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"Nutritional Status of Young Children with Inherited Blood Disorders in Western Kenya"

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Abstract Cover Page

"Nutritional Status of Young Children with Inherited Blood Disorders in Western Kenya"

By

Becky Tsang

B.A., University of California, Berkeley, 2006

Thesis Committee Chair: Parminder S. Suchdev, MD, MPH

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
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Abstract

"Nutritional Status of Young Children with Inherited Blood Disorders in Western Kenya"

By Becky Tsang

There is demonstrated high prevalence of inherited blood disorders in Africa; however, it is uncertain how these disorders affect nutrition status. To determine the burden of anemia, iron and vitamin A deficiency (VAD), stunting, wasting, and underweight amongst young children in western Kenya with one or more blood disorders (haptoglobin 2-2, glucose-6-phosphate dehydrogenase [G6PD] deficiency, sickle cell disease [SCD], and α-thalassemia), we conducted a cross sectional survey of 882 children aged 6-35 months, randomly selected from 60 villages. Hemoglobin (Hb), ferritin, transferrin receptor (TfR), C-reactive protein (CRP), α-1-acid glycoprotein (AGP), retinol binding protein (RBP), anthropometry, and blood disorders using PCR were measured. Of 861 children with Hb results, 71.7% were anemic (Hb<11 g/dL), 27.2% iron deficient (serum ferritin<12μg/L and CRP <5), 16.8% vitamin A deficient (RBP<0.7 μmol/L and CRP<5). In crude analysis of the four blood disorders, anemia burden only differed between α-thalassemics: homozygote (-α/-α) (82.3%), heterozygotes (-α/αα) (75.6%), or normal (αα/αα) (66.8%) (p=0.002). Compared to αα/αα individuals, homozygous thalassemics were less likely to have VAD (p=0.05). There was no relationship between α-thalassemia and iron deficiency, stunting, wasting, and underweight. Logistic regression was conducted to assess the relationship between anemia and low RBP levels with α-thalassemia; after adjusting for all significant independent, confounding, and interaction variables, α-thalassemia remained significantly associated with anemia (OR=1.77, p<0.01) but not low RBP (OR=0.61, p=0.07). The results suggest that in young children in rural western Kenya, α-thalassemia is associated with anemia, while haptoglobin 2-2, G6PD deficiency and SCD are not associated with poor nutrition status.

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Abbreviations

α_1-glycoprotein	AGP
Acute Hemolytic Anemia	AHA
Body Mass Index	BMI
C-reactive Protein	CRP
Centers for Disease Control and Prevention	CDC
Chronic Non-spherocytic Hemolytic Anemia	CNSHA
Demographic Health Survey	DHS
Dried Blood Spot	DBS
Enzyme-linked Immunosorbent Assay	ELISA
Food and Agriculture Organization	FAO
Glucose-6-Phosphate Dehydrogenase	G6PD
Hemoglobin	Hb
Haptoglobin	Hp
Human Leukocyte Antigen	HLA
Insecticide Treated Net	ITN
Iron Deficiency Anemia	IDA
Mean Corpuscular Hemoglobin	MCH
Mean Corpuscular Volume	MCV
Mid-upper Arm Circumference	MUAC
Ministry of Public Health and Sanitation	MOPHS
Nyando Integrated Child Health and Education	NICHE
Polymerase Chain Reaction	PCR
Prevalence Odds Ratio	POR
Prevalence Ratio	PR
Red Blood Cell	RBC
Retinol Binding Protein	RBP
Sickle Cell Anemia (HbSS)	SCA
Sickle Cell Disease (HbS)	SCD
Sickle Cell Trait (HbAS)	SCT
Socioeconomic Status	SES
Transferrin Receptor	TfR
United Nations Children's Fund	UNICEF
Variance Decomposition Proportions	VDP
Vitamin A Deficiency	VAD
World Health Organization	WHO
Zinc Protoporphyrin	ZP

Chapter 1: Introduction

Background

The Food and Agriculture Organization (FAO) estimated in 2010 that 925 million people, or 13.1% of the world's population, were undernourished, defined by the agency as food consumption below energy requirement norms [1]. However, inadequate energy intake may not take into consideration that micronutrient deficiencies, or inadequacies of essential vitamins and minerals such as vitamin A and iron, are also a major contributor of undernutrition. The World Health Organization (WHO) estimates that anemia (Hb<11g/dL), largely due to iron deficiency anemia (IDA), may affect as much as 30% of the world's population, higher than the FAO's estimate of the undernourished population. There are 69 countries with an anemia burden considered by WHO to be a "severe public health problem" (anemia prevalence at population level >40%) [2]. Vitamin A deficiency (VAD) is just as prevalent, at a worldwide estimate of 33.3% of preschool children with low serum retinol concentrations (<0.7μmol/L) [3].

These conditions are considered significant contributors to morbidity, causing delayed growth and blindness as well as indirect costs to society in the form of productivity loss. Estimates from the 1999 National Micronutrient Survey of anemia and VAD amongst children 0-5 in Kenya are 73.4% and 84.4% respectively, both considered at "severe" levels by WHO [3, 4]. Prevalence of stunting, wasting and underweight are also high at 37%, 6%, and 27% respectively [5]. Interventions to combat micronutrient deficiencies include supplementation (syrups, pills, powders), staple food fortification, and broader dietary improvement programs such as conditional cash transfers, dietary diversification and illness prevention. However, in order to adequately plan, monitor, and

evaluate programs to target micronutrient deficiencies, accurate assessment of the burden of disease is necessary.

Assessment of micronutrient deficiencies is complicated by the fact that effects are often subclinical. Clinical symptoms are may be uncommon, difficult to diagnose, and/or represent a long-term deficiency. For example, iron deficiency may be exhibited through eyelid and tongue pallor, but identification and standardization may vary amongst several enumerators in a survey setting. Reliance on clinical symptoms for diagnosis is also an insensitive marker of deficiency; by the time diagnosis is made, there may be irreversible damage. For example, though India's VAD prevalence is amongst the highest in the world at 57%, only 0.7% exhibit clinical symptoms such as night blindness, Bitot's spots, and xerophthalmia [6]. For more sensitive diagnosis of micronutrient deficiencies, blood samples are typically required.

However, micronutrient status assessment via blood samples is not without complications either. Anemia is most commonly and most cost-effectively field-measured using a HemoCue® B-Hemoglobin device (Ängelholm, Sweden). The causes of anemia are numerous and may be nutritional (poor dietary intake), infectious (malaria, parasitic diseases), blood loss, and inherited blood disorders. As a result, complete micronutrient assessment would ideally also take into consideration diet and local endemic conditions; not doing so may lead to inaccurate conclusions of a dietary intervention. In the case of flour fortification, a pre-post assessment may not demonstrate any impact of added micronutrients in the flour if the main contributor of anemia in a population is malaria. Inherited blood disorders (e.g. hemoglobinopathies, glucose-6-phosphate-dehydrogenase [G6PD] deficiency, and haptoglobin [Hp] polymorphism) may affect red blood cell (RBC)

mechanisms, with implications for iron metabolism, malarial infection susceptibility, and absorption of supplemental micronutrients.

Inherited blood disorders in the scope of this thesis include two hemoglobinopathy variants (sickle cell disease [SCD, HbS] and α-thalassemia[heterozygous -α/αα and homozygous -α/-α]), G6PD deficiency, and Hp polymorphisms. Hemoglobinopathies and G6PD deficiency combined are the most common single-gene mutations affecting the world population; WHO estimates as much as 7% of the population are gene carriers, with a hemoglobinopathies incident between 0.25 and 25/1,000 births [7].

Understanding the prevalence of inherited blood disorders in a target population would be useful when conducting micronutrient research in order to interpret findings, guide intervention programs, and interpret monitoring and surveillance results. While it is understood that inherited blood disorders affect anemia status through multiple mechanisms, it is unknown what contributions they may have towards macro-nutritional status (e.g., growth) and specific micronutrients. The focus of this thesis is to explore whether there is a relationship between the four aforementioned inherited blood disorders and anemia, iron deficiency, VAD, stunting, wasting, and underweight in a cross-sectional survey of children 6-35 months of age in western Kenya. The significance of the findings will have implications on the analysis of nutrition status in children living in areas with high prevalence of inherited blood disorders and poor nutrition status.

