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The Assessment of Iron Status of Kenyan Preschool Children in Rural Western Kenya

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Doctor of Philosophy

Division of Biological and Biomedical Sciences

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The Assessment of Iron Status of Kenyan Preschool Children in Rural Western Kenya

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Bachelor of Science (Nutrition & Food Science), University of Ghana, 2001

Master of Philosophy (Nutrition), University of Ghana, 2006

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An abstract of

A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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2011

ABSTRACT

The Assessment of Iron Status of Kenyan Preschool Children in Rural Western Kenya

By

Frederick Kobina Ebo Grant

The assessment of the true burden of iron deficiency (ID) has been plagued by the influence of infection, especially in developing countries. Common and recurrent infections, such as malaria and human immunodeficiency virus (HIV) infection may impact the evaluation of the iron status of the population. This makes ID monitoring in these areas difficult, as inflammation influences hemoglobin (Hb), ferritin (SF), zinc protoporphyrin (ZP), and to some extent soluble transferrin receptors (TfR). Further, in the absence of a non-invasive or feasible gold standard, the stages of ID are best characterized by the use of multiple-indices. However, the choice of an appropriate single iron biomarker to replace the multiple-criteria model (use of ≥3 tests of iron status) for screening for ID at the population level in resource poor and remote field settings continues to be a critical need.

Diarrhea and respiratory illnesses are the leading causes of death in children less than 5 years of age. Sprinkles have been shown to be effective against diarrhea and febrile illnesses. However, issues surrounding the use of sprinkles in resource poor settings include the need for daily supplementation.

Our study suggests that subclinical inflammation (indicated by C-reactive protein/CRP and alpha-1-acid-glycoprotein/AGP) affects TfR, ZP and SF and not

correcting for such inflammation alters the measures of ID. In the absence of a gold standard for iron status indicator the multiple-criteria model can be used to assess iron deficiency, and that TfR was as accurate as the multiple-criteria model in assessing the prevalence of iron deficiency in preschool children. Additionally, when multiple-micronutrient powder, Sprinkles, is distributed under non-experimental conditions as part of a health products package it helps reduce the incidence of both diarrhea and fever in preschool children.

Studies aimed at assessing the iron status of children in resource poor, high inflammation settings should include both CRP and AGP as inflammatory biomarkers to accurately determine the true prevalence of ID. Also, Sprinkles distribution through an integrated health promotion and income-generating program should be considered in an effort to improve child health in resource poor, and high inflammation settings.

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ACKNOWLEDGMENTS

The achievement of this dissertation was made possible through contributions from various individuals and entities. First, I give thanks to the Almighty God for making this possible.

I would like to sincerely thank my academic advisor and mentor, Dr. Reynaldo Martorell for his excellent guidance and support throughout my studies here at Emory University. My wife and I are very grateful for his encouragement, guidance and support during some difficult periods we encountered. He was prepared always to listen to me and offer his help during times I sought for guidance and support for both academic and non-academic purposes. I will also like to express my sincere appreciation to the members of my dissertation committee including Dr. Usha Ramakrishnan, Dr. Conrad Cole, Dr. Rafael Flores and Dr. Parminder Suchdev for their academic guidance and understanding during the period of this dissertation.

My acknowledgements also go to the Nevin Scrimshaw International Nutrition Foundation and the International Micronutrient Malnutrition Prevention and Control (IMMPaCt) Program of the Centers for Disease Control and Prevention (CDC) for funding this research. And to the women and children of Nyando Division in Kenya, and also the workers of the NICHE study office in Kisumu, Kenya, I say thank you for agreeing to participate in the study and making this possible.

I also appreciate the support and encouragement I received from my course mates, especially Jean Welsh and Kirsten Herrick during our stay in Atlanta. I am also indebted to my family back home in Ghana for their support and prayers which made this achievement possible.

Finally, I will like to say a big thank you to the most important person in my life, my wife Paschalina Ampofo Grant, for her undying love, support, encouragement and understanding during our time here at Emory University. Without her, this achievement would not have been possible.

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CHAPTER 1

INTRODUCTION

Micronutrient malnutrition (particularly of iron, vitamin A, zinc, and iodine) is a major public health problem especially in the developing world (1). More than 3 billion persons globally are affected by iron deficiency (ID), vitamin A deficiency, or iodine-deficiency disorders. Together, deficiencies of iron, vitamin A and iodine constitute a devastating public health problem which contributes greatly to the vicious cycle of underdevelopment and hinders the attainment of education, health, and productivity goals globally. Infants and children are more seriously affected. For example, current global estimates of anemia indicate that up to 31% of school-going children are affected (2). Iron deficiency continues to be the most prevalent nutritional deficiency worldwide and responsible for about 50% of all cases of anemia globally (3, 4). Anemia due to iron deficiency affects up to 60% of all children globally (5, 6). Iron deficiency alone, with or without anemia, has important health consequences, including increased perinatal and maternal mortality, delayed child mental and physical development, and reduced physical work capacity and productivity during adulthood (7-10).

Iron deficiency and anemia is of public health concern in Kenya as in many developing countries. The largest burden of anemia in Kenya is in children under 3 years of age and pregnant and lactating women (11). The 1999 Kenya National Anemia Survey estimated 77% of children under 30 months of age to be anemic. In Western Kenya, the burden of anemia is particularly significant with over half of pediatric hospital deaths attributable to anemia with 25% of all children hospitalized having severe anemia (Hb <

5.0 g/dL) (12). A cross-sectional survey of children less than 3 years of age in Nyanza Province found anemia prevalence of up to 76% (13).

The assessment of iron deficiency in developing countries and/or high inflammation settings is problematic due to the influence of inflammation on most indicators of iron status such as hemoglobin, ferritin, zinc protoporphyrin, and to a lesser extent, transferrin receptors (14-16). In areas of high inflammation burden, the World Health Organization recommends the assessment of one or more of the acute phase proteins, C-reactive protein and alpha-1-glycoprotein, (CRP and AGP) together with the above-mentioned iron indicators to examine their relationships at different stages of inflammation (17).

In children, ID can be prevented and treated with increased intake of iron. This may include consumption of high iron-content foods, fortification of common staple and complementary foods, or provision of therapeutic doses of iron in supplements. The World Health Organization, WHO, recommends a blanket supplementation of iron to children between the ages of 6 to 24 months in areas where the prevalence of anemia exceeds 20-30% (18, 19). However, despite the well-recognized benefits of iron supplementation for control of anemia, its implementation is hindered by poor adherence to daily dosing, inadequate iron supplies, low coverage, potential dose-related gastrointestinal side effects, and possible increased risk of malaria morbidity and mortality (20). However, a multiple micronutrient mix powder, Sprinkles, can be added to any home prepared complementary food to provide a daily dose of essential micronutrients (21). The lipid encapsulation coating prevents iron and other nutrients

from dissolving into the food and therefore prevents any change in color, flavor, or taste to the food thus enhancing compliance with intake. Sprinkles have been known to be efficacious in treating anemia and also effective against diarrhea and febrile illnesses in young children in developing countries (22-27).

In March 2007, the CDC in collaboration with the Safe Water and AIDS Project (SWAP) and other partners implemented the Nyando Integrated Child Health and Education Project (NICHE), an effectiveness study that combines social marketing with mobilization of local institutions to promote the sale of Sprinkles along with other health products (28, 29). Sprinkles were marketed to households with children 6-59 months by vendors, who sell health products to their neighbors. The primary objective of NICHE project was to assess the feasibility and effectiveness of Sprinkles distribution in western Kenya when integrated with an existing health promotion and income-generating program and to measure the impact of Sprinkles sales on iron deficiency and anemia among young children.

The current study was conducted to address the main hypothesis that correcting for the effect of inflammation on iron indicators using the correction factor approach will improve the estimation of iron deficiency among preschool children. We also assessed the diagnostic efficiency of three independent iron status tests (SF, TfR, or ZP) with a multiple criteria model in assessing iron deficiency in Kenyan children using capillary blood. In the absence of a gold standard for assessing iron status such as stainable bone marrow iron, the use of an appropriate single biomarker for screening for ID at the population level in high inflammation, resource-poor and remote field settings continues to be debated (30, 31). Additionally, we examined the effect of a multiple-micronutrient

powder, including iron and zinc, on the incidence of diarrhea, fever and cough in preschool children in rural western Kenya. Even though supplemental zinc and Sprinkles have been shown to be efficacious in reducing diarrhea and respiratory illness under experimental conditions, issues surrounding the use of sprinkles in resource poor settings include compliance for daily supplementation (32-36).

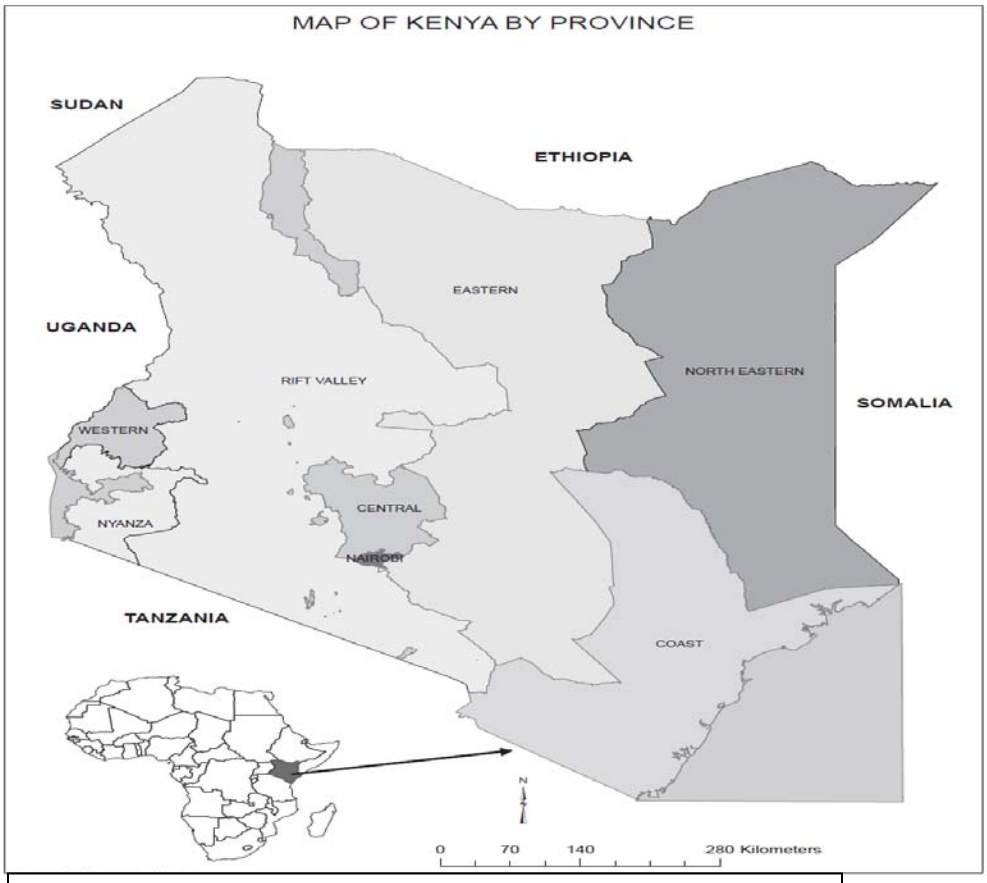
This dissertation includes: (a) a detailed literature review of the effect of inflammation on iron status assessment, assessment of iron deficiency using various iron status indicators, and the effect of micronutrients on childhood illnesses such as diarrhea and respiratory infections (Chapter 2); (b) a detailed description of the study population and setting, methodological considerations and data management and statistical procedures (Chapter 3); (c) reports on the analyses and findings of the dissertation (Chapters 4, 5, and 6); (d) a summary of the overall findings, conclusions, and implications of this research (Chapter 7).

CHAPTER 2

LITERATURE REVIEW

2.1. HEALTH AND NUTRITION CHALLENGES IN KENYA

2.1.1. Basic Indicators of Kenya



Source: 2008 Kenya Demographic and Health Survey, 2010. (37).

The Republic of Kenya lies along the equator in East Africa and is bordered by Ethiopia (north), Somalia (northeast), Tanzania (south), Uganda and Lake Victoria (west), and Sudan (northwest). With a total land surface area of 571,466 square kilometers, it is divided into 8 provinces. Geographically, Kenya is diversely elevated into lowlands and highlands. Children less than 5 years of age constitute 15.7% of the population with

average household size of 4.2 persons, whilst 34% of households are headed by women (37). According to the most recent estimates, 80% of the population fall below the highest wealth quintile with poverty being concentrated in the rural areas; one quarter of rural population fall within the lowest quintile with only 6% in the highest quintile (37). The proportion of women and men with no education are 19% and 13%, respectively; and 40% of children of school-going age are not enrolled in school (37). About 36% of Kenyan households get their drinking water from a non-improved source, mainly surface water from lakes, streams, and rivers (24% of households). However, only 6% of urban households use non-improved sources for drinking water, compared to 46% for rural households. Only 23% of households have improved toilet facilities that are not shared with other households; 23% have access to electricity which is unequally distributed between urban (66%) and rural (8%) households (37). The maternal mortality ratio is estimated at 488 per 100,000 live births (37).

2.1.2. Health and Nutrition Indicators in Nyanza Province, Kenya

Nyanza Province is situated in rural south western Kenya and bordered by Lake Victoria. Nyanza includes part of the eastern edge of Lake Victoria and is inhabited predominantly by the Luo. The province lacks adequate transport and communication infrastructure. The inhabitants are mainly subsistent farmers who cultivate mainly maize, sorghum, millet, cassava, and vegetables. Families live in compounds (“bomas”) that consist of a single main house surrounded by families of the main house. As one of the poorest provinces in the country, Nyanza has high maternal and infant mortality rates as well as HIV prevalence. Current infant and under-five mortality rates are estimated at

95/1000 and 149/1000 live births, respectively, whilst the prevalence of HIV is estimated at 13.9% compared to the national prevalence of 6.3% (37). According to the 2008 Demographic and Health Survey, Nyanza province has the lowest proportion of children (65%) fully vaccinated. Two-week diarrhea prevalence appeared to have declined from 20% in the 2003 Demographic and Health Survey to 16% in the 2008 survey (37, 38). The prevalence of stunting, wasting, and underweight in children under five years old were reported as 31%, 4%, and 11% respectively (37). Nyanza province lack adequate access to improved water sources which contributes to the high diarrheal disease in the province. Diarrhea is reported to be the second leading cause of death among children 1 to 12 months old, and fourth leading cause of health facility visits and cause of death among children 1 to 12 years old (39). Malaria constitutes the largest burden on health and is considered as a cause and consequence of poverty in most developing countries, especially in sub-Saharan Africa (40, 41). In western Kenya malaria transmission is holoendemic with peak periods occurring between June to August and November to December which are the major rainy season periods. The mean entomological inoculation rate is between 60 and 300 bites per person a year. Helminth infestation is common in developing countries such as Kenya and responsible for child morbidity and mortality, as well as undernutrition, iron deficiency anemia, and impaired cognitive performance (42). The prevalence of soil-transmitted helminth infection in western Kenya is 63% in school children (43).

Various micronutrient deficiencies contribute to reduced child growth and development in western Kenya and other developing countries and are of important public health concern. These deficiencies include anemia and iron deficiency.

2.2. ANEMIA AND IRON DEFICIENCY

Anemia is a condition in which the body does not have enough healthy red blood cells and hence their oxygen-carrying capacity is insufficient to meet physiologic needs. The physiologic needs usually vary by age, sex, pregnancy status, altitude, or smoking status. Anemia occurs at all stages of the life cycle, but is more prevalent in pregnant women and young children (2). Iron deficiency is one of the most commonly known forms of nutritional deficiencies and iron deficiency anemia is the final and most severe stage of iron deficiency. It is characterized by hypochromic, microcytic red blood cells, decreased hemoglobin and hematocrit level and a reduction in oxygen delivery to body cells and tissues.

The concentration of hemoglobin, the oxygen carrying pigment in the red blood cell, is normally used to indicate the presence or absence of anemia in an individual. Normal hemoglobin distributions vary with age, sex, and physiological status such as infancy and during pregnancy (2). The World Health Organization classifies individuals as anemic using hemoglobin thresholds (Table 1.1).

Table 2.1: Hemoglobin thresholds indicative of anemia

Age or gender group	Hemoglobin threshold (g/L)
Children (0.50-4.99 years)	110
Children (5.00-11.99 years)	115
Children (12.00-14.99 years)	120
Non-pregnant women (≥15.00 years)	120
Pregnant women	110

Men ((≥15.00 years)	130

Source: Adapted from reference (3).

2.2.1. Causes of anemia and iron deficiency

Iron deficiency is mainly caused by inadequate dietary intake, increased losses from the body and/or increased requirements of iron (44). In developing countries, the main dietary sources of iron are plant based foods such as cereals and legumes which are high in phenolic compounds or phytates, with little access to animal products such as red meat which are good sources of the more bioavailable heme iron (45, 46). Iron deficiency is the main cause of anemia accounting for about 50% of all anemia cases (2, 3).Other causes of anemia that may coexist with iron deficiency include folate, vitamin B12, riboflavin, copper, and vitamin A deficiencies, parasitic infections such as schistosomiasis, ascaris, and hookworms, acute and chronic inflammation such as malaria, HIV, cancer, and tuberculosis, and inherited disorders such as sickle cell anemia and other hemoglobinopathies (2). Lack of knowledge about the importance of food group diversity for adequate growth and health of young children, as well as poverty, and lack of availability or decreased accessibility to certain foods, may limit the inclusion of micronutrient-rich foods in diets of children from low-income families (47).

2.2.2. Consequences of anemia and iron deficiency

There is enough evidence to suggest that iron deficiency with or without anemia adversely affect physical, cognitive, and mental functions and exposure to risks for infectious diseases in children, as well as reproductive, physical work capacity, exposure

to risks for several adult-onset chronic diseases, and increased perinatal and maternal mortality (48–57).

2.2.3. Assessment of anemia and iron deficiency

At the population level, hemoglobin concentration continues to be the most reliable and cost effective indicator of anemia (2, 3). Hemoglobin is usually estimated using the Hemocue photometer. Measuring hemoglobin concentration with the Hemocue hemoglobinometer is relatively easy and inexpensive, and this measurement is frequently used as a proxy indicator of iron deficiency in most clinical practices. However, because the causes of anemia are most often than not multi-facetted other than iron deficiency alone, this should be cautiously interpreted if the only indicator used is hemoglobin (3).

Iron deficiency is commonly estimated with the use of ferritin and, transferrin receptors from serum or plasma, and zinc protoporphyrin from whole blood (17, 58, 59).

Serum ferritin

Serum ferritin at ranges of 20 - 200 µg/L relates quantitatively with iron stores, with 1 µg/L of serum ferritin being indicative of 8 mg of stored iron (60). It is therefore the single best non-invasive measure of iron stores except where inflammation is prevalent (17, 60). The WHO define serum ferritin concentrations <12 µg/L to indicate depleted iron stores in children less than 5 years of age, and concentrations <15 µg/L indicate iron deficiency in those older than 5 years of age (3).

Serum soluble transferrin receptors

Soluble transferrin receptor, TfR, is a trans-membrane glycoprotein that is expressed on cell surfaces and regulated by post-transcriptional regulation of the iron-mediated iron-reactive element. It is important for iron uptake of the cell; expression of TfR levels increases during iron deficiency and decrease when there is iron overload. Body iron status can thus be reflected in TfR cellular uptake of iron (61). It is generally believed that serum or plasma TfR levels are unaffected during an acute phase response because reduced erythropoietin production and suppression of erythropoiesis by cytokines may prevent elevation (14, 62). Lack of international standards as well as its assessment by different assays inhibits direct comparison of TfR values between studies (63). Cut-offs of TfR indicative of ID are usually based on the assay manufacturer.

Zinc protoporphyrin (ZP)

Zinc protoporphyrin (ZP) is a measure of bone marrow iron availability for erythropoiesis. During iron deficiency, excess ZP is synthesized as a by-product of the heme biosynthetic pathway. The elevated ZP/ heme ratio in circulating red cells indicates a condition of relative iron-deficiency erythropoiesis (59). Therefore, elevated ZP is a sensitive diagnostic indicator comparable to serum ferritin for detecting iron deficiency anemia and preanemic iron depletion. However, ZP requires a smaller volume of blood and may be more field-friendly. The levels of ZP are usually measured from capillary whole blood using a hematofluorometer with a cutoff value of >80 μmol/mol indicating iron deficiency (58, 59).

