitative evaluation:
        7.2.1. Determine the relative percent of each hemoglobin band by scanning the dried gels in the Quickscan using acid blue filter. This will be recorded by the Laboratory technician in the lab results book in the Laboratory and the same information collected by the research assistant on a weekly basis for sharing with the clinician on record.
    7.3. Stability of the end product:
        7.3.1. The dried gels are stable for an indefinite period of time.

VII. References
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    2. Schneider, R.G., Methods for Detection of Hemoglobin Variants and Hemoglobinopathies in the Routine Clinical Laboratory, CRC Critical Reviews in Clinical Laboratory Sciences, 1978.

VIII. Attachments
None

6

EPITOMISE

Title: Hemoglobin Electrophoresis Testing

IX. Attestations

If you are onsite at Homa Bay, please sign this document stating you have read and understood the information contained in this SOP. This documentation will be held in the study file on site in the study office at Homa Bay, Kenya. If you are not onsite, please send an email to the project manager stating that you have read and understood the SOP and that email will be filed with this SOP in the study file.

Name	Signature	Dates (DDMonthYYYY)

7

EPiTOMISE

Appendix C

Standard Operating Procedure for *P. falciparum* Detection and Quantification

SOP #: EPiTOMISE1	Version: 1.0	Effective date: 10October2018	Page 1 of 5
Title: DBS gDNA testing for *P falciparum*			

Author(s): **Steve Taylor**

Revision History:	**Version**	**Effective date**	**Note**
	1.0	10October2018	New SOP

I. Introduction
This SOP describes the project-specific procedures for testing plates of genomic DNA that was extracted from EPiTOMISE dried blood spots for *Plasmodium falciparum*.

II. Background
In the EPiTOMISE study, dried blood spots (DBSs) were collected from all participants at each clinic visit. The participants were enrolled, randomized to a malaria chemoprevention regimen, and followed monthly for 12 months. DBS were collected at each visit, as well as at acute-care visits in between routine monthly visits.

All participants donated DBS, and these were punched into 96-well plates (90 in each plate), and extracted with Chelex-100. These plates of genomic DNA are then tested for *P falciparum*.

This SOP describes how to implement the duplex real-time PCR assay targeting both *P falciparum pfr364* and human β-tubulin that is described in SOP *DMC 2017-6*. By amplifying both of these targets from the EPiTOMISE samples along with a set of controls at known parasite densities, we can both detect and quantify *P falciparum* in each EPiTOMISE sample.

This SOP also describes how to perform initial data processing from the real-time PCR amplification run. This primarily involves exporting the data and re-formatting these data so that they can be compiled for downstream analyses.

III. Materials needed
- Reagents for SOP *DMC 2017-6*
- Two EPiTOMISE DBS gDNA plates
- EPiTOMISE Mock Quant Controls gDNA
- 384-well real-time plate
- 25mL reagent reservoir
- Excel workbook "EPiTOMISE export formatter QS6 readonly.xslx"

IV. Procedure
1. Prepare the reagents
1.1. Lab reagents
    1.1.1. Defrost two EPiTOMISE DBS gDNA sample plates that you want to test
    1.1.2. Defrost enough primer and probe that you will need in the amounts described below. Protect the probe from exposure to light.
    1.1.3. Confirm that you have enough Taqman mastermix.

1

      1.1.4. Confirm that you have extracted gDNA of the "EPiTOMISE Mock Quant Controls." This is stored on a 96-well plate as 10 individual samples, 6 in row A1-A6 and 4 in B1-B4.

      1.1.5. Confirm that nobody else is using the Quantstudio6 machine, and that the 384-well block is currently installed. Its best to have signed up for the use of the instrument.

1.2. Prepare the electronic file to upload the sample names to the Quantstudio6.

      1.2.1. Copy 2 sets of up to 96 sample names from electronic database to designated columns on the sheet "384x – Raw data" in the file "Plate Map Converter QS6." This file is located in Box in Duke Malaria Collaboratory \ Lab operations \ Protocols \ Active protocols.

      1.2.2. Verify that the names were transferred correctly by spot-checking random wells between the database and the simulated plate maps on the first sheet of the file.

      1.2.3. Click to the sheet "384w – Output setup."

      1.2.4. Save this sheet as a tab-delimited text file with the format EPiTOMISE DBS Plate X-X.

      1.2.5. Open the txt file and add to the top of this file:

          "* Instrument Type = QuantStudio 6

          * Passive Reference = ROX

          [Sample Setup]"

      1.2.6. Save this altered txt file.

      1.2.7. Transfer the setup file to a memory stick in order to import to the Quantstudio6 machine below.

2. Prepare the template-free reaction plate

2.1. The reaction plate is prepared in the dead-air box in Sands 313.

      2.1.1. Wipe the p20 multichannel pipet down with ethanol and bring into the box.

      2.1.2. Work quickly while preparing the mastermix, in order to prevent prolonged exposure to room temperature and to prevent exposure of the Taqman probes to light.

2.2. Prepare the following amount of mastermix. Because the total volume is nearly 5mL, it is prepared in 4 separate but identical 1.5mL tubes.

	ea (µL)		Mastermix	Mastermix (/4)
TaqMan Environmental MasterMix	6		2484	621
Pfr364F (20uM) (250nM final)	0.15		62.1	15.5
Pfr364_R2 (20uM) (250nM final)	0.15		62.1	15.5
Pfr364 probe (20uM) (300nM final)	0.18	x414	74.5	18.6
HbtubF (20uM) (250nM final)	0.15	→	62.1	15.5
HbtubR (20uM) (250nM final)	0.15		62.1	15.5
Hbtub probe (20uM) (300nM final)	0.18		74.5	18.6
Template	1		<1>	<1>
H₂O	4.04		1672.6	418.2
Total	12		4968	1242

2.3. Pipet the contents of all 4 tubes of identical mastermix into a 25mL reservoir.

2.4. Distribute 11uL of mastermix to each well of a 384-well reaction plate.

      2.4.1. Place the reaction plate on a cold block while doing this.

      2.4.2. You can re-use tips at this step, but keep an eye on the ability of the individual tips to aspirate and to dispense.

2.5. Using the same water used for the mastermix, add 1uL water to wells P23 and P24.

2.6. When finished, place a real-time cover atop the reaction plate to protect from direct light.

2.7. Put reagents back in the freezer.

2.8. With the reaction plate atop a cold block, transport the reaction plate to Sands 319.

Title: DBS gDNA testing for *P falciparum*

3. **Add template to reaction plate**
   3.1. Wipe down the interior of the PCR hood with ethanol.
   3.2. Ensure that you have 4 full boxes of p2 tips, as well as the p10 multichannel, in the hood.
   3.3. Add the positive controls from the plate "EPiTOMISE Mock Quant Controls."
      3.3.1. Use the p10 multichannel pipet.
      3.3.2. With just 6 tips, aspirate 1uL from A1-A6 and dispense this gDNA to H13 – H15 – H17 – H19 – H21 – H23.
      3.3.3. Get 6 new tips.
      3.3.4. Aspirate 1uL again from A1-A6, but dispense to H14 – H16 – H18 – H20 – H22 – H24.
      3.3.5. Get 4 new tips.
      3.3.6. With these, aspirate 1uL from B1-B4 and dispense this gDNA to P13 – P15 – P17 – P19.
      3.3.7. Get 4 new tips.
      3.3.8. Aspirate 1uL again from B1-B4, but dispense to P14 – P16 – P18 – P20.
      3.3.9. Re-cover and close this control plate.
   3.4. Add the EPiTOMISE gDNA from the sample plate to the reaction plate.
      3.4.1. The samples will be tested in duplicate on the reaction plate, in columns next to each other. Therefore, column 1 on the sample plate will become columns 1 and 2 on the reaction plate. Sample plate column 2 → reaction plate columns 3 and 4, and so forth.
      3.4.2. Samples from the first plate (usually the odd-numbered one) will be tested on the top, in rows A-H, and those from the second plate (usu. Even-numbered one) on the bottom, in row I – P.
      3.4.3. Open the first sample plate.
      3.4.4. Using 12 fresh tips, aspirate 1uL from sample plate row A and dispense to the odd-numbered columns of reaction plate row A.
      3.4.5. Get fresh tips, and again aspirate 1uL from sample plate row A, but dispense to the even-numbered columns of reaction plate row A.
      3.4.6. Get fresh tips, and repeat 3.4.4 – 3.4.5 for rows B – H. Remember that sample plate row H has only 6 samples, so only use 6 tips.
      3.4.7. When finished with the first sample plate, re-cover it securely.
      3.4.8. Repeat the above steps for the second sample plate, but add to reaction plate rows I – P. Therefore, sample plate row A → reaction plate row I, sample plate row B → reaction plate row J, and so forth.
      3.4.9. Remember that sample plate row H has only 6 samples so only use 6 tips.
      3.4.10. When finished with the second sample plate, re-cover it securely.
      3.4.11. Cover securely the reaction plate with a real-time plate cover and spin it in the plate spinner.
      3.4.12. While it is spinning:
         • Replace sample plates in the fridge/freezer.
         • Return controls to fridge/freezer.
         • Clean up the PCR hood.
      3.4.13. When it has spun 2-3 minutes, remove, place on cool block, and cover to protect from light. Carry to Sands 337.