Nyando Integrated Child Health and Education Project (NICHE)

The NICHE project was a longitudinal cohort study to evaluate the effectiveness of community-based distribution of health products, including micronutrient Sprinkles, with data collection from 2007-2009. In 2010, a cross-sectional follow-up survey took place in

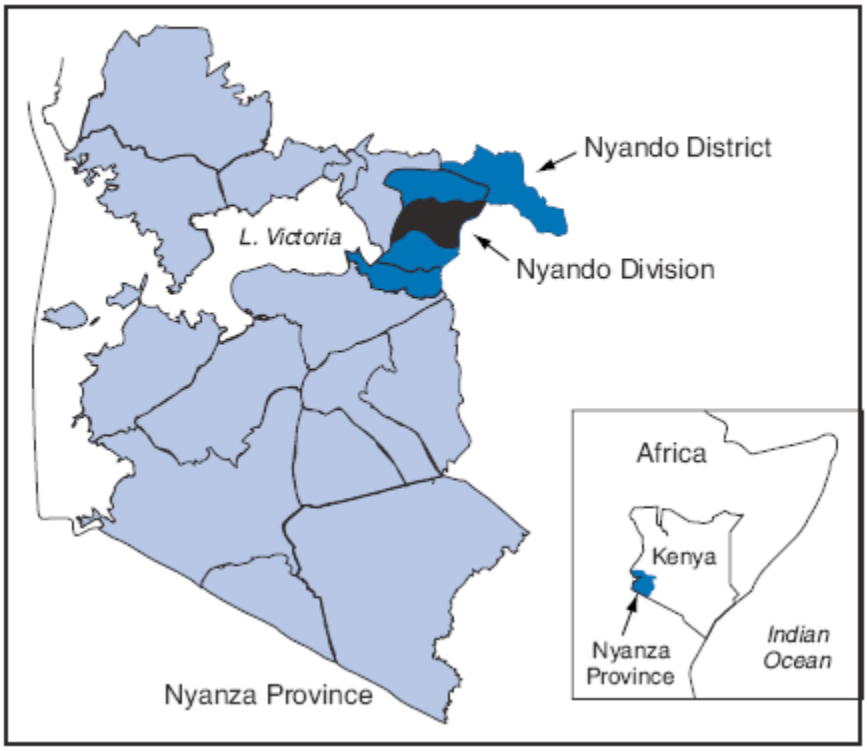
the study area 18 months after formal study activities ended to assess the sustainability of the previous years' interventions. The data for this thesis draws upon the follow-up survey data.

NICHE took place in Nyando Division (Figure 1) in western Kenya and was conducted by multiple partners, including Centers for Disease Control and Prevention (CDC), the Kenya Ministry of Public Health and Sanitation (MOPHS), Safe Water and AIDS Project, Kenya Medical Research Institute (KEMRI), and the Sprinkles Global Health Initiative,. The project's objectives were to assess the effectiveness of social marketing and community-based distribution to implement multiple evidence-based public health interventions. Targeted risk factors for mortality included parasitic infections, diarrhea, malaria, and micronutrient deficiencies. The integrated intervention included distribution of insecticide treated nets (ITN), hand washing promotion with soap, Sprinkles® (a micronutrient powder) distribution, and de-worming of primary-school children with albendazole [8].

Baseline characteristics of 30 intervention and 30 control villages was collected in 2007 with subsequent data collection in 2008. Marketing and household monitoring was finished in 2009. The 2010 follow-up survey was conducted to assess the effectiveness and sustainability of the NICHE project to better inform the MOPHS regarding a national Sprinkles® program. From two strata with 30 randomly selected villages each, 882 children between 6-35 months were enrolled; sample calculations were based on an expected estimated change in anemia prevalence from 45% (March 2009) to 53% (August 2010) [9]. Data collection commenced and finished in August 2010.

The survey instrument collected data regarding household characteristics and assets, maternal education, and caretaker report of child health indicators such as recent illness and feeding practices. Data collected included anthropometric measurements (height/length, weight, mid-upper arm circumference [MUAC]) and a finger stick blood sample. Blood analysis yielded Hb, a field malaria smear, zinc protoporphyrin (ZP), transferrin receptor (TfR), serum ferritin, α-1-acid glycoprotein (AGP), c-reactive protein (CRP), and retinol binding protein (RBP). HbS, α-thalassemia, G6PD deficiency and Hp polymorphism was assessed using polymerase chain reaction (PCR) from packed RBC's. Dried blood spots (DBS) were also collected for isoelectric focusing testing for sickle cell hemoglobin.

Figure 1: Nyando Division (pop. 80,000), in rural Nyando District in Nyanza Province – Kenya, 2007 [10]



Literature Review Methodology

A comprehensive review of the literature was conducted by searching PubMed for each of the blood disorders (thalassemia, Hp, G6PD deficiency, sickle cell anemia [SCA, genotype HbSS]) and one of the following: anemia, iron, vitamin A, nutrition, and malaria. For G6PD deficiency, 41 articles were reviewed, for Hp, 48, SCA, 33, and thalassemia 47, for a total of 171 articles reviewed.

The disorders selected were based on currently available epidemiology – while there are many sickle hemoglobin and thalassemia variants, HbAS/HbSS and α-thalassemia are present in the sub-Saharan Africa region.

Table 1: PubMed Search Terms

SCA + anemia	SCA + iron	SCA + vitamin A	SCA + nutrition	SCA + malaria
Thalassemia + anemia	Thalassemia + iron	Thalassemia + vitamin A	Thalassemia + nutrition	Thalassemia + malaria
Hp + anemia	Hp + iron	Hp + vitamin A	Hp + nutrition	Hp + malaria
G6PD + anemia	G6PD + iron	G6PD + vitamin A	G6PD + nutrition	G6PD + malaria

Chapter 2: Comprehensive Review of the Literature

Overview of Inherited Blood Disorders

Hemoglobinopathies, or mutations and/or deletions of a hemoglobin gene, include thalassemias and abnormal hemoglobin syndromes. A large proportion of the latter category is made up of variants of SCD. G6PD deficiency, as an enzyme mutation, is classified as an enzymopathy. Since Hp is a universally inherited protein, it is not considered a blood disorder. However, Hp subtypes vary amongst individuals and the various subtypes confer differential morbidity risk. Due to its significance in iron metabolism, it has been included in analysis. Reference to "blood disorders" in this thesis includes all four conditions. Symptomatic of SCD, thalassemia, and G6PD deficiency is hemolytic anemia. Hemolysis is the removal or destruction of red blood cells before the end of their normal 120-day life span [11]. When measuring anemia status in a population, it may also be relevant to assess for applicable blood disorders in order to later control for any attributable anemia in analysis. This comprehensive review of the literature will briefly discuss the following information regarding each disorder: biological mechanisms, clinical significance, diagnosis and treatment, epidemiology in sub-Saharan Africa (and specifically Kenya, if the information is available), any preexisting associations with nutrition status and body composition.

G6PD Deficiency

G6PD is an enzyme with a central role in cellular metabolism; G6PD deficiency refers to a group of sex-linked disorders that leads to decreased enzymatic activity. Gene coding for G6PD activity is located on the X-chromosome, resulting in hemizygous deficient males as the predominant phenotype. Deficient homozygous females are rare and occurring more frequently are heterozygous females.

G6PD is a catalyst in the first step to reduce NADP to NADPH, an electron carrier complex that is a required cofactor in several biosynthetic reactions [12]. Of consequence to RBCs is NADPH's maintenance of tri-peptide glutathione in a reduced state, allowing it to act as an antioxidant. Deficiency is expressed in all cellular activity but RBCs are particularly vulnerable due to their long life span and lack of nucleus. As a result, G6PD deficiency commonly manifests itself in shorted RBC life span due to lessened tolerance to oxidative stress.

There are three major clinical manifestations of G6PD deficiency: neonatal jaundice, acute hemolytic anemia (AHA), and chronic non-spherocytic hemolytic anemia (CNSHA). G6PD-related neonatal jaundice differs from classical neonatal jaundice in terms of clinical peak (2-3 days vs. at birth) and lack of severe anemia. Hemizygous males and homozygous females are twice as likely to experience neonatal jaundice compared to the general population, but the condition is rare in heterozygous females. AHA is typically triggered by fava bean ingestion (favism), infection or certain drugs, all of which are oxidative stressors. Given that fava beans are a common dietary ingredient in regions with high G6PD deficiency prevalence, favism is theorized to be the most common hemolysis trigger [13]. Symptoms of AHA typically occur several hours to 2-3 days after trigger exposure and include malaise, weakness, abdominal and/or lumbar pain, jaundice and dark urine due to hemoglobinuria. The most adverse side effect in adults is acute renal failure; however, with proper treatment and lack of co-morbidity, events are usually self-limiting. In CNSHA, anemia occurs without oxidative stress and in a much smaller minority of deficient individuals. Anemia severity ranges from mild to transfusion-dependent and symptoms may include jaundice, enlarged spleen and gallstones. Individuals with CNSHA

are vulnerable to the same oxidative stress agents that individuals with AHA are, and thus must also be cognizant of trigger substances [14].

G6PD deficiency is diagnosed through measuring red cell enzyme activity. There are multiple semi-quantitative screening tools available, including a fluorescence spot test, but these tests are inappropriate for individuals with complications or active AHA; sensitivity can also be lacking, particularly in heterozygous females [15]. If resources are available, positively screened G6PD-deficient individuals should be confirmed by a quantitative assay. Normal G6PD enzymatic activity in red blood cells is 7-10 IU/g Hb; in most variants, activity is reduced by at least 20% - in the most severe variants, enzyme activity may be unnoticeable. Diagnosis in heterozygous females is more difficult as enzyme activity is more variable than in hemizygous males and homozygous females. Enzyme activity levels of greater than 70% are considered unlikely to have clinical repercussions [14]. The American Academy of Family Physicians recommends neonatal screening for G6PD according to family history, ethnic background, or neonatal jaundice [16].

G6PD deficiency is the most common inherited enzymopathy in humans, which supports evolutionarily selection bias for the condition. Currently, a low estimate of the number of individuals affected worldwide is 400 million [14]. Geographically, G6PD deficiency is most prevalent in Africa, Southern Europe, the Middle East, Southeast Asia and Oceania; however, due to migration from areas of high prevalence, deficiency is also commonplace in the Americas and Northern Europe. Within a region, prevalence differs by terrain; studies have found differing prevalence of deficiency in highland vs. coastal areas, correlating directly with malarial endemicity [17]. Multiple studies have associated

G6PD deficiency with a degree of malaria protection, particularly *plasmodium falciparum* [18]. A theorized mechanism of hemolytic anemia and malaria protection in G6PD-deficient individuals is the release of ferriheme, a cytolytic agent, by erythrocytes [19]. There is conflicting evidence whether deficient hemizygous males and heterozygous females share similar levels of protection against *p. falciparum*, but a more recent study suggests that heterozygous females experience little to no protection [20, 21] despite sharing the same deficiency level as hemizygous males [14].

More than 80 variants of G6PD deficiency have been identified but only about half result in enzyme deficiency of clinical importance. Of those 40, about half are associated with CNSHA, and the other half cause AHA [22]. Variant A- is considered the most common, as its frequency is as high as 20% in sub-Saharan Africa, where it is also the most common variant in that population [18]. Other common regional variants include G6PD B and G6PD A, but variant A- is the only variant with deficiency levels where hemolytic anemia may occur [12]. Given this detail, this thesis will limit its analysis of G6PD deficiency variants to type A-. The frequency of occurrence of A- amongst individuals of African descent is 10-15%. The "red cell G6PD activity" for A- subjects is 10-20% of normal, compared to almost normal functioning for A [12]. The deficiency level of A- is considered mild/moderate in relation to more severe variants such as Mediterranean, which causes a G6PD deficiency of <1% of normal activity [13]. While most G6PD-deficient individuals are asymptomatic and do not require micronutrient supplementation to correct the condition, a hemolytic event may have serious consequences in malaria-endemic, resource-poor environments.

While population prevalence is not available on a country level, research in the western Kenya city of Kisumu found a G6PD deficiency prevalence of 6% in anemic children compared to 4% in non-anemic children [23].

With relation to the other blood disorders of interest, there does not appear to be an additive or multiplicative protective effect of HbS and G6PD deficiency against *p. falciparum* infection [24]. Nor are individuals with HbAS more likely to be G6PD deficient [25]. Studies have found little interaction between HbSS and G6PD deficiency [26]; however, G6PD A- may be related to decreased hemoglobin levels – but not increased hemolysis – in HbAS individuals.

This literature review did not find previous research studying G6PD deficiency's associations to non-hemolytic anemia, IDA, VAD, or body composition.

Table 2 refers to a list of drugs that are known to induce hemolytic anemia in G6PD-deficient individuals; avoidance or careful administration is recommended in screened individuals [16].

Table 2: Drugs/Substances at Risk for G6PD-deficient Individuals [27]

Dimercaptosuccinic acid†	Phenazopyridine
Furazolidone	Phenylhydrazine
Glibenclamide†	Primaquine‡
Isobutyl nitrite	Sulfacetamide
Lawsone (henna)	Sulfanilamide
Methylene blue‡	Sulfapyridine
Nalidixic acid‡	Thiazolesulfone
Naphthalene	Toluidine blue†
Niridazole	Trinitrotoluene
Nitrofurantoin	Urate oxidase

‡Antimalarial

Sickle Cell Hemoglobin

SCD refers to the inheritance of the sickle cell hemoglobin gene (HbS) and another abnormal hemoglobin. SCA (genotype HbSS) is the most common and severe form of SCD. Since this disease is the most predominant form in sub-Saharan Africa, this

literature review will focus on SCA and sickle cell trait (SCT, genotype HbAS), which is the inheritance of only one abnormal β-globin gene.

HbSS individuals have affected RBCs that are morphologically different from healthy RBCs, shaped like the eponymous sickle tool or crescent moon. The irregular shape combined with the firm and sticky nature of these cells makes transportation through blood vessels difficult, creating blood flow blockages and oxygen deprivation.

Anemia occurs in HbSS individuals due to the shortened lifespan of sickle cells (10-20 days) compared to the standard 120 days of healthy RBCs. A reduced number of healthy RBCs to transport nutrients and oxygen is thought to be one of the mechanisms behind delayed growth and puberty in children and adolescents [28]. Sickle cell blockages in blood vessels may cause painful episodic pain called "crises," commonly located in the chest, abdomen, joints, and bones. The frequency and pain intensity differs according to individual and may vary from only a few crises in a lifespan to a dozen or more a year [29]. Adding to the variability, crises may last from a few hours to weeks, with severe episodes potentially requiring hospitalization. In the hands and feet, the same blood flow blockages may cause swollen appendages, and blockage of the optic blood vessels can damage the retina [30].

Diagnosis is performed through hemoglobin analysis via protein electrophoresis or chromatography [30]. Established antenatal screening programs in the USA, England, some European countries, and gradually in Africa [31] of importance since early detection of HbS combined with patient follow-up and education improves survival through prophylactic penicillin, and improved care-seeking practices to recognize complications [32].

Although the disease was first described in 1910 [33], disease management options are still limited: the cytotoxic drug hydroxycarbamide, blood transfusions, and hemopoietic cell transplantation. Hydroxycarbamide has the effect of increasing hemoglobin concentrations while decreasing multiple SCD symptoms [30]. Blood transfusions, also used for treatment of another hemoglobinopathy, β-thalassemia, corrects anemia, decreases the proportion of sickle cells in circulating RBCs by suppressing synthesis, and reduces hemolysis. However, since individuals are at risk for alloimmunization, donor erythrocytes must be screened thoroughly for ABO, Rhesus (Cc/D/Ee) and Kell blood groups [34-36]. Chronic transfusion also puts individuals at risk for hemosiderosis, a form of iron overload; chelation is necessary to avoid liver damage [30]. Hemopoietic cell transplantation is the only curative treatment available but rarely performed due to restrictions: patients must have severe disease complications requiring chronic transfusions and most successful with a sibling who meets human leukocyte antigen (HLA) donation requirements. Studies have shown overall survival rates ranging between 92-94%, with event-free survival rates slightly lower: 82-86% [37].

There are over a dozen HbS genotypes that cause characteristic clinical symptoms but only three are specific to sub-Saharan African populations: HbSS, HbSC, and HbSβ. HbSS is by far the most common variant, accounting for as much as 70% of the SCD in African populations [30], followed by HbSC and HbSβ. Of the three, HbSS is also the most severe. HbSC and HbSβ produce comparatively moderate and mild forms of SCD.

Much like G6PD and thalassemias, HbS allele distribution is geographically dictated by malaria endemicity and population migration from malaria endemic areas. Areas of original prevalence include the Mediterranean, India, Middle East and Africa;

there is typically a specific variant for each location [30]. Though lack of diagnostic resources are caveats in all disease estimates, WHO calculates that sub-Saharan Africa accounts for 80% of the global total SCD births at 230,000 affected children born each year [38]. Prevalence in a Tanzanian population has been found to be associated with altitude and ranged from 0-14% [39].

HbSS is associated with growth retardation and multiple micronutrient deficiencies, most markedly vitamin B6, D, zinc, folic acid, and iron [40-46]. With respect to vitamin A status, there has been conflicting evidence regarding the relationship between vitamin A and HbSS or HbAS. In a small (n=50) comparison between growth retarded (GR), growth normal (GN) patients and non-patients from the hospital's "well-child population," a study found lower serum retinol and RBP in SCA children compared to HbAS children. However, given that GR and GN patients had similar serum RBP levels, its relationship to growth is unknown [45]. Serum vitamin A status was low in 66% of American HbSS children while 17% were deficient. Body mass index (BMI) z-scores were also low, but not less than two standard deviations [47].

Recent work has associated lower than normal anthropometric measurements in adult and adolescent HbSS patients, with a larger effect in males than females [43]. A small dietary intervention trial demonstrated clinical improvement in body composition through macronutrient supplementation, suggesting that HbSS individuals may have higher energy needs than β-globin-normal individuals [48]. A review of growth retardation and HbSS found that HbSS individuals were more severely affected than children with other sickle cell hemoglobinopathies; growth failure in HbSS subjects occurred worldwide but with more severity in resource poor environments. At birth, cases had normal birth

weight and length, but growth retardation began between six months and two years [44]. Compared to healthy counterparts, HbSS children in America and Ghana have a higher prevalence of undernutrition. In the US, 41% of subjects were moderately underweight, 25% severely underweight [49], and 11% stunted [50]; in Ghana 44% [51] were stunted and all underweight. Amongst children in Tanzania, HbSS is associated with stunting and wasting, with the greatest deficits seen in adolescents and boys. Lower Hb concentration is found to be associated with increased odds of malnutrition in SCA patients [52].

In another study of nutrition status and HbSS, body composition, serum vitamin A and ferritin were measured alongside three-day dietary intake records. Compared to controls, HbSS individuals had a higher proportion of growth retardation (22% of population $<5^{th}$ percentile in height) and weighed less despite consuming more calories and protein according to dietary intake data [53]. Higher resting basal metabolic rates (BMR) in HbSS children may be an explanation, though potential mechanisms behind increased BMR are unclear. Serum vitamin A was lower in SCA children whereas serum ferritin was higher despite comparable dietary intake of both micronutrients. The only indicators where the two groups did not differ were RBC count and serum folate, both being in the normal range. Though contradictory to the previous study's finding, there is other evidence that SCA individuals may also be susceptible to folic acid deficiency [44].

Due to the excess availability of iron in the blood system from RBC destruction and potentially increased iron absorption as a coping mechanism, it has been suggested that HbAS/HbSS individuals may be relatively less susceptible to IDA; however this was not found to be the case in a coastal Kenyan population [54]. While studies have found no significant IDA in non-transfused SCA patients, studies in India and Nigeria have found

the opposite: 36-50% of SCA patients with IDA [42, 55]. This suggests that the differing environments between developing and developed countries may be a modulating factor. In some scenarios, it is suggested that IDA may actually be beneficial for SCD individuals, though this is not well understood in comparison to the accompanying negative effects [44].

There is strong evidence that HbAS is protective against severe malaria [56, 57]. Young (2-16 months) HbAS individuals experience a protective advantage over HbAA infants, with reduced all-cause mortality, severe malarial anemia, and high density parasitemia [58]. This early advantage seems to disappear by adolescence as HbAA individuals develop natural immunity towards malaria through continued exposure [59].

Co-inheritance of HbSS and α-thalassemia is a common occurrence, with estimations as high as 30% in SCD individuals of African descent [60]. There is evidence that the combination of hemoglobinopathies has the beneficial effect of increasing survival [61] and decreasing hemolysis rates, stroke incidence, gallstones and priapism [30]. On the other hand, there has been scant evidence that crises are increased in individuals with combined HbSS and α-thalassemia, though not conclusive [29].

Similarly, a study of co-afflicted individuals with α-thalassemia and HbAS in coastal Kenya suggested an interaction between the two hemoglobinopathies, the effect being a reduction of α-thalassemia's hematological effects [62]. However, there may be a loss of malarial protection when α-thalassemia and HbAS are co-inherited [63].

Thalassemia Syndromes

Thalassemias are a result of genetic defects affecting hemoglobin synthesis. Thalassemias differ from SCD disorders in that hemoglobin structure is maintained and

instead refer to non-operational synthesis of one of two hemoglobin chains. There are two main thalassemia syndromes, differentiated as α and β to refer to the defective hemoglobin chain affected [64], and detailed in Table 3. Clinical symptoms of the thalassemias arise from an imbalance between α-globin or β-globin chains due to suppressed synthesis of one or the other [65].

Clinically, α-thalassemia is expressed in four different manners as a result of partial (α^+) or total (α^0) α chain deletions in one of its four genes: *minima*, *minor, HbH,* and *Hb Barts [66]*. *Minima* individuals are generally asymptomatic heterozygous α^+ (–α/αα) carriers and experience a very mild reduction in Hb values. α-Thalassemia *minor* is inherited as heterozygous total deletion (--/αα) or homozygous partial deletion (-α/-α) and expressed in individuals as mild anemia, hypochroma, or microcytosis. *HbH* is a result of compound heterozygous α^+/α^0 thalassemia with three inactive α genes (--/-α) [64]. The clinical effects of this form are anemia crises triggered by oxidants and infections and complications that include folic-acid deficiency, gallstones, leg ulcers in the lower extremities, and cardiac complications [64]. *Hb Barts* is a form of hydrops fetalis resulting from homozygous α^0 (--/--), which is complete lack of α-globin chain synthesis. The condition causes severe hemolytic anemia apparent *in utero* and is fatal without both *in utero* and chronic transfusion treatment.

Table 3: Thalassemia Variants [64]

Phenotype	Gene Arrangement	Hemoglobin Range	RBC Effects/ Symptoms
α-thalassemias			
Normal	αα/αα	Normal	None
α-thalassemia *minima*	-α/αα	Normal	None Mild changes to blood count: MCH<27 pg*
α-thalassemia *minor* (heterozygous)	--/αα	Normal/low	None Significant changes to blood count: MCH<24 pg

α-thalassemia *minor* (homozygous)	-α/-α	Normal/low	None Significant changes to blood count: MCH<26 pg
HbH disease	--/-α	8-10 g/dL	Chronic hemolytic anemia of varying severity MCH<22 pg
Hb Barts hydrops fetalis	--/--	<6 g/dL	Fetal anemia fatal if untreated; Hydrops MCH<20 pg
β-thalassemias			
Normal	β/β	Normal	None
β-thalassemia *minor*	β/β^{+}	9-15 g/dL	Mild anemia: MCH 19-25 pg MCV 55-75 fl **
β-thalassemia *minor*	β/β^{0}		
β-thalassemia *intermedia*	β^{+}/β^{+}	6-10 g/dL	Moderate disease; chronic anemia of varying transfusion dependency; MCH 15-23 pg MCV 55-70 fl
β-thalassemia *intermedia*	β^{+}/β^{0}		
β-thalassemia *intermedia*	β^{0}/β^{0}		
β-thalassemia *major*	β^{+}/β^{+}	<7 g/dL	Chronic anemia requiring lifelong blood transfusions; MCH 14-20 pg MCV 14-20 fl
β-thalassemia *major*	β^{0}/β^{0}		
β-thalassemia *major*	β^{+}/β^{0}		

*MCH = Mean Corpuscular Hemoglobin; Normal range = 27 picograms/cell [67]
**MCV = Mean Corpuscular Volume; Normal range = 80-100 femtoliter [67]

Unlike the α-hemoglobin chain, the β-hemoglobin chain only features two genes. Thus, the differences between the β-thalassemia subtypes are due to a wider range of variations in the level of β-globin production suppression. The multiple combinations are expressed in three phenotypes: *minor, intermedia,* and *major* [65]. Individuals with thalassemia *minor* are heterozygous for defective β-globin synthesis (β/β^{+}, β/β^{0}) and exhibit mild microcytic hypochromic anemia. Thalassemia *intermedia* features mild homozygous or mixed heterozygous β-thalassemia (β^{+}/β^{+}, β^{+}/β^{0}, $\beta^{0/}\beta^{0}$); common complications are skeletal deformities and tumorous masses due to RBC hyperplasia, but erythrocyte transfusion need is variable. Thalassemia *major* is inheritance of severely defective β-globin chain genes (β^{+}/β^{+}, β^{+}/β^{0}, $\beta^{0/}\beta^{0}$) and always requires life-long blood transfusions for chronic anemia treatment [65]. This transfusion dependence places patients at risk for iron overload and requires chelation to remedy. Failure to treat the

general condition results in childhood death by age 10. In high-resource settings equipped with treatment facilities, patients have projected life spans of 50-60 years [64].

Diagnosis of milder forms of α-thalassemia may be difficult since clinical symptoms are often lacking and hemoglobin values are only slightly reduced. It is suggested that the condition be considered in the presence of mild microcytosis absent of IDA or β-thalassemia [68]. For all major thalassemia forms, *in utero* diagnosis is possible. The large reduction in new cases of β-thalassemia in common carrier regions such as the Mediterranean is attributed to genetic counseling and terminations [65, 69].

The mild thalassemias do not require treatment (α-thalassemia *minima* and *minor*, β-thalassemia *minor*) [64]; because iron supplementation will not correct thalassemia-attributed anemia, supplementation is not recommended unless IDA is present. The moderate forms require varying forms of treatment according to severity. For example, α-thalassemia *HbH* treatment involves regular folic acid supplementation and in rare cases requires transfusions [64]. The most severe forms – α-thalassemia *Hb Barts* and β-thalassemia *major* – require life-long transfusions. For β-thalassemia, as with HbSS, the only curative treatment is hematopoietic stem-cell transplantation. Co-inheritance of both thalassemias has the effect of equalizing the alpha and beta globin chain imbalance and dilutes clinical symptoms of both conditions [65].

WHO estimates that of the 350,000 children born with hemoglobinopathies per year, 20% have a form of thalassemia [38]. Geographically, α-thalassemia primarily occurs in Africa, Arabic regions, and South-East Asia [68]; documented prevalence in sub-Saharan Africa ranges from 25-67% [39, 62, 70], and is higher in Asia and Oceania [71]. β-Thalassemia distribution overlaps with α-thalassemia in Arabic regions and South-

East Asia but is limited in sub-Saharan Africa; it also extends more broadly into Asia and the Mediterranean. Prevalence of β-thalassemia ranges between 2-19% depending on region [72].

Though there is less epidemiological evidence for β-thalassemia, both thalassemias appear to provide a measure of protection against severe malaria and anemia caused by *p. falciparum* [70, 73-79] but not parasitemia density or infection. Because of this higher prevalence in malaria-endemic areas, 90% of thalassemia births take place in lower-middle income countries [38]. Mechanisms behind the protective effects of both thalassemias remain unclear. Unlike SCD, α-thalassemia also appears to be protective of infection by another malarial parasite, *p. vivax.* Because the *p. vivax* parasite appears to preferentially invade reticulocytes [80] and α-thalassemia increases RBC turnover and thus decreases the average age of an individual's RBC population, the parasite has high rates of infections in young α-thalassemic children [81]. Early exposure may be a mechanism behind improved malaria protection later on in life [82]. α-Thalassemia has also been documented to be protective against non-malarial diseases, including respiratory diseases, gastroenteritis, and meningitis [83].

Beyond potential contraindication with iron supplements, literature pertaining to the relationship between α-thalassemia, VAD, IDA, and body composition was not found.

Haptoglobin Polymorphism

Hp is a plasma protein with strong antioxidant capabilities through its high affinity rate for binding free circulating Hb ($k= 5.5x10^5 M^{-1} sec^{-1}$ [84]). Hp is primarily synthesized in the liver under conditions of infection or inflammation with a half-life of 2-4 days. Free circulating hemoglobin causes oxidative tissue damage by promoting the accumulation of

hydroxyl radicals. However, in the bound Hp-Hb complex, these oxidative properties are lost. On an IC_{50} antioxidant ranking scale, all Hp polymorphisms are a stronger antioxidant than probucol and vitamin E [85].

While Hp is present in animals, only in humans are there subtypes of the protein, with three major variants: Hp1-1, Hp2-1, and Hp2-2. The three proteins are of differing molecular morphology, which has implications for its heme binding and antioxidant abilities. Hp1-1 is the smallest at a defined 86kDa; Hp2-1 and Hp2-2 have variable ranges, 86-300kDa and 170-900kDa respectively [86]. All Hp proteins have the same affinity for Hb, but differential affinity for the Hp-Hb complex receptor, CD163. The Hp2-2 complex has 10x the binding affinity to CD163 as the Hp1-1 complex [87]. Despite this, Hp1-1 is the most effective antioxidant protein of the three molecules, possibly due to the highest cell membrane permeability [84]. In addition to a larger mass, Hp2-2's decreased antioxidant functionality may be due to increased prevalence of the A-16C allele in the Hp2-2 polymorphism relative to Hp1-1 individuals [88].

Hp-Hb is removed from circulation by macrophages, facilitating iron recycling. Since Hp is not recycled after endocytosis and acute events rather than low Hp levels trigger synthesis, it may take several days to reach original Hp levels if there is an excess of free circulating hemoglobin. Due to Hp's role in mediating iron metabolism, it is considered to have a clinical importance in diseases such as cancer, infections, atherosclerosis, and neurological disorders [89].

In addition to reducing oxidative stress, Hb capture provides a bacterial defense against certain organisms, as demonstrated by inoculation of pathogenic *e. coli* in ahaptoglobinemic mice [90]. However this is not a complete defense, as other pathogens

(*n. meningitides, c. jejuni, b. fragilis and v. vulnificans.*) have a more sophisticated heme uptake system, allowing circumvention of Hp's binding mechanism [91-94]. By acting as a bacteriostatic agent, increased Hp levels during acute disease phases may potentially lessen severity of some diseases. Hp polymorphisms are related to multiple disorders, from psychiatric to autoimmune, with differential associations for each allele or subtype [86].

The Hb binding process also provokes an anti-inflammatory response through inhibition of prostaglandin synthesis. As with antioxidant capabilities, the subtypes are not equally capable anti-inflammatory agents: Hp1-1 is the most effective inhibitory variant [95].

The Hp variants are the product of two related alleles, HP1 and HP2. The frequency of the two alleles – and thus the variants – is regional and related to ethnicity. The H1 allele is more predominant in Africa and S. America, with the lowest prevalence in India [96]. The H2 allele is thought to have originated in India but its current low frequency in the region suggests a selective bias for H1. In some areas, there is a clear genetic selection for a specific polymorphism: Polynesian populations on Easter Island had a very high frequency for Hp1-1 (72.2%), but no evidence of Hp2-2 [86]. In sub-Saharan Africa the subtypes were more widely distributed, with Hp1-1 ranging from 27.9-53.5%, 11.1-47.9% for Hp2-1 and 3.0-38.2% for Hp2-2 in west African and Burundian populations [86, 97]. In coastal Kenya, Hp2-1 has the highest frequency (46.8%), followed by Hp1-1 (27.9%) and Hp2-2 (25.3%) [98].