2.2.4. Prevention and treatment of anemia and iron deficiency

Iron deficiency in children can be prevented and treated through the increased intake of iron. This may include consumption of high iron-content foods, through fortification of common staple and complementary foods, or provision of therapeutic doses in supplements

A new strategy for enhancing micronutrient intake such as zinc and iron among young children is through home-fortification. Supplementation of children with zinc has been shown to decrease the risk of morbidity and childhood mortality related to diarrheal diseases, whilst iron supplementation of children may improve cognitive development (64). Micronutrient deficiencies such as iron, zinc, vitamin B12, thiamin, and niacin have been linked with poorer cognitive outcomes in children (65).

Sprinkles as home-fortificant

Sprinkles have been developed to be used in 'home fortification' of complementary foods to deliver iron and other essential micronutrients to young children particularly in developing countries (23). Sprinkles are single-dose packets of dry powder containing lipid encapsulated iron and other micronutrients intended for daily consumption by children 6-24 months of age (25). The composition of each 2g sachet of Sprinkles include encapsulated ferrous fumarate (12.5 mg), zinc gluconate (5 mg), vitamin A (375 µg), iodine (50 µg), copper (0.6 mg), vitamin C (35 mg), vitamin D3 (5 µg), vitamin B12 (0.9 mg), vitamin B1 (0.5 mg), vitamin B2 (0.5 mg), vitamin B6 (0.5 mg), vitamin E (6.0 mg), niacin (6.0 mg) and folic acid (150 mg).They can be sprinkled onto any home-prepared complementary food. The lipid encapsulation coating prevents iron and other nutrients from dissolving into the food and therefore prevents any change

in color, flavor, or taste to the food. Sprinkles were developed in 1996 by Stanley Zlotkin's research group at the Hospital for Sick Children, University of Toronto as a novel approach for delivering iron and other micronutrients. Sprinkles were developed to be a form of "home fortification" in response to the operational constraints of other forms of iron delivery for young children, such as commercially fortified food and supplementation with iron drops. Sprinkles, containing iron and zinc, have demonstrated efficacy in treating and preventing recurrence of anemia in infants and young children (24). The cost-effectiveness of Sprinkles has also been demonstrated, with incremental gains in lifetime earnings estimated at over $450 (64).

2.3. EFFECT OF INFLAMMATION ON IRON STATUS INDICATORS

Even though there are a number of indicators of iron status, the practicability of their measurement and their theoretical advantage as a measure of iron status stipulates that only five of these indicators are considered in most research studies (14, 17). These indicators are hemoglobin for the measure of anemia (3), zinc protoporphyrin for assessing adequacy of iron supply (58, 59), mean cell volume for determination of the size of red blood cells either as too large (i.e. macrocytic anemia) or too small (i.e. microcytic anemia) (14, 66), serum/plasma soluble transferrin receptors (TfR) for assessing erythropoietic intensity and hence iron requirement by erythrocytes (67), and serum/plasma ferritin as an indicator of stored iron (3).

Currently, the World Health Organization / Centers for Disease Control and Prevention (WHO/CDC) recommends that the best approach to assess the iron status of a population is to determine the concentration of hemoglobin together with measurements

of ferritin, and soluble transferrin receptors (3). However, the accurate assessment of iron deficiency is hampered by inflammation especially in developing countries where both iron deficiency and infection coexist. During the onset of inflammation, the synthesis of ferritin is stimulated and thus the true prevalence of iron deficiency as assessed by serum/plasma ferritin may be masked. This is because the concentration of ferritin may be higher than that which truly exists (14).

Plasma ferritin concentration increases proportionally with acute phase proteins, C-reactive protein (CRP) and alpha-1-acid-glycoprotein (AGP), during the onset of inflammation thus rendering its interpretation of iron status problematic (14). Within 10 hours of onset of acute inflammation, CRP levels increase and normalize rapidly, usually within 1 week (16), whereas AGP levels begin to increase only after 24 hours after the onset of inflammation, but remain elevated well into convalescence (14). Levels of CRP and AGP may thus identify different but overlapping groups of people with respect to their inflammation status (16). Inflammation has a smaller influence on plasma TfR (an indicator of erythropoietic intensity and iron requirements) compared to its influence on SF (62, 67). Therefore TfR may be useful when estimating the prevalence of ID in the presence of inflammation. Thus, in areas of high inflammation burden such as in developing countries, the WHO/CDC recommends the assessment of one or more of the acute phase proteins (CRP and AGP) together with the above-mentioned iron indicators to examine their relationships at different stages of inflammation (17).

2.4. EFFECT OF IRON AND ZINC SUPPLEMENTATION ON CHILDHOOD ILLNESS

Multiple micronutrient deficiencies such as zinc and iron are prevalent in developing countries, with devastating effects observed in poor segments of the society. Micronutrient malnutrition (particularly of iron, vitamin A, zinc, and iodine) is a major public health problem especially in the developing world (1). More than 3 billion persons globally are affected by iron deficiency, vitamin A deficiency, or iodine-deficiency disorders. Together, deficiencies of iron, vitamin A and iodine constitute a devastating public health problem which contributes greatly to the vicious cycle of underdevelopment and hinders the attainment of education, health, and productivity goals globally. Infants and children are more seriously affected. For example, current global estimates of anemia indicate that up to 31% of school-going children are affected (2). Vitamin A deficiency is responsible for the compromised immune status of about 40% of children in developing countries resulting in the deaths of 1 million children annually with iron deficiency impairing cognitive development of up to 60% of children and other health issues (68). Deficiency of iodine causes goiter, cretinism and mental impairment of children (68), whereas zinc deficiency has been associated with poor growth, reduced immune function and increased mortality in children (69).

Causes of micronutrient malnutrition include poor nutrition and infections. Poverty may stifle food choices and therefore affect the adequacy of diets. Also, unfavorable ecology together with seasonal variations may limit the availability of foods rich in micronutrients. Deficiency of one nutrient may result in the deficiency of another due to the synergistic interaction of nutrients, thus absorption and metabolism of nutrients are affected by total diet quality. Furthermore, parasitic infections and infestations may

result in loss of blood, decreased appetite, or reduced absorption of nutrients, thus adversely influencing micronutrient status.

Pneumonia and diarrhea are responsible for an estimated 40% of all child deaths around the world each year (70). Zinc deficiency is common in areas where pneumonia and diarrhea are prevalent (71), and children with diarrhea have been shown to have poor nutritional status and are at increased risk of micronutrient deficiencies such as iron and zinc (27). Studies have indicated the effect of micronutrients especially zinc, in reducing diarrheal morbidity and mortality in young children (72-75). Zinc deficiency impairs cellular mediators of innate and acquired immunity which results in increased susceptibility to infection (76). Zinc is also essential for the integrity of the gut mucosal cells by blocking the baso-lateral potassium channels and therefore inhibiting cAMP-induced chloride-dependent fluid secretion which is a major control point for fluid secretions in the large intestines (77). Apart from the well known functions of iron as being essential for psychomotor development, maintenance of physical activity and work capacity, it is also needed for resistance to infection (78). Iron is important for immune-surveillance due to its growth-promoting role for immune cells and its interference with cell-mediated immune-effector pathways and cytokine activities (79). In iron deficiency there are subtle effects on immune status through the alteration of the proliferation and activation of T-, B- or NK-cells (79).

There is considerable evidence of the effect of micronutrient supplementation on diarrhea prevention and reduction: 11% reduction in longitudinal prevalence of diarrhea among young Pakistani children given daily micronutrients including zinc as Sprinkles for two months compared to the placebo (27), 18% diarrhea reduction in young children

treated with zinc in India (80), 16% decrease in mean diarrhea duration in a meta-analysis of oral zinc effect in diarrhea treatment of young children (81) and 17% reduction in incidence of pneumonia among Bangladeshi infants given weekly zinc supplements for 12 months (36). The supplementation of young children with micronutrients such as zinc and iron are therefore essential in preventing and treating childhood disease morbidities such as diarrhea and pneumonia.

CHAPTER 3

EXPANDED METHODOLOGY

This chapter introduces the methods and design of the studies, as well as the laboratory analyses used. It also discusses the relevant statistical issues and key predictors and outcome measures involved in the various studies.

3.1. STUDY METHODOLOGY

Detailed description of the study procedure for the main study, the Nyando Integrated Child Health and Education (NICHE) project has been reported elsewhere (83).

3.1.1. Study area and study population

The data for the current study are from the Nyando Integrated Child Health and Education (NICHE) project (82, 83). Nyanza Province, a largely rural region with poor transportation and communication infrastructure, is situated in Western Kenya and is one of the poorest provinces in Kenya. Over 95% of the people in Nyanza Province are of the *Luo* ethnic group. Our study focused on a smaller division of this province called Nyando Division. The Division has approximately 80,000 people and 15,000 households. Ahero, the capital of Nyando Division, houses the district hospital; community clinics also provide routine vaccinations, growth monitoring, and nutrition counseling for children. Written informed consent was obtained from all participating households. The Ethics Committee of the Kenyan Medical Research Institute (KEMRI) in Nairobi, Kenya (protocol 1176) and the Institutional Review Board of the Centers for Disease Control

and Prevention (CDC) in Atlanta, GA (protocol 5039) approved the study. The study is registered with ClinicalTrials.gov (Identifier NCT01088958).

In March 2007, the Centers for Disease Control and Prevention (CDC) joined with the Safe Water and AIDS Project (SWAP) and other partners to implement the Nyando Integrated Child Health and Education Project (NICHE). The Safe Water and AIDS Project (SWAP) has been delivering health products through a community-based social marketing program in Nyanza Province for seven years. The SWAP approach is based on an ecological framework that mobilizes formal and informal community institutions to support community vendor groups who sell health products to their neighbors. This system combines household, clinic, school, and local commercial distribution and promotion approaches to increase access to various evidence-based health products. Examples of SWAP health products include water storage containers, water disinfectant products, soap, bednets, contraceptives and deworming tablets. Community based groups and HIV groups within the project area are encouraged to register to become SWAP vendor groups. SWAP groups then receive training on the purpose and use of health products, business practices, and microcredit. There are approximately 775 active SWAP groups in Nyanza Province (83).

3.2. STUDY DESIGN AND DATA SOURCES

The NICHE study was a cluster-randomized, longitudinal cohort trial with the primary objectives to measure the effectiveness of Sprinkles distribution through an integrated health promotion and income-generating program, and to measure the impact

of Sprinkles sales on iron deficiency and anemia among young children. The secondary objectives include: to estimate the impact of Sprinkles sales on disease morbidity such as diarrhea, fever, and cough incidence in young children. Monitoring of sprinkles sales and use, and biological impact took place in both arms of the study.

Sources of monitoring data are presented in Table 3.1 and included: 1) cross-sectional baseline and follow-up surveys, including measurement of selected biomarkers and anthropometry; 2) SWAP office records of Sprinkles sales to vendors; 3) biweekly household monitoring of the selected cohort to determine Sprinkles use and health status; and 4) qualitative data collection, including focus groups and key informant interviews with vendors. Quantitative and qualitative data monitoring were integrated to increase understanding of the intervention delivery and utilization as well as confidence in the validity of the findings (83).

Table 3.1: Sources of Monitoring Data for Sprinkles Intervention

Data Source	Target participants	Type of research	Date
Baseline Household Survey	Mothers of children 6-35 months of age (n=550)	Quantitative	March 07
Follow-up Household Survey	Mothers of children selected at baseline available for follow-up (n=451)	Quantitative	March 08
Follow-up Household Survey	Mothers of children selected at baseline available for follow-up (n=712)	Quantitative	March 09
Office record of sales & incentives distribution	Vendors (n=243)	Quantitative	June 07-May 09

Biweekly household monitoring	Mothers of children 6-35 months old (n=550)	Quantitative	June 07-May 09
Focus group discussions (FGD), Key informant interviews (KII) with vendors	Vendors (n=7 FGD; n=39 KII); Additional sample of high-selling vendors (n=11) and low selling vendors (n=8) interviewed at two time points	Qualitative	August 07 – June 09
Observations of vendor training and launches	Trainers (n=14) and vendors (n=14)	Qualitative & Quantitative	June, July, October, November 07

3.3. SAMPLE SIZE CALCULATION AND SUBJECT SELECTION

3.3.1. Sample size calculation

Sample size estimates for the larger NICHE study were based on predicted change of hemoglobin among households consuming Sprinkles. Based on prior efficacy trials, it was estimated that Sprinkles would decrease anemia in this population of children aged 6-35 months from 60% to 50% over a 12 month period (22, 25). At a confidence level of 95%, power of 80%, and design effect of 1.5, we determined that a minimum of 583 children would need to be followed in the intervention and comparison groups at baseline and follow-up. We adjusted the sample size for an estimated 20% non-response and loss to follow-up rate to produce a final estimated sample size of 729 children per arm.

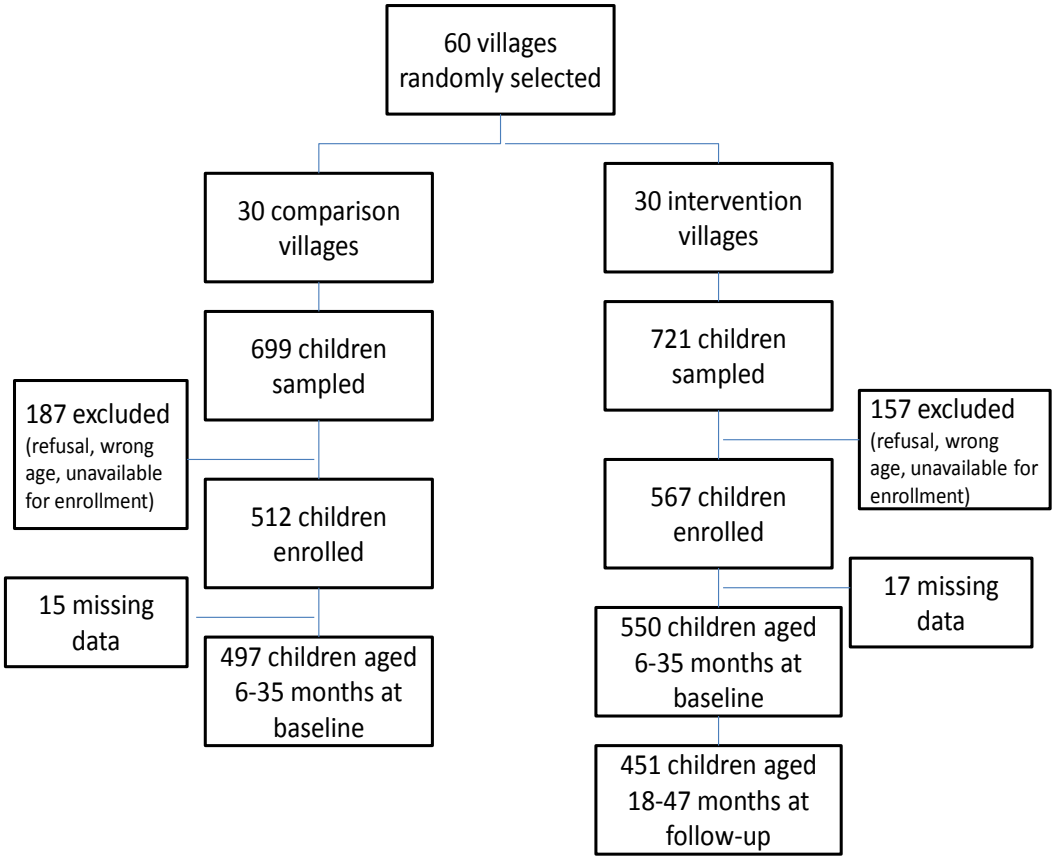
3.3.2. Subject selection for quantitative data collection

We used a two-stage cluster-sampling strategy to select 30 intervention and 30 comparison villages from Nyando Division. Villages were chosen from separate political jurisdictions (i.e., sublocations) to inhibit interventions in one village from influencing

conditions in the other. Villages in and near the urban centers of Ahero and Awasi (n=38) and villages with preexisting SWAP groups (n=4) were excluded from selection. At the first sampling stage, 30 intervention and 30 comparison villages were selected randomly, with probability proportional to size (PPS), using the 1999 Nyando Division census. A household census was performed in all 60 villages selected to be in the study. Village maps with Geographical Positioning Systems (GPS) coordinates of all households and important landmarks were created. During the second sampling stage, 25 children aged 6 to 35 months were randomly selected from each intervention and comparison village. In villages with less than 25 children, all children in the target age group were sampled.

Figure 3.1 describes the selection of the cohort. Of 1420 sampled children, 1079 were enrolled, indicating an enrollment rate of 75.9%. There were no differences in enrollment rates between intervention and comparison areas. Among the 341 excluded from the study, 33.3% of children were outside of the age range (due to discrepancies in date of births reported during census), 2.9% of parents did not give consent, and 63.8% of children were unavailable for enrollment on 3 separate household visits. This resulted in an enrollment of 567 children in the intervention villages, and 512 children in the comparison villages. During the course of the study, any person found to be ill, severely anemic (hemoglobin < 7 g/dL), or febrile was referred for treatment to the nearest hospital or clinic.

Figure 3.1. Selection of study subjects for the NICHE study.



A total of 497 and 550 children aged 6-35 months in the comparison and intervention villages respectively, had complete data at baseline (Figure 3.1). Approximately 18% were lost to follow-up, mainly due to respondents moving from the study area during the political violence following the Kenyan elections as well as some deaths. The mothers (or primary caretakers) of these children were the respondents of the administered survey questions.

3.4. OVERVIEW OF SPRINKLES DISTRIBUTION

Vendors purchased Sprinkles at wholesale for 1 Kenya Shilling (KES) (~1.3 US cents) from the SWAP offices and were instructed to resell them at retail in their village and surrounding areas for 2 KES (~2.7 US cents) per sachet. The profit served as incentives that individual vendors either kept for their personal use or contributed to the activities of their affiliated community groups.

Sprinkles training and orientation meetings were provided to vendor groups, hospital/clinic staff, and community leaders prior to the start of the intervention to increase their Sprinkles knowledge and build support for the intervention. Trained vendor groups were registered by SWAP, and only members of trained groups were supposed to have access to wholesale Sprinkles at the SWAP field offices. A promotional launch took place in each village or at the border of two villages, to introduce vendor groups and Sprinkles to community members. Skits and songs performed by a local theatre group encouraged audience participation and informed community members about Sprinkles. Promotional songs about Sprinkles were also played on vehicle loudspeakers, testimonials and demonstrations were provided by mothers who gave Sprinkles to their children during the formative research phase (84) and free samples of Sprinkles were distributed to participants who attended the launches. Additional promotional materials with visual and written instructions describing correct Sprinkles preparation were also distributed. After the launches, repeated peer-to-peer communication among vendors and community members reinforced the rationale, benefits and appropriate use of Sprinkles. It also provided opportunities for neighbors to follow up with vendors if there were problems or concerns. The SWAP field officers

also supported vendors by conducting regular site visits to all villages throughout the study period (83).

Incentives were provided to vendors and consumers. Vendor incentives, which included t-shirts, promotional stickers, and free sachets, were distributed during vendor meetings and trainings. T-shirts were given to every vendor who purchased 200 sachets wholesale. Vendors received promotional stickers to post on their house to identify them as vendors. First time vendors were given a loan of 200 sachets of Sprinkles as start-up stock given low cash supply. In early 2008, an additional set of 200 sachets were distributed at no cost to active vendors to enable them to mitigate the effects of political and economic crisis after several weeks of post-presidential election violence. Incentives for consumers were also developed and distributed to both educate and incentivize the purchase of Sprinkles. These materials included a calendar and leaflet with visual and written instructions on use, a plastic cup, an initial 2 free sachets to introduce Sprinkles, and periodic promotions, such as “buy one get one free” sachet to boost sales. When purchasing Sprinkles wholesale, vendors received consumer incentives for free and were instructed to give them to consumers to motivate sales. Consumers were expected to receive the incentives free of charge from vendors after purchasing the specified quantity of Sprinkles, for example, a free calendar after the consumer purchased 10 sachets.

3.5. LABORATORY DATA ANALYSES

Blood collection: Trained laboratory technicians obtained capillary blood from children 6-35 months between 0800 and 1600 hours by the use of single-use sterile micro-lancets (Becton Dickinson, Franklin Lakes, NJ) into a purple top microtainer capillary blood collector with EDTA (Becton Dickinson, Franklin Lakes, NJ).

Hemoglobin concentration: This was determined within three minutes of blood collection with the use of the HemoCue® B-Hemoglobin machine (Ängelholm, Sweden).