4. **Run the reaction plate on the Quantstudio6**
   4.1. Open a New Experiment.
   4.2. On the Setup – Experiment Properties tab:
      4.2.1. Name the file "EPiTOMISE DBS plateX-X Taqman duplex MM-DD-YY"
      4.2.2. Choose 384-well block.

3

Malaria Collaboratory   Duke University

Title: DBS gDNA testing for *P falciparum*

    4.2.3. Choose Standard Curve as the experiment type

    4.2.4. Choose "TaqMan reagents" as the chemistry

    4.2.5. Choose Standard as the cycling.

4.3. On the Setup – Define tab:

    4.3.1. Choose "Import" at the top of the screen, and import your .txt file with the sample names that you created above in Step 1.2.

        4.3.1.1. When imported, this should populate the "Sample" pane on the top right.

        4.3.1.2. Scan through the Sample pan list and delete any "placeholder" names like "blank" that were also imported.

    4.3.2. Add two targets by clicking New twice in the top-left Targets pane.

        4.3.2.1. "pfr364", choose FAM as reporter, "None" as quencher, and red as color.

        4.3.2.2. "Hbtub", choose VIC as reporter, "None" as quencher, and black as color.

4.4. On the Setup – Assign tab:

    4.4.1. Select all wells by left-clicking the clear box at the top-left of the plate map and then choose both targets for all in the top left. Task for all is U (for unknown).

    4.4.2. Add positive and negative controls.

        4.4.2.1. Change the following wells to these settings for the positive controls:

Wells	Target	Task	Quantity
H13/14	pfr364	S	2000
H15/16	pfr364	S	1000
H17/18	pfr364	S	200
H19/20	pfr364	S	100
H21/22	pfr364	S	20
H23/24	pfr364	S	10
P13/14	pfr364	S	2
P15/16	pfr364	S	1
P17/18	pfr364	S	0.2
P19/20	pfr364	S	0.1

        4.4.2.2. For the above wells, leave Hbtub selected and set to U.

        4.4.2.3. Change the following wells to these settings for the negative controls:

Wells	Target	Task
P23/24	pfr364 and Hbtub	N

4.5. On the Setup – Run Method tab:

    4.5.1. Change to 12uL the "Reaction volume per well"

4.6. Start the run

    4.6.1. Open the plate tray on the instrument and place the reaction plate on it.

    4.6.2. Ensure that the reaction plate is in the correct orientation and then close the tray.

    4.6.3. Save the file to "Malaria Collaboratory \ EPiTOMISE \ Experiment run files"

    4.6.4. On the Run tab, click the green START RUN button and choose the machine.

    4.6.5. You will hear a faint mechanical sound and the screen on the instrument will show the remaining time in large numbers.

5. Get the data

5.1. On the Analysis tab, underneath the amplification plot, un-tick "Auto" for both targets.

5.2. For each target, drag the threshold line above the background fluorescence. Set each threshold at the same y-value for the two targets.

Title: DBS gDNA testing for *P falciparum*

5.3. Click green Analyze button on the top right.
5.4. Export the data
    5.4.1.   Click to the Export tab
    5.4.2.   Click Load Export Set. Choose "EPiTOMISE DBS export"
    5.4.3.   For Export File Location, click the Browse button, and navigate to "Malaria Collaboratory \ EPiTOMISE \ Exported results."
    5.4.4.   For Export File Name, type "EPiTOMISE DBS Plate x-x MM-DD-YY"
    5.4.5.   In the top right, click the Export button.
    5.4.6.   Close the run file.
5.5. Re-format the export data
    5.5.1.   Open the file in Excel "Malaria Collaboratory \ EPiTOMISE \ EPiTOMISE export formatter QS6 readonly.xslx"
    5.5.2.   Also open the xslx file that you created in Step 5.4.4.
        5.5.2.1.   In this second xslx file, right-click the tab "Results," and left-click "Move or Copy."
        5.5.2.2.   Under the resulting menu, for the field "To book:" select "EPiTOMISE export formatter QS6 readonly.xslx." Click OK.
    5.5.3.   In the file "EPiTOMISE export formatter….", click to sheet "Formatted."
        5.5.3.1.   Copy everything on this sheet.
    5.5.4.   Re-open the xslx file created in Step 5.4.4. (Excel automatically closed this a few steps back)
    5.5.5.   In this file, create a new sheet and paste the Values that are on the clipboard into it.
        5.5.5.1.   Save this file and close it.
    5.5.6.   Click to the file "EPiTOMISE export formatter…" and delete the "Results" tab. Close this file without saving.
5.6. Compile the results
    5.6.1.   Create a new Excel file called "Compiled…."
    5.6.2.   Copy and paste the entire contents of each of the individual exported, reformatted results sheets created in Step 5.5.5.

V. References

Appendix 1

Appendix 2

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