Reduced or null expression of Hp is referred to as hypo-haptoglobinemia and ahaptoglobinemia (Hp0) respectively [99]. A survey in the Nyanza district of Kenya found

very high levels of Hp0 (40.4%) [100]. Hp0 prevalence elsewhere ranges between 22.9%-48% [97]. Hp0 and malaria are considered positively associated, as evidenced by correction of Hp0 status by antimalarial treatment [101]. However, lowered or lack of Hp production cannot be fully explained by malarial infection since the condition has also been found with high prevalence in uninfected individuals in the Gambia [97]. Interestingly, the same study in the Gambia also found Hp0 frequencies to vary by season, suggesting that cross sectional studies may not accurately capture Hp polymorphism inheritance in individuals [97].

Hp's relationship to malaria and anemia susceptibility is unclear. A longitudinal study in the Gambia found Hp2-2 a risk factor for anemia – individuals with Hp2-2 had a larger drop in hemoglobin levels compared to Hp1-1 and Hp2-1 individuals after the malarial season, possibly due to Hp2-2's reduced ability to scavenge free iron. However, Hp did not influence other iron status measurements [102]. A conflicting study also in the Gambia did not find the same results [103]. Whereas Hp1-1 may be a risk factor for severe malaria [104], a study in a Kenyan cohort found reduced incidence of clinical malarial episodes in Hp2-2 children as age increased, suggesting increased immunity from earlier exposure to *p. falciparum* [98]. Again, this evidence is questioned by another study which found no relationship between Hp phenotypes and susceptibility to severe malaria [105].

There are few studies on Hp's relationship to SCD and thalassemias, and none were found for G6PD deficiency. Available research suggests a higher prevalence of Hp1-1 amongst HbS subjects [66, 106, 107]. A case-control study in Brazil found that Hp1-1 was more common than Hp2-2 in HbS patients of all ages relative to non-patients [108]. Given that oxidative stress exacerbates SCD, it is thought that individuals with different

Hp polymorphisms may have differing clinical SCD expression. This supports the suggestion that Hp polymorphism may be involved in the pathophysiology of SCD, although to what degree is unknown.

Studies on Hp's effect on iron biomarkers are contradictory. A study on a healthy European population showed differing iron marker levels according to phenotype [109]. With Hp1-1 and Hp2-2 individuals as reference, Hp2-2 males had significantly higher levels on all iron indicators – serum iron, transferring saturation, ferritin, and sTfR. Though female participant biomarkers were also higher than the relative population, the difference in levels was not statistically significant ($p<0.05$). The authors suggest this difference between sexes may be due to menstrual bleeding. However, another study suggested that the difference found in this study was actually due to an interaction with C282Y mutation of the HFE gene – in the presence of controlling for this gene, Hp polymorphisms were not found to have an effect on iron status markers [110]. Other studies also have reported no difference between Hp subtypes and iron status in a mixed European population and black Zimbabwean population [111, 112].

While there is no preexisting literature on any of the Hp polymorphism's effects on vitamin A and body composition, Hp2-2 is associated with a negative effect on vitamin C metabolism [113-115] due to higher circulation of free hemoglobin in Hp2-2 individuals. Malnourished individuals may also have lower serum Hp levels [86].

Chapter 3: Manuscript

Nutritional Status of Young Children with Inherited Blood Disorders in Western Kenya[1-3]

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Abstract

There is demonstrated high prevalence of inherited blood disorders in Africa; however, it is uncertain how these disorders affect nutrition status. To determine the burden of anemia, iron and vitamin A deficiency (VAD), stunting, wasting, and underweight amongst young children in western Kenya with one or more blood disorders (haptoglobin 2-2, glucose-6-phosphate dehydrogenase [G6PD] deficiency, sickle cell disease [SCD], and α-thalassemia), we conducted a cross sectional survey of 882 children aged 6-35 months, randomly selected from 60 villages. Hemoglobin (Hb), ferritin, transferrin receptor (TfR), C-reactive protein (CRP), α-1-acid glycoprotein (AGP), retinol binding protein (RBP), anthropometry, and blood disorders using PCR were measured. Of 861 children with Hb results, 71.7% were anemic (Hb<11 g/dL), 27.2% iron deficient (serum ferritin<12μg/L and CRP <5), 16.8% vitamin A deficient (RBP<0.7 μmol/L and CRP<5). In crude analysis of the four blood disorders, anemia burden only differed between α-thalassemics: homozygote (-α/-α) (82.3%), heterozygotes (-α/αα) (75.6%), or normal (αα/αα) (66.8%) (p=0.002). Compared to αα/αα individuals, homozygous thalassemics were less likely to have VAD (p=0.05). There was no relationship between α-thalassemia and iron deficiency, stunting, wasting, and underweight. Logistic regression was conducted to assess the relationship between anemia and low RBP levels with α-thalassemia; after adjusting for all significant independent, confounding, and interaction variables, α-thalassemia remained significantly associated with anemia (OR=1.77, p<0.01) but not low RBP (OR=0.61, p=0.07). The results suggest that in young children in rural western Kenya, α-thalassemia is associated with anemia, while haptoglobin 2-2, G6PD deficiency and SCD are not associated with poor nutrition status.

Introduction

Previous research has shown a high prevalence of inherited blood disorders in young children in Kenya; coastal studies have found a prevalence of 39% heterozygote α−thalassemia *minima* (−α/αα)and 14.7% homozygous α−thalassemia *minor* (−α/−α) [78]. Sickle cell hemoglobin prevalence estimates vary across the country and ethnic groups; for example, amongst the Luo tribe of western Kenya, sickle cell trait (SCT, HbAS) prevalence is 28% [116] whereas prevalence is 15% in the coastal city of Kilifi [63]. Other inherited blood disorders, such as G6PD deficiency and haptoglobin (Hp) 2-2 genotype may be as common as 20% and 25.3% respectively [18, 98]. While research on the four disorders' relationships to anemia and malaria is available, less studied is how blood disorders affect the overall nutrition status of young children.

Given high prevalence of these genetic polymorphisms, associations between inherited blood disorders and anemia, iron deficiency, vitamin A deficiency (VAD), and poor anthropometry outcomes (stunting, underweight, wasting) are of interest to better target and monitor nutrition interventions. Sickle cell anemia (SCA, genotype HbSS) and α−thalassemia, caused by mutations or deletions of a β-globin chain, reduce red blood cell (RBC) lifespan [64]. G6PD deficiency puts individuals at risk of hemolytic anemia events if a trigger event occurs, such as consumption of fava beans or exposure to certain drugs, including many common anti-malarial drugs [27]. While this condition has limited consequences for deficient individuals in regions of non-malaria endemicity, it is possible that such hemolytic events are key contributors to anemia elsewhere. Haptoglobin (Hp) polymorphism, consisting of the Hp1-1, Hp2-1, and Hp2-2 genotypes, acts as one of the body's important antioxidant agents [85]. However, of the three polymorphisms, Hp2-2 is

the least effective anti-oxidant and individuals with this subtype may be at higher risk of oxidative stress than individuals of the other two variants [84]. Given SCA and α-thalassemia's protective role against severe *f. plasmodium* infection [56, 70] it is unknown if in areas of malaria endemicity and poor dietary quality if SCA and α-thalassemia reduce the prevalence of anemia.

Of secondary interest is the disorders' relationship to growth indicators, such as stunting, wasting, and underweight. Previous research has linked SCA with growth retardation, micronutrient deficiencies, and higher macronutrient requirements than normal individuals [48, 52, 53], but there is little known regarding the associations between nutrition status and the other blood disorders.

Therefore, the purpose of this study was to analyze whether there are associations among anemia, iron deficiency, VAD, stunting, wasting, and underweight amongst young children in Western Kenya with one or more of the following inherited blood disorders: Hp2-2, G6PD deficiency, HbS, and α-thalassemia.

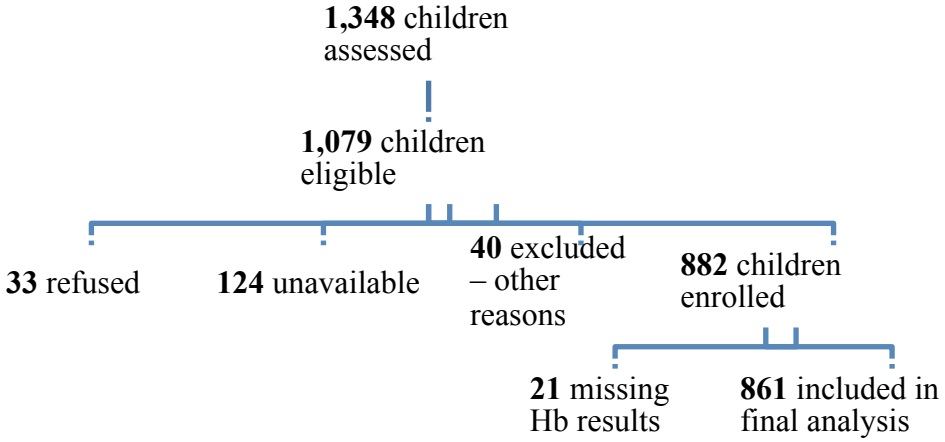
Methods

Study Setting and Design

These relationships were assessed in 2010 using data from a three-year follow-up cross-sectional survey of the Nyando Integrated Child Health and Education (NICHE) project. The NICHE project was a cluster-randomized, longitudinal cohort trial, conducted from 2007 to 2009 in Nyando Division, located in rural Nyanza Province in western Kenya. A complete description of NICHE is discussed elsewhere [10, 117], as well as methodology and findings from the follow-up survey [118].

Rural Nyando Division is populated by the Luo tribe, who largely rely on subsistence farming; data from NICHE indicate high morbidity from malaria and poor nutrition status [118]. Villages were selected by probability proportional to size, with the 1999 Nyando District census as reference. A household census was conducted prior to this survey and 19 compounds per village were randomly selected. All children aged 6-35 months from each compound were eligible for inclusion; from 1,348 total assessed children, 861 were included in final analysis (**Figure 2**). Due to inadequate blood samples in some children, not all 861 had available lab data and the smallest sample size for some analyses was 826. Age was verified using a birth certificate or an immunization record. Sample size calculations were calculated using an expected change in anemia prevalence from 45% (March 2009) to 53% (August 2010).

Figure 2: Selection of Survey Participants in Nyando Division, Kenya



Assessment of Nutrition Status, Hemoglobin Genotype, and Socioeconomic Status

Enumerator training and data collection occurred during August 2010. Field workers administered a questionnaire to the parents or primary caretaker of the index child to collect information on age, sex, illness and micronutrient supplementation. The questionnaire was written in English, translated into the local Dholuo language, and back-

translated into English for accuracy. Illness in the last 24 hours (diarrhea, respiratory disease, fever) was reported by the caretaker, and clinical malaria diagnosed via a positive malaria blood smear and report of fever in the last 24 hours. Multiple socioeconomic status indicators were also collected, including maternal education, and household assets and characteristics.

Anthropometry indicators measured included height and length (for children <24 months) and weight. Height/length was measured to the nearest 0.1cm using a wooden measuring board (Irwin Shorr Productions, Olney MD, USA). Weight was measured to the nearest 0.1 kg using a digital scale (Seca Corp, Hanover MD, USA). Stunting, underweight, and wasting were defined by height-for-age *z*-score (HAZ), weight-for-age *z*-score (WAZ), and weight-for-height (WHZ) *z*-score of <- 2 standard deviations, respectively, below the mean values of the 2006 WHO growth reference data.

Finger prick blood samples were collected and analyzed for hemoglobin (Hb) genotype, Hb, ferritin, transferrin receptor (TfR), C-reactive protein (CRP), α-1-acid glycoprotein (AGP), retinol binding protein (RBP), and malaria smears.

Anemia was field measured using a HemoCue® B-Hemoglobin device (Ängelholm, Sweden) and defined using the WHO cut-off point of <11.0 g/dL [119]. Parasitemia was quantified by Giemsa-stained thick blood films and analyzed by the KEMRI/CDC lab in Kisian, Kenya. Children with severe anemia (Hb <7.0 g/dL) or clinical malaria (reported fever with positive malaria smear) were referred to the closest hospital or clinic. Capillary blood samples of 500 μl were collected in heparinized microcontainers and stored in cold boxes during the day until centrifugation in the evening. Ferritin, CRP, TfR, AGP, and RBP were assessed using a sandwich ELISA

technique (DBSTech, Germany [120]) Iron deficiency was defined using ferritin, using the CDC and WHO defined cut point of <12μg/L where CRP<5 mg/L [121, 122]. Ferritin was used since it is the most sensitive and specific marker of iron deficiency in individuals without inflammation [123]; however, as an acute phase protein, levels rise in the presence of inflammation. Though TfR was collected, it was not included in this analysis due to potential bias by malaria, inflammation, and hemoglobinopathies [124]. Vitamin A deficiency was defined at RBP levels <0.7 μmol/L where CRP<5 mg/L as recommended by WHO [125]. Acute and chronic inflammation were defined respectively by CRP >5 mg/L or AGP > 1 g/L [121].

Hemoglobin genotype (HbA and HbS) and 3.7-kilobase α-globin chain deletion were typed by PCR Sandwich ELISA. Hp genotype was determined by allele-specific PCR. On the basis of recent phenotypic studies (Shivang Shah, unpublished data), males were classified as G6PD deficient if they were hemizygous for the $G6PD^{A-}$ allele, and females as G6PD deficient if homozygous for the $G6PD^{A-}$ allele (A−/ A−), or compound heterozygous for the $G6PD^{A-}$ and $G6PD^{A}$ alleles [126, 127]. Analysis was conducted at the KEMRI-Kilifi laboratory; details of analysis are available elsewhere [70, 128, 129].

Micronutrient supplementation of interest in the survey included Sprinkles®, which contained 14 micronutrients, including 12.5 mg iron as microencapsulated ferrous fumarate, 375 μg vitamin A, and other micronutrients [130].

Socioeconomic status was assessed with the use of a principal components analysis (PCA) wealth index developed by the World Bank to allocate the study population into socioeconomic quintiles as a measure of relative poverty [131, 132].

Statistical Analysis

Age was categorized as 6-23 months and 24-35 months since age by months to capture those within the "critical" nutrition period of less than 24 months compared to those 24 months and over [133]. Hb, low ferritin and low RBP levels were dichotomized using cut-off points indicated above because the primary objective was to identify whether the disorders vary by nutrition status at internationally accepted definitions. Iron deficiency and VAD were referred to as "low ferritin" and "low RBP" respectively because the values were left uncorrected for inflammation in regression analysis. Inflammation was accounted for by controlling for CRP/AGP in the adjusted model in order to avoid a biased sub-set analysis.

Univariate and Multivariable Modeling

To identify crude associations between the blood disorders and nutrition outcomes, contingency tables of sex, anemia, low ferritin, low RBP, elevated TfR, inflammation, stunting, underweight, wasting, and clinical malaria by the four disorders were tested using the Rao-Scott chi-square test [134]. The Rao-Scott test yields slightly more conservative interpretations than the Wald chi-square.

Multivariable logistic regression analysis was conducted for a blood disorder's relationship to anemia, low ferritin, low RBP, stunting, wasting, and underweight if the contingency tables identified a significant association ($p<0.05$). Secondary exposure variables were identified from the literature in order to control for confounding and/or to account for interaction. Covariates included male sex, low socioeconomic status (SES, quintiles 1-3 vs. 4-5) child morbidity (diarrhea, respiratory illness, fever) in the last 24 hours, child-feeding practices in the last 24 hours (breastfeeding, tea consumption, Sprinkles® use, pica behavior), inflammation (elevated CRP and/or AGP), positive

malaria smear, blood disorders (homozygous and heterozygous α-thalassemia, HbS, G6PD deficiency, Hp 2-2) and nutritional status (low ferritin, low RBP, stunting, wasting, and underweight). For any given model, all non-primary exposure of interest blood disorders were included to control for co-inheritance of disorders.

Due to low numbers of wasted individuals (n=30), wasting was not included as a secondary exposure variable. Due to low numbers of HbSS individuals (n=14), HbSS and HbAS individuals were combined in analysis.

Covariates were included in the full logistic regression model if they were considered a significant independent variable (bivariate analysis p-value <0.05), affected the primary exposure's odds ratio by greater than 10%, or if the interaction between the primary exposure and the secondary exposure was significant ($p<0.05$). Backwards regression eliminated covariates in the following order: interaction terms (if any), independent covariates, and confounding covariates. Interaction terms and independent variables were eliminated if $p>0.05$; at each step, the primary exposure's odds ratio (OR) was monitored to ensure that covariate removal was not confounding (10% change in OR). Previously confounding terms were assessed last. Age and sex remained in the model as a potential confounding covariate regardless of p-value if there was a previously identified association. The same process was conducted for all significant associations between blood disorders and nutrition status. Collinearity between covariates was defined as Condition Indices (CI) >30 and assessed with a SAS collinearity macro [135] in both the full and final models. Final models included all remaining significant two-way interactions, significant independent variables, and confounding variables as defined above.

All four blood disorders were included in all models to adjust for the co-inheritance of multiple conditions, regardless of p-value. For HbS, HbAA individuals were used as reference; normal αα/αα genotype served as reference for α-thalassemia homozygous and heterozygous genotypes. G6PD-normal individuals served as reference for G6PD-deficient individuals, and Hp1-1/Hp2-1 served as reference for Hp2-2. Hp1-1 and Hp2-1 individuals have demonstrated relative advantage against Hp2-2 individuals due to lowered anti-oxidant capabilities [84].

Data was analyzed using SAS version 9.3 (SAS Institute Inc., Cary, NC) taking into account the complex survey design of 60 primary sampling units (villages) by using PROC SURVEYFREQ and PROC SURVEYLOGISTIC. *P* values less than 0.05 were considered statistically significant. All p-values were two-sided.

Ethical Considerations

All study protocols were approved by the Scientific Steering Committee and the Ethical Review Committee of the Kenyan Medical Research Institute in Nairobi (protocol number SSC 1176) and the CDC Institutional Review Board (protocol number 5039). This trial is registered by clinicaltrials.gov, identifier NCT01088958.

Results

Sociodemographics, household characteristics, child morbidity, anthropometric status, and Hb genotype.

Of a total sample size of 882 children, 861 children had valid Hb measurements and 826 had valid samples for Hb genotyping (**Table 4,**

Table 5). The percentage of girls in the population was 50%, and 54% of the children 6-24 months of age. The majority (82%) of mothers had attended some primary school. The majority of households owned a radio (80.2%), but few had electricity (1.6%) or a refrigerator (0.8%). Household characteristics were rudimentary: 93.9% had dung/mud flooring, 31.6% a thatched roof as opposed to an iron sheet, and 25.4% did not have access to a latrine.

Among the children surveyed, 30% were stunted (height-for-age z score [HAZ] <-2), 12.1% underweight (weight-for-age z score [WAZ] <-2), and 3.5% were wasted (weight-for-height z score [WHZ] <-2). Iron deficiency prevalence was 19.1% and VAD 16.9%.

Caretaker report of child illness in the last 24 hours included fever (42%), respiratory infection (38%) and diarrhea (24%). Clinical malaria was diagnosed in 14.5% of children and 32.6% children had positive parasitemia results. A greater percentage exhibited chronic inflammation as indicated by elevated AGP compared to elevated CRP(60.9% vs. 34.4% respectively).

In general, micronutrient supplementation intake was low – 6.3% reported ever giving the child iron supplements (e.g. R.B. Tone) while 11% reported Sprinkles® use in the last 24 hours prior to the survey. The majority of children had consumed tea (83%) and/or breast milk (54%) in the previous 24 hours. Pica behavior was reported among almost half the children within the last 24 hours (47.8%).

Malnutrition prevalence was high; 45% of children were stunted, wasted, or underweight; of these stunted was the most common at 30%. Acute malnutrition in the

form of wasting (3.5%) was not common. Anemia was present in 71.7% of children, VAD in 16.9%, and iron deficiency in 27.2%.

More than half of the population (66.1%) carried a genotype for one or more of the following: Hp2-2, G6PD deficiency, heterozygous or homozygous α-thalassemia and/or HbS. Heterozygous α-thalassemia was the most common (38.6%) of children, compared to 9.6% for homozygous α-thalassemia. Hp2-1 was the most common form of the polymorphism (45.3%). HbS appeared most commonly in the HbAS genotype (17.0%). G6PD deficiency affected 6.8% of the population; there was no significant difference between the sexes despite the sex-linked nature of the deficiency.

Univariate analysis: iron, vitamin A status, infection and anthropometric indicators by blood disorders

Anemia prevalence did not significantly differ between the Hp subtypes, the HbS genotypes, or between G6PD-deficient or normal individuals. However, the prevalence of anemia differed among the three strata of α-thalassemia (p=0.0027). There was a significant difference in prevalence of anemia between homozygous and heterozygous genotypes (p=0.0143) as well as between normal and homozygous recessive genotypes (p=0.0031). In both cases, α-thalassemia homozygous and heterozygous individuals were more likely to have anemia relative to normal genotype individuals (crude ORs=1.5 and 2.3 respectively) **(**Table 7).

There was no difference in low ferritin between the various strata of each disorder; iron deficiency as defined by low ferritin and adjusted for inflammation did not differ either. With normal genotype individuals as reference, there was a negative relationship between low RBP and homozygous α-thalassemia (p=0.0239); this was borderline when

low RBP was adjusted for inflammation (p=0.0522). There was no difference in low RBP prevalence between heterozygous or normal genotypes (p=0.6001). There was no significant relationship between low RBP status and any of the other disorders.

There was no difference between each disorder and prevalence of stunting, wasting, or underweight, with the exception of Hp 2-1 variant and stunting (p=0.0373). Inflammation in the study population did not differ among disorder strata; elevated CRP varied from 21.4%-37.2% while elevated AGP varied from 53.6%-64.3% amongst the disorders

Association Between Homozygous α-Thalassemia (-α/-α) *and Low RBP*

In a multivariable logistic regression model assessing homozygous α-thalassemia's relationship with low RBP, in the presence of other significant covariates and confounders, homozygous α-thalassemia was found to be protective of low RBP status (OR=0.61). However, this result was borderline (p=0.0651) (Table 9). Covariates in the adjusted model included young age (6-23 months), underweight, inflammation, positive malaria smear, G6PD deficiency, HbS, and Hp 2-2. In the adjusted model, significant predictors included underweight, inflammation, and positive malaria smear. Age was retained in the model in order to control for any confounding as a result of survival bias and HbS, Hp and G6PD deficiency remained in the model as potential confounders due to co-inheritance of blood disorders. No evidence of significant collinearity (CI>30) amongst the independent variables was found.

Association Between Homozygous (-α/-α) *or Heterozygous* (-α/αα) *α-Thalassemia and Anemia*

In the presence of other significant independent variables of anemia and potential confounding covariates, α-thalassemia remained a significantly associated with of anemia (p=0.0037) (Table 8). Children with α-thalassemia have 1.77 times the odds of anemia relative to normal Hb αα/αα genotype children (CI=1.20-2.59). Other significant variables in the adjusted model included young age (6-23 months), low SES, male sex, low ferritin, inflammation, and positive malaria smear. Of these, positive malaria smear (OR=6.93), low ferritin (OR=4.40), and inflammation (OR=3.26) had the strongest associations with anemia. Breastfeeding in the last 24 hours was a confounding variable. HbS, Hp and G6PD deficiency remained in the model as potential confounders due to co-inheritance of blood disorders. No evidence of significant collinearity (CI>30) amongst the covariates was found.