Malaria parasitemia: A single blood drop was placed on a microscope slide (Thermo Fisher Scientific Inc, Waltham, MA) for thick malaria smear examination for detection of malaria parasitemia for malaria infection. Testing for presence of malaria parasites and the level of parasitemia were performed by the CDC lab in Kisian, Kenya. The number of malaria parasites per 300 white blood cells (WBCs) was counted on Giemsa-stained thick blood films. Malaria parasite counts were converted to parasite loads or densities on the basis of 8000 WBCs/μL of blood (85). Severity of malaria infection was defined by malaria parasite loads >5000 parasites/μL. All of the children who met criteria for active malaria infection were notified and referred to the nearest government clinic for anti-malarial treatment according to the local standard of care.

Zinc protoporphyrin: We collected an additional 400 to 500 μL of capillary blood into a microtainer with EDTA (Becton Dickinson, Franklin Lakes, NJ). The blood was then transported on ice to the KEMRI/CDC laboratory in Kisian, Kenya, where with the use of a pipette with a disposable tip, a drop of the whole blood was placed on a disposable glass cover slip (Aviv Biomedical, Lakewood, NJ) to assess zinc protoporphyrin (ZP) using the Aviv ZP Hematofluorometer (Aviv Biomedical, Lakewood,

NJ). The hematofluorometer was standardized daily with control solutions provided by Aviv Biomedical. Testing was done in duplicates. A correction factor was applied to the results of the ZP on the advice of the manufacturers and the CDC quality assurance lab.

Plasma preparation: The rest of the blood was then centrifuged at 1500 x *g* for 5 minutes at 27ºC, and plasma was removed and stored in cryovials (Thermo Fisher Scientific Inc, Waltham, MA) at -20ºC within 12 h after blood collection.

Ferritin, transferrin receptors, AGP, and CRP: The plasma samples were transported to Germany for subsequent laboratory analysis of SF, TfR, AGP, and CRP at the laboratories of the DBS – Tech (Willstaett, Germany) by a procedure that combines all four analytes using sandwich enzyme-linked immunosorbent assay technique (86). All samples were measured in duplicate and the intra- and interassay coefficient of variations, CoVs, were less than 10%.

Definition of iron deficiency and anemia: The thresholds for defining abnormal values for the biochemical indicators under consideration were as follows: SF, <12 µg/L; TfR, >8.3 mg/L (TfR®, Ramco Laboratories, Inc., Houston, TX); CRP, >5 mg/L; and AGP, >1.0 g/L (86). The ratio of TfR to SF (TfR/SF index) was calculated by dividing TfR (µg/L) by SF (µg/L), with elevated TfR/SF index defined as > 500 (87).

Anemia was defined according to the World Health Organization threshold as Hb< 110 g/ L for children 0.5-4.99 years (88). The HemoCue machines were calibrated and checked for accuracy twice daily (morning before fieldwork and evening after fieldwork) using Hemocontrols and control cuvettes respectively, records of which were kept in a secured place.

A cutoff value of 80 µmol/mol heme was used to indicate elevated ZP level and hence iron deficiency (ID) (89).

3.6. DATA COLLECTION AND MANAGEMENT

The study team oversaw all field operations, including enrolling subjects and monitoring Sprinkles use and health outcomes. A Personal Digital Assistant device (PDA) was used for all household quantitative data collection, including the cross-sectional surveys and biweekly household monitoring. PDAs were mounted with GPS, which were used to map a variety of locations, including the wholesale SWAP sales office, vendors' houses, and households of study participants. Data were entered in customized electronic forms using Visual CE software version 10.0 (Syware, Cambridge, MA) and stored in an Access 2007 database (Microsoft Corporation, Redmond, WA) on a daily basis. A master list of all people living in the survey households (including respondents, children and their siblings, and anyone else residing in the home) was updated at each household monitoring visit.

Vendor wholesale Sprinkles purchases were collected in SWAP office record books, which documented the date; name of the vendor; name of SWAP group; village of origin; quantity purchased; and number of vendor and/or consumer incentives received, including free Sprinkles sachets. These data were double entered into an Excel database.

Data were collected from 36 biweekly household monitoring visits to measure household Sprinkles purchases, individual use, and reported illness. Individual Sprinkles sachet use was estimated by dividing the reported biweekly household Sprinkles

purchases or gifts by the number of children aged 6 to 59 months living in that household (population to which Sprinkles were promoted). Due to problems with a skip pattern on the PDA questionnaire, the questions on individual Sprinkles use were not available for all monitoring rounds; furthermore, it was later determined that there were also translation and comprehension issues with those questions. However, the correlation between estimates of individual Sprinkles use and reported use was good (correlation coefficient of 0.48, $p<0.01$), so household purchases were used as a proxy for use. Data on Sprinkles use in the last 24 hours and last 7 days was also collected during the follow-up surveys.

Socioeconomic status was assessed using a principal component analysis wealth index developed by the World Bank to allocate the study population into socioeconomic quintiles of Kenya as a measure of relative poverty (90).

3.7. ANALYTICAL CONSIDERATIONS

The data for this dissertation were obtained through a cluster randomized, longitudinal cohort trial as well as community-based cluster surveys. This stipulates that issues of design effects as well as correlated data be taken into consideration during the analysis stage.

3.7.1. Design effects

The design effect represents the cumulative effect of design components such as stratification, unequal weighting, and clustering, and will differ for each design. Sampling variability increases when cluster sampling is used rather than simple random sampling. The design effect is the ratio of the true variance of a statistic under the actual design divided by the variance that would have been obtained from a simple random sample of the same size:

$$DEFF(\hat{u}) = \frac{Var(\hat{u})}{SRS\ Var(\hat{u})}$$

- where SRS Var(u) is the variance that would have resulted under simple random sampling assumptions
- Var(u) is the variance of the statistic under the actual design

A design effect of 1 indicates that the standard errors are the same, whilst that greater than 1 indicates that the standard error has been increased because of sample design (91). Because our study design was of cluster sampling, it was important that we make adjustments to the variance estimates by using the design effect (91).

3.7.2. Two-stage, stratified cluster sampling design

Because the study samples were not simple random samples as explained above, we adjusted for the variance estimates related to the statistics derived from the dataset for the sampling design. We accounted for the survey design by specifying, within the statistical procedure used, the value of the stratum or cluster that was associated with each observation.

3.7.3. Correlated data

Longitudinal data, a form of correlated data, are important for describing dynamic processes which may be poorly estimated by a single outcome or 'point prevalence' estimation. During the analysis of correlated data, it is usually assumed that the observations made within a cluster are correlated whilst those in different clusters are not. In our analyses, we considered a village, the primary sample unit, as the cluster. Our assumption was that observations within each cluster were more similar to each other than to observations in other cluster because of similar social and environmental influences.

Next, we specified the type of variance-covariance or correlation that exists between the observations, as well as the working correlation matrix. We applied exchangeable working correlation matrix to support our assumption that the correlation between the outcomes of any two observations within the same cluster were the same. We also used the robust standard error option in our analytical procedures to allow for the possible incorrect specification of the correlation matrix.

The statistical analysis software version 9.2 (SAS Institute Inc., Cary, NC) was used to account for the complex survey design, and correlated data. We included the survey design variable, CLUSTER, in the SAS procedure statements such as PROC SURVEYFREQ or PROC SURVEYMEANS for within cluster models.

3.8. STATISTICAL ANALYSES

All statistical analyses were performed using SAS, version 9.2 (SAS Institute Inc., Cary, NC), taking into account complex survey design. The data were assessed for normality by the use of box plots, stem-and-leaf plots, normality plots and Kolmogorov-Smirnov tests. Whenever outcome variables were observed to be non-Gaussian, we tried to normalize them through log-transformation of such variables. Both parametric and nonparametric approaches were used for analyses and reported as medians and interquartile ranges or geometric means and standard deviations. We used Spearman's rank correlation coefficients to assess relationships between outcome variables such as the iron status indicators (Hb, SF, TfR, ZP, and TfR/SF index), and the acute phase proteins (APPs), CRP and AGP. Baseline characteristics across treatment groups were compared using t-test, ANOVA, Wilcoxon and Kruskal-Wallis tests for continuous variables and chi-square or fisher's exact test for categorical variables. We computed Kappa statistic, K, to assess the extent of agreement between estimates of iron deficiency prevalence in the study population using different iron status indicators.

After completing the univariate analysis, we analyzed the data using the generalized estimating equations (GEE) models. The effect of treatment on outcome variables were assessed using the GEE models for the categorical outcomes with a REPEATED statement to account for repeated measures within the same subject. We applied exchangeable correlation to account for the correlation among repeated observations for a given subject. In the GEE models, we step-wise included age, sex, household socio-demographic characteristics such as sanitation and hygiene variables as covariates to account for any confounding effects.

CHAPTER 4

Correcting for the influence of inflammation modifies the estimation of iron status among preschool children in rural western Kenya

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Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Reprints are not available.

This work is supported in part by an Ellison Medical Foundation / Nevin Scrimshaw International Nutrition Foundation fellowship (FKEG), and the Centers for Disease Control and Prevention (PSS).

Running head: Inflammation and iron status of preschoolers

ABSTRACT

Iron status estimation in areas with high rates of infection is complicated by the effects of inflammation on iron indicators. Using data on iron indicators [(hemoglobin (Hb), zinc protoporphyrin (ZP), ferritin, transferrin receptor (TfR), and TfR/ferritin index)] and subclinical inflammation [(acute phase proteins (APPs); C-reactive protein (CRP) and alpha-1-acid glycoprotein (AGP)] on preschoolers, we compared approaches to adjusting estimates of iron deficiency (ID) by inflammation. In this cross-sectional study, blood was obtained from 680 children (aged 6–35 mo), and iron indicators, subclinical inflammation (i.e. CRP>5mg/L and/or AGP>1g/L) and malaria parasitemia were measured. Children were divided into 4 groups based on APP levels: reference (normal CRP and AGP); incubation (raised CRP and normal AGP); early convalescence (raised CRP and AGP) and; late convalescence (normal CRP and raised AGP). Correction factors (CF) were estimated as the ratios of the geometric means of the iron indicators for the reference group of those for each inflammation group. Corrected values of the iron indicators within inflammation groups were obtained by multiplying values by their respective group CFs. C-reactive protein correlated with AGP (r=0.65; p<0.001), ferritin (r=0.38; p<0.001), Hb (r= -0.27; p<0.001) and ZP (r=0.16; p<0.001); AGP correlated with ferritin (r=0.39; p<0.001), Hb (r= -0.29; p<0.001) and ZP (r=0.24; p<0.001). Use of the CFs to adjust for inflammation increased the prevalence of ID based on ferritin<12 μg/L by 33.9%. Applying the CFs strengthened the expected relationship between Hb and ferritin (r = 0.095, p=0.013 vs. r = 0.203, p<0.001, before and after adjustment, respectively). CRP and AGP influence the estimation of iron status among preschoolers. The use of CFs to adjust for inflammation may be indicated in assessing ID. However,

further work is warranted to identify if the CF approach proposed in this study improves the accuracy of assessment of ID.

INTRODUCTION

The World Health Organization / Centers for Disease Control and Prevention consultative group (WHO/CDC) recommends that the concentration of hemoglobin (Hb), ferritin, and soluble transferrin receptors (TfR) be used in the assessment of iron status (1). Additionally, zinc protoporphyrin (ZP), a measure of bone marrow iron availability for erythropoiesis, is useful in identifying preanemic iron deficiency (ID) (2, 3).

The assessment of the true burden of ID is complicated by the influence of infections, such as malaria and human immunodeficiency virus (HIV), on iron indicators, especially in developing countries (4). This makes ID monitoring among children in these areas difficult, as inflammation influences Hb, ferritin, ZP, and to a lesser extent TfR (5, 6). Serum ferritin concentration, an indicator of iron body stores, spikes during infections , even in the case of subclinical infections whose occurrence is reflected in elevated acute phase proteins, C-reactive protein (CRP) and alpha-1-acid-glycoprotein (AGP); this makes interpretation of iron status problematic (4). CRP levels increase within 10 hours of the onset of acute inflammation and normalize rapidly, usually within 1 week (6), whereas AGP levels begin to increase only after 24 hours after the onset of inflammation, but remain elevated well into convalescence (4). Levels of CRP and AGP may thus identify different but overlapping groups of people with respect to their inflammation status (6). Inflammation has a smaller influence on plasma TfR (an indicator of erythropoietic intensity and iron requirements) compared to its influence on ferritin (7, 8). Therefore TfR may be useful when estimating the prevalence of ID in the presence of inflammation. Thus in areas of high inflammation burden such as in developing countries, the WHO/CDC recommends the measurement of one or more of

the acute phase proteins (CRP and AGP) together with the above-mentioned iron indicators to assess iron status (1).

Three approaches have been proposed to adjust for inflammation when assessing iron status: 1) Upward adjustment of the commonly used cut-off for low ferritin values from 12-15 µg/L to 30-50 µg/L; 2) Exclusion of individuals with inflammation as indicated by elevated values of one or more of the acute phase proteins (APP) or; 3) The use of correction factors (CF) that use APP biomarker(s) to adjust iron status indicators for the effects of inflammation (6, 9, 22).

The first and third approaches do not exclude data, which could bias results if iron deficient individuals are more prone to infection (6, 10-13, 22). Furthermore, in areas where there are high rates of inflammation, exclusion of a large number of people may be impractical (6, 10-13, 22). The use of CF's incorporates the effects of the APPs on iron status indicators, information which is not fully captured by simply modifying the cut-off point for ferritin. The CF approach has not been explored among preschool children in areas of high inflammatory stress, nor has it been applied to multiple iron indicators. Additionally, we are not aware of research exploring whether the use of CF modifies the relationship between malaria infection and iron indicators.

The objectives of this study were to: 1) document the relationship between inflammation as indicated by the APPs, (CRP and AGP) and iron indicators (Hb, ZP, ferritin, TfR, and TfR/ferritin index); 2) assess the effect of the use of the CF on the relationship between malaria infection and iron indicators and; 3) explore various approaches for estimating ID in the presence of inflammation among preschool children in rural western Kenya.

SUBJECTS AND METHODS

Study area and study population

The data for this study are from the Nyando Integrated Child Health and Education (NICHE) project (14, 21) and were obtained from a cross-sectional survey of children 6-35 months of age in 60 randomly selected villages from Nyando Division (population 80,000) in the Nyanza Province of western Kenya between March and May 2009. After developing community maps and completing a household census, households with children aged 6-35 months were selected by population proportion to size (PPS) cluster sampling using updated population registries of Nyando District. Children were selected if they were between the ages of 6-35 months at the time of enrollment, and lived within the catchment area of the study; children with hemoglobin concentrations (Hb) < 70 g/L were referred to the nearest clinic for treatment of severe anemia.

Children were excluded if they were unavailable for enrollment on 3 separate household visits, or parental refusal to give informed consent. Data were recorded in the field using Dell Axim personal digital assistants (PDA) and downloaded into an Access 2007 database daily. All children participating in the survey, whether febrile or not, had thick and thin smears made from capillary blood samples along with the other biochemical laboratory testing. History of fever 24-hours prior to the interview was obtained through caregiver recall.

Written informed consent was obtained from all participating households. The Ethics Committee of the Kenyan Medical Research Institute (KEMRI) in Nairobi, Kenya (protocol 1176) and the Institutional Review Board of the Centers for Disease Control

and Prevention (CDC) in Atlanta, GA (protocol 5039) approved the study. The study is registered with ClinicalTrials.gov (identifier NCT01088958).

Laboratory analysis

Trained laboratory technicians obtained capillary blood from the children between 0800 and 1600 hours by the use of single-use sterile micro-lancets (Becton Dickinson, Franklin Lakes, NJ) into a purple top microtainer capillary blood collector with EDTA (Becton Dickinson, Franklin Lakes, NJ). Hb was determined within three minutes of blood collection with the use of the HemoCue® B-Hemoglobin machine (Ängelholm, Sweden). A single blood drop was placed on a microscope slide (Thermo Fisher Scientific Inc, Waltham, MA) for thick malaria smear examination for detection of malaria parasitemia for malaria infection. Testing for presence of malaria parasites and the level of parasitemia were performed by the CDC lab in Kisian, Kenya. The number of malaria parasites per 300 white blood cells (WBCs) was counted on Giemsa-stained thick blood films. Malaria parasite counts were converted to parasite loads or densities on the basis of 8000 WBCs/μL of blood (15). Severity of malaria infection was defined by malaria parasite loads >5000 parasites/μL. Caretakers of all children who met the criteria for active malaria infection were notified and referred to the nearest government clinic for anti-malarial treatment.

An additional 400 to 500 μL of capillary blood was collected into a microtainer with EDTA (Becton Dickinson, Franklin Lakes, NJ). The blood was then transported on ice to the KEMRI/CDC laboratory in Kisian, Kenya, where with the use of a pipette with a disposable tip, a drop of the whole blood was placed on a disposable glass cover slip

(Aviv Biomedical, Lakewood, NJ) to assess zinc protoporphyrin (ZP). Testing was done in duplicates. The rest of the blood was then centrifuged at 1500 x *g* for 5 minutes at 27ºC, and plasma was removed and stored in cryovials (Thermo Fisher Scientific Inc, Waltham, MA) at -20ºC within 12 hours after blood collection. Samples were transported to Germany for subsequent laboratory analysis of ferritin, TfR, AGP, and CRP at the laboratories of the DBS – Tech (Willstaett, Germany) by a procedure that combines all four analytes using sandwich enzyme-linked immunosorbent assay technique (17). All samples were measured in duplicate and the intra- and interassay CVs were less than 10%. The thresholds for defining abnormal values for the above biochemical indicators were as follows: ferritin, <12 µg/L; TfR, >8.3 mg/L (TfR®, Ramco Laboratories, Inc., Houston, TX); CRP, >5 mg/L; and AGP, >1.0 g/L (17). The ratio of TfR to ferritin (TfR/ferritin index) was calculated by dividing TfR (µg/L) by ferritin (µg/L), with elevated TfR/ferritin index defined as > 500 (18).

Anemia was defined according to the World Health Organization threshold as Hb< 110 g/ L for children 0.5-4.99 years (16). The HemoCue machines were calibrated and checked for accuracy twice daily (morning before fieldwork and evening after fieldwork) using Hemocontrols and control cuvettes respectively, records of which were kept in a secured place. ZP was measured at the laboratory of the KEMRI/CDC in Kisian, Kenya, with the Aviv ZP Hematofluorometer (Aviv Biomedical, Lakewood, NJ). The hematofluorometer was standardized daily with control solutions provided by Aviv Biomedical. A cutoff value of 80 µmol/mol heme was used to indicate elevated ZP level and hence iron deficiency (ID) (3). A correction factor was applied to the results of the ZP on the advice of the manufacturers and the CDC quality assurance lab.

Statistical analysis

All statistical analyses were done using SAS 9.2 (SAS Institute Inc., Cary, NC). Statistical significance was defined as $p < 0.05$. The distributions of the various biomarker concentrations were assessed for normality by the use of normality plots and Kolmogorov-Smirnov tests. The distributions of ferritin, TfR, ZP, CRP, and AGP were non-Gaussian. Both parametric and nonparametric approaches were used for analyses and reported as medians and interquartile ranges or geometric means and standard deviations.

Spearman's rank correlation coefficients were determined to assess relationships among the iron status indicators (Hb, ferritin, TfR, ZP, and TfR /ferritin index), and also between the iron indicators and the acute phase proteins (APPs), CRP and AGP. Where correlations between any iron indicator and an APP were significant, we adjusted values by multiplying them by group-specific correction factors (CF). Four groups were defined: 1) non-elevated state or reference (CRP ≤5mg/L and AGP≤ 1g/L); 2) incubation (CRP > 5mg/L and AGP ≤ 1g/L); 3) early convalescence (CRP > 5mg/L and AGP > 1g/L), and 4) late convalescence (CRP ≤ 5mg/L and AGP > 1g/L). The CFs were defined as the ratios of the geometric mean values of the iron indicator for the reference group to those at groups 2, 3, and 4 (13, 22). We calculated "adjusted" *corrected* concentrations of the iron indicators by multiplying the individual values by their group-specific CF (10, 12, 13, 22). Spearman's rank correlations were computed again between the *corrected* iron indicator concentrations and the APPs. We then tested the equality of the two observed correlations (between *uncorrected* indicators and APPs; and between *corrected* indicators and APPs) with Fisher's *z* transformation using PROC CORR with the FISHER option in SAS

(version 9.2; SAS Institute Inc., Cary, NC). The objectives were to check that the effect of inflammation on iron indicators was attenuated by use of the correction factors and to explore whether expected relationships among iron indicators were masked by inflammation. We hypothesized *a priori* that correcting for inflammation would uncover or strengthen the following relationships: 1) a positive relationship between Hb and ferritin and between TfR and ZP and 2) an inverse relationship between Hb and TfR, Hb and ZP, ferritin and TfR, and ferritin and ZP.

We evaluated the extent to which correcting for inflammation changed estimates of iron deficiency by comparing the median values and proportions of abnormal values (i.e. anemia or ID) of the *uncorrected* indicators with those of the *corrected* indicators. We also explored if correcting for inflammation modifies the association between malaria infection and iron status indicators. Finally, we compared the prevalence of ID generated by each of the three approaches generally used to adjust for inflammation effects on iron indicators.

RESULTS

The characteristics of study participants are shown in **Table 1**. Fifty-one percent were males, and the mean (SD) age was 21(9.2) months. Twenty-eight percent reported recent fever whilst the prevalence of malaria parasitemia, all as a result of *P. falciparum*, was 12%. Elevated CRP and AGP were present in 23% and 46% of participants, respectively.

There were positive correlations between malaria parasite density and CRP (r=0.33, p<0.001) and AGP (r=0.26, p<0.001). Children who tested positive for malaria had significantly higher CRP [median (25^{th}, 75^{th} quartiles): 6.6 (1.7, 20.5) vs. 1.1 (0.3, 4.1) *mg/L*, p<0.001] and AGP [median (25^{th}, 75^{th} quartiles): 1.5 (1.1, 1.8) vs. 1.0 (0.8, 1.3) *g/L*, p<0.001] levels than non- infected children.

There were positive correlations between CRP and AGP; and among CRP, AGP and ferritin (see uncorrected values, **Table 2**). Hb (negatively), ZP (positively), and TfR (positively) correlated with the two acute phase proteins. The relationships between the iron indicators and Hb indicated a strong to moderate inverse (Hb with ZP, TfR, and TfR/ferritin index) correlations but a weak positive correlation with ferritin (see uncorrected values, Table 2).

Geometric mean concentrations of iron indicators were assessed by inflammation status defined by four APP groups: reference, incubating, early and late convalescence. There were significant differences (p<0.05) among groups for all indicators (**Table 3**). As an example, we found significantly higher geometric mean ferritin concentrations in the incubation (27.4), early convalescence (42.1), and late convalescence (23.9) groups than in the reference group (14.9) (all p <0.001). These values translated into correction

factors of 0.54, 0.35, and 0.62, respectively, to correct ferritin values in the three inflammation stages. The use of the CF resulted in attenuation of the correlation coefficients between all iron indicators and the APPs (Table 2). The correlation between CRP and ferritin was reduced from 0.384 to 0.079 after correction ($p<0.001$) and that between AGP and ferritin from 0.388 to 0.041 ($p<0.001$).

Malaria parasitemia was significantly associated with the iron indicators (**Table 4**). The presence of malaria parasitemia was generally associated with lower levels of Hb and TfR/ferritin index but higher levels of ferritin, ZP, and TfR, regardless of whether the indicators had been *corrected* for inflammation.

There were differences in both the median concentrations and the estimated prevalence of anemia and ID in all indicators when the *corrected* and *uncorrected* indicators were compared (**Table 5**). After correcting for inflammation, the ferritin concentration decreased by 6.8*μg/L* or 47% and the prevalence of ID (ferritin <12 *μg/L*) increased by 34%. Applying the correction factor strengthened the expected relationship between hemoglobin concentration and ferritin that was obscured by inflammation ($r=0.095$, $p=0.013$ vs. $r=0.203$, $p<0.001$, before and after correction, respectively) (Table 2). The estimated prevalence of ID varied depending on the approach used to correct for inflammation, particularly for ferritin (**Table 6**). The use of correction factors increased the prevalence of ID (ferritin < 12 *μg/L*) from 26.9 to 40.7%. Exclusion of inflammation cases resulted in prevalence values that ranged from 32 to 40%, and use of a higher ferritin cutoff point (ferritin <30 *μg/L*) resulted in a prevalence of 66%. Taking the ID prevalence of 40.7% obtained by using CFs as the reference, the uncorrected prevalence was 33% lower, and that obtained by using a higher cut-off point was 62% higher.

DISCUSSION

Our data indicate that correcting for the influence of inflammation increases the estimated prevalence of ID in children, particularly when the iron indicator is ferritin. The prevalence of subclinical inflammation as indicated by elevated APPs was high in this sample of preschool children in western Kenya. We applied cutoff values commonly used to facilitate comparison with other studies (6). The proportion of children with malaria parasitemia was 12% and similar to those reported in this setting (19). The elevated levels of AGP and CRP in the current study may be explained by the inflammatory response to malaria as well as other common childhood infections in the population, such as diarrhea, upper respiratory infections and HIV (4, 23). The iron indicators Hb, ferritin, and ZP correlated significantly with the APPs (Table 2).

The concentration of ferritin increases rapidly in parallel to CRP in the first 20 hours after the onset of an acute infection during which AGP levels show only small elevations; and in chronic infection, continues to a maximum level at about 48 hours during which AGP level peaks and CRP declines (4, 9, 22). CRP is therefore a better indicator of how early inflammation influences ferritin and other iron indicators such as ZP and Hb, while AGP may better predict this influence in the latter infection stages (5, 10-13). Thus, both CRP and AGP levels should be measured in studies, since they identify different but overlapping groups of people with respect to infection status in a community (6, 22). However, our data support that AGP is more important in its association with the various iron indicators we assessed.

A correction factor (CF) was used in this study to correct for the effect of inflammation on iron indicators and thus adjust the estimation of the prevalence of ID in preschoolers in an area of high inflammation without discarding data (i.e. cases with elevated APPs), altering the cutoff levels for low ferritin values or masking potentially important associations (22). Various studies have proposed the usefulness of CFs in adult populations with little or no evidence of its usefulness in preschool children (10-13, 22). Some investigators have argued that in areas of high inflammation, there may not be enough children to form a reference group to enable the estimation of reliable CFs (5). However, in our study this was not an issue since about one-third of the sample did not show inflammation (i.e. APPs were not elevated). At incubating and convalescence stages, ZP and ferritin levels were high; an indication of their sensitivity to the influence of inflammation. During an acute infection, redistribution of iron into the liver and mononuclear phagocyte system mediated by cytokines may occur resulting in increased ferritin levels and low serum iron (4). This limited iron supply to developing red cells results in excess ZP formation as a by-product of the heme biosynthetic pathway (3). In our study, ferritin and ZP levels increased about 3 and 1.5 fold respectively in the early convalescence (both elevated CRP and AGP), compared to the reference group. Similar trends for ferritin levels were observed among HIV positive adults in Kenya (11), in young Zanzibari children (5), and in Indonesian infants (6).

In our study, the proportions of children in the incubating, early convalescence, and late convalescence groups were 1.6%, 22% and 33%, respectively. Similar percentages by group were made in Pakistani preschoolers (20) and by Thurnham et al

(22). This is because the incubation period is very short (~48 hours), and a high intensity of infection is required for numbers in this group to be large; whilst the late convalescence period is usually longer, especially in situations of poor nutritional status and slow recovery from disease (22). This indicates the presence of chronic infection in the children in our study area, probably due to the seasonality of malaria infection, and anemia due to chronic infection per the low Hb levels in the early and late convalescence acute phase groups in our study. Thus, the seasonal exposure of children to malaria and other infections may have resulted in longer periods of convalescence from previous malaria episodes and other infections in this area (11). This situation is normally reversed in adults who have reduced convalescence periods probably due to a more fully developed humoral immunity and hence quicker recovery from infection (22). Therefore, using CFs from pooled data (e.g., meta-analysis) may be unreliable because of heterogeneity across populations due to differences in age, intensity and burden of infection, location and seasonality that all affect measures of inflammation.

Our results suggest that malaria infection may be a major contributor to the observed elevated APPs, because the geometric mean levels of the *corrected* iron indicators in non-malaria infected children were generally similar to those of the reference group. In malaria endemic areas, most infections in children are due to malaria (19) although intestinal worm infestation, diarrhea, respiratory infections, as well as HIV infection may also contribute their quota (4, 23). Data from this study indicate that malaria infection results in reduced Hb levels and increased ferritin, ZP, and TfR levels. The inflammatory response to malaria is demonstrated by elevated serum levels of the

APPs and ferritin with malaria-associated hemolysis resulting in decreased Hb (19). This influence has important implications for the interpretation of ID. Malaria may also induce ID through reduced iron absorption or iron loss in urine after hemolysis, as well as sequestration of iron in macrophages of the mononuclear phagocyte system. In our study, most children were either convalescing from malaria or were asymptomatic as evidenced by low fever and malaria parasitemia prevalence.

The relationships between Hb levels and ZP, TfR, and TfR/ferritin index were not significantly affected by the APPs. After correcting for the influence of CRP and AGP on these iron indicators, the inverse correlations between them and Hb levels remained significant. However, this was not the case for ferritin; the weak direct association between Hb and ferritin became stronger after correcting for the influence of the APPs on ferritin, indicating a strong influence of inflammation on ferritin levels as explained. Similar results have been reported elsewhere after adjusting for recent fever, CRP and AGP (5). Thus, in the absence of inflammation, ferritin levels reflect iron stores (5).

As expected, when we assessed the prevalence of ID in the reference group (by discarding data for subjects with inflammation based on both elevated CRP and AGP, as is the practice in most studies), we obtained a prevalence of ID similar to that in the corrected data. However, these reference subjects constitute only 44% of the sample and may not be representative of the population. Often, investigators carry out analyses of the information, such as measuring effects of iron interventions or exploring risk factors for ID. In such circumstances it is not desirable to discard the majority of the data. Since the objective of the use of the CF is to correct the data of the inflamed group and thus restore

the data distribution to that of the community as represented by the reference group, our internally-generated CF produces identical geometric means across the three groups of inflammation (incubation, early and late convalescence). However, as noted, the CF approach retains all cases. The fact that associations among iron indicators are unmasked by the use of CFs suggests that the CF approach generates iron status variables closer to what would have been observed in the absence of inflammation; unfortunately, we do not have a means for testing this directly.

A limitation of the study is its cross-sectional nature and thus we could not estimate how the participants will respond to an iron intervention based on their infection status before and after correcting for such influence. Again, the CFs estimated in this study may not be generalizable or applicable to other populations with different severity and frequency of infections and malnutrition status; thus the CF may be population-specific. The CF also does not take into account nutritional status, age or other socio-demographic characteristics; however, we did not find any significant differences in wasting prevalence, socioeconomic status or age of the children by inflammation group (data not shown). The strengths of this study include the large sample size which enabled the calculation of reliable CFs in the reference group for correcting the effect of inflammation. We used the ratios of the geometric means of iron indicators in estimating the CFs as suggested by the literature and also because it allows for comparison with future studies in other settings (22). Also we measured a wide range of iron indicators and subclinical inflammation biomarkers, which allowed for the assessment of the influence of inflammation on all available iron biomarkers.

This study showed that the use of a CF can modify the levels and prevalence of anemia and ID, using various iron indicators known to be affected by inflammation. The CF approach helps in retaining all biochemical data from the study population without discarding essential data which may unmask associations between iron indicators. Using the CF approach may help interpret the assessment of iron status in populations with high rates of subclinical inflammation.

The variability across ID prevalences as measured by the different approaches may be important in public health terms (Table 6), especially for ferritin. Our study goes beyond the specific results in proposing the CF approach for addressing the issue of ID measurement in areas of high inflammation.

In conclusion, subclinical inflammation affected all the iron biomarkers (and not only ferritin), and not correcting for such inflammation altered the measures of ID. However, further work is needed to identify whether the CF approach proposed in this study improves the accuracy of assessment of ID.

ACKNOWLEDGMENTS

PSS, LJR, FKEG, RM and RF were responsible for the study design. FKEG, LJR, and PSS were responsible for conducting the study and the data collection. FKEG, PSS, RF, CRC, UR, and RM were responsible for data analysis. All authors were responsible for preparing the manuscript. None of the authors had a personal or financial conflict of interest.

The authors are indebted to the children and their parents who agreed to take part in this study.

This study is dedicated to the memory of Alfredo Obure, the NICHE study coordinator.

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Table 1: Characteristics of study participants (*n*=680)[1, 2]

Characteristic	
Sex, % *male*	51.4
Age, *months*	21.1 ± 9.2
WAZ, % < -2SD	12.4
HAZ, % < -2SD	28.3
WHZ, % < -2SD	5.2
Recent fever, *%*	28.0
Malaria parasitemia, *%*	12.4
Hemoglobin, *g/L*	
Median (IQR)	111.0 (100.0, 119.0)
Mean ± SD	108.6 ± 15.4
Ferritin, *µg/L*	21.2 (11.3, 39.3)
ZP, *µmol/mol* heme	124.3 (89.9, 186.9)
TfR, *mg/L*	9.0 (7.4, 11.3)
TfR/ferritin index	399.0 (210.7, 855.4)
CRP, *mg/L*	0.9 (0.2, 4.6)
AGP, *g/L*	1.0 (0.8, 1.4)

[1]Values are mean ± SD, median (25^{th}, 75^{th} percentiles), and %.

[2]Abbreviations: WAZ (weight-for-age z-scores); HAZ (height-for-age z-scores); WHZ (weight-for-height or length z-scores); ZP (zinc protoporphyrin); TfR (soluble transferrin

receptors); CRP (C-reactive protein); AGP (alpha-1-acid-glycoprotein); TfR/ferritin index (soluble transferrin receptors to ferritin index).

Table 2: Spearman's correlation coefficients relating biomarkers of inflammation to iron-status indicators in preschool children before and after correcting for inflammation (n=680) [1]

	Ferritin	**ZP**	**TfR**	**TfR/ferritin index**	**CRP**	**AGP**
Hb						
Uncorrected	0.095*	-0.637**	-0.559**	-0.239**	-0.268**	-0.289**
Corrected	0.203**	-0.576**	-0.527**	-0.333**	-0.069	-0.044
Ferritin						
Uncorrected	-	-0.300**	-0.284**	-0.946**	0.384**	0.388**
Corrected	-	-0.395**	-0.254**	-0.941**	0.079*	0.041
	-					
ZP						
Uncorrected	-	-	0.755**	0.475**	0.162**	0.244**
Corrected	-	-	0.753**	0.572**	0.107*	0.038
TfR						
Uncorrected	-	-	-	0.530**	0.145**	0.149**
Corrected	-	-	-	0.610**	0.043	0.032
TfR/ferritin index						
Uncorrected	-	-	-	-	-0.298**	-0.295**
Corrected	-	-	-	-	-0.059	-0.040
CRP	-	-	-	-	-	0.647**

[1] Values are Spearman's correlation coefficient. Abbreviations: Hb (hemoglobin), ZP (zinc protoporphyrin); TfR (soluble transferrin receptors); CRP (C-reactive protein); AGP (alpha-1-acid-glycoprotein); TfR/ferritin index (soluble transferrin receptors to ferritin index); *r* (Spearman's correlation coefficient).

* P<0.05, ** P <0.001.

Table 3: Estimation of correction factors using ratios of geometric mean of iron indicators by inflammatory status*

Biomarker	Concentration (Geometric mean ±SD)	Inflammation versus reference group	Ratio (95% CI)[1]	Correction factor[2]
Hemoglobin				
Reference	110.7 ± 11.5			
Incubation	111.2 ± 10.9	Incubation vs. reference	0.99 (0.87, 1.13)	1.01
Early convalescence	98.9 ± 12.2	Early convalescence vs. reference	0.89 (0.86, 0.93)	1.12
Late convalescence	108.4 ± 11.5	Late convalescence vs. reference	0.97 (0.94, 1.02)	1.03
Ferritin				
Reference	14.9 ± 2.2			
Incubation	27.4 ± 1.5	Incubation vs. reference	1.84 (0.88, 3.86)	0.54
Early convalescence	42.1 ± 2.7	Early convalescence vs. reference	2.83 (2.22, 3.61)	0.35
Late convalescence	23.9 ± 2.4	Late convalescence vs. reference	1.61 (1.30, 1.99)	0.62
ZP				
Reference	126.2 ± 1.7			
Incubation	113.3 ± 1.6	Incubation vs. reference	0.90 (0.56, 1.43)	1.11
Early convalescence	165.8 ± 1.7	Early convalescence vs. reference	1.31 (1.13, 1.53)	0.76
Late convalescence	130.4 ± 1.7	Late convalescence vs. reference	1.03 (0.90, 1.18)	0.97

TfR				
Reference	9.2 ± 1.5			
Incubation	8.9 ± 1.3	Incubation vs. reference	0.97 (0.69, 1.35)	1.03
Early convalescence	10.6 ± 1.6	Early convalescence vs. reference	1.15 (1.03, 1.28)	0.87
Late convalescence	9.6 ± 1.4	Late convalescence vs. reference	1.04 (0.94, 1.14)	0.96
TfR/ferritin index				
Reference	619.6 ± 2.7			
Incubation	325.3 ± 1.6	Incubation vs. reference	0.53 (0.22, 1.28)	1.89
Early convalescence	251.2 ± 3.3	Early convalescence vs. reference	0.41 (0.30, 0.54)	2.44
Late convalescence	400.2 ± 2.7	Late convalescence vs. reference	0.65 (0.50, 0.84)	1.54

* Sample sizes per inflammatory groups: Reference, n=299; Incubation, n=11; Early convalescence, n=147; and Late convalescence, n=223.

[1] Geometric mean ratio that compares the iron biomarker concentration at each inflammatory stage with that of the reference group (22).

[2] Correction factors were estimated from the ratios of the geometric mean as explained in reference (22). Example: ferritin correction factor at *incubation* stage (i.e. incubation vs. reference) = 1.0/1.84= 0.54; *Early convalescence* stage=1.00/2.83=0.35; and *Late convalescence* stage=1.00/1.61= 0.62.

Table 4: Association of malaria infection with iron indicators in preschool children*

Iron status indicators	*Uncorrected*			*Corrected*		
	Malaria			**Malaria**		
	Present	**Absent**	***p*[1]**	**Present**	**Absent**	***p*[1]**
Hemoglobin, *g/L*	96.7±16.5 (*84*)	110.2±14.5 *(596*)	<0.001	103.3±16.4 *(84*)	113.7±14.9 *(596*)	<0.001
Ferritin, *µg/L*	28.5±2.9 (*84*)	21.2±2.5 (*596*)	0.019	25.2±2.4 (*84*)	13.9±2.3 (*596*)	<0.001
ZP, *µmol/mol* heme	182.7±1.6 (*84*)	129.5±1.7 (*596*)	<0.001	147.4±1.6 (*84*)	119.0±1.7 (*596*)	0.001
TfR, *mg/L*	10.8±1.5 (*84*)	9.4±1.5 (*596*)	0.008	11.4±1.6 (*84*)	8.9±1.4 (*596*)	<0.001
TfR/ferritin index	378.9±3.5 (*84*)	444.7±2.9 (*596*)	0.267	450.6±3.0 (*84*)	648.3±2.8 (*596*)	0.005
CRP	4.8±5.3	1.02±6.4	<0.001	--	--	--
AGP	1.4±1.4	1.04± 1.4	<0.001	--	--	--

*Values are geometric mean±SD except hemoglobin, with *n* in parenthesis.

[1] Student's *t* test: contrasting mean concentrations of iron status indicators between children with malaria and those without malaria.

Table 5: Comparison of median concentrations and proportions with abnormal values of *uncorrected* and *corrected* iron-status indicators (*n*= 680)*.

Biomarker			***p*[2]**	**Abnormal value threshold**	**Proportion abnormal[3]**	***p*[4]**
Hemoglobin (g/L)	*Uncorrected*	111.0 (100.0, 119.0)[1]	<0.001	<110*g/L*	46.0 (313)	<0.001
	Corrected	114.0 (104.2, 122.1)			37.4 (254)	
Ferritin (*µg/L*)	*Uncorrected*	21.2 (11.3, 39.3)	<0.001	<12 *µg/L*	26.9 (183)	<0.001
	Corrected	14.4 (8.4, 25.5)			40.7 (277)	
ZP (*µmol/mol*)	*Uncorrected*	124.3 (89.9, 186.9)	<0.001	>80 *µmol/mol*	82.8 (562)	<0.001
	Corrected	116.4 (85.7, 174.4)			79.9 (543)	
TfR (*mg/L*)	*Uncorrected*	9.0 (7.4, 11.3)	<0.001	>8.3 *mg/L*	61.2 (416)	<0.001
	Corrected	8.7 (7.1, 10.7)			56.6 (385)	
TfR/ferritin index	*Uncorrected*	399.0 (210.7, 855.4)	<0.001	>500	43.1 (293)	<0.001

Corrected	578.7 (307.3, 1117.5)	55.9 (380)

* Correction of data achieved using CFs estimated from the ratios of geometric means.

[1] Values are *Median (25^{th}, 75^{th} percentiles)*

[2] Paired Wilcoxon test of medians.

[3] Percent with *n* in parenthesis.

[4] McNemar's chi-square of proportion with abnormal iron-status values and anemia.

Table 6: Comparison of estimated prevalence of ID in the presence of inflammation based on different methods (n=680)[1]

Iron status indicator	**Full Sample (Uncorrected)** ***n*=680**	**Full Sample (CF approach, this study)** ***n*=680**	**Exclusion of inflammation cases: elevated CRP and AGP (CRP ≤ 5mg/L; AGP ≤ 1g/L)** ***n*=299**	**Exclusion of inflammation cases: elevated CRP only (CRP ≤ 5mg/L)** ***n*=522**	**Exclusion of inflammation cases : elevated AGP only (AGP ≤ 1g/L)** ***n*=310**
Ferritin < 12 *µg/L*	26.9 (183)	40.7 (277)	39.8 (119)	32.0 (167)	38.4 (119)
ZP >80 *µmol/mol*	82.8 (562)	79.9 (543)	79.6 (238)	81.1 (424)	79.4 (247)
TfR >8.3 *mg/L*	61.2 (416)	56.6 (385)	55.5 (166)	60.0 (313)	55.2 (171)
TfR/ferritin index >500	43.1 (293)	55.9 (380)	56.2 (168)	49.0 (256)	54.8 (170)

[1] Values are percent with *n* in parenthesis.

CHAPTER 5

The assessment of iron deficiency in Kenyan children from capillary blood

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Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Reprints are not available.

This work is supported in part by an Ellison Medical Foundation / Nevin Scrimshaw International Nutrition Foundation fellowship (FKEG), and the Centers for Disease Control and Prevention (PSS).

Running head: Assessment of iron deficiency in Kenyan preschoolers

ABSTRACT

Background: In the absence of a feasible, non-invasive gold standard, the stages of iron deficiency (ID) are best characterized by the use of multiple indicators. However, the choice of an appropriate single iron biomarker to replace the multiple-criteria model for screening for ID at the population level in resource poor and remote field settings continues to be debated.

Objective: We compared ID defined as ≥2 of 3 abnormal values in ferritin, soluble transferrin receptor (TfR), or zinc protoporphyrin (ZP) concentrations (i.e. multiple-criteria model) to ID defined by abnormal levels in any of the independent candidate iron status biomarkers (ferritin alone, or TfR alone, or ZP alone), body iron stores (ID, < 0 *mg/kg*.) and TfR/ferritin index (ID, >500). Values were adjusted for inflammation, as measured by C-reactive protein (CRP >5mg/L) and alpha-1-acid-glycoprotein (AGP >1g/L) before applying cut-offs for ID.

Design: This was a community-based cluster survey in which capillary blood was obtained from 680 children (aged 6–35 mo) for measurement of iron status using ferritin, TfR, and ZP. Based on the multiple-criteria model, ID was defined as ≥2 of 3 abnormal values in ferritin (<12 µg/L), TfR (>8.3 mg/L), or ZP (>80 µmol/mol).

Results: Based on the multiple-criteria model, ID prevalence (±SE) was 62.1±1.9%, whilst based on abnormal values in ferritin, TfR, ZP, body iron, and TfR/ferritin index were 40.7±1.9%, 56.6±1.9%, 79.9±1.6%, 34.0±1.8%, and 55.9±1.7%, respectively. The κ statistics for agreement between the multiple-criteria model and the other iron status

indicators ranged from 0.5 to 0.8; with TfR having the best agreement (κ=0.84) with the multiple-criteria model. The positive predictive values (proportion ±SD) of ID based on TfR, ferritin, and ZP, in predicting ID based on the multiple-criteria model were 0.97±0.02, 0.93±0.02, and 0.78±0.02, respectively; the negative predictive values were 0.85±0.02, 0.59±0.02, 0.94±0.02, respectively. Receiver-operating characteristic (ROC) curve analysis demonstrated that TfR (area-under-the-curve (AUC) = 0.69) was superior to ferritin (AUC=0.61), and ZP (AUC=0.63) in predicting iron deficiency anemia ($p<0.001$).

Conclusion: Soluble transferrin receptor (TfR), compared to ferritin and ZP, more accurately estimates the prevalence of ID in preschool children based on multiple-indices in a high inflammation, resource-poor setting.

INTRODUCTION

Anemia, mainly caused by iron deficiency (ID), globally affects up to 60% of children less than 48 months, with the highest prevalence found in developing countries (1, 2). Several adverse effects on health and development have been attributed to ID without anemia, including reduced cognitive, mental and physical functions in children, and increased perinatal and maternal mortality (3-7). In children, ID can be prevented and treated with increased intake of iron. This may include consumption of high iron-content foods, fortification of common staple and complementary foods, or provision of therapeutic doses of iron in supplements. As a consequence of the current WHO iron supplementation guidelines, a non-invasive screening tool for ID is urgently needed (8, 9). The use of feasible approaches for screening for ID at the population level in high inflammation, resource-poor and remote field settings continues to be a critical need (10, 11). Iron deficiency anemia (IDA) is generally characterized by three stages: depletion of iron stores reflected by a fall in serum ferritin concentration; iron-deficient erythropoiesis reflected through increased soluble transferrin receptors (TfR) and zinc protoporphyrin / heme ratio concentrations (ZP) and; anemia reflected by reduction in hemoglobin concentration (Hb) due to restricted supply of iron to the bone marrow for erythropoiesis (12). In the absence of a non-invasive or feasible gold standard, these stages are best characterized by the use of multiple-criteria indicators as well as the body iron model and the TfR/ferritin index (13). Iron status estimates are potentially improved through the use of a combination of several (at least 3) tests of iron status rather than a single indicator. The presence of two or more abnormal values indicates impaired iron status (12, 13). In developing country field-settings however, multiple-criteria models may be problematic

due to the need for venous blood collection, which is difficult to do in children, and the presence of inflammation, which may adversely alter results of ID (11). Some workers have thus investigated the feasibility of capillary blood for assessing iron status in field-studies for a number of analytes such as ferritin (14), ZP (15), and TfR (11). Measures of inflammation from capillary blood are also required to assess their effect on these iron biomarkers.

The goal of this study is the identification of a simple, inexpensive, single tool for assessing ID as compared to the multiple-criteria model (ferritin, TfR, and ZP). This single test must approximate the multiple-criteria model in its ability to detect modifications in iron status of preschool children in areas of high infection burden if it is to replace the multiple-criteria model. The costs of laboratory assessments are similar for ferritin, TfR, and to some extent ZP and are a function of the number of individuals and indicators measured. A further desirable characteristic is that the indicator be the least affected by inflammation as indicated by CRP and/or AGP. The objectives of this study were to: 1) assess the diagnostic efficiency of 3 independent iron status tests (ferritin, TfR, or ZP), the body iron model and the TfR/ferritin index by comparing the prevalence of ID in children aged 6-35 months as defined by ≥2 of 3 abnormal values in ferritin, TfR, or ZP concentrations (i.e. multiple-criteria model) to ID defined by abnormal levels in any of the independent candidate iron biomarkers (ferritin alone, or TfR alone, or ZP alone) or body iron model and TfR/ ferritin index; and 2) determine the predictive value of the above iron indicators in identifying anemia among preschool-age children in areas of high inflammation burden.

SUBJECTS AND METHODS

Study area and study population

The data for the current study are from the Nyando Integrated Child Health and Education (NICHE) project (16, 17). The data were obtained by a cross-sectional survey of children 6-35 months of age in 60 randomly selected villages from Nyando Division (population 80,000) in Nyanza Province of western Kenya from March to May 2009. After developing community maps and a complete household census, the clusters (villages) were selected. Children were selected by simple random sampling from each village, following the census. Children were selected if they were 6-35 months of age at the time of enrollment and lived within the catchment area of the study. Children with hemoglobin concentrations (Hb) < 70 g/L were referred to the nearest clinic for treatment of severe anemia, but were still approached for enrollment.

Children were excluded if they were unavailable for enrollment on 3 separate household visits or if their parents refused to give informed consent. Data were recorded in the field using Dell Axim personal digital assistants (PDA) and downloaded into an Access 2007 database on a daily basis.

Written informed consent was obtained from all participating households. The Ethics Committee of the Kenyan Medical Research Institute (KEMRI) in Nairobi, Kenya (protocol 1176) and the Institutional Review Board of the Centers for Disease Control and Prevention (CDC) in Atlanta, GA (protocol 5039) approved the study. The study is registered with ClinicalTrials.gov (Identifier NCT01088958).

All children participating in the survey, whether febrile or not, had thick and thin malaria smears made from capillary finger-sticks. Reports of fever 24-hours prior to the interview were obtained through caregiver recall.

Laboratory analysis

Capillary blood was obtained from children 6-35 months by trained laboratory technicians with single-use sterile micro-lancets (Becton Dickinson, Franklin Lakes, NJ) into a purple top microtainer capillary blood collector with EDTA (Becton Dickinson, Franklin Lakes, NJ) to prevent clotting. Hemoglobin concentration was determined within three minutes of blood collection using the HemoCue® B-Hemoglobin machine (Ängelholm, Sweden). Two drops of blood were placed on a microscope slide (Thermo Fisher Scientific Inc, Waltham, MA) for thick and thin malaria smears for detection of malaria parasitemia. An additional 400-500 μL of capillary blood was collected into a microtainer with EDTA (Becton Dickinson, Franklin Lakes, NJ). The blood was transported on ice to the KEMRI/CDC laboratory in Kisian, Kenya, where a drop of whole blood was transferred from microcontainers to a disposable glass cover slip (Aviv Biomedical, Lakewood, NJ) using a pipette with a disposable tip, to assess ZP. The rest of the blood was centrifuged at 1500 x *g* for 5 minutes at 27ºC, and plasma was removed and stored in cryovials (Thermo Fisher Scientific Inc, Waltham, MA) at -20ºC within 12 hours of blood collection. The plasma samples were transported to Germany for subsequent laboratory analysis of ferritin, TfR, alpha-1-acid-glycoprotein (AGP) and C-reactive protein (CRP), using a simple sandwich enzyme-linked immunosorbent assay

technique (22). The acute phase proteins, AGP and CRP, were used to identify children with infection and inflammation that could confound measures of iron status especially, ferritin and ZP (11, 19, 20). Body iron stores were estimated based on TfR and ferritin concentrations as: Body iron stores = - (LOG_{10} (TfR/ (ferritin/1000))-2.8229)/0.1207 (24). All indicators were measured twice and the average of the duplicate measures used; the intra- and interassay coefficients of variation were less than 10%.

Anemia was defined according to the World Health Organization threshold as Hb< 110 g/ L for children 0.5-4.99 years (1). The HemoCue machines were calibrated and checked for accuracy twice daily (morning before fieldwork and evening after fieldwork) using Hemocontrols and control cuvettes respectively, records of which were kept in a secured place. Zinc protoporphyrin was measured at the laboratory of the KEMRI/CDC in Kisian, Kenya, with the Aviv ZP Hematofluorometer (Aviv Biomedical, Lakewood, NJ). The hematofluorometer was standardized daily with control solutions provided by Aviv Biomedical. We applied a correction factor to the results of the ZP on the advice of the manufacturers and the CDC quality assurance lab. A cutoff value of 80 µmol/mol heme was used to indicate elevated ZP level and hence iron deficiency (21).

The thresholds for defining abnormal values for the above biochemical indicators were as follows (23): ferritin, <12 µg/L; TfR, >8.3 mg/L (TfR®, Ramco Laboratories, Inc., Houston, TX); body iron stores, < 0 mg/kg (24); CRP, >5 mg/L; and AGP, >1.0 g/L. For the multiple-criteria model, ID was considered present if individuals had two or more abnormal values from among ferritin, TfR, and ZP (12, 13). The ratio of TfR to ferritin

(TfR/ferritin index) was calculated by dividing TfR (μg/L) by ferritin (μg/L), with elevated TfR/ferritin index defined as > 500.

Statistical analysis

We used SAS 9.2 (SAS Institute Inc., Cary, NC) for all statistical analyses. Statistical significance was defined as $p < 0.05$. Plasma ferritin, TfR, and ZP were found to have non-Gaussian distributions when they were assessed for linearity using plots and Kolmogorov-Smirnov tests and were therefore log-transformed for all statistical analyses and reported as geometric means and standard deviations or as medians and interquartile ranges. The analyses for this study were carried out for 680 children who had all biochemical data available. There were no statistically significant differences in demographic characteristics between excluded (n=32) and included children ($p>0.05$) for this analysis.

The iron biomarkers (Hb, ferritin, TfR, and ZP) used for this study were corrected for the effect of inflammation as described previously (25). In summary, these iron indicators were observed to be significantly correlated with inflammation biomarkers (CRP and AGP) and thus correction factors were calculated to attenuate such relationships. Corrected concentrations of the iron indicators were estimated by multiplying the concentrations by their appropriate correction factors. These corrections were done before applying the cut-offs for the iron biomarkers.

To identify the best single estimate of ID, the proportion of children that were iron deficient based on the multiple-criteria model (defined as ≥2 of 3 abnormal values in

ferritin, TfR, or ZP), the standard, was compared using chi-square and fisher tests to the proportion of children with abnormal values for each of the 3 independent tests (i.e. ferritin alone, or TfR alone, or ZP alone), body iron stores and the TfR/ferritin index.

Kappa statistics, K, were computed to assess the extent of agreement between estimates of ID prevalence on the basis of the multiple-criteria model and each of the 3 independent tests, body iron stores and the TfR/ferritin index using PROC FREQ with the AGREE option in SAS 9.2 (SAS Institute Inc., Cary, NC). We characterized K> 0.75 as indicating excellent agreement, 0.40-0.75 as fair to good agreement, and <0.40 as poor agreement (26).

The relative accuracy of the 3 individual tests, the body iron model and the TfR/ferritin index in predicting ID based on the multiple-criteria model was assessed by examining the sensitivity, specificity, positive and negative predictive values of the various indicators. Also, we applied receiver-operating characteristic (ROC) curves for the diagnosis of anemia using the 3 different independent tests, body iron model and the TfR/ferritin index.

RESULTS

Approximately equal proportions of boys and girls were enrolled in this study with mean (SD) age of 21.1 (9.2) months (**Table 1**). The prevalence of malaria parasitemia and reported fever within 24-hours were 12% and 28%, respectively. Mean levels of Hb, ferritin, and body iron stores were within normal ranges, whereas mean levels of ZP, TfR and TfR/ferritin index were higher than expected. The prevalence of ID as assessed by the multiple-criteria model was significantly different from those estimated by the TfR/ferritin index, the body iron model, ferritin, TfR, and ZP ($p<0.001$). Elevated CRP and AGP were present in 23% and 46% of participants, respectively.

The Κ statistics for agreement between the multiple-criteria model and the other iron indicators in the estimation of ID prevalence among the preschool children ranged from 0.5 to 0.8, i.e., fair to excellent agreement with the combined model (**Table 2**).

The sensitivity for identifying ID (proportion ± SE) as defined by the multiple-criteria model was greater for ZP (0.98±0.01) and TfR (0.90±0.01), but least for TfR/ferritin index (0.78±0.02), body iron model (0.67±0.02), and ferritin (0.60±0.02) (**Table 3**); the specificity for identifying ID was however higher in TfR, body iron model and ferritin, but least in TfR/ferritin index and ZP.

The ROC curves for the various iron indicators were used to predict ID as defined by the multiple-criteria model (**Figure 1**). The area-under-the-curve, AUC, values (95% CI) indicated that the diagnostic accuracy of TfR (0.927 (0.908, 0.946)) in predicting iron deficiency was superior to that of the other iron status indicators ($p<0.001$).

The ability to accurately diagnose anemia on the basis of the 3 independent iron indicators, the body iron model and the TfR/ferritin index were compared (**Table 4**). The proportion of anemic children who were correctly diagnosed using the iron indicators, i.e., positive predictive value, was greatest for TfR (51%) and similar to that of body iron model (50%) and ferritin (49%), but appears to differ from that of ZP (44%) and TfR/ferritin index (43%). The area-under-the-curve (AUC) values indicated that TfR was the best predictor of anemia among these preschool children in comparison to the other iron indicators ($p<0.01$).

There was an increased risk of anemia among children with low body iron stores, low ferritin, elevated TfR, and elevated ZP than in children with adequate levels of body iron, ferritin, and normal TfR and ZP values; however, significantly greater risks were only observed for TfR and ZP indicators (**Table 5**).

DISCUSSION

Multiple criteria indicators that require a combination of at least 3 tests of iron status are used to characterize the stages of iron deficiency anemia (IDA) (12, 13). Recent assay techniques allow for the measurement of these indicators using small quantities of blood from capillary finger-sticks (11, 14, 15, 22). Costs per test are US$8-26 for ferritin, US$8-30 for TfR, US$1-6 for ZP, US$6-22 for CRP, and US$2-10 for AGP; however, the costs of all these instruments are high. Venous versus capillary blood testing cost differences are minimal for these indicators. Thus, it is more expensive to measure all three indicators required for the multiple-criteria model (US$24-90) versus just one indicator (US$1-30) (22). Therefore, the identification of a single iron indicator that provides similar information as the multiple-criteria model will be cost-effective and hence, is of importance.

The prevalence of ID in this preschool population ranged from 34% to 80% based on the different iron indicators used. The prevalence of ID was highest for ZP and least for the body iron model, as reported by others (13). The high ID prevalence by ZP may be due to interfering substances in the plasma produced by hemolysis and inflammation, which can increase ZP concentrations 3-4-fold in the absence of ID (29). Further, the specificity of ZP in identifying ID may be limited by increased blood lead levels, hemolytic anemias, malaria, or hemoglobinopathies (21, 29).

The prevalence of anemia was 43% compared with 69% reported in the same setting among 2-36 mos old children by Verhoef *et al* (19). Although similar methods for obtaining blood as well as similar cutoffs for hemoglobin were applied in both studies,

the children were younger and the prevalence of malaria was greater than in our study (18% vs. 12%). The lower prevalence of malaria, and consequently of anemia, in our study is probably due to the existence of an active control program (19).

The Kappa statistics, κ, for agreement between the multiple-criteria model and the other iron indicators for ID was fair to excellent; TfR had the best agreement with the multiple-criteria model, whilst ferritin had the least agreement with the multiple-criteria model. This confirms results from earlier studies that indicated TfR to be a more sensitive index of IDA and preanemic iron deficiency eryrthropoeisis (11, 19, 20, 37, 38).

The trans-membrane glycoprotein, TfR, is expressed on cell surfaces and regulated by post-transcriptional regulation of the iron-mediated iron-reactive element. It is important for iron uptake of the cell; expression of TfR levels increases during iron deficiency and decreases when there is iron overload. Body iron status can thus be reflected in TfR cellular uptake of iron (28). Transferrin receptor as an indicator has many strengths; it appears to be a specific indicator of iron deficiency erythropoeisis not significantly confounded by inflammation although it may be influenced by malaria, age and ethnicity (29). Additionally, it has been shown to be more sensitive than ZP in detecting functional ID (28) with greater changes being observed earlier in TfR levels compared to other iron indicators (30), and more reliable in reflecting early-stage tissue ID (31). However, lack of international cutoffs as well as its assessment by different assays inhibits direct comparison of TfR values between studies (29).

Plasma ferritin, an indicator of iron stores in healthy individuals, is an acute phase reactant and is affected by inflammation. However, for this study we adjusted for

the effect of inflammation using the correction factor method (25, 32-34) to reduce the potential confounding effects of inflammation. The low prevalence of ID based on ferritin in this study may have been found in children with depleted iron stores who were yet to progress to iron deficient erythropoeisis (35).

The results in the present study support the finding that TfR, compared to ZP, ferritin, and body iron model and TfR/ferritin index, is more strongly associated with the multiple-criteria model as an indicator of ID. Fewer children were misdiagnosed as iron deficient when TfR was used compared to ZP and ferritin. We used ROC curves to evaluate the predictive value of each of the candidate iron indicators for identifying anemia. Although we found a significant association between low ferritin, low body iron, elevated TfR/ferritin index, elevated TfR, and elevated ZP with anemia; ferritin, TfR/ferritin index and body iron model had low sensitivities for predicting anemia compared to TfR and ZP. Thus, 46%, 39% and 32% of the anemic children would not have been identified on the basis of the ferritin, body iron model and TfR/ferritin index cutoffs, respectively compared to 18% for TfR.

Our results show that children with abnormal iron status values were more likely to be anemic; however, significantly greater risks were observed only for TfR/ferritin index, TfR and ZP. Children with abnormal levels of TfR/ferritin, TfR and ZP were 2 to 4 times more likely to be anemic compared to those with normal values. This indicates that TfR/ferritin, TfR and ZP may be good predictors of anemia. Similar results were obtained for TfR among US preschoolers but only when a cutoff of >10mg/L was applied (27).

Although the body iron model is being considered to replace the ferritin model (which defines ID as an abnormal value of at least 2 or 3 indicators [ferritin, erythrocyte protoporphyrin, and transferrin saturation] (36)) in the estimation of ID among the US population (13), in our study of African preschool children the body iron model was only better than ferritin alone in predicting ID based on the multiple-criteria model. Both TfR and ZP performed better than the body iron model in estimating ID based on the multiple-criteria model.

A limitation of our study was that blood lead and hemoglobinopathies (e.g. sickle-cell anemia, thalasemias) were not measured, which may have confounded the ZP levels. The strengths of the study include the use of CRP and AGP to address potential confounding by inflammation in ZP and ferritin measurements. Additionally, we used field-friendly capillary blood methods to minimize discomfort among the children and to measure multiple iron status indicators from capillary blood using the sandwich ELISA technique that requires a small volume of plasma.

Our study showed that plasma TfR, obtained from capillary blood, can accurately estimate ID based on the multiple-criteria model when screening for ID in preschool children in high inflammation settings prior to an iron intervention. Further, TfR was least affected by inflammation compared to ferritin and ZP. In fact, the American Academy of Pediatrics (AAP), the WHO, and the European Society for Pediatric Gastroenterology, Hepatology and Nutrition also support the use of TfR for ID screening in children once the method has been validated and normal assay values established (38).

In conclusion, in this high inflammation setting, our study indicates excellent agreement between TfR and the multiple-criteria model for identifying prevalence of ID in preschoolers; and that TfR misdiagnosed the least percent of children as iron deficient based on the multiple-criteria model compared to the other indicators (body iron model, ferritin, ZP and TfR/ ferritin index). Additionally, TfR was least affected by inflammation and the most accurate predictor of anemia compared to the other iron indicators.

ACKNOWLEDGMENTS

PSS, LJR, FKEG, RF and RM were responsible for the study design. FKEG, LJR, and PSS were responsible for conducting the study and the data collection. FKEG, PSS, RF, CRC, UR, and RM were responsible for data analysis. All authors were responsible for preparing the manuscript. None of the authors had a personal or financial conflict of interest.

The authors are indebted to the children and their parents who agreed to take part in this study.

This study is dedicated to the memory of Alfredo Obure, the NICHE study coordinator.

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Table 1: Characteristics and iron status of study participants (*n*=680)[1]

Characteristics	
Sex, *% male*	51.4
Age, *months*	21.1 ± 9.2
Recent fever, *%*	28.0
Malaria parasitemia, *%*	12.4
CRP > 5*mg/L*, *%*	23.0
AGP > 1*g/L*, *%*	46.0
Hemoglobin, *g/L*	113.0 ± 17.5
Plasma ferritin, *µg/L*	14.9 ± 2.3
Transferrin receptor, *mg/L*	9.3 ± 1.5
Zinc protoporphyrin, *µmol/mol heme*	122.7 ± 1.7
Body iron stores[2], *mg/kg*	1.5 ± 0.15
TfR/ferritin index	635.3 ± 2.81
Prevalence of anemia and iron deficiency[3]	
Anemia[4]	42.8 ± 2.3
Multiple-criteria model[5]	62.1± 1.9
Body iron model[6]	34.0 ± 1.8
Low ferritin, < 12 *µg/L*	40.7 ± 1.9
Elevated TfR, > 8.3 *mg/L*	56.6 ± 1.9
Elevated ZP, > 80 *µmol/mol*	79.9 ± 1.6

TfR/ferritin index, >500	55.9 ± 1.7

[1]Values are geometric means ± SD for ferritin, soluble transferrin receptors, zinc protoporphyrin, and TfR/ferritin index; arithmetic means ± SD for age, hemoglobin, and body iron stores; and percent for sex, recent fever, and malaria parasitemia prevalence. All iron status indicator values were corrected for the effect of inflammation using the correction factor approach (25).

[2]Body iron stores = - (LOG_{10} (TfR/ (ferritin/1000))-2.8229)/0.1207 (24).

[3] Values are prevalence ± SE. Significant differences were observed between the combined model and each of the other indicators in assessing the proportions of children with iron deficiency; Chi-square tests, $p<0.001$.

[4]Anemia defined as hemoglobin < 110 *g/L*.

[5] Iron deficiency was considered present if individuals had two or more abnormal values from among ferritin (<12*µg/L*), TfR (>8.3*mg/L*), and ZP (>80*µmol/mol*) (12, 13).

[6] Depleted body iron defined as body iron < 0 *mg/kg*.

Table 2: Agreement between the multiple-criteria model and other indicators to define iron deficiency among preschool-age children[1]

Iron deficiency by	Iron deficiency by multiple-criteria model[2]		K Statistic
	Yes	No	
	%		
Body iron model[3]			
Yes	41.9±2.3 (285)	2.4±0.6 (16)	0.56 (0.50, 0.62)
No	20.2± 1.9 (137)	35.6±2.1 (242)	
Low ferritin, < 12 *µg/L*			
Yes	37.2±2.0 (253)	2.8±0.7 (19)	0.47 (0.41, 0.53)
No	24.9±1.9 (169)	35.2±2.1 (239)	
Elevated TfR, > 8.3 *mg/L*			
Yes	55.9± 2.3 (380)	1.8± 0.6 (12)	0.84 (0.79, 0.88)
No	6.2± 1.0 (42)	36.2± 2.2 (246)	
Elevated ZP, > 80 *µmol/mol*			

Yes	60.5± 2.4 (411)	16.8±1.4 (114)	0.58 (0.52, 0.64)
No	1.5± 0.4 (10)	21.2± 1.9 (144)	
TfR/ferritin index, >500			
Yes	48.4± 2.3 (329)	7.9 ± 1.2(54)	0.55 (0.49, 0.62)
No	13.7± 1.7 (93)	30.0 ± 2.0 (204)	

[1] Values are mean percent ± SEs with *n* in parenthesis or K Statistics with 95% confidence intervals in parenthesis.

[2] Iron deficiency was considered present if individuals had two or more abnormal values from among ferritin (<12*µg/L*), TfR (>8.3*mg/L*), and ZP (>80*µmol/mol*) (12, 13).

[3] Body iron < 0 *mg/kg*. Body iron = - (LOG_{10} (TfR/ (ferritin/1000))-2.8229)/0.1207 (24).

Table 3: Accuracy and predictability of iron deficiency based on the multiple-criteria model using the body iron model, TfR/ferritin index and 3 different independent tests in preschool children

Iron deficiency defined by	Combined model[1]		Accuracy, and predictive values[3]	Percent misclassified[4]
	Yes[2]	No[2]		
Body iron model[5]				%
Yes	41.9±2.3(285)	2.4±0.6 (16)	Ŝe = 0.67±0.02(422)	16.5
No	20.2± 1.9(137)	35.6±2.1(242)	Ŝp = 0.94±0.02(258)	
			PPV = 0.95±0.01(301)	
			NPV = 0.64±0.02(379)	
Low ferritin, < 12 *µg/L*				
Yes	37.2±2.0(253)	2.8±0.7(19)	Ŝe =0.60±0.02(422)	15.1
No	24.9±1.9(169)	35.2±2.1(239)	Ŝp =0.93±0.02(258)	
			PPV =0.93±0.02(272)	
			NPV =0.59±0.02(408)	
Elevated TfR, >8.3 *mg/L*				

Yes No	55.9± 2.3(380) 6.2± 1.0(42)	1.8± 0.6(12) 36.2±2.2(246)	Ŝe =0.90±0.01(422) Ŝp =0.95±0.01(258) PPV =0.97±0.02(392) NPV =0.85±0.02(288)	5.7
Elevated ZP, >80 *µmol/mol* Yes No	 60.5± 2.4(411) 1.5± 0.4(10)	 16.8±1.4(114) 21.2±1.9(144)	 Ŝe =0.98±0.01(421) Ŝp =0.56±0.03(258) PPV =0.78±0.02(525) NPV =0.94±0.02(154)	 8.6
TfR/ferritin index, >500 Yes No	 48.4±2.3 (329) 13.7± 1.3 (93)	 7.9 ± 1.1(54) 30.0±2.1(204)	 Ŝe =0.78±0.02 (422) Ŝp =0.79±0.03(258) PPV =0.86±0.02(383) NPV =0.69±0.03(297)	 16.9

Ŝe, sensitivity = [children with iron deficiency as defined by the single iron indicator test and multiple-criteria model (true positives)/ children with iron deficiency as defined by the single iron indicator test (true positives + false negatives)]; Ŝp,

specificity = [children without iron deficiency as defined by both the single iron indicator test and multiple-criteria model (true negatives)/ children without iron deficiency as defined by the single iron indicator test alone (true negatives + false positives)]; PPV, positive predictive value = [children with iron deficiency as defined by the single iron indicator test and multiple-criteria model (true positives)/ children with iron deficiency as defined by the single iron indicator alone (true positives + false positives)]; NPV, negative predictive value = [children without iron deficiency as defined by both the single iron indicator test and multiple-criteria model (true negatives)/ children without iron deficiency as defined by the single iron indicator test alone (true negatives + false negatives)] (13).

[1]Iron deficiency was considered present if individuals had two or more abnormal values from among ferritin (<12$\mu g/L$), TfR (>8.3mg/L), and ZP (>80$\mu mol/mol$) (12, 13).

[2] Values are mean percent ± SEs with *n* in parenthesis.

[3] Values are proportions ± SDs (*n* in parenthesis).

[4] Percent misclassification = (1 - sensitivity) x (percent iron-deficient children) + (1 - specificity) x (percent iron replete children) (27).

[5] Body iron < 0 *mg/kg*. Body iron = - (LOG_{10} (TfR/ (ferritin/1000))-2.8229)/0.1207 (24).

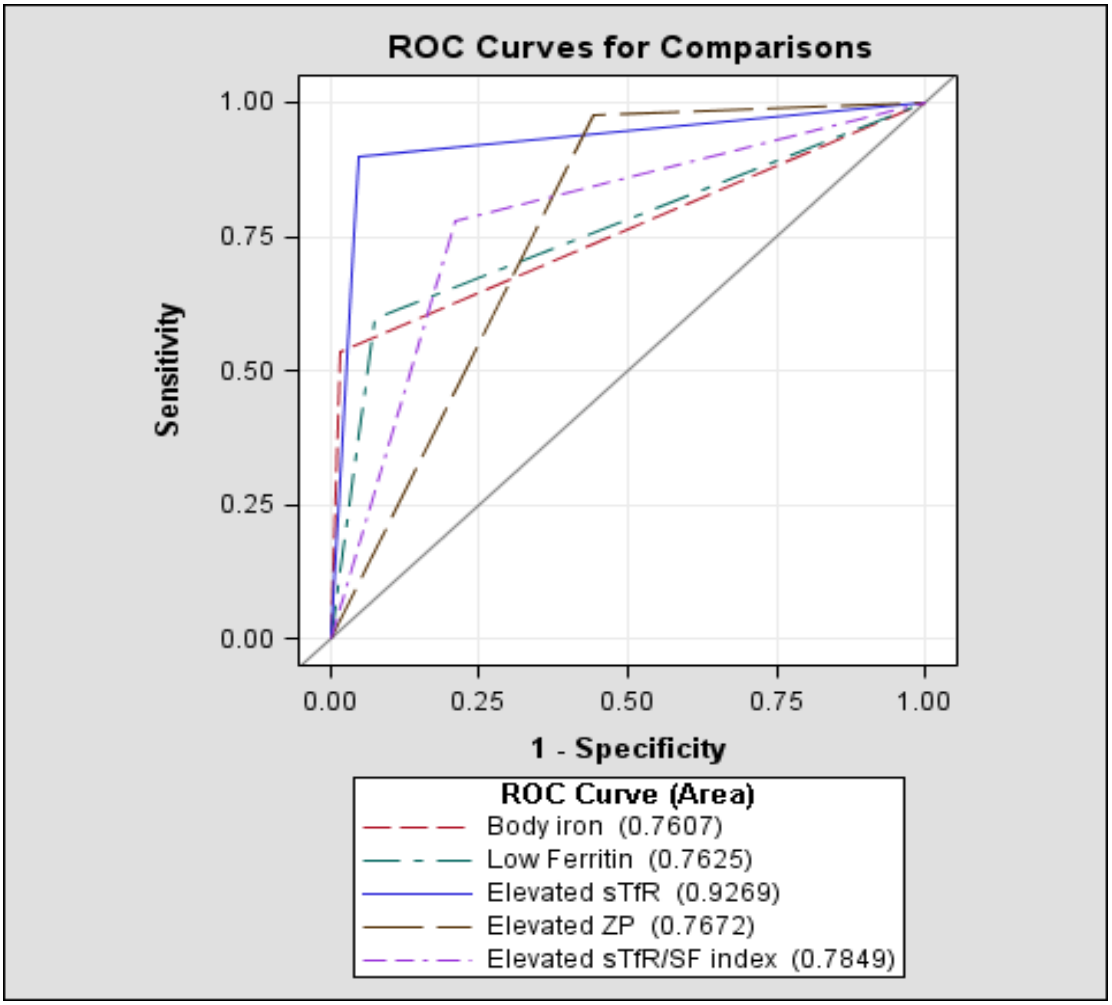

Figure 1: Receiver operating characteristic (ROC) curve analysis for the diagnosis of iron deficiency (ID) (based on the multiple-criteria model) using body iron model and 3 different independent tests. ID was defined per the multiple-criteria model as individual having two or more abnormal values from among ferritin (<12*µg/L*), TfR (>8.3*mg/L*), and ZP (>80*µmol/mol*) (12, 13). Area-under-the-curve (AUC) values (95% CI) for body iron model (< 0 *mg/kg*), low ferritin (<12*µg/L*), elevated TfR (>8.3*mg/L*), elevated ZP (>80*µmol/mol*), and elevated TfR/ferritin index (>500) were 0.761 (0.736, 0.786), 0.763

(0.734, 0.791), 0.927 (0.908, 0.946), 0.767 (0.736, 0.798), and 0.785 (0.753, 0.817) respectively; TfR was the most accurate estimator of ID; $p<0.0001$; $n=679$.

Table 4: Accuracy and predictability of iron status indicators for identifying anemia in preschool children

Iron deficiency by	Anemia[1]		Accuracy and predictive values[3]	AUC[4]
	Yes[2]	No[2]		
Body iron model[5]				
Yes	21.9±1.6(149)	22.4±2.0(152)	Ŝe=0.61±0.03(243)	0.63(0.59, 0.67)
No	13.8±1.6(94)	41.9±2.3(285)	Ŝp=0.65±0.02(437)	
			PPV=0.50±0.03(301)	
			NPV=0.75±0.02(379)	
Low ferritin, < 12 *μg/L*				
Yes	19.4±1.5(132)	20.6±1.7(140)	Ŝe=0.54±0.03(243)	0.61(0.57, 0.65)
No	16.3±1.8(111)	43.7±2.1(297)	Ŝp=0.68±0.02(437)	
			PPV=0.49±0.03(272)	
			NPV=0.73±0.02(408)	
Elevated TfR, >8.3 *mg/L*				
Yes	29.4±2.1(200)	28.2±2.0(192)	Ŝe=0.82±0.02(243)	0.69(0.66, 0.73)

No	6.3±1.0(43)	36.0±2.3(245)	Ŝp=0.56±0.02(437) PPV=0.51±0.03(392) NPV=0.85±0.02(288)	
Elevated ZP, >80 *µmol/mol*				
Yes	33.7±2.3(229)	43.6±2.2(296)	Ŝe=0.95±0.01(242)	0.63(0.61, 0.66)
No	1.9±0.5(13)	20.8±2.0(141)	Ŝp=0.32±0.02(437) PPV=0.44±0.02(525) NPV=0.92±0.02(154)	
TfR/ferritin index, >500				
Yes	24.3±1.6(165)	32.1±2.2(218)	Ŝe =0.68±0.03(243)	0.59 (0.55, 0.63)
No	11.5±1.5(78)	32.2±2.2(219)	Ŝp =0.50±0.02(437) PPV=0.43±0.03(383) NPV=0.74±0.03(297)	

Ŝe, sensitivity = [children with iron deficiency and anemia (true positives)/ children with anemia (true positives + false negatives)]; Ŝp, specificity = [children without iron deficiency or anemia (true negatives)/ children without anemia (true negatives + false positives)]; PPV, positive predictive value = [children with iron deficiency and anemia (true positives)/

children with iron deficiency (true positives + false positives)]; NPV, negative predictive value = [children without iron deficiency or anemia (true negatives)/ children without iron deficiency (true negatives + false negatives)] (13).

[1] Anemia was defined as hemoglobin <110 g/L.

[2] Values are percent ± SEs with *n* in parenthesis.

[3] Values are proportions ± SDs (*n* in parenthesis).

[4] AUC, area-under-the-curve, values are means (95% confidence intervals)

[5] Body iron < 0 *mg/kg*. Body iron = - (LOG_{10} (TfR/ (ferritin/1000))-2.8229)/0.1207 (24).

Table 5: Odds ratios (ORs) and 95% CIs for anemia in children with abnormal values for different iron status indicators, controlling for age and sex of children[1] (*n*=680)

	OR (95% CI)	p-value[7]
Body iron model[2]	1.97 (0.88, 4.44)	0.102
Ferritin[3]	1.55 (0.82, 2.91)	0.176
Transferrin receptors[4]	3.52 (2.29, 5.44)	<0.001
Zinc protoporphyrin[5]	4.24 (2.22, 8.11)	<0.001
TfR/ferritin index[6]	2.44 (1.27, 4.76)	0.008

[1] Anemia was defined as hemoglobin <110 g/L.

[2]Body iron<0 *mg/kg*. Body iron=-(LOG$_{10}$(TfR/(ferritin/1000))-2.8229)/0.1207(24).

[3] Low ferritin, < 12 *µg/L*

[4] Elevated TfR, >8.3 *mg/L*

[5] Elevated ZP, >80 *µmol/mol*

[6] TfR/ferritin index, > 500

[7] Wald Chi-square test.

CHAPTER 6

Selling Sprinkles as part of a health products package may reduce diarrhea incidence but not respiratory illness in preschool children in western Kenya

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Reprints are not available.

This work is supported in part by an Ellison Medical Foundation / Nevin Scrimshaw International Nutrition Foundation fellowship (FKEG), and the Centers for Disease Control and Prevention (PSS).

Running head: Home fortification of children's food may reduce diarrhea incidence

ABSTRACT

Background: Diarrhea and respiratory illnesses are the leading causes of death in children less than 5 years of age. Supplementation with zinc and vitamin A has been associated with reduced duration and severity of diarrhea and respiratory illnesses.

Objective: We measured the association of community-based sales of a multiple micronutrient powder (Sprinkles) with incidence of diarrhea, fever and cough in children 6-59 months.

Design: The study was a cluster-randomized, longitudinal cohort trial in 60 villages for the first 9 months (July 2007-March 2008), and expansion of access to Sprinkles to both study groups in the second 9 months (July 2008-March 2009). Hence, in addition to the experimental analyses using the first year's data, an as-treated analysis to compare users and non-users of Sprinkles across all 60 villages using all available data was carried out. The incidence of diarrhea, cough, and fever were compared between children who used Sprinkles and those who did not. Children's morbidity was self-reported to fieldworkers who interviewed them biweekly for 24 months.

Results: Of the 1079 children enrolled, 78% (n=847) had data on Sprinkles use. Analyses using the experimental design (July 2007- March 2008) suggested null effects on diarrhea and cough. However, children in the intervention villages had 32% reduced fever (relative risk, RR=0.68; 95% CI=0.62, 0.74). Although some baseline characteristics differed between users and non-users in the as-treated analysis, the association of Sprinkles use with morbidity incidence controlled for these variables. Incidence of diarrhea and fever among users was significantly lower than among non-users (0.97 vs.

1.03, p=0.012; and 3.93 vs. 4.66, p=0.040, respectively). Use of Sprinkles was associated with a 30% reduction in diarrhea (relative risk, RR=0.70; 95% CI=0.57, 0.86) and 18% reduction in fever (RR=0.82; 95% CI=0.72, 0.95). There was no association with respiratory illness.

Conclusion: The sale of Sprinkles may reduce diarrhea and fever incidence among children 6-59 months in western Kenya.

INTRODUCTION

Diarrheal disease is the second leading cause of death among children under five globally, and in developing countries it is responsible for an estimated 21% of all deaths in preschool children (1, 2). In western Kenya, diarrhea is the fourth leading cause of health facility visits, the second leading cause of death among infants, and the fourth leading cause of death among children less than 5 years of age (3). Diarrheal diseases impair nutritional status and increase the risk of micronutrient deficiencies such as vitamin A, iron and zinc (4). Micronutrients, especially vitamin A and zinc, are known to reduce diarrheal morbidity and mortality in young children (5-9). Zinc deficiency, which is common in areas of high prevalence of diarrhea and respiratory illness, impairs cellular mediators of innate and acquired immunity which results in increased susceptibility to infection (10, 11). Worldwide, diarrhea and pneumonia are responsible for a combined estimate of 40% of all child deaths annually (12).

However, in most countries, multiple micronutrient deficiencies such as zinc, iron and vitamin A, rather than a single isolated micronutrient deficiency are common (13). This is mainly due to low intake of animal foods and/or poor bioavailability of micronutrients from the local diet. In addition to zinc, iron and vitamin A have also been associated with proper immune function (9, 14). Iron is required for normal immune function due to its growth-promoting role for immune cells and its interference with cell-mediated immune effector pathways and cytokine activities (14). The co-administration of zinc and iron supplementation reduces diarrhea duration among preschool children (15).

Additionally, vitamin A up-regulates T-helper type 2 (Th2) humoral response whilst down-regulating the T-helper type 1 (Th1) cellular response (9). Supplementation of zinc and vitamin A may thus affect the innate and adaptive immune responses which are important for protection against infections (16).

The optimal delivery of multiple micronutrients including vitamin A, iron and zinc through a common vehicle to prevent diarrhea and respiratory illness continues to be urgently needed.

Sprinkles are single-dose packets of dry powder containing lipid encapsulated iron, vitamin A, zinc, and 11 other micronutrients which can be sprinkled onto any home-prepared complementary food, thus, providing a daily dose of essential micronutrients to treat and prevent recurrent anemia in children (17-22). Sprinkles have been shown to be effective against diarrhea and febrile illnesses under experimental conditions (4); however, operational research and program evaluation of Sprinkles use for the prevention of diarrhea and other childhood illnesses is limited. Evidence of the effectiveness of Sprinkles as a delivery vehicle for vitamin A, iron and zinc to control diarrhea and other childhood illnesses is lacking. Thus, our goal was to evaluate the effect of the sale of Sprinkles on the incidence of diarrhea, fever and cough among preschool children in rural western Kenya.

SUBJECTS AND METHODS

Enrollment, intervention, and follow-up procedures

The data for the current study are from the Nyando Integrated Child Health and Education (NICHE) project (23). The children were participants of a cluster-randomized, longitudinal cohort trial in 60 villages which evaluated the effectiveness of the delivery of household-based interventions through local institutions and its potential for increased utilization and improved health outcomes in rural settings of Nyando Division (population 80,000) in Nyanza Province of western Kenya between March 2007 and March 2009. This setting is characterized by endemic malaria transmission, high rates of diarrheal diseases due to poor access to safe water, and anemia (23). A study describing the sales, demand, and promotion of Sprinkles, one of these intervention products, has been published elsewhere (24). Details of the formative research on cultural conceptions of morbidity and study methodology have also been previously reported (25). A two-stage cluster-sampling strategy was used to select 30 intervention and 30 comparison villages and children aged 6-35 months from Nyando Division (24). Children were selected if they were within the ages of 6-35 months at the time of enrollment, and lived within the catchment area of the study. Following a baseline survey in March 2007, Sprinkles distribution was initiated in intervention group households and, from July 2007 through March 2008, biweekly home visits were made to study households to assess product use and occurrence of diarrhea, respiratory illness, or fever. A follow-up survey was conducted in March 2008, Sprinkles distribution was expanded to comparison group households, and biweekly home visits were continued from July 2008 through March

2009. Throughout the study, children with hemoglobin concentrations (Hb) < 70 g/L were referred to the nearest clinic for treatment of severe anemia and children with active diarrhea were given free oral rehydration salts (ORS). Children were excluded if they were unavailable for enrollment on 3 separate household visits or parental refusal to give informed consent.

Written informed consent was obtained from all participating households. The Ethics Committee of the Kenyan Medical Research Institute (KEMRI), Nairobi, Kenya (protocol 1176) and the Institutional Review Board of the Centers for Disease Control and Prevention (CDC), Atlanta, GA (protocol 5039) approved the study. The study is registered with ClinicalTrials.gov (Identifier NCT01088958).

From July 2007- March 2008, Sprinkles were promoted and distributed as part of an integrated health package in 30 intervention villages but not in 30 comparison villages. Beginning in July 2008 the distribution was scaled up to all 60 villages. The composition of each 2g sachet of Sprinkles included encapsulated ferrous fumarate (12.5 mg), Zinc gluconate (5 mg), Vitamin A (375 µg), Iodine (50 µg), Copper (0.6 mg), Vitamin C (35 mg), Vitamin D3 (5 µg) Vitamin B12 (0.9 mg), Vitamin B1 (0.5 mg), Vitamin B2 (0.5 mg), Vitamin B6 (0.5 mg), Vitamin E (6.0 mg), Niacin (6.0 mg), Folic acid (150 mg). Folic acid was removed from the formulation in year 2. Sprinkles were sold retail for 2 KES (~$0.03 US cents) a sachet and promoted for daily use by children 6-59 months of age.

Data were collected by trained fieldworkers from 36 biweekly household monitoring visits to measure household Sprinkles purchases, individual use, and reported

illness which included reported diarrhea, cough/difficulty breathing, and subjective fever in the last 24 hours. All morbidity measures were based on caretaker's reports. Diarrhea was defined as ≥3 loose stools in 24 hours and/or hospital/clinic visit within past 2 weeks for diarrhea episode, whilst respiratory illness was defined as reported cough/difficulty breathing in 24 hours and /or hospital/clinic visit within past 2 weeks for respiratory illness. Baseline assessments included household sociodemographic characteristics, history of child feeding practices, and anthropometry.

Capillary blood was obtained with single-use sterile micro-lancets (Becton Dickinson, Franklin Lakes, NJ) into a purple top microtainer capillary blood collector with EDTA for determinations of hemoglobin, plasma ferritin, C-reactive protein (CRP), and retinol binding protein (RBP) levels. Hemoglobin concentration was determined within one minute of blood collection on the second drop of blood using the HemoCue® B-Hemoglobin machine (Ängelholm, Sweden). Two drops of blood were placed on a microscope slide (Thermo Fisher Scientific Inc, Waltham, MA) for thick and thin malaria smears for detection of malaria parasitemia. Plasma samples were prepared and transported to Germany for subsequent laboratory analysis of ferritin, RBP and CRP, using a simple sandwich ELISA technique (26). Iron deficiency was defined as ferritin <12 µg/L and CRP < 10mg/L, whilst vitamin A deficiency was RBP <0.70µg/L and CRP < 10mg/L.

Data management and statistical analyses

Data were recorded in the field using Dell Axim personal digital assistants (PDAs) by trained field workers. Data were entered in customized electronic forms using Visual CE software version 10.0 (Syware, Cambridge, MA) and stored in an Access 2007 database (Microsoft Corporation, Redmond, WA) on a daily basis. Individual Sprinkles sachet use was estimated by dividing the reported biweekly household Sprinkles purchases or gifts by the number of children aged 6 to 59 months living in that household (24). Baseline anthropometric measures were calculated using the WHO child growth standards for underweight (weight-for-age Z-score <- 2), stunting (height / length-for-age Z-score < -2), and wasting (weight-for-height / length Z-score <-2) (27).

The original experimental design included randomization of villages into intervention and comparison groups for the first 9 months of data collection (July 2007-March 2008), and expansion of access to Sprinkles to both study groups in the second 9 months (July 2008-March 2009). Consequently, we carried out two types of analyses: 1) an intent-to-treat analysis for the randomized trial using the first year's data and, 2) an as-treated analysis to compare users and non-users of Sprinkles across all 60 villages using all available data. For the as-treated analysis, the children were divided into two groups based on Sprinkles use over the entire 2-year follow-up period: group 1= those ever reporting consuming Sprinkles (n=727children; contributing 1003 child-years) and, group 2= those reporting never to have consumed Sprinkles during the 2-year follow-up period (n=120 children; contributing 167 child-years). However, we controlled for the experimental group and period of follow-up (July 2007- March 2008 vs. July 2008 –

March 2009) as well as other baseline variables that differed between users and non-users of Sprinkles such as age, sex, anthropometry, and sanitation and hygiene as potential confounders (28). To assess dose-response relationships between use of Sprinkles and morbidity, outcomes were compared across three groups: non-users, users with less than the median intake of ~0.7 sachets/week and those at or above the median intakes (≥0.7 sachets/week).

If a child was absent during a visit by the fieldworker, that surveillance period was excluded from the analysis due to absence of both morbidity and Sprinkle use data (n=1619 excluded, or 11.2% of total visits). The analysis for this study was carried out for 847 children who had reported individual Sprinkles use and morbidity data available. We censored children who were lost to follow-up due to withdrawal or migration from the study area; the data were included in the analysis up to the point of withdrawal.

Initial analyses involved examining the data for normality using plots and Kolmogorov-Smirnov tests. The number of morbidity episodes, based on caretaker reports over the follow-up period was estimated for reported diarrhea, cough/difficulty breathing, subjective fever, and any illness. The incidence of diseases was estimated by dividing the number of disease episodes by follow-up period contributed by each child in the study. Because disease rates (particularly, diarrhea) among children were relatively low during the surveillance period, incidence rates were compared by Poisson regression for relative risks, controlling for multiple observations (clustering) within the same child and/or household. In the generalized estimating equation models, we step-wise included age, sex, household sociodemographic characteristics such as sanitation and hygiene

variables as covariates to account for any potential confounding effects. All data analyses were done by SAS 9.2 (SAS Institute Inc., Cary, NC); $p<0.05$ was considered significant for hypothesis testing.

RESULTS

At baseline, 1420 children were sampled and 1079 were enrolled (enrollment rate of 75.9%) (**Figure 1**). There were no differences in enrollment rates between intervention and comparison areas. Among 341 children excluded from the study, 33.3% were outside the age range (due to discrepancies in date of births reported during the census), 2.9% of parents did not give consent, and 63.8% were unavailable for enrollment on 3 separate household visits. This resulted in an enrollment of 567 children in the intervention villages, and 512 children in the comparison villages (24). Of the 1079 children enrolled at baseline, data on Sprinkles use were available for 78% (n=847). There were 14474 total surveillance visits for these 847 children for the 2-year follow-up period; 7719 and 6755 visits in the intervention and comparison villages, respectively. The median (inter-quartile range, IQR) visit per child was 17.0 (9.0, 26.0), with approximately equal average (median (IQR)) number of visits for the intervention and comparison groups (17.9 (9.0, 26.0) vs. 17.4 (9.0, 26.0), respectively). About 11% of total surveillance visits were excluded due to missing Sprinkles use and morbidity data. The trial profile is shown in figure 1.

Randomized field trial data (July 2007- March 2008)

At baseline, there were more boys (52.4% vs. 48.3%, p=0.04) and underweight children (16.4% vs. 10.1%, p=0.01) as well as a lower proportion of vitamin A deficient children (13.5% vs. 18.0%, p=0.01) in the comparison villages compared to the intervention villages. There were no significant differences in other child demographic

characteristics (**Table 1**). Mothers of study children were older in the intervention villages but poorer in the comparison villages (Table 1).

Intent-to-treat analyses comparing intervention and comparison group data from July 2007- March 2008 follow-up period indicated null effects, except for fever incidence (**Table 2**); children in the intervention villages had 32% reduced rates of fever (Relative risk, RR=0.68; 95% confidence interval (CI)=0.62, 0.74) compared to those in the comparison villages. The analyses presented are adjusted by the variables that differed across the two experimental groups: sex, underweight, vitamin A deficiency, maternal age, and household SES. Similar findings were found before adjustment of these variables (data not shown).

As-treated analysis (Sprinkles users vs. non-users; July 2007-March 2009)

At baseline, mothers of Sprinkles non-users were older (mean [standard deviation (SD)]: 27.8 (7.5) vs. 26.9 (6.9) yrs, $p<0.01$) and poorer (mean [SD] 53.2% vs. 47.1%, $p<0.01$) than Sprinkles users. There were also more underweight (17.0% vs. 12.4, $p<0.01$) and male (58.2% vs. 48.7%, $p<0.01$) children and a lower proportion of vitamin A deficient children (18.4% vs. 23.8%, $p<0.01$) in the Sprinkles non- users compared to the Sprinkles users. Median (Inter-Quartile Range) duration of follow-up per child was 238 (126, 364) days for Sprinkles users and 210 (112, 350) days for non-users. There were significantly fewer reports of diarrhea (0.97 vs. 1.03, $p=0.01$) and fever (3.93 vs. 4.66, $p=0.01$) incidence in the Sprinkles group than in the non-Sprinkles group; no differences were observed for cough or difficulty breathing (**Table 3**). Use of Sprinkles was

associated with a 30% reduction in diarrhea (RR=0.70; 95% CI=0.57, 0.86) and an 18% reduction in fever (RR=0.82; 95% CI=0.72, 0.95) rates. Adjustment of the Poisson model for enumeration area (randomized intervention or comparison villages) and period of follow-up (July 2007- March 2008 vs. July 2008 – March 2009) had no impact on this outcome.

Median consumption of Sprinkles per week was 0.7 sachets (~ 1 sachet per week). Compared to non-users, children who consumed at least 0.7 sachets of Sprinkles per week (RR: 0.60, 95% CI: 0.46, 0.80) and children who consumed fewer than 0.7 sachets per week (RR: 0.75, 95% CI: 0.62, 0.93) had a lower risk of diarrhea (**Table 4**).

DISCUSSION

Our study observed a health impact in a non-experimental setting in which Sprinkles distribution was completely up to the subjects: an intent-to-treat analysis using the experimental design (July 2007- March 2008) indicated that children in the intervention villages had 32% reduced rates of fever compared to those in the comparison villages; whilst the as-treated analysis of Sprinkles users and non-users indicated that the use of micronutrient Sprinkles, 0.7 sachets per week on average, was associated with 30% and 18% reduced incidence of diarrhea and fever respectively, but not respiratory illness/cough in children less than 5 years old. The dose response analyses are also consistent with this observation; compared to non-users, children who consumed at least 0.7 sachets of Sprinkles per week (RR: 0.60, 95% CI: 0.46, 0.80) and children who consumed fewer than 0.7 sachets per week (RR: 0.75, 95% CI: 0.62, 0.93) had lower risk of diarrhea. The data suggest that you can have health impact with relatively infrequent of Sprinkles in a really poor population.

Our finding is consistent with 11% reduction in incidence of diarrhea among Pakistani children given daily micronutrients including iron, vitamin A and zinc compared to a placebo (4), 18% reduction in diarrhea incidence in young children treated with zinc in India (29), 16% decrease in mean diarrhea duration in a meta-analysis of oral zinc for diarrhea treatment of young children (30), and 19% reduced incidence of severe diarrhea in Bangladeshi infants who received intermittent simultaneous iron and zinc supplements (31). In our study, we found a greater reduction in diarrhea incidence

through a market-based Sprinkles program compared to other studies of zinc supplementation or fortification (8, 32).

Previous studies on the association of intermittent or preventive zinc supplementation have mostly shown a reduction in incidence of childhood morbidity such as diarrhea and pneumonia when administered for 2-12 months under experimental conditions (4, 5, 8, 29). However, this may not be practical under non-experimental conditions due to issues of compliance and economics. Thus, an alternatively feasible strategy to provide multiple micronutrients including zinc and vitamin A under non-experimental conditions to improve zinc, vitamin A and other micronutrient status in young children with potential beneficial associations with childhood diarrhea, fever, and cough is warranted. In our study, Sprinkles were distributed by vendors who sold them to households with children 6-59 months. Under these conditions Sprinkles were taken sporadically and infrequently.

The positive association of Sprinkles use and diarrhea prevention observed in our study is perhaps due to the additive and interactive effects of the micronutrients in Sprinkles. Zinc deficiency impairs both humoral and cellular immune functions by reducing the number of B and T lymphocytes through increased apoptosis, and reduces their functional capacity; subsequent supplementation in deficient individuals improves the compromised immune function as indicated by increased CD4 lymphocytes as well as delayed cutaneous hypersensitivity (11). Further, zinc deficiency has direct effects on the gastrointestinal tract such as the impairment of the intestinal brush border, increased secretion in response to bacterial enterotoxins, and modifications in intestinal

permeability (29, 33). Adequate vitamin A status is also essential in maintaining the integrity of the epithelial barrier, the first line of defense against many infections, with adequate vitamin A stores being positively associated with measures of innate immune activity which suggests protection against diverse pathogens (9, 16, 34). Zinc and vitamin A supplementation can therefore prevent diarrhea and pneumonia mainly through their ability to restore immunity in children who are deficient in zinc and vitamin A. Prevention of diarrhea by optimizing intake of micronutrients including zinc and vitamin A may therefore be biologically plausible. Our data showed that there was a 30% reduction in diarrhea rates among children who consumed Sprinkles compared to those who did not use Sprinkles. An average dose of 0.7 sachets of Sprinkles per week was associated with the reduced diarrhea and febrile illness observed in these preschool children. A dose-response association was observed for only diarrhea incidence; children who had higher intake of Sprinkles (at least 0.7 sachets per week) benefitted more from diarrhea prevention than those with low intake (less than 0.7 sachets per week) or non-users (Table 4).

There was no association of Sprinkles use and respiratory illness as indicated by incidence of cough or difficulty breathing. A study of vitamin A supplementation of children did not find any beneficial association with respiratory infection (35) and zinc supplementation is more efficacious in diarrheal prevention than respiratory infections (36). Studies suggest that either daily zinc supplements of 20 mg/day (33) or high weekly dose of zinc (70 mg) (8) for children aged 2-24 months can prevent respiratory illness, whilst beneficial effect of vitamin A on respiratory infection was only observed in

underweight children who received 10,000 IU weekly (37). In contrast, our study participants included children 6-59 months old who consumed on average 0.7 sachets of Sprinkles per week (corresponding to ~4 mg zinc; and ~263 µg or 866 IU vitamin A per week). Thus, the low intake of zinc and vitamin A in our study population may not have been enough to have an impact on respiratory illness. Younger children are more vulnerable to acute respiratory illnesses (8, 38); zinc and vitamin A have been shown to protect against more invasive and severe acute respiratory illnesses which is common in younger and underweight children (8, 37). However, a sub-analysis of the association of Sprinkles intake with cough among underweight (weight-for-age z-score < -2SD) and young (6-24 month-old) children in our study did not show any beneficial association (data not shown).

Our study has some limitations. Sprinkles were distributed as part of a health product 'package', which included insecticide treated bed-nets (ITNs), soap, and point-of-use water treatment, many of which have proven efficacy in preventing diarrhea. Therefore, our finding on diarrhea prevention might have been due to an additive effect of these health products, and not Sprinkles use. Morbidity was also self-reported by caretakers. We may therefore have missed more severe cases of diarrhea, since there was no active surveillance at the hospitals. Also there was low overall intake of Sprinkles through this market-based distribution system, which may have minimized the impact on our outcomes of interest. Finally, due to the exclusion of children with missing data (Sprinkles intake data), selection bias may potentially be an issue in the study; however, we did not detect any significant differences in socio-demographic characteristics

between children with reported Sprinkles intake and those with no intake (data not shown). The strengths of the study include the distribution of Sprinkles by vendors in a non-experimental setting and active biweekly surveillance during the follow-up period of the study to assess Sprinkles intake and occurrence of diarrhea, fever, and cough in the population. Another strength of the study was the use of mixed analysis, i.e. intent-to-treat analyses and as-treated analyses, for the different study designs in the first year (experimental) and second year (observational) of follow-up. We found them consistent in terms of fever.

In conclusion, our findings suggest that the health product 'package' intervention with Sprinkles is associated with reduced incidence of diarrhea and fever among children 6-59 months in a resource-poor setting. We also demonstrated that a dose of approximately 1 sachet of Sprinkles per week appeared to be associated with reduced incidence of diarrhea and fever in this population.

ACKNOWLEDGMENTS

PSS, LJR, FKEG, PCJ, RQ, RM and RF were responsible for the study design. FKEG, LJR, PCJ, MKP, and PSS were responsible for conducting the study and the data collection. FKEG, PSS, RF, CRC, UR, and RM were responsible for data analysis. All authors were responsible for preparing the manuscript. None of the authors had a personal or financial conflict of interest.

The authors are indebted to the children and their parents who agreed to take part in this study.

This study is dedicated to the memory of Alfredo Obure, the NICHE study coordinator.

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Figure 1: Trial profile during experimental phase of the study (July 2007–March 2008)

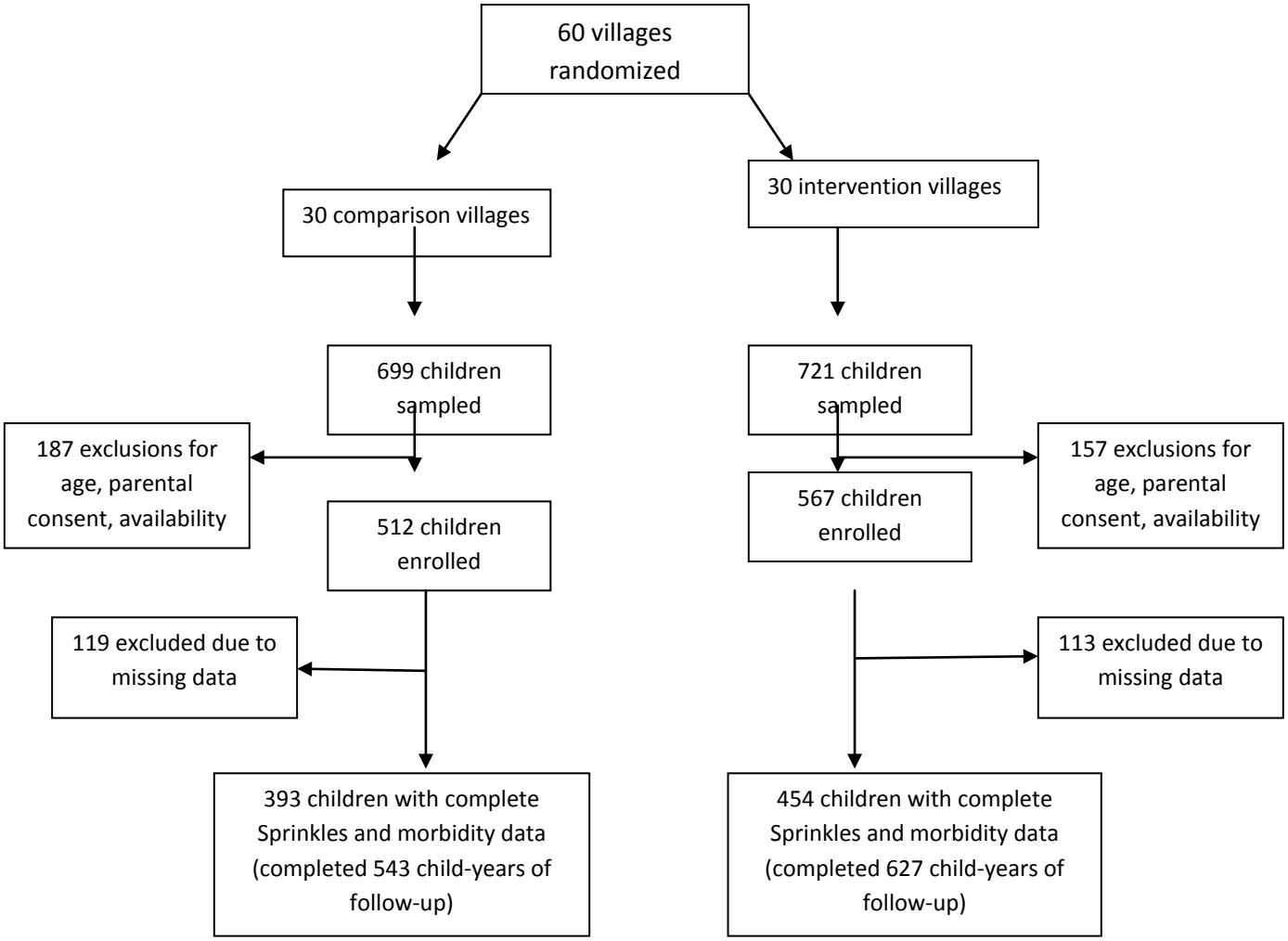

Table 1: Demographic characteristics of children and mothers, and anthropometric, feeding history, and morbidity indicators of children, by study group, NICHE Field Trial, Nyando District Kenya, July 2007- March 2008 (*n*=847)[1, 2]

	Intervention (n=454)	**Comparison (n=393)**	**p-value**
Age (months)[*]	19.3 (8.3)	19.4 (8.1)	0.79
Boys[#]	219 (48.3)	205 (52.4)	0.04
Anthropometric measures[#]			
Underweight	45 (10.1)	64 (16.4)	0.01
Stunted	136 (30.2)	115 (29.0)	0.87
Wasted	21 (4.4)	23 (5.6)	0.06
Feeding history[#]			
Ever breast fed (%)	434 (95.7)	362 (92.1)	0.70
Breast fed yesterday	264 (58.1)	234 (59.4)	0.28
Duration of breastfeeding			
≤9 months	10 (2.2)	13 (3.0)	0.59
>9 months	178 (39.2)	151 (38.3)	0.66
Still breastfeeding	266 (58.6)	230 (58.5)	0.87
Morbidity indicators[#]			
Positive malaria parasitemia	87 (19.2)	78 (19.7)	0.66
Iron deficiency (ferritin<12ug/L and normal CRP)	185 (40.8)	170 (43.4)	0.05
Anemic (Hb< 110g/L)	315 (69.3)	277 (70.4)	0.32
Vit. A deficiency (RBP<0.7ug/L and normal CRP)*	82 (18.0)	53 (13.5)	0.01
Maternal characteristics			
Maternal age(yrs)[*]	27.5 (7.2)	26.5 (6.7)	<0.01
Poor (bottom 2 SES quintiles)[#]	196 (42.9)	213 (54.1)	<0.01
Completed primary education[#]	252 (55.3)	213 (54.1)	0.31

* Mean (SD).

Number (%).

Table 2: Total illness episodes and incidence (episodes per person-year) NICHE Project, Nyando District, Kenya, July 2007-March 2008[1].

	Intervention (782 child-years of follow-up)	**Comparison (707 child-years of follow-up)**	**Risk ratio[2] (95% CI)**	**P value**
Diarrhea				
Total episodes, *n*	377	350		
Incidence, *episodes/yr*	0.482	0.495	1.07 (0.94, 1.21)	0.288
Cough				
Total episodes, *n*	1125	1164		
Incidence, *episodes/yr*	1.439	1.646	0.98 (0.91, 1.05)	0.576
Fever				
Total episodes, *n*	1332	1545		
Incidence, *episodes/yr*	1.703	2.185	0.68 (0.62, 0.74)	0.001

[1] Comparing incidences of morbidity among intervention and comparison villages during the July 2007- May 2008 follow-up period, i.e. 17 biweekly surveillance rounds.

[2] Generalized estimating equation (GEE) model for longitudinal analysis employed to account for correlation of outcomes (diarrhea, fever, cough incidences and any illness) within the same child. The GEE multivariate analyses adjusted for only factors which differ in Table 1 at baseline: sex, underweight, vitamin A deficiency, maternal age, and household SES.

Table 3: Total illness episodes and incidence (episodes per person-year) by Sprinkles use group, among enrolled children, NICHE Project, Nyando District, Kenya, July 2007-March 2009[1].

	Ever used Sprinkles (1003 child-yrs of follow-up)	**Never used Sprinkles (167 child-yrs of follow-up)**	**Risk ratio[2] (95% CI)**	**P value**
Diarrhea				
Total episodes, *n*	974	121		
Incidence, *episodes/yr*	0.971	1.034	0.70 (0.57, 0.86)	0.001
Cough				
Total episodes, *n*	3211	540		
Incidence, *episodes/yr*	3.201	3.234	0.96 (0.87, 1.04)	0.315
Fever				
Total episodes, *n*	3941	778		
Incidence, *episodes/yr*	3.928	4.659	0.82 (0.72, 0.95)	0.010

[1] Comparing incidences of morbidity among users of Sprinkles vs. non-users of Sprinkles over the 2-year follow-up period.

[2] Generalized estimating equation (GEE) model for longitudinal analysis employed to account for correlation of outcomes (diarrhea, fever and cough incidences) within the same child. Model adjusted for sex, experimental group/enumeration area, follow-up period (July 2007- March 2008 vs. July 2008 – March 2009), household SES, sanitation and hygiene variables.

Table 4: Dose response impact of Sprinkles use on morbidity incidence[1, 2].

Dose response association of Sprinkles use	**Diarrhea**	**Cough**	**Fever**
	Risk ratio (95% CI)	Risk ratio (95% CI)	Risk ratio (95% CI)
Reference group: Non-users (0 sachets/wk; *167 child-yrs*)	1.00	1.00	1.00
Low users (>0 and <0.7 sachets/wk; *411 child-yrs*)	0.75 (0.62, 0.93)	0.96 (0.83, 1.10)	0.75 (0.65, 0.86)
Medium users (≥ 0.7 sachets/wk; *591 child-yrs*)	0.60 (0.46, 0.80)	1.15 (1.02, 1.29)	0.93 (0.80, 1.10)

[1] Comparing incidences of morbidity among those who consumed below vs. above the weekly median Sprinkles intake over the entire follow-up period (July 2007- March 2009).

[2] Generalized estimating equation (GEE) model for longitudinal analysis employed to account for correlation of outcomes diarrhea, fever and cough incidences) within the same child. Model adjusted for sex, experimental group/enumeration area, follow-up period (July 2007- March 2008 vs. July 2008 – March 2009), household SES, sanitation and hygiene variables.

CHAPTER 7

SUMMARY AND CONCLUSIONS

7.1. KEY FINDINGS AND POTENTIAL SIGNIFICANCE

Using data from both community-based cross sectional surveys and a cluster-randomized, longitudinal cohort trial in 60 villages in rural Nyanza province in western Kenya, we examined the alternative approaches to estimate iron deficiency (ID) during states of subclinical inflammation using various iron status indicators such as hemoglobin concentration (Hb), zinc protoporphyrin (ZP), plasma ferritin (SF), plasma soluble transferrin receptors (TfR), and transferrin receptors / ferritin index (TfR/SF index). The acute phase proteins (APPs) which were used as indicators of subclinical inflammation in our study were C-reactive protein (CRP), and alpha-1-acid glycoprotein (AGP). We also examined the best iron indicator for assessing the prevalence of iron deficiency in preschool children as compared to the multiple-criteria model in a resource poor setting with high inflammation. Our final analysis looked at the effect of community-based sales of a multiple-micronutrient powder, Sprinkles, on diarrhea and cough incidence in children 6-59 months of age.

In examining the alternative approaches to estimate ID during subclinical inflammation, it was evident that the influence of inflammation on iron status indicators needs to be corrected in order for an accurate estimation of the true prevalence of ID in the population. Our data indicated that the prevalence of subclinical inflammation as indicated by elevated APPs was high in this sample of preschool children in western Kenya, and that when the influence of inflammation is not corrected, the prevalence of ID (SF <12 $\mu g/L$) among anemic and nonanemic children is underestimated. We used the

correction factor (CF) approach to adjust for this inflammatory effect since this approach makes use of both CRP and AGP. This is important since our data and a recent meta-analysis suggest that if either one of the APPs is used alone in adjusting for inflammation the risk of under-reporting ID is even greater than when both are used together (92). In our study, the Use of the CFs to adjust for inflammation increased the prevalence of ID based on ferritin <12 μg/L by 33.9%, and up to 6% over-estimation (ZP>80 *μmol/mol*, and TfR >8.3 *mg/L*) of ID prevalence. Further, applying the CF improved the expected relationship between Hb and SF that was masked by inflammation ($r = 0.095$, $p=0.013$ vs. $r = 0.203$, $p<0.001$) as well as other associations.

Investigating the best iron indicator (in comparison to the multiple-criteria model) for assessing the prevalence of iron deficiency in preschool children from capillary blood, our findings indicate that TfR was more efficient in estimating ID compared to the other independent candidate iron status indicators, SF and ZP. Transferrin receptors also had an excellent agreement ($\kappa=0.84$) with the multi-criteria model in assessing ID. Receiver-operating characteristic (ROC) curve analysis also demonstrated that TfR (area-under-the-curve (AUC) = 0.69) was superior to both SF (AUC=0.61) and ZP (AUC=0.63) in predicting anemia ($p<0.001$). In summary, TfR, compared to SF and ZP, more accurately estimates the prevalence of ID in preschool children based on multiple-indices and least affected by inflammation in a high inflammation, resource-poor setting.

For the effect of community-based sale of Sprinkles on diarrhea, fever, and cough incidence, our findings indicate that incidence of diarrhea and fever among users was significantly lower than among non-users (0.97 vs. 1.03, $p=0.012$; and 3.93 vs. 4.66, $p=0.040$, respectively). Use of sprinkles was associated with a 30% reduction in diarrhea

(relative risk, RR=0.70; 95% CI=0.57, 0.86) and 18% reduction in fever (RR=0.82; 95% CI=0.72, 0.95). There was no association with respiratory illness. A dose of approximately 1 sachet of Sprinkles per week appeared to be associated with the reduced incidence of diarrhea and fever in this population. Our results show that the sale of low cost sprinkles under non-experimental conditions is effective in reducing diarrhea and fever incidence among children 6-59 months in western Kenya.

7.2. LIMITATIONS AND STRENGTHS OF THE STUDY

7.2.1. Study limitations

The present study has some important limitations. These include the fact that we obtained some of the data from cross-sectional surveys and therefore could not infer causality (Chapters 4 and 5). Our results are also specific to our population and may not be generalizable to other populations. In terms of data collection, while the use of personal digital assistants, PDAs, may have many advantages, one disadvantage we experienced was data loss due to technical errors. For example, we lost the health outcomes for surveillance round 29 for reasons which remain unclear. Some important confounding factors were also not collected during our surveys; example, blood lead and hemoglobinopathies were not measured, which may have confounded the ZP levels of the children during the estimation of iron deficiency (Chapter 5). Again, other important variables such as serum / plasma zinc were not measured to help determine the impact of Sprinkles use on zinc status and its association with the morbidity outcomes we assessed. We only relied on self-report of morbidity by caretakers, and therefore may have missed more severe cases of morbidity incidences such as diarrhea, fever, and cough. There was

low overall intake of Sprinkles through this market-based distribution system, which may have minimized the impact on our outcomes of interest. Further, due to the integrated sale of other health products which might have contributed to the reduction of diarrhea, cough, fever and other morbidity incidences in our population, the impact of Sprinkles on such morbidity might have been attenuated. However, we were able to detect a 30% reduction in diarrhea and 18% reduction in fever incidences among children who consumed Sprinkles during the follow-up period compared to those who did not use Sprinkles (Chapter 6). Finally, even though our data could have been influenced by the Hawthorne effect, during our second follow up, we evaluated this and did not detect any evidence of the Hawthorne effect in our data at the 2009 survey (manuscript in press).

7.2.2. Strengths of the study

This study also has several strengths. The design of the project was a community-based cluster-randomized, longitudinal cohort trial which has the potential to nullify any systematic differences between the intervention and comparison villages and thus minimize the presence of possible confounding factors. The study also had a large sample size which was very essential in our first analysis when we estimated the correction factors for adjusting for inflammation on iron status indicators (Chapter 4). Additionally, the study measured a wide range of biological outcomes such as iron status and subclinical inflammatory biomarkers including hemoglobin, plasma ferritin, transferrin receptors, zinc protoporphyrin, C-reactive protein, and alpha-1-acid- glycoprotein. This enabled us to assess the association between inflammation and all available iron status biomarkers. We also used field-friendly capillary blood methods to minimize discomfort among the children and to measure multiple iron status indicators from capillary blood

using the sandwich ELISA technique that requires small volume of plasma (Chapters 4 and 5). Another important strength was the distribution of Sprinkles by vendors in a non-experimental setting and bi-weekly active surveillance during the follow-up period of the study to access sprinkles intake and occurrence of diarrhea, fever, and cough episodes in the population (Chapter 6). Our study was able to detect a beneficial association of community-based sales of Sprinkles with childhood diarrhea (relative risk, RR=0.70; 95% CI=0.57, 0.86) and fever (RR=0.82; 95% CI=0.72, 0.95), which to our knowledge, is the first of its kind.

This study was carefully and rigorously implemented at every level from training to data collection and supervision. There was a comprehensive data collection and data monitoring. Both quantitative and qualitative data collections, including focus group discussions and key informant interviews with vendors, were integrated to enhance the understanding of the intervention delivery and utilization as well as confidence in the validity of the findings.

7.3. FUTURE RESEARCH

Our study assessed various biochemical outcomes such as iron status biomarkers to investigate the influence of subclinical inflammation using an approach that takes into account two important acute phase reactants, CRP and AGP, the correction factor. However, we were not able to determine if the proposed correction factor really improved the accuracy of estimation of iron status in this population. This is because we did not have access to a gold standard iron biomarker such as bone marrow iron or liver iron

stores through biopsies. Future educational and research opportunities may include having a more standard reference of iron status such as stainable bone marrow iron with which the corrected iron indicators, obtained through the use of the correction factor approach, can be compared to detect if the correction factor accurately improves the assessment of iron status. It is also known that the specificity of zinc protoporphyrin, ZP, as indicator of iron status may be limited by increased blood lead concentration, and other interfering substances such as bilirubin produced by hemolysis in hemolytic anemias, and other hemoglobinopathies which tend to increase the level of blood ZP (59, 63). Future studies should therefore explore these potential confounding variables in this population by measuring sickle cell anemia and other hemoglobinopathies as well as level of interfering substances such as bilirubin.

The current study also indicated that in a high inflammatory setting the prevalence of iron deficiency in preschool children as assessed by TfR was in excellent agreement with that estimated using the multiple-criteria model. Future studies should look to compare the validity of TfR in relation to stainable bone marrow iron, which is the gold standard for iron deficiency assessment.

Further, our findings suggest that the sale of low cost sprinkles under non-experimental conditions is effective in reducing diarrhea and fever incidence among children 6-59 months in a resource poor setting. A weekly dose of one sprinkles sachet was associated with reduced diarrhea and fever episodes in this population. Future longer-term longitudinal studies should investigate the length of protection after this periodic supplementation of multiple-micronutrient including zinc in similar populations under non-experimental conditions.

7.4. POLICY IMPLICATIONS

The findings from this dissertation suggest that the various iron status biomarkers normally used to assess iron status in high inflammatory settings are affected by subclinical inflammation. Surveys aimed at assessing the iron status of children in resource poor, high inflammation settings should include both CRP and AGP as inflammatory biomarkers to accurately determine the true prevalence of iron deficiency. Further, the community-based sale of multiple-micronutrient Sprinkles was associated with a reduction in the incidence of diarrhea and fever among preschool children. Programs aimed at reducing childhood illnesses such as diarrhea should therefore include distribution of Sprinkles which has also been shown to reduce anemia and iron deficiency in most developing countries. Countries in sub-Sahara Africa can consider the distribution of Sprinkles as a critical aspect of helping reduce both anemia and childhood diarrhea. However, there should be a sentinel surveillance system in place to detect any impact this may have on malaria morbidity since this critical issue is still under consideration as a consequence of the current WHO iron supplementation guidelines (93, 94).

7.5. CONCLUSION

Our study suggests that subclinical inflammation affects TfR, ZP and SF and not correcting for such inflammation alters the measures of ID. In the absence of a feasible, non-invasive gold standard for iron status indicator the multiple-criteria model can be

used to assess iron deficiency, and that TfR was as accurate as the multiple-criteria model in assessing the prevalence of iron deficiency in preschool children. Additionally, when multiple-micronutrient powder, Sprinkles, is distributed under non-experimental conditions it helps reduce the episodes of both diarrhea and fever in preschool children. Therefore, Sprinkles distribution through an integrated health promotion and income-generating program should be considered in an effort to improve maternal and young child health in resource poor, and high inflammation settings.

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