The Role of Sulfur Amino Acids in Risk of Kwashiorkor

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by:

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In memory of the children of Mugunga Refugee Camp

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Abstract

Kwashiorkor is a form of severe acute malnutrition identified more than 80 years ago as a syndrome and still affects hundreds of thousands of children each year, yet its etiology remains unknown. A better understanding of its etiology is necessary to develop effective preventive strategies. Although kwashiorkor is only found in regions where protein intake and sanitation are poor, study findings comparing the dietary protein intake of children with and without kwashiorkor have been conflicting. Protein quality has not been explored and the large array of signs characterizing kwashiorkor can plausibly be explained by a deficiency of sulfur amino acids (SAAs). Children with kwashiorkor have low circulating sulfur amino acids (methionine and cysteine) and their metabolites, and their metabolism of SAAs is altered. The aim of this research is to provide evidence upon which to design preventive interventions, hypothesizing that children in higher-prevalence populations will have lower intakes of SAAs, and that low intake of SAAs will be a stronger predictor of the risk of kwashiorkor than other factors. Additionally, due to its low nutrient content and potentially high cyanide content, cassava intake was hypothesized to be a stronger predictor of risk than food security.

An anthropometric survey of all children in one Health Area of eastern Democratic Republic of the Congo located all cases of kwashiorkor among children 12 to 59 months old to aid in the selection of two populations with very different prevalence. A commercial laboratory was used to provide amino acid profiles on market samples of key staples and sources of proteins. Household samples of cassava flour and cooked cassava leaves were tested for cyanogens. Interviews with caregivers of children 36 to 59 months old recorded multiple factors of the household and the child and a 24-hour quantitative recall of the child's diet. Among the many signs characterizing kwashiorkor, only bipedal pitting edema is used for admission to treatment, though light-colored brittle hair and facial edema have been reported to appear before edema in the feet, therefore all visible signs were recorded. One urine sample was taken to compare urinary sulfate and another for thiocyanate. Analysis included GIS mapping, direct statistical comparisons of diets, and path analysis modeling of the multiple potential causal factors.

Together, the findings showed children in a population with a higher prevalence of kwashiorkor had lower intakes of sulfur amino acids, methionine in particular, more children in this population were at risk of inadequate methionine intake than other amino acids, and methionine was the limiting amino acid in both populations. Median intake of both methionine and cysteine was above the WHO requirement, but true adequacy of a nutrient must take into consideration factors that raise the requirement. Children in the HPP were more stunted, were ill more often, and their families were more food insecure than those in a lower-prevalence population. SAA intake, followed by illness, was the strongest predictor of a family history of kwashiorkor. A family history of kwashiorkor, followed by illness, was the strongest predictor of early signs of kwashiorkor. It appears that low SAA intake makes children especially vulnerable to kwashiorkor, but illness is often the event that triggers the onset of the syndrome. Intervention trials to reduce prevalence of kwashiorkor should simultaneously reduce exposure to infection and increase intake of both cysteine and methionine.

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Acronyms

CMAM	Community-based Management of Acute Malnutrition
CN (or HCN)	Cyanide
CSI	Coping Strategies Index
DRC	Democratic Republic of the Congo
EED	Environmental Enteric Dysfunction
FAO	Food and Agriculture Organization of the United Nations
FCS	Food Consumption Score
FH	Family History of kwashiorkor
GAG	Glycosaminoglycan
GSH	Glutathione
HAZ	Height for Age Z-score
HE	Hair Changes and Facial Edema
HPP	High Prevalence Population
IOM	International Organization of Medicine
IQR	Inter-quartile Range
LPP	Low Prevalence Population
MUAC	Middle-Upper Arm Circumference
MoH	Ministry of Health
PRONANUT	National Program for Nutrition
SAA	Sulfur Amino Acids
SAH	S- Adenosyl Homocysteine
SAM	Severe Acute Malnutrition
SAMe	S-Adenosyl Methionine
SEM	Structural Equation Modeling
SCN	Thiocyanate
WAZ	Weight for Age Z-score
WFH	Weight for Height Z-score
WHO	World Health Organization

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Chapter 1: Introduction

Overview

Kwashiorkor[1] is a type of severe acute malnutrition that is often fatal and is estimated to affect hundreds of thousands of children annually (Alvarez, Dent, Brown, Myatt, & Briend, 2016). Despite more than 80 years of research, its etiology is largely unknown. Major signs and symptoms of kwashiorkor include bipedal pitting edema, skin lesions, pale brittle hair, irritability, high oxidative stress (low total anti-oxidant status), fatty liver, and low circulating albumin (Gopalan, 1955; Jahoor, Badaloo, Reid, & Forrester, 2006b; Kamalu, 1993; Manary, Leeuwenburgh, & Heinecke, 2000). Although mortality rates for kwashiorkor vary, they are often three to four times higher than for marasmus without edema (Dramaix et al., 1993; Prudhon, Briend, Laurier, Golden, & Mary, 1996).

Children diagnosed with kwashiorkor consistently show low blood concentrations of sulfur amino acids (SAAs) and their substrates (Ittyerah, Pereira, & Dumm, 1965; Jahoor, Badaloo, Reid, & Forrester, 2005; Jahoor et al., 2006b). Additionally, all symptoms of kwashiorkor can plausibly be attributed to a physiological insufficiency of circulating SAAs and their substrates.

Research over the past 50 years has supported the development of effective treatment for kwashiorkor but there are currently no effective, evidence-based preventive interventions (Bahwere et al., 2006). Local ministries of health where

[1] The terms "kwashiorkor" and "edematous malnutrition" are often used to describe the same state. This study will use the term "kwashiorkor" because there are many other symptoms that distinguish kwashiorkor than just edema.

kwashiorkor is endemic are therefore unable to fully address malnutrition in their regions. This study seeks evidence on the relative influence of SAAs, other nutritional components and environmental factors on risk of kwashiorkor in order to design public nutrition strategies to prevent it.

Overall Aim - *Provide evidence upon which preventive interventions for kwashiorkor can be designed*. The association of multiple potential environmental and dietary factors to risk of kwashiorkor, with special attention to sulfur amino acids, will be established, a key step toward designing preventive interventions likely to have the greatest impact[2].

Hypothesis #1: *Children living in an area with a high prevalence of kwashiorkor will have lower levels of sulfur amino acids available for metabolic processes than children in areas with a lower prevalence of kwashiorkor*.

Hypothesis #2: *Risk of kwashiorkor in eastern Democratic Republic of the Congo will have a stronger association with intake of SAAs, once the requirements for the detoxification of CN have been accounted for, than with other factors such as recent infection, sanitation, water source, shelter, access to health care, displacement history or past medical history*. For this hypothesis, risk of kwashiorkor was operationalized using two different outcome measures: 1) having light-colored, brittle hair or facial edema; 2) living in a family that has

[2] While this study hopes to reveal associations between kwashiorkor prevalence and specific factors, it will not prove causation. A follow-on intervention-based study using the results of this study would be required to prove causation.

had a case of kwashiorkor in the previous five years. A family history of kwashiorkor is arguably both an outcome and a potential risk factor. The development of cases within a particular family is the outcome of a past dynamic. A continuation of that dynamic could increase risk of additional cases. The association of each outcome measure of risk was analyzed separately. The analysis with hair changes or facial edema as an outcome used family history as a risk factor.

Hypothesis #3: *Cassava consumption will be a stronger predictor of risk of kwashiorkor than food insecurity*. Cassava is being widely promoted in Africa even in food secure regions as a resilience strategy. Due to its very low nutrient density and potentially high levels of cyanide requiring SAAs for detoxification, its effect on nutritional outcomes needs to be taken into account in programs promoting cassava production.

If this study confirms the hypothesized associations, trial interventions to reduce incidence of kwashiorkor should aim to reduce the proportion of calories derived from cassava or to increase SAA consumption in complementary foods.

The rest of this chapter provides a literature review, describing the unique aspects of kwashiorkor, the progression of theories relating to the etiology of kwashiorkor, and an explanation of the metabolic pathways of sulfur amino acids and their possible connection to the signs of kwashiorkor, and the design of past dietary research on kwashiorkor. Chapter 2 describes a finding of the study not

addressing one of the stated hypotheses, but that lays a foundation for Chapter 3. It describes the uneven, clustered distribution of kwashiorkor within a small geographic area. Chapter 3 addresses Hypothesis 1, the relative intakes of SAAs in two populations with very different prevalence of kwashiorkor. Chapter 4 addresses Hypotheses 2 and 3, examining the relative effect of multiple factors on risk of kwashiorkor. Chapter 5 provides a summary of the findings and conclusion.

Background and Significance

Kwashiorkor is a type of severe acute malnutrition (SAM) found in some of the most under-developed parts of the world, where diet and environmental sanitation are poor (Gopalan, 1955; Manary, Heikens, & Golden, 2009). A recent large-scale data review conducted by the Community-Based Management of Acute Malnutrition (CMAM) Forum estimates that "hundreds of thousands of children" suffer from kwashiorkor annually but there are no estimations of its prevalence at national or global levels because it is not systematically included in standard national nutrition surveys (Ahmed, Rahman, & Cravioto, 2009; Alvarez et al., 2016; Briend, Myatt, Dent, & Brown, 2013). Two major surveys used globally, the national Demographic and Health Surveys and UNICEF's Multiple Indicator Cluster Surveys measure wasting but not edema (Frison, Checchi, & Kerac, 2015; The DHS Program, 2017; UNICEF, 2017b). As Frison points out, in countries or regions with a higher prevalence of kwashiorkor their overall burdens of acute malnutrition can be severely underestimated if edema is not taken into

account, potentially leaving the health system under-resourced in addressing malnutrition.

Most prevalence data come from nutrition surveys and treatment admissions records covering scattered geographic areas. Children with kwashiorkor, much like wasting, sometimes spontaneously recover or die without treatment so prevalence surveys and admissions data may actually be underestimating the true scale of the problem, especially during crises when malnutrition rises and access to medical care is reduced (Alvarez et al., 2016; Williams, 1933). In 2012, more than 11,600 cases of kwashiorkor were treated in North Kivu Province, Democratic Republic of the Congo (DRC) alone (PRONANUT, 2012), while coverage surveys in one of the better-covered zones estimated that only 61% of SAM cases had been treated (ACF, 2011a). During conflict, when malnutrition is arguably much higher than normal, coverage is especially low. The Kasai Region Nutrition Cluster reported only 13.3% of the 350,463 estimated SAM cases had been treated from January to September 2017, a time when malnutrition spiked and humanitarian access to affected populations was restricted because of insecurity (Kasai Regional Nutrition Cluster, 2017).

Survey and treatment data on kwashiorkor usually cover children under five years of age because they are generally the most affected by kwashiorkor, though cases among older children and adults are not unusual in some regions (Gupte & Mehta, 1971; Newman, 1995; PRONANUT, 2013). The typical age groups most vulnerable to kwashiorkor do vary somewhat by region. In Malawi, for example, the primary age group is 6 to 24 months, most often at the time

babies are weaned from breast milk, and rarely seen in individuals older than 48 months (Ciliberto et al., 2005; Lin et al., 2007; Newman, 1995). On the other hand, Gupte and Mehta found that in India in 1970, the peak age of incidence was 36 to 59 months, with 22% of cases older than 59 months (Gupte & Mehta, 1971). In 2012, in North Kivu Province, DRC, 53% of all cases were 24 to 59 months-old, and 14% were older than 59 months (PRONANUT, 2013). The difference in affected age groups between regions suggests there may be different root causes in different contexts, but the similarities in the signs of the syndrome in all contexts imply a common biological pathway.

Kwashiorkor in Nutrition Policy and Research

The Lancet article suggesting the Wellcome Trust definition of kwashiorkor and marasmus, and the two recent *Lancet* series on child undernutrition have had an enormous impact on policy relating to research on and treatment of kwashiorkor (Lancet, 1970, 2008, 2013). The impact of the former is related to its very narrow diagnostic criteria and the latter through its omission of kwashiorkor.

Definition Defines Research Priorities and Study Design

Early research on kwashiorkor noted that many children in high-prevalence populations who do not present with bipedal pitting edema still have many of the biomarkers of kwashiorkor and labeled this phenomenon "subclinical kwashiorkor" or "pre-kwashiorkor" (Brock & Autret, 1952; Scrimshaw & Viteri, 2010; Whitehead & Dean, 1964). A number of these researchers reported that edema in the face and changes to the hair usually precede edema in the feet

(Brock & Autret, 1952; Demaeyer, 1958; Gopalan, 1968; Whitehead & Dean, 1964). Gopalan noted that, "there is a continuous and insidious transition from apparent normality to the full-fledged disease [kwashiorkor], and cases can be encountered in various stages" (Gopalan, 1955). Scrimshaw noted that edema in the feet appeared only in "the advanced state" (Scrimshaw & Béhar, 1961). Indeed, kwashiorkor is considered a form of severe acute malnutrition (SAM); unlike wasting and stunting, there is no official classification for "moderate" kwashiorkor and therefore no recommended treatment protocol until the child is severely malnourished (WHO, 2013).

In 1970, *The Lancet* published the Wellcome Trust classifications for marasmus and kwashiorkor to ensure that all research on kwashiorkor or marasmus would refer to the same conditions (Lancet, 1970). After a discussion among experts on kwashiorkor, they selected only bipedal pitting edema and wasting from among the multiple signs associated with kwashiorkor as the criteria for diagnosing kwashiorkor because "edema is the single clinical feature which is universally present". In the years since the publication of this classification, other signs have been largely ignored in research designs and treatment policy. The WHO guidelines have more recently dropped the criteria of wasting, as many children with overt kwashiorkor are not wasted (Frison et al., 2015; WHO, 1999). But WHO does clarify that the use of edema alone without other signs is indicative only of "oedematous malnutrition" and not "kwashiorkor", a syndrome with many other signs. The CMAM forum's review states that this

single criterion of edema has now mistakenly become synonymous with the full syndrome of "kwashiorkor" in general usage (Alvarez et al., 2016).

One consequence of the use of bipedal pitting edema as the sole criterion for diagnosis and treatment is that research on kwashiorkor classifies children based solely on the current presence or lack of bipedal pitting edema; in other words, only those in the "advanced state" (Badaloo, Forrester, Reid, & Jahoor, 2006; Hendrickse et al., 1982; Jahoor et al., 2005; Kismul et al., 2015; Kismul, Van den Broeck, & Lunde, 2014; Lin et al., 2007; Odigwe, Smedslund, Ejemot-Nwadiaro, Anyanechi, & Krawinke, 2010; Phadke et al., 1995; Sullivan, Ndekha, Maker, Hotz, & Manary, 2006). Children with other signs, but not edema in their feet, are therefore potentially mistakenly classified as not being affected by kwashiorkor, even though research shows those with other signs have many of the same biomarkers (Whitehead & Dean, 1964).

Omissions in Surveys Become Neglect in Policy

The 2008 and 2013 Lancet Series on Maternal and Child Nutrition have had enormous impact on recent nutrition policy, making recommendations for effective interventions to prevent and treat undernutrition, but completely neglecting kwashiorkor (Lancet, 2008, 2013). In 2008, when two different letters to the editor objected to this omission, the series' authors justified it by pointing out that "there are no reliable data available at country level to quantify the number of children with oedematous malnutrition." (Bhutta, Black, Cousens, & Ahmed, p. 1749; Ndekha; von Schoen-Angerer, Shepherd, Lokuge, Mills, & Fournier)

This seemingly minor omission has had a tremendous ripple effect, focusing attention of both funding, policy and programming on wasting and stunting to the exclusion of kwashiorkor as they exclusively target the interventions cited in these series (Briend et al., 2013; ELRHA, 2017). The Scaling Up Nutrition Movement Framework for Action, endorsed by over 100 institutions, including national governments, was initiated based on the initial Lancet Series and is the basis for much of the direction of international nutrition funding and policy, all of which address stunting, wasting, and micronutrient deficiencies but not kwashiorkor (DFID, 2011; U.S. Government, 2016; UNICEF, 2015; WHO, 2014). The World Health Assembly Global Nutrition Targets for 2025 focus on wasting and stunting, but not kwashiorkor (WHO, 2012). Sustainable Development Goal 2.2 aims to "end all forms of malnutrition", yet the targets do not include kwashiorkor (UN-DESA, 2015). While this omission has led to a nutrition policy focus that excludes kwashiorkor, the completeness of the omission has highlighted the gradual fading of kwashiorkor from nutrition surveys and has stimulated some discussion of the true burden of kwashiorkor and has spurred a small resurgence in the kwashiorkor research.

The Geographic Distribution of Kwashiorkor

Spurred by the Lancet Series' omission of kwashiorkor, the CMAM Forum has attempted "to strengthen the evidence base and support advocacy for inclusion of kwashiorkor in relevant methodology discussions at global level (Alvarez et al., 2016)". While existing data cannot provide a robust estimate of the global prevalence of kwashiorkor, there is some data on its geographical distribution, as

defined by bilateral pitting edema. The CMAM forum recently reviewed 2,515 datasets from surveys and treatment admission records containing information on a total population of about 1.7 million children (Alvarez et al., 2016). They present the proportion of SAM cases identified which were edematous, broken down by country. In ten countries, they found more than 20% of SAM was edematous. Among these, 32% of SAM in the DRC and 50% in Malawi was edematous.

Although this is the most comprehensive review of data on the burden of kwashiorkor, the nationally aggregated figures can hide areas within a country where kwashiorkor forms a higher proportion of SAM, obscuring the gravity of the nutritional status in those regions. Courtright & Canner showed neighboring districts in Malawi had very different prevalences of kwashiorkor (Courtright & Canner, 1995), and in Jamaica, Fonaroff noted that cases were more common in villages with particular child care habits, parental support, and landholding patterns (Fonaroff, 1969). The CMAM review estimated that 32% of SAM cases identified in the DRC were edematous. Within the DRC, the Ministry of Health (MoH) National Program for Nutrition (PRONANUT) reported that in 2012 about 55% of children treated for SAM in North Kivu province were edematous (PRONANUT, 2013) and yet surveys in other parts of the country in that same year found that only 4 to 6% of SAM was edematous (ACF, 2012a, 2012b).

Even within a single province, the proportion of SAM that is edematous can vary widely. Data from PRONANUT in North Kivu Province, DRC, indicate that in 2012 this ranged from 36% to 75% in the 25 Health Zones, with more than 100

cases of SAM (PRONANUT, 2013). Similar clustering patterns have been noted elsewhere (Newman, 1995; Newman & Gulliver, 1979; Trowell & Davies, 1952). The fact that high prevalence of kwashiorkor tends to cluster locally indicates a clustering of risk factors that, if they do not specifically cause kwashiorkor, then at least provide the underlying vulnerability that allows its progression (Newman, 1995; Trowell & Davies, 1952).

This clustering of cases may lead to both challenges and opportunities. The tendency of large surveys or large treatment catchment populations can hide pockets where kwashiorkor clusters, preventing the effective targeting of resources. On the other hand, differences in the prevalence of kwashiorkor, or even the proportion of SAM that is edematous, provides an opportunity for comparing population-level differences in risk factors. For example, if two adjacent regions share many environmental and economic characteristics but very different prevalence of kwashiorkor, these shared factors would be controlled for naturally in comparisons between them, narrowing down those factors that would have the most impact on risk of kwashiorkor.

Previously Proposed Etiologies of Kwashiorkor

Many biological mechanisms have been proposed but few can explain the full spectrum of symptoms. Hendrickse found that Sudanese children with kwashiorkor had higher serum aflatoxin levels and lower urinary output of its detoxified metabolites than Sudanese children without kwashiorkor (Hendrickse et al., 1982). As the affected children already had kwashiorkor when the measurements were taken, it is difficult to say if they were exposed to higher

levels, or if the metabolic changes due to kwashiorkor prevented the child from being able to detoxify the aflatoxin. In a letter in responding to Hendrickse's article, Long noted that a guinea-pig study unrelated to kwashiorkor, using a diet with sufficient protein but low in methionine, was twice halted because it resulted in kwashiorkor-like symptoms (Long, 1982). The effect was ascribed to aflatoxin in the diets, though the letter did not report data on the aflatoxin load.

Children with kwashiorkor show high oxidative stress, leading to the theory that edema was due to low antioxidant status (Manary et al., 2000). Anti-oxidant supplements have not effectively prevented kwashiorkor, leading to the tentative conclusion that the oxidative stress is secondary to some other cause (Ciliberto et al., 2005; Fuchs, 2005; Odigwe et al., 2010). A promising new development is the theory that the microbiome mediates digestion and can inhibit the absorption of some nutrients, including possibly SAAs (M. I. Smith et al., 2013). Smith's research on the gut biome found some differences in the biomes of twins when one twin developed kwashiorkor and the co-twin did not (M. I. Smith et al., 2013). It remains unclear how the microbiota originally formed in the children with kwashiorkor and its causal relationship to kwashiorkor.

Because kwashiorkor appears almost exclusively in areas with a low-protein diet, protein deficiency is one of the longest-standing proposed mechanisms, but evidence has been conflicting (Gopalan, 1955, 1968; Gupte, 1975; Gupte & Mehta, 1971; Nambisan, 2011; Scrimshaw & Viteri, 2010; Trowell & Davies, 1952). The relationship of kwashiorkor with protein intake is complicated by its relationship to energy. Where energy intake is insufficient, protein will be broken

down for its energy content. Therefore, if protein intake is adequate but marginal, and energy intake is insufficient, protein deficiency may still result (Gopalan, 1968; Scrimshaw & Viteri, 2010). One protein energy theory proposed the opposite, that the origin of kwashiorkor lies with overconsumption of calories in proportion to protein, but this theory was quickly disproven when Gopalan showed children with kwashiorkor in India consumed too few calories (Gopalan, 1968; Scrimshaw & Viteri, 2010).

Another theory, first proposed by Gopalan, was that children with kwashiorkor were maladapted to low-protein diets, whereas children with marasmus had successfully adapted to those diets (Gopalan, 1968). Kurpad questioned the theory that undernourished individuals adapt to low-protein diets, showing that chronically malnourished Indian men did not have reduced lysine requirements, which would have indicated a "metabolic adaptation to low lysine intakes. Yet it is clear that children with kwashiorkor show differences in protein metabolism from children with marasmus. Jahoor and Manary both found that children with kwashiorkor mobilized body proteins slower than those with marasmus (Jahoor et al., 2005; Jahoor, Badaloo, Reid, & Forrester, 2006c; Manary, Broadhead, & Yarasheski, 1998).

It has long been established that the mucosa of children with kwashiorkor is damaged, with stunted villi reducing the ability to absorb nutrients (Brunser, Reid, Mönckeberg, Maccioni, & Contreras, 1966). But it remains unclear whether this is due to chronic exposure to an infectious environment (manifested as

environmental enteropathy), through nutritional deficiencies, or a combination of factors (Brunser et al., 1966; Jahoor et al., 2006c).

It may be that the origin of kwashiorkor has been particularly elusive because it depends on the combined influence of many different environmental, dietary and physiological factors that vary from one context to the next (M. I. Smith et al., 2013). A social factor found to be significant in one area, such as weaning practices in Malawi and South Africa, may not apply in other areas, such as DRC where the most affected age is older (Courtright & Canner, 1995; Newman & Gulliver, 1979). Rather than interpreting this difference to mean that poor weaning practices do not contribute to kwashiorkor at all, it may be that the combination of factors leading to kwashiorkor may vary between contexts though stressing the same metabolic pathways and resulting in similar outcomes (Fuchs, 2005).

Sulfur Amino Acids in the Etiology of Kwashiorkor

As more has become known about amino acids, the theory about protein deficiencies has been revised and narrowed down to particular amino acids (Phadke et al., 1995; Roediger & Waterlow, 1995). As early as 1995, Roediger proposed a deficiency in SAAs (specifically methionine and cysteine but not taurine) as a key factor in the etiology of kwashiorkor based on their plausible roles in many of its characteristic signs (Roediger & Waterlow, 1995). It has also been shown that people with kwashiorkor consistently have low blood concentrations of SAAs and their substrates (Ittyerah et al., 1965; Jahoor et al., 2005, 2006b). Lowered intakes of methionine in guinea pigs result in biological

changes similar to kwashiorkor (Long, 1982). Jahoor and Badaloo found differences in SAA kinetics[3] between children with and without kwashiorkor, though they were unable to determine whether this was a causal factor or as a result of kwashiorkor (Jahoor, Badaloo, Reid, & Forrester, 2006a; Jahoor et al., 2006b, 2006c).

In Smith's study on micro-biota, two of three mice receiving the kwashiorkor micro-biota excreted less fecal methionine and cysteine and lost weight when they were also put on a typical Malawian diet (M. I. Smith et al., 2013). Smith concluded from his results "the combination of a kwashiorkor micro-biota and a Malawian diet may contribute to abnormal sulfur metabolism". The micro-biota in the colon can change the composition of amino acids excreted, especially in a high-fiber diet, and may not reflect true absorption and excretion of individual amino acids (Hendriks, van Baal, & Bosch, 2012). Hendriks explains that the "fermentation of nitrogen-containing components in the large intestine" and subsequent reabsorption of nitrogen as ammonia (NH_3), so alters the amino acid profile and total nitrogen excreted in the feces that examination of feces "would not provide accurate estimates of the amino acids absorbed by the intestine". Therefore, other evidence examining a potential link between SAAs and kwashiorkor is necessary.

Sulfur Amino Acid Metabolism

In order to understand how SAAs may result in the variety of seemingly unrelated signs observed in the syndrome, it is helpful to first review some of the

[3] The rate at which SAAs and particular SAA metabolites are produced or catabolized.

basic metabolic pathways of SAAs, the metabolites or processes related to SAAs that are known to be altered in children with kwashiorkor, and how deficiencies in methionine and cysteine would likely result in particular signs of kwashiorkor.

The human body's primary source of sulfur is from sulfur amino acids (Henry & Ammerman, 1995). The three major SAAs in human nutrition are methionine (Met), cysteine (Cys) and taurine (Tau), of which only methionine is considered an indispensible amino acid (i.e., it must be obtained through the diet) (IOM, 2005). Cysteine can be synthesized from methionine and taurine from cysteine (Stipanuk & Ueki, 2011). During rapid growth, illness or recovery from physical trauma, the requirements for cysteine may be greater than the rate by which it can be converted from methionine, rendering cysteine "conditionally" indispensible (WHO, FAO, & UNU, 2007). Evidence also indicates that transulfuration is also limited in premature infants (Viña et al., 1995). Inorganic sulfur has been shown in poultry to contribute to the production of taurine, but not cysteine or methionine (Gordon & Sizer, 1955; Henry & Ammerman, 1995). Sulfur amino acids are eventually catabolized and excreted, primarily in the form of urinary sulfate (SO_4), while excess cysteine may also be excreted as taurine (Stipanuk & Ueki, 2011).

Though the homeostasis of the cysteine and methionine are very tightly interconnected, each serves very different metabolic functions and will be examined separately.

Methionine Metabolism

Methionine functions primarily as a methyl group donor, though it also serves other critical functions. As shown in figure 1, methionine is a precursor of S-

adenosyl methionine (SAMe) which is then transmethylated through the intermediary S-adenosyl homocysteine (SAH) to form homocysteine (Hcy) (Stipanuk & Ueki, 2011). Homocysteine may either be transulfurated in a one-way reaction to form cystathione (the precursor for cysteine) or remethylated to form methionine (Jahoor et al., 2006b).

When circulating methionine levels are low, the highest priority use of methionine is protein synthesis rather than conversion to SAMe (Courtney-Martin, Ball, & Pencharz, 2012). Methionine is the key initiating amino acid for RNA translation; therefore, no cellular protein synthesis or cellular reproduction is possible in the absence of methionine (Bickler, Ring, & Maio, 2011). S-adenosyl methionine (SAMe) is necessary for many methylation reactions (Mato, Martínez-Chantar, & Lu, 2008) and for regulating the sodium potassium pump (Brown, Dudeja, & Brasitus, 1988; Roediger & Waterlow, 1995). Children with kwashiorkor have low intracellular potassium and high intracellular sodium (Golden, 1998).

Cellular turnover, and therefore cellular reproduction, is highest in the mucosa of the intestines, causing very high demand for SAAs in these tissues. Chen demonstrated that supplementation of methionine improved the integrity of the mucosa and "improved villus architecture" in weanling piglets (Chen et al., 2014). The mucosa is known to be depleted in children with kwashiorkor and their villi truncated (Patrick & Golden, 1977).

Methionine also acts as a substrate in the formation of key metabolites in lipid and energy metabolism. When insufficient carnitine or choline are consumed in

the diet, the body can synthesize them in the liver if the necessary substrates, including methionine, are available (Borum, 1983). Carnitine is necessary to transport fatty acids across the mitochondrial membrane and is necessary for cellular energy metabolism (Borum, 1983). Borum explains that a shortage of carnitine results in muscle weakness due to poor energy metabolism, and a fatty liver due to poor lipid oxidation in the liver. Choline, a precursor for phosphatidylcholdine and betaine, is produced through methylation of phosphatidylethanolamine by SAMe (Fukagawa, 2006; Zeisel, 2000). As with carnitine, a shortage of choline leads to poor lipid mobilization for energy metabolism, as well as cellular apoptosis (Zeisel, 2000). Acylcarnitine and phosphatidylcholine acyl-alkyls have been found to be low in children with kwashiorkor (Di Giovanni et al., 2016). A shortage of methionine in a diet also limited in carnitine and choline could plausibly contribute to the lethargy, poor lipid mobilization leading to fatty liver, and oxidative damage from the cellular apoptosis observed in children with kwashiorkor.

Cysteine Metabolism

Cysteine, acquired either through the diet or transulfuration of methionine, also has a number of potential uses affecting a surprising number of metabolic processes. Cysteine is required for the production of coenzyme A, necessary for the metabolism of fatty acids in the liver, a shortage of which also results in a fatty liver (Leonardi, Zhang, Rock, & Jackowski, 2005). Cysteine is very reactive and potentially toxic; therefore, the body does not store cysteine beyond the limited amounts in the circulating pool of amino acids required for immediate use (Nimni, Han, & Cordoba, 2007). Instead, it is stored in the form of glutathione

(GSH), the most ubiquitous anti-oxidant in the body, present in every cell (Nimni et al., 2007). As Nimni explains, when there is a shortage or increased demand for cysteine beyond what can be supplied through intake or transulfuration, GSH is preferentially converted back to cysteine. A shortage of GSH results in high levels of oxidative damage which can compromise cell wall integrity (Satpute, Hariharakrishnan, & Bhattacharya, 2010). GSH augments "the innate and the adaptive immunity as well as conferring protection against microbial, viral and parasitic infections" and GSH levels are often low after an infection, especially a viral infection (Morris et al., 2012). Mammals on a protein-deficient diet have exceptional difficulty maintaining GSH homeostasis when challenged with an infection as compared to those with adequate SAA intake (Courtney-Martin et al., 2012). The detoxification of some medications used to moderate fever, such as paracetamol, requires sulfur from cysteine further increasing dietary requirements (Glazenburg, Jekel-Halsema, Scholtens, Baars, & Mulder, 1983). Children with kwashiorkor have low levels of GSH and high levels of oxidative damage (Lenhartz et al., 1998; Manary et al., 2000).

Cysteine is an essential ingredient in glycosaminoglycans (GAGs) and is responsible for GAG sulfation (Manary et al., 2009). Sulfated GAGs form the core of the glycocalyx, a fine fur-like covering on the outer cell membrane on endothelial cells which retains large amounts of water in a gel-like medium in the interstitial space (Golden, 2015).

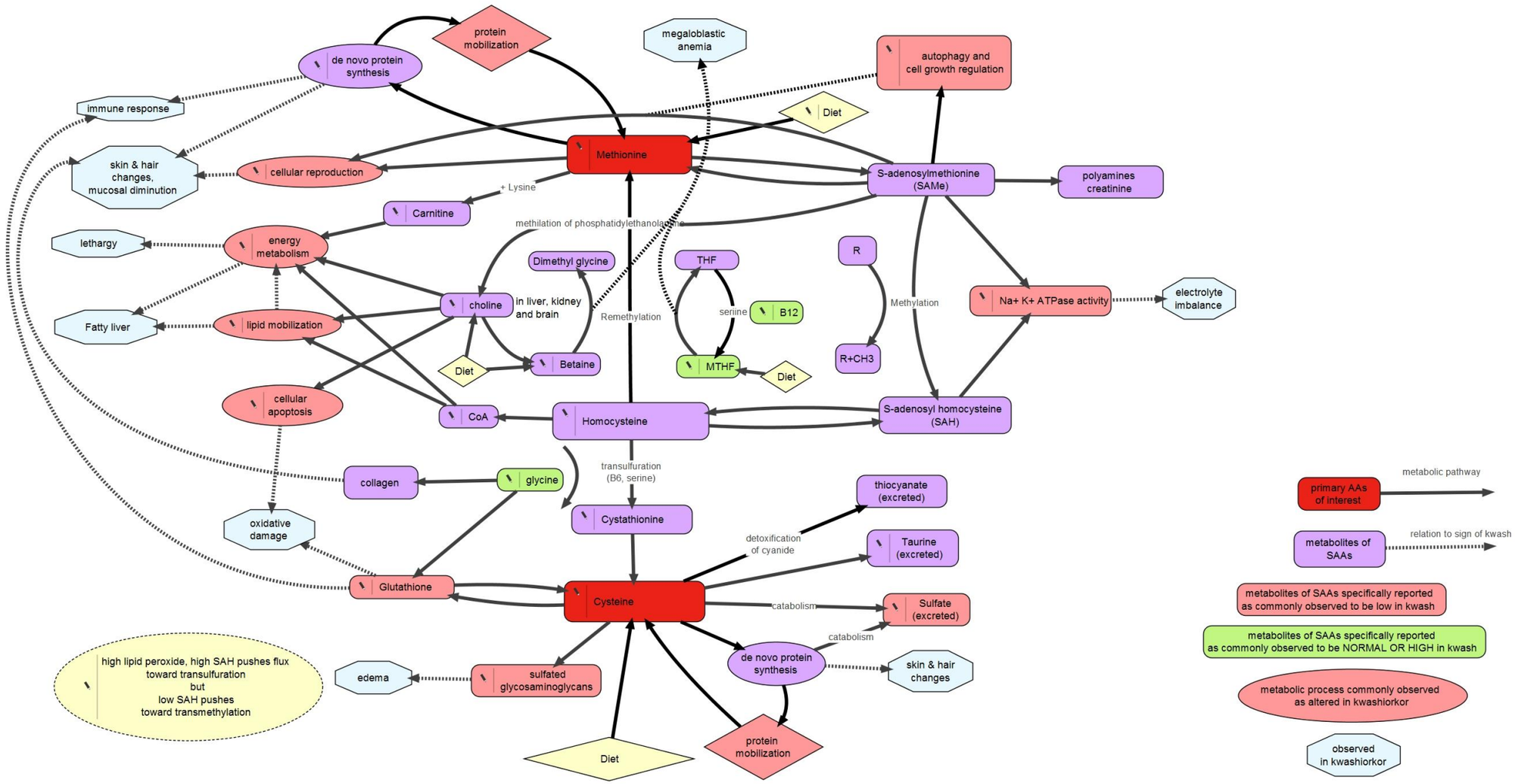

Figure 1: SAA metabolic pathways and metabolites in the presence of low SAA intake and high CN intake

Sulfated GAGs are noted to be low in children with kwashiorkor (Amadi et al., 2009; Chandrasekaran, Mukherjee, & Bachhawat, 1971). When the GAGs lose their sulfur, they also lose their hold on the water and the water then moves more freely in the interstitial space, collecting in lower areas such as the feet and hands, a mechanism that has been proposed as one explanation for the pitting edema seen in kwashiorkor (Golden, 2015).

Evidence indicates that the body requires sulfur from cysteine to detoxify cyanide (CN). When exposed to cyanide, the body renders it less reactive and therefore less toxic through a multi-step process using rhodanese and thiosulfate, both of which use sulfur from cysteine, to form the excreted metabolite thiocyanate (SCN) (Nagahara & Sawada, 2006; Swenne et al., 1996; Wing & Baskin, 1992). Importantly, CN detoxification takes priority use for sulfur above all other uses (Adamolekun, 2011; Banea-Mayambu, Tylleskar, Tylleskar, Gebre-Medhin, & Rosling, 2000; Stipanuk & Ueki, 2011). Swenne showed that even with low SAA intake, rats exposed to cyanide excreted sulfur in the form of urinary thiocyanate (SCN) with a corresponding drop in urinary sulfate (Swenne et al., 1996). The detoxification of high chronic intakes of CN therefore shifts the use of cysteine away from other metabolic uses, even when cysteine is in short supply, resulting in increased overall intake requirement for methionine and cysteine (Banea-Mayambu et al., 2000; Cardoso et al., 2004).

In many parts of eastern DRC, cassava-based food products with high levels of cyanide are dietary staples, requiring extensive processing to reduce the cyanide content (Banea-Mayambu, Poulter, & Rosling, 1992; Mlingi, Poulter, &

Rosling, 1992). When families are displaced by conflict, short of food, or short of time, the cassava root processing is cut from about three days to one, cassava consumption increases, while simultaneously the amount of SAA-rich foods like fish is reduced (Banea-Mayambu et al., 1992; Chabwine et al., 2011). This increases the cyanide load while reducing the cysteine available to detoxify it, potentially leading to SAA deficiencies.

Modulating levels of Methionine and Cysteine

As figure 1 demonstrates, methionine and cysteine are critical to numerous metabolic outcomes, and yet their reactive properties make them toxic, limiting the amount that can safely circulate. Excess methionine raises levels of SAH which push homocysteine toward transulfuration to cystathionine and then to cysteine, a step requiring cystathionine synthetase, and a safety mechanism to keep methionine below toxic levels (Stipanuk & Ueki, 2011). Supplementation of methionine in a low-protein diet increases the demand for cystathionine synthetase beyond its normal availability, blocking further transulfuration to cysteine (Sugiyama, Kushima, & Muramatsu, 1987). Sugiyama found that the resulting high levels of methionine caused liver disease in rats (Sugiyama et al., 1987). These effects are attenuated if glycine and serine are supplemented alongside methionine (Garlick, 2006; Sugiyama et al., 1987).

Excess cysteine can be converted to GSH, then reconverted back to cysteine when cysteine levels are low, but methionine has no equivalent except the catabolism of protein in body tissues, a process that is inhibited in children with kwashiorkor (Badaloo, Reid, Forrester, Heird, & Jahoor, 2002; Jahoor et al.,

2006c; Manary et al., 1998). Conservation of methionine is prioritized when methionine intake and circulating levels are low. Synthesis of proteins is also generally prioritized over conversion to SAMe (Golden, 2009). Low intake of methionine or high demand for protein synthesis, such as during an immune response, reduces SAMe and SAH synthesis. Low levels of SAH will push flux toward remethylation of homocysteine rather than transulfuration (a one-way path out of the methionine cycle) to conserve methionine for replenishing SAMe (Courtney-Martin et al., 2012).

When cysteine levels are high, in addition to increased synthesis of both GSH, production of taurine or oxidation of cysteine to sulfate, both of which can be safely excreted, keep cysteine below toxic levels (Stipanuk & Ueki, 2011). When cysteine levels are low, GSH is preferentially converted back to cysteine. GSH is a major intracellular anti-oxidant, so when GSH drops to a certain point, oxidative stress rises and lipid peroxidase pushes the flux at homocysteine toward transulfuration to replenish cysteine levels (Morris et al., 2012). Children with kwashiorkor are known to have low levels of GSH as well as low cysteine (Badaloo et al., 2002).

Supplementation of cysteine resulted in a rapid rise in the erythrocyte levels of GSH in children with kwashiorkor, indicating that cysteine is limiting in GSH synthesis for these children (Badaloo et al., 2002). Supplementation of methionine did not result in a rise of GSH synthesis until after 5 days of supplementation, even though cysteine was being synthesized, possibly

indicating a deficit in cysteine for other non-GSH needs that first needed to be met (Green et al., 2014).

Methionine and Cysteine Deficiency Related to Kwashiorkor Signs

In summary, the potential outcomes of a deficiency of methionine and cysteine fit very closely with the signs and biomarkers observed in kwashiorkor, as demonstrated in figure 1. Low SAA intake reduces the capacity for *de novo* protein synthesis, reducing production of low priority proteins such as keratin and melanin in the hair and skin, resulting in brittle pale hair, rough skin and lesions. With low methionine intake, the use of methionine directly for protein production takes precedence over conversion to SAMe and may even cause the reconversion of SAMe back to methionine (Courtney-Martin et al., 2012), reducing the levels of SAMe available for one-carbon metabolism and regulation of the Na/K pump. This slows cellular reproduction and impairs electrolyte balance (Roediger & Waterlow, 1995). Retardation of cellular reproduction would be seen first where cellular reproduction is most rapid - in the mucosa - leading to degradation of the mucosa and shortened villi (Bickler et al., 2011).

Labile methyl groups can be provided in the diet through many sources, but all connect with methionine. Disruption of methionine remethylation results in many signs seen in kwashiorkor, such as megaloblastic anemia, lethargy associated with hindered fatty acid metabolism, a fatty liver, and poor membrane health leading to cellular apoptosis and heightened oxidative stress. High oxidative stress increases oxidation of GSH, shifting homocysteine flux toward production of cystathione, a precursor of cysteine and GSH (Nagahara &

Sawada, 2006). This push towards transulfuration competes with remethylation, increasing methionine intake requirements and enlarging the gap between methionine intake and requirement. Low levels of cysteine, meanwhile, demand that some of the remaining GSH be reconverted back to cysteine, further reducing circulating levels of GSH (Jahoor, 2012).

Past Research Designs on the Etiology of Dietary Deficiency Diseases

In developing research strategies on what may be a dietary deficiency disease, it is helpful to review the history of research on other dietary deficiency diseases, how the diseases were distributed within populations, and how researchers went about searching for associations before testing hypotheses through experimentation.

Comparisons of Populations Before Comparisons of Individuals

The first step toward understanding scurvy, beriberi, pellagra and vitamin A deficiency was noting that particular communities, households or vulnerable groups tended to have a higher incidence than other seemingly similar groups (Barrett, 1849; BMJ, 1886, 1894; Carpenter, 1986, 2000; De Mertans, 1778; Durham, 1904; Shapter, 1847). In every case, not all individuals within high-incidence populations were equally vulnerable to the deficiency diseases, even when living on the same diets. Certain individuals or families might also have repeated bouts of the deficiency disease as seasonal changes to the diet reduced the amount of the missing nutrient in the diet, while others appeared to cycle in and out of the disease periodically for other reasons (Carpenter, 1986,

2000; Jarrow, 2014). It was determined that within high-incidence populations, normal interpersonal differences made one individual develop more overt symptoms of deficiency before others while the key underlying vulnerability was a common diet. Researchers compared otherwise similar populations with high- and low-prevalence populations such as residents of institutions, and occupational groups like mill workers in different mills, or sailors on different ships (Carpenter, 1986; Goldberger, Waring, & David, 1915).

The initial successful, informative interventions were changes in the common diet, a blanket supplement or a fortification of the diet at the group level to lower incidence, rather than focusing initially on which individuals within high-incidence populations might be most vulnerable (Carpenter, 1986, 2000). For example, the standardized diets of residents in high-prevalence orphanages and prisons were altered to test the effect of specific dietary changes on incidence of pellagra and beriberi in those populations (Cooper, 1913; Goldberger et al., 1915). Even today, if 5% of children in a population are found to have night blindness, vitamin A deficiency is considered a serious public health problem and all of the young children in that population are given a high-dose vitamin A supplement based on the assumption that all are at high risk of being deficient, just not yet to the point of presenting overt signs (Sphere Project, 2011; WHO, 1996).

Unlike research on other dietary deficiency diseases, it is common in observational studies of kwashiorkor research to select subjects from clinics without regard to their communities of origin, or to compare individual children within communities or even within the same households (Gopalan, 1968; Kismul

et al., 2014; Lin et al., 2007; Sullivan et al., 2006). The unspoken assumption is that because some children within these high-incidence communities or households have developed kwashiorkor and others have not, the primary etiological factors can be found by comparing individuals within those units. When a factor such as diet is generally common to a community or household with only minor differences, then it is discarded as a potential causal factor even if that community has a chronic high prevalence (Golden, 1998; Gopalan, 1968; Kismul et al., 2014; Lin et al., 2007; Sullivan et al., 2006). Instead of trying to detect differences between individuals within a high prevalence population, it may be more productive to follow the pattern of previous successful research on dietary deficiency diseases and first compare factors between high- and low-prevalence populations.

Taking into Account Requirements as well as Intake

Adequacy of a diet is a balance of both intake and requirement, with the requirement varying within a population, hence the use of distribution curves in dietary requirements (WHO et al., 2007). As WHO explains, we do not yet have the ability to determine the true requirement of an individual; the requirement for each individual will change over time depending on a multitude of factors, only a fraction of which are known or can be measured. If, as hypothesized for this study, kwashiorkor is due to a dietary deficiency, intake may be similar throughout a community but interpersonal differences in requirement may result in differences in outcomes. In such cases, comparisons between the diets of cases and non-cases within high-incidence communities might not detect the role

of diet. Therefore, any study considering the role of diet in the etiology of kwashiorkor must also consider other health, environmental and individual factors that could affect requirement. Just as the WHO uses population distributions to describe requirement, it may be more useful to compare intake and exposure to environmental factors affecting requirement at the population level, rather than comparing individuals.

Approaches to Surmounting Difficulties in Measuring Diets in Kwashiorkor

Children with edematous malnutrition very often have reduced appetites, so judging differences in diets becomes very complicated and there are no ideal solutions. Lin assessed the diets of 1,651 children in a longitudinal study in which 43 children developed kwashiorkor using a two-month food frequency recall only at the time of enrollment (Lin et al., 2007). While this allowed measurement of the diet prior to development of the condition, the recall period was abnormally long and in some cases preceded diagnosis by two to three months, long enough for seasonal changes to affect the diet. Sullivan's ingenious solution was to assess the diets of healthy siblings of affected children four weeks after the affected child's admission to treatment, based on the assumption that siblings share the same diet and that diets had not changed during the time between onset of kwashiorkor and the later dietary measurement (Sullivan et al., 2006).

While prospective studies have the benefit of describing the diets leading to kwashiorkor, the low incidence of kwashiorkor in a population with 2% prevalence (common in endemic populations) quickly becomes prohibitively

resource intensive for relatively little statistical power. Gopalan monitored the nutritional status and diets of 300 children every two weeks during their first year of life and monthly thereafter (Gopalan, 1968). After 3 years of following a cohort that started with 300 children, only 7 developed kwashiorkor. In a larger prospective study, Kismul assessed diets of a 4,235 child cohort quarterly over 15 months, identifying just 41 children with kwashiorkor (Kismul et al., 2014). The very high burden of monitoring so many diets meant that Kismul only gathered data on whether or not the child had eaten particular foods, not frequency or amounts of foods eaten.

Use of Bipedal Pitting Edema Complicates Etiological Study Design

There are multiple signs of kwashiorkor that are generally evident before bipedal pitting edema and which are associated with biological changes seen in kwashiorkor (Brock & Autret, 1952; Demaeyer, 1958; Gopalan, 1955, 1968; Whitehead & Dean, 1964). Bipedal pitting edema is therefore a sign of the "advanced state" of kwashiorkor (Scrimshaw & Béhar, 1961). A complication of using a measure that is indicative of an advanced state of malnutrition is that once the child is so ill, it is difficult to determine what characteristics lead to the illness versus those that are caused by the illness. Researchers have responded with creative approaches around this difficulty. For example, Smith et al. fed the fecal micro-biota of three Malawian children with and three without kwashiorkor into gnotobiotic mice (born and raised in a sterile environment) (M. I. Smith et al., 2013). He found that mice with feces from two of three donor children with kwashiorkor lost weight when they were also fed the typical Malawian diet.

Prospective studies appear to be the most logical approach to studying individuals developing kwashiorkor, but as noted above, with the relatively low incidence of advanced kwashiorkor in a population, prospective studies quickly become prohibitively expensive and resource intensive. In Gopalan's study mentioned above, although only seven developed bipedal pitting edema, many more developed other signs of kwashiorkor, specifically facial edema and hair changes (Gopalan, 1968). To ensure a large enough sampling of cases with bipedal pitting edema, researchers have, by and large, resorted to selecting cases from treatment programs.

Many more children tend to present with facial edema and hair changes than those who progress on to the advanced state and few who develop bipedal pitting edema do not also have the facial edema and hair changes. The use of other signs commonly recognized to accompany kwashiorkor, much as was done prior to the establishment of the Wellcome criteria, may be a way to access a large enough sample of children who closely resemble those with advanced kwashiorkor.

Another possibility is to consider the family history of children. The majority of the factors in the UNICEF Framework are household factors that are unlikely to vary significantly in the short to medium term (UNICEF, 2015). Among the underlying causes, sanitation and access to health services depend heavily on infrastructure that does not change frequently. As public nutrition advocates will attest, childcare practices are very difficult to change and one caregiver is likely to use similar practices for each of her children at particular stages of their

development (Adeyemi & Oyewole, 2014). Among the root causes in the framework are factors that depend on the household's human, social and natural capital, which are in large part determined by the character of the members of the households, and long-standing policies, institutions and practices. Therefore, most of the factors affecting the nutritional status of a child are based on the household and are unlikely to change rapidly unless there are strong, sudden changes in the general context or composition of a household. This means that a household that has had malnourished children in the past may be at higher risk of additional cases of malnutrition. In other words, households that have had cases of kwashiorkor in the past may be more likely to have current cases of kwashiorkor. If so, then a household history of past cases of kwashiorkor may well be considered an indicator of risk of kwashiorkor for all children residing in that household.

Summary

Kwashiorkor research has a long history but has failed to identify the etiology of the condition. A lack of data on the global prevalence of kwashiorkor has prevented policy and research from making kwashiorkor a priority. While we do not know its global prevalence, we do know there are many regions where kwashiorkor constitutes a large proportion of SAM. We also know that although kwashiorkor tends to cluster in particular areas, recent research on the etiology of kwashiorkor has often selected cases of kwashiorkor and controls without regard to their communities of origin. Additionally, the decision to use bipedal pitting edema as the sole criterion for diagnosing kwashiorkor has also shifted

research away from children that show other signs of the syndrome, even though studies show that children with other signs share many of the biomarkers with children who have bipedal pitting edema. Ignoring these other signs may lead to misclassification of children at high risk of developing kwashiorkor, potentially confounding research on its etiology, and allowing cases to progress into the advanced stages of the syndrome. A review of study designs that were successful in past dietary deficiency diseases indicates that comparisons of populations are likely to provide many key insights that will make later comparisons of individuals more fruitful.

Although the precise etiology of kwashiorkor remains unclear, we know that it is found only where the general diets are marginal, especially in protein content and quality. Studies comparing protein intake between children with and without kwashiorkor have been conflicting, some showing differences and others not. Nonetheless, studies comparing the metabolic pathways of children with and without kwashiorkor indicate that their SAA metabolism is altered. There are also plausible metabolic pathways linking a deficiency of SAAs with the signs characteristic of kwashiorkor.

Dietary intake of a nutrient is deficient when intake is insufficient to meet requirement. Requirement varies between individuals and even within the same individual, being affected by many factors. Research designs comparing the adequacy of a nutrient between populations therefore must include consideration of factors that will affect requirement as well as dietary intake.

Combining research designs that have served investigation of dietary deficiency disease well in the past with recent advances in the understanding of amino metabolism, factors affecting requirement and a broader definition of kwashiorkor may shed new light on an unsolved riddle.

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Chapter 2

Lost in Aggregation: The Geographic Distribution of Kwashiorkor in Eastern Democratic Republic of the Congo

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Abstract

Background: Kwashiorkor, a major classification of severe acute malnutrition whose etiology remains elusive. It is estimated to affect hundreds of thousands of children annually but no accurate global prevalence figures are available. Little is known how prevalence varies within populations, an important undocumented aspect of kwashiorkor obscured by the aggregation of prevalence or incidence of the condition across large populations and geographic areas.

Objective: To estimate the prevalence of kwashiorkor in select neighboring villages of the eastern Democratic Republic of the Congo and assess if prevalence can vary dramatically among neighboring villages.

Methods: An anthropometric census survey evaluated 1,328 children 12 to 59 months old within all 19 villages in one Health Area of eastern DRC, recording all cases of kwashiorkor, diagnosed by bipedal pitting edema.

Results: Village-level prevalence of kwashiorkor varied from 0% to 14.9% in the study area. Interviews with health services staff in the study area and across two provinces confirmed that current differences in prevalence reflect a long-term pattern and are a common feature of kwashiorkor throughout this region.

Conclusions: Aggregation of kwashiorkor prevalence and incidence data across large populations or geographic regions poses several risks to understanding the epidemiology of kwashiorkor. If clustering of kwashiorkor is not taken into account: 1) nutritional crises in particular villages may go undetected 2) the real effect of interventions may be underestimated; 3) interventions may be inappropriately targeted, leading to reduced coverage, efficacy and cost-efficiency; and 4) important insights into the root causes of kwashiorkor may be lost.

Keywords: kwashiorkor, edematous malnutrition, geographic distribution, prevalence, Congo

Background

Severe Acute Malnutrition (SAM) is classified into marasmus, kwashiorkor, or marasmic kwashiorkor (a mix of the two) (Bahwere et al., 2006; WHO, 2006)[4]. Kwashiorkor was first documented as a specific syndrome in the 1930s (Alvarez et al., 2016), yet despite more than 80 years of research, the specific causes and mechanisms leading to the onset and progression of kwashiorkor remain elusive (Briend et al., 2013; Heikens & Manary, 2009; Manary et al., 2009; Scrimshaw & Viteri, 2010). The epidemiologic distribution of kwashiorkor cases at a local level has not been thoroughly explored, yet may be a key component to understanding the nature of the disease and its treatment, prevention and etiology. This paper examines the geographic distribution of the prevalence of kwashiorkor in one Health Area[5] of eastern DRC and hypothesizes that the aggregation of prevalence data at a high level may mask communities in nutritional crisis.

It is estimated that "hundreds of thousands of children" suffer from kwashiorkor annually, but there is a lack of accurate estimations of its global prevalence (Alvarez et al., 2016). According to Briend, this has allowed kwashiorkor to become a neglected disease (Briend et al., 2013). Although Sustainable Development Goal 2.2 aims to "end all forms of malnutrition," the targets measure wasting and stunting, but not kwashiorkor (UN-DESA, 2015). Neither the initial 2008 Lancet Series on Maternal and Child Nutrition, nor the 2013 update to the series addresses kwashiorkor. In 2008, when two

[4] Marasmus is diagnosed by a weight for height less than 3 standard deviations of the WHO 2006 Growth Standards. Kwashiorkor is diagnosed by bipedal nutritional edema.

[5] A Health Area is the smallest rural administrative unit used by the DRC Ministry of Health, often containing 15 to 25 villages or one small town.

contributors expressed concern at this omission, the authors of the series pointed to the fact that "there are no reliable data available at country level to quantify the number of children with oedematous malnutrition." (Bhutta et al., p. 1749; Ndekha; von Schoen-Angerer et al.)

The Community-Based Management of Acute Malnutrition (CMAM) Forum has attempted to increase recognition of the scale of the problem of kwashiorkor as well as to illuminate its geographical distribution by conducting a review of 2,515 datasets representing more than 1.7 million children, calculating the proportion of SAM cases identified which were edematous, that is, either kwashiorkor or a mix of kwashiorkor and marasmus (Alvarez et al., 2016). Although the data are broken down by country, there can be large differences within a country and the aggregate figures can hide areas where kwashiorkor is especially common. Courtright showed prevalence varied greatly between neighboring districts in Malawi (Courtright & Canner, 1995), and Fonaroff noted in 1969, that in Jamaica kwashiorkor tended to cluster in villages of a particular typology related to childcare habits, presence of parents, adequacy of landholding, and other similar factors (Fonaroff, 1969). The CMAM forum calculated that 32% of SAM cases in the DRC were edematous. Breaking down this data from the Ministry of Health in North Kivu Province, DRC showed that in 2012 more than 55% of children treated for SAM in that province were edematous (PRONANUT, 2013), while surveys at the same time in other parts of the country reported only 4% to 6% of SAM was edematous (ACF, 2012a, 2012b).

The most common nutritional survey method uses a cluster sampling design based on assumptions about the level of clustering among cases of marasmus and gives a single prevalence figure for the entire sample population (Katz, 1995). Sampling strategies in standard nutrition surveys do not allow comparisons of survey sub-populations and cannot identify pockets where cases cluster, leaving communities experiencing nutritional crises undetected. Additionally, studies to detect risk factors leading to kwashiorkor also do not currently take into account the potential for chronic, uneven distribution of kwashiorkor at the local level. They are therefore unable to detect community level factors that may increase risk of kwashiorkor.

This study provides evidence of a highly clustered distribution pattern for kwashiorkor common in eastern DRC and highlights the potential opportunities and risks associated with understanding or ignoring that pattern. The analysis presented here aims to provide evidence to improve the design of studies on factors leading to high risk of kwashiorkor through a better understanding of its distribution within populations.

Methods

The study design was a cross-cutting census survey, screening all children 12 to 59 months old in one Health Area. The DRC Ministry of Health uses Provinces as its largest administrative unit, then Health Zones that are further broken down into Health Areas. The study area was the Murambi/Malehe Health Area of Kirotshe Health Zone, North Kivu Province. This Health Area was selected because the Ministry of Health estimated there was a relatively high

general prevalence of kwashiorkor, it was easily accessible and there was sufficient physical security for study staff.

Children 12 to 59 months old were eligible regardless of the child's nutritional status but excluded if they were under treatment for an illness lasting more than 6 months as this could have affected height and weight. If there were more than one eligible child in a household, all were included. This age population was selected because it contained the highest proportion of all cases treated, according to provincial Ministry of Health (MoH) records. In this region, cases younger than 12 months are relatively rare. It is also much more difficult to diagnose pitting edema among these younger children and their inclusion would have increased measurement error. Eight children were missed where the caregiver was absent or unwilling to give consent. A total of 1,328 children, 99.6% of the total population of 12 to 59 months old, were registered and screened. The figures given include 96% of available children and therefore is considered a census or population measurement rather than a sample measurement. If this was a sample, the number of children would be large enough to detect a difference in proportions between 4.5% and 3% with a power of 0.8.

The survey was conducted in June 2016, at the start of the one short, two-month dry season. The "dry season" is drier only relative to the "rainy season" and does receive enough rain throughout to continue cultivation. Many crops continue almost without seasonal variation, and there was no indication whether

from secondary data, interviews with healthcare staff or with parents, that incidence of kwashiorkor varied seasonally in the study area.

Using the normal admissions protocol, only bipedal pitting edema was used in the classification of cases of kwashiorkor (Bahwere et al., 2006; Golden et al., 2006). Following standard diagnostic methods, edema was detected by pressing the skin at particular points for 5 seconds, then releasing to see if a dimple remained (Bahwere et al., 2006). Age was recorded to the nearest month and middle upper-arm circumference (MUAC) was recorded to the nearest millimeter with the cutoff for severe wasting being 11.5 cm (WHO & UNICEF, 2009). Measurements from three subjects were discarded as either having an age outside of the inclusion range or a physically improbable measurement.

The prevalence of kwashiorkor was calculated as the number of children with bipedal pitting edema, divided by the total number of children screened. The prevalence of wasting was calculated as the number of children with a MUAC less than or equal to 11.5 cm divided by the total number of children screened. SAM prevalence was calculated as the number of children with either kwashiorkor or MUAC less than or equal to 11.5 cm, divided by the total number of children. If a child had mixed kwashiorkor and wasting, he was counted in both the kwashiorkor and wasting prevalence, but only once in the SAM prevalence.

Ethical Reviews and Consent

Ethical reviews of the study protocol were conducted by the Tufts University Social, Behavioral, and Educational Research Internal Review Board

in Medford, Massachusetts, and the *Université Catholique de Bukavu (UCB) Commission Institutionnelle d'Ethique* in Bukavu, DRC. Informed consent was obtained from the caregivers of all registered children.

Results

A total of 1,328 children 12 to 59 months old were registered and screened. Because we evaluated all but a few children present, we were able to calculate the prevalence of kwashiorkor at the village level. Table 1 provides a summary of the results.

Table 1: Population Characteristics

Village/Quartier	**Children 12-59 months**	**Mean age in months** (SD)	**SAM %** (n)	**Edema in feet %** (n)	**Severe Wasting** (MUAC<=11.5) (n)
Kihuhuma	19	38.3 (12.9)	0.0% (0)	0.0% (0)	0.0%(0)
Kachene Malehe	55	36.4 (15.1)	0.0% (0)	0.0% (0)	0.0%(0)
Kautu	36	36.5 (16.0)	0.0% (0)	0.0% (0)	0.0%(0)
Katabiro	82	34.5 (13.5)	1.2% (1)	0.0% (0)	1.2%(1)
Rugeyo	143	32.7 (11.9)	0.0% (0)	0.0% (0)	0.0%(0)
Buganjo I	83	39.5 (15.4)	2.4% (2)	1.2% (1)	2.4%(2)
Kabira	67	37.5 (14.1)	3.0% (2)	1.5% (1)	1.5%(1)
Kanyatsi	61	37.5 (14.9)	1.6% (1)	1.6% (1)	0.0%(0)
Kitingitira	108	39.4 (14.5)	3.7% (4)	1.9% (2)	1.9%(2)
Kavumu	46	32.8 (11.5)	2.2% (1)	2.2% (1)	0.0%(0)
Jerusalem	35	36.5 (14.9)	2.9% (1)	2.9% (1)	0.0%(0)
Bunyangabo	94	33.6 (13.5)	3.2% (3)	3.2% (3)	0.0%(0)
Kaniga	114	31.4 (11.3)	4.4% (5)	4.4% (5)	0.0%(0)
Buganjo II	79	35.1 (13.6)	5.1% (4)	5.1% (4)	0.0%(0)
Kachene Murambi	76	34.1 (12.4)	6.6% (5)	6.6% (5)	0.0%(0)
Kahocho	60	34.9 (12.8)	6.7% (4)	6.7% (4)	1.7%(1)
Mwanza	62	31.9 (14.5)	11.3% (7)	9.7% (6)	1.6%(1)
Rushengeshi	58	32.7 (11.9)	12.1% (7)	12.1% (7)	1.7%(1)
Maendeleo	47	33.3 (13.0)	14.9% (7)	14.9% (7)	0.0%(0)
Health Area total	**1325**	**35.1 (13.6)**	**4.1% (54)**	**3.6% (48)**	**0.68% (9)**
5 villages with highest prevalence	303	33.9 (13.1)	9.9% (30)	9.5 (29)***	0.3% (3)
5 villages with lowest prevalence	335	34.5 (13.4)	0.3% (1)	0%***	1.0 (6)

*** p<0.001 using a chi squared test, difference between the 5 highest and 5 lowest prevalence villages

The overall prevalence of kwashiorkor for this population was 3.6%, but within that area village prevalence ranged from 0% to 14.9%. A prevalence of 3.6% for kwashiorkor would generally be considered high but not a crisis, and would not attract unusual attention (WHO, 2017). A prevalence of 14.9%, on the other hand, indicates a community under extreme nutritional stress [ibid].

Kwashiorkor is usually found in populations where wasting is also found, but both wasting and edema are not always found in the same child and its distribution within a population can be very different. Of the 48 children with edema, only three children had both edema and severe wasting (MUAC$\leq$11.5) and six children had severe wasting but no edema.

The map in Figure 2 provides an idea of the proximity of these villages and their respective kwashiorkor prevalence. The total Health Area with its 19 villages can be contained within a box 4km by 4km. The region is mountainous and houses run roughly in one or two rows to either side of a road or path that follows either a ridge or a valley. GPS coordinates taken at either end of a village and perhaps the center of a curve in the road with a line connecting the points and a buffer to either side of the line give a close approximation of the residential areas of each village. The villages are shaded according to their kwashiorkor prevalence, with lighter shades indicating lower prevalence and darker shades for higher prevalence.

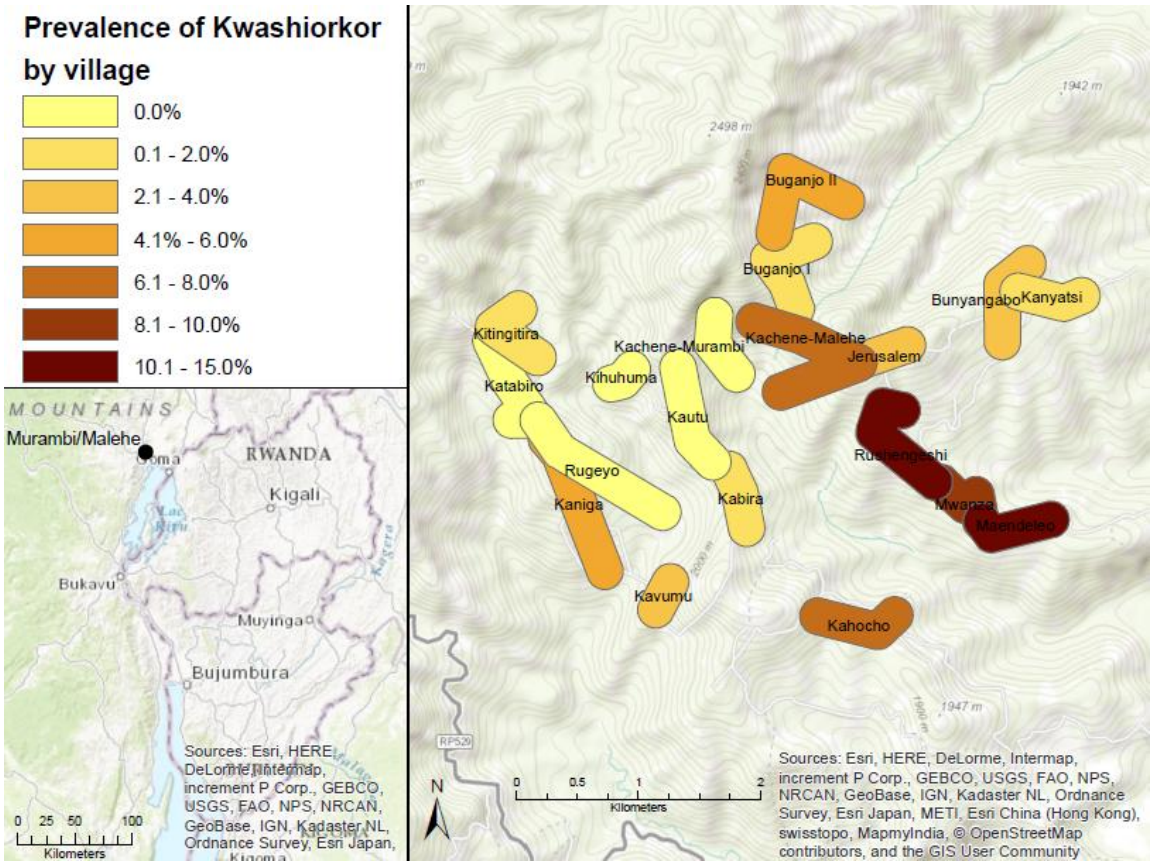

Figure 2: Map of villages in the study area, colored by prevalence of kwashiorkor

When the villages are listed in rank order by their prevalence of kwashiorkor, the differences appear to be gradual. But a visual inspection of the map shows there are definite spatial trends to the distribution of the cases. The map clearly shows one area (outlined with a solid line) with the five villages devoid of cases. The area indicated by the dashed line encompasses the five villages with the highest prevalence, containing 29 of the 48 cases screened in the entire study area. Taken as sub-populations, these groups had similar numbers of total children screened, 335 and 303 respectively, with prevalence of 0% and 9.5% respectively ($p<0.0001$).

Interviews with healthcare staff in ten Health Areas across five health zones in two provinces suggest that the presence of high and low pockets, as shown on this map, may be generalizable at least to eastern DRC. In each Health Center, staff members were able to easily name villages or groups of villages that consistently produced the greatest and least number of cases and often did so before the question was asked. In the Murambi/Malehe Health Area (the study area), the Health Center's initial observation of which villages had the highest and lowest prevalence of kwashiorkor over an extended period was borne out almost exactly by the data in the prevalence survey.

Discussion

The five highest-prevalence villages, with 29 of the 48 cases identified, are clustered together while immediately adjacent another five villages with a similar-size population had no cases (Figure 2). The tight grouping of kwashiorkor cases may be more common than the two documented contexts (Jamaica and eastern DRC) but has gone unnoticed because it is continually obscured by standard nutritional survey techniques. Nutritional surveys in areas where kwashiorkor is most prevalent are generally carried out by agencies with very limited resources. To minimize the staff, time and transportation needed to conduct a survey, most use a standardized approach in which about 30 randomly positioned clusters of about 30 children each are measured, for a total of around 900 children (Golden et al., 2006). This method produces a single prevalence estimate for the entire survey area. That area is often vast, representing tens of thousands of children, but this sampling method is widely accepted as effective

for providing a single but accurate aggregate figure of prevalence of wasting for such large populations (Deitchler, Valadez, Egge, Fernandez, & Hennigan, 2007; FSNAU, 2011; UNHCR, 2009). It is, however, insufficient to allow comparisons of subpopulations within the full survey population (UNHCR, 2009), hiding the fact that there may be communities with crisis levels of malnutrition within the larger sample population and leading to the conclusion that the nutrition situation of a population does not warrant a nutritional intervention.

The last nutrition survey conducted in the Kirotshe Health Zone, the Zone in which the Murambi/Malehe Health Area is located, was conducted by Action Against Hunger (ACF) in August 2014. It gave a prevalence estimate of 0.3% (CI 0.1-1.3%) SAM (all edematous) for the entire Health Zone and was similar to the previous survey in March 2011 which gave a prevalence estimate of 0.2% SAM (CI 0.0-0.7%) (all edematous) (ACF, 2011b). This consistency and the lack of major changes in the area between 2014 and 2016 make this a reasonable estimate for the current prevalence in Kirotshe Health Zone as a whole. Progressing from the more highly aggregated figures to the more disaggregated figures, we have 0.3% kwashiorkor prevalence for the Health Zone, 3.6% for the Murambi Health Area within that Zone, and a range of 0% to 14.9% by village within that Health Area. Each figure gives a different impression of the nutritional status of the population, largely due to the clustering of cases.

These different impressions have a direct impact on resource allocation. With an estimated general SAM prevalence of 0.3%, ACF withdrew from the Kirotshe Health Zone in 2014. The Health Zone reports data aggregated data

from all 15 to 20 Health Areas within the Health Zone to the Provincial MoH level. Provincial MoH officials stated they were unaware of the clustering of kwashiorkor and unaware of the gravity of the situation in the high-prevalence communities prior to the anthropometric survey conducted for this study. Several MoH staff from the provincial office participated in this survey and were so shocked by these concentrated pockets of kwashiorkor that they immediately reallocated resources to the Murambi Health Area to treat them.

Most kwashiorkor study samples are taken from children admitted to treatment programs with kwashiorkor and are compared to children without kwashiorkor, without regard to their communities of origin (Attia et al., 2016; Phadke et al., 1995; Roy Ittyerah, 1969; Rytter et al., 2015) or the studies compare children from within the same community (or even the same family) (Lin et al., 2007; M. I. Smith et al., 2013). This sample selection method implies two assumptions that this paper challenges: 1) that only those children currently affected by kwashiorkor are exposed to key risk factors, 2) risk factors for kwashiorkor must lie at the household or individual level because not all children in a community or family have kwashiorkor at the same time.

This paper provides a case study where certain communities consistently generate many more cases than other nearby communities, and not always from the same households. It is therefore likely that there are factors common within high-prevalence communities that increase risk of kwashiorkor, or other factors common in low-prevalence communities that are protective against kwashiorkor. In essence, all children in the high-prevalence communities (both those currently

with and without kwashiorkor) may be subject to heightened risk. If so, comparisons of children from within the same community or household may not detect differences in particular factors because the two study populations have more similar risk than assumed.

The area studied was small, contained within a 3km radius, and presents a single case-study. Although interviews with healthcare staff indicate that its generalizability very likely extends throughout North and South Kivu provinces, the general tendency to aggregate nutritional data throughout the world limits our ability at this point to determine how generalizable these results are. Understanding that this clustering effect exists, at least in some regions, provides an opportunity to increase the effectiveness of treatment through better targeting in those regions and to explore potential risk factors for kwashiorkor. All villages in this survey shared the same climate, watershed, markets, language, Health Center, religion and schools. Understanding why certain villages or groups of villages continually generate large numbers of cases of kwashiorkor, while others sharing the same general environment and services do not, allows us to control naturally for many potential confounders while not losing the ability to detect risk factors that may be common to households within a community. A detailed examination of childcare practices, diets and other factors that are common in low-prevalence communities, but not in high-prevalence communities, may be able to guide us toward new, durable, low cost, socially acceptable solutions to prevent kwashiorkor. By first recognizing that certain communities may have a high burden of kwashiorkor while neighboring communities do not, then

comparing the differences between those communities, we may gain new insights into the etiology of kwashiorkor and make steps toward its prevention.

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Chapter 3

Dietary intake of sulfur amino acids and risk of kwashiorkor malnutrition in eastern Democratic Republic of the Congo

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Abstract

Background: Kwashiorkor is an often-fatal type of severe acute malnutrition affecting hundreds of thousands of children annually, but whose etiology is still unknown. Evidence suggests that inadequate sulfur amino acids (SAAs) status may explain many signs of the condition but studies evaluating dietary protein intake in the genesis of kwashiorkor have been conflicting. There are no studies on kwashiorkor that measure dietary SAA.

Objective: To determine whether children in a population with a high prevalence of kwashiorkor (HPP) have lower dietary intakes of SAAs than children in a low-prevalence population (LPP).

Design: A cross-sectional census survey design of 358 children was used to compare two adjacent populations of children 36 to 59 months old in North Kivu Province of the Democratic Republic of the Congo. One population had a high prevalence of kwashiorkor (4.5%) and the other had a low prevalence (1.7%). Data collected included urinary sulfate and thiocyanate, linamarin in cassava-based food products, recent history of illness, and a 24-hour quantitative diet recall for the child.

Results: Median intake of SAAs was 32.4 mg/kg for the LPP and 29.6 mg/kg for the HPP ($p<0.05$). A higher percentage of children in the HPP were at risk for inadequate intake of SAAs than in the LPP (23.2% versus 13.3%, $p<0.05$). Methionine was the first limiting amino acid in both populations, with the highest risk for inadequate intake among children in the HPP, (35.1% versus 23.6%, $p<0.05$).

Conclusions: Children in a population with a higher prevalence of kwashiorkor have lower dietary intake of SAAs than children in a population with a lower prevalence. Interventions to reduce incidence of kwashiorkor should consider increasing SAA intake with particular attention to methionine.

Key Words: kwashiorkor, edematous malnutrition, methionine, cysteine, protein, diet, Congo

Introduction

Kwashiorkor, also known as edematous malnutrition, is one of two major classifications of severe acute malnutrition (SAM) and is estimated to affect hundreds of thousands of children each year (Alvarez et al., 2016; Briend et al., 2013; Frison et al., 2015; Kismul et al., 2014). In eastern Democratic Republic of the Congo (DRC), the majority of SAM is kwashiorkor, with more than 10,000 cases of kwashiorkor treated annually in North Kivu Province alone (PRONANUT, 2012, 2013).

Although formally described more than 80 years ago, kwashiorkor's etiology remains uncertain and there are no effective preventive interventions (Alvarez et al., 2016; Briend et al., 2013; Frison et al., 2015; Kismul et al., 2015; Rytter et al., 2015). Protein deficiency is the longest standing proposed causal factor because kwashiorkor exists only in areas where protein intake is low, but the role of protein intake is still debated and evidence has been inconclusive (Annegers, 1973; Courtright & Canner, 1995; Gopalan, 1955, 1968; Kamalu, 1993; Lindtjørn, 1987; Newman & Gulliver, 1979; Scrimshaw & Viteri, 2010; Trowell & Davies, 1952). Gopalan found that 30 children developing kwashiorkor were not eating "qualitatively different diets" than those not developing kwashiorkor, but were at the "lower limit of the range of intakes" recorded by the study (p.53)(Gopalan, 1968). Subsequent studies using food frequency measures did not find differences in protein intake, but two studies using quantitative 24-hour recalls found households with a case of kwashiorkor had lower protein intakes than those without a case (Gupte, 1975; Gupte & Mehta, 1971; Kismul et al., 2014; Lin et al., 2007; Sullivan et al., 2006).

Roediger suggests kwashiorkor may be due to "a patterned deficiency of amino acids" rather than a "global deficiency of amino acids", proposing a deficiency of sulfur amino acid (SAA) intake, methionine and cysteine (Roediger & Waterlow, 1995, p. p. 130) (p.130). Children with kwashiorkor have low circulating SAAs, and altered SAA metabolism (Arroyave, Wilson, De Funes, & Béhar, 1962; Badaloo et al., 2002; Holt, Snyderman, Norton, Roitman, & Finch, 1963; Ittyerah et al., 1965; Jahoor et al., 2005, 2006b, 2006c; Manary et al., 1998). The richest sources of SAAs are animal-source proteins while common vegetable sources of protein such as beans and soy are particularly low in SAAs (USDA, 2017). In regions where very low protein starchy tubers or plantains are used as the primary complement to beans rather than a grain, SAAs are likely to be the first limiting amino acids. Cassava, a starchy tuber and major staple often associated with kwashiorkor, naturally contains cyanogens, which require sulfur from cysteine to detoxify it, further increasing the SAA intake requirement (Annegers, 1973; Bradbury, Egan, & Bradbury, 1999; Graham, Lembcke, & Morales, 1988; Kamalu, 1993; Newman, 1995; Newman & Gulliver, 1979).

To our knowledge, other than the two studies noted above, no studies on the relationship between diet and kwashiorkor have used quantitative 24-hour recalls and none at all have considered the adequacy of individual AAs in those diets. The aim of this study was specifically to test the hypothesis that SAA intake is inadequate for more children in populations with a high prevalence of kwashiorkor than those with a low prevalence.

Subjects and Methods

This study was conducted in the Malehe/Murambi Health Area, Kirotshe Health Zone, North Kivu Province, DRC, comparing two adjacent populations of children 36 to 59 months old, using a cross-sectional census survey design. July and August are the one period when the least rain falls, though still sufficient to cultivate root crops, the primary staples. Due to local administrative delays, the dietary data was collected during one of the two annual bean harvests, possibly giving higher than usual intakes of beans and therefore protein in general, weakening the ability to detect deficiencies in the diets. However, historic admissions data from the Ministry of Health (MoH) and Action Against Hunger (ACF) did not show seasonal variation in admissions of kwashiorkor, so it is not likely that the timing of the recalls critically affected the conclusions (ACF, 2011a; PRONANUT, 2012).

Ethical Reviews and Consent

Ethical reviews of the study protocol were conducted by the Tufts University Social, Behavioral, and Educational Research Internal Review Board in Boston, Massachusetts, and the *Université Catholique de Bukavu, Commission Institutionnelle d'Ethique* in Bukavu, DRC. Caregivers provided verbal informed consent and permission for all registered children.

Recruitment and Sample Selection

A separate anthropometric census survey conducted in June 2017 identified two neighboring villages in the Murambi Health Area, one with a kwashiorkor prevalence of 0% and another with 9.6% among children 12 to 59

months old. This study compares these two populations, with the addition of one small area contiguous with the higher-prevalence population to ensure a large enough sample size. The two populations shared the same climate, market, water table, Health Center, transportation, language, school system, and often even the same churches, naturally controlling for these factors. To minimize within-group variation, the age range for this study was narrowed to 36 to 59 months old (PRONANUT, 2013). Age was the only inclusion criteria. The only exclusion criteria was if the caregiver reported the child was being treated for an illness that had lasted for more than six months, for example, tuberculosis, but no children fit this criteria. If a household contained more than one eligible child, all were included.

Figure 3: Map with the study area indicated by the star

Of all variables to be measured, urinary thiocyanate required the largest estimated sample size. A sample size of 145 per group (n=290) is needed in order to detect a difference in urinary thiocyanate (SCN) of 26 mMol/L (+/-SEM 80) using two-sided alpha = 0.05 and power = 0.8, similar to those found in other studies on cassava-consuming populations (Banea-Mayambu et al., 2000; Banea et al., 2012; Cliff, Muquingue, Nhassico, Nzwalo, & Bradbury, 2011; Okafor,

Okorowkwo, & Maduagwu, 2002). To account for missing samples, the target sample size was increased by 25% to 180 children per group.

Household Data Collection

Enumerators passed from door-to-door in the targeted areas, seeking all eligible children and their caregivers. If a caregiver was not present, the enumerator passed again at a later time. Upon receiving verbal consent from the caregiver, the enumerator enrolled the child, recording the caregiver's name and the child's name, age and sex.

The enumerators measured each child's height to the nearest one millimeter using standard UNICEF height/length boards. Birth date, as reported by the caregiver, was recorded to the nearest month. Children were weighed without shoes or heavy clothing to the nearest 100 grams using digital scales (model TIAN SHAN -2003B). Middle Upper Arm Circumference (MUAC) was measured to the nearest millimeter. Weight was measured three times and MUAC twice with the averages used for analysis. If there was a difference of more that 200g or 2mm between measurements, the surveyors were instructed to repeat the measurements.

Light-colored unkinked hair, facial edema, rough darkened skin, visible lethargy, and bilateral pitting edema in the feet, legs and hands were recorded as potential signs of kwashiorkor (Brock & Autret, 1952). Diagnosis of kwashiorkor used only bilateral pitting edema in the feet, in accordance with the criteria for admission to nutrition treatment (Bahwere et al., 2006). At the end of the

registration process, the enumerator scheduled an appointment to return for a longer interview.

24-hour Diet Recall and Caregiver Interview

We administered a quantitative 24-hour diet recall of the child. The diet recall used the USDA Multi-Pass 24-hour recall method with minor modifications to accommodate local meal preparation and eating habits (Blanton, Moshfegh, Baer, & Kretsch, 2006). Caregivers were asked to use their own pots and plates along with models in order to demonstrate volumes of each ingredient used in the preparation of each dish, the prepared food for the family, and the cooked food that went to the child's plate as well as amounts left uneaten on the child's plate. If multiple people ate from the child's plate, adult male equivalents were used to estimate the child's portion of that serving, calculated based on equivalents of energy by age and sex, following the procedures used by Smith and Subandoro (L. C. Smith & Subandoro, 2007). Enumerators also asked specifically about food the child ate outside of meals.

Other data collected from the caregiver included socio-demographic data of the household (the household being defined as those who eat and sleep regularly in the house), as well as the child's recent health history, defecation habits, and attendance at vaccination and growth monitoring days (Maxwell & Caldwell, 2008; WFP, 2009b).

Urine Samples

A morning urine sample was requested from each child within seven days of the diet recall. Cyanide is quickly metabolized and excreted in the urine as thiocyanate (Haque & Bradbury, 1999). During focus group discussions and key

informant interviews, caregivers reported that most cyanide-containing foods (i.e., cassava products) were eaten at the evening meal; therefore, a urine sample was collected from the child between the main evening meal and going to bed.

On the evening of the urine collection, ice in coolers was prepositioned near the children's homes. On the afternoon of the sample collection, local Ministry of Health (MoH) staff delivered a receptacle with the child's unique identifier on it and instructed the mothers how to collect the urine samples. Caregivers were instructed to take each sample to a collection point as soon as it was collected. The urine samples were collected from the villages in the morning and kept on ice until they could be frozen later in the morning.

Food Sample Collection

To assess cyanide exposure, enumerators collected samples of raw cassava flour from 28 randomly selected households in the HPP and 27 households in the LPP. Ten households from each population provided samples of cooked cassava leaves.

Samples of staples, greens and primary protein sources were purchased in the market where both study populations buy and sell produce. Samples of fresh, immature beans were purchased in the study area because they were not available in the market. A portion of the immature beans, taro and sweet potatoes were cooked in an open pot of boiling water. Volumes before and after cooking were recorded. Cassava and amaranth leaves were air dried to prevent spoilage prior to analysis, with fresh and dried weights recorded.

Nutrient Analysis and Calculations

Samples of cassava flour, taro, sweet potatoes, potatoes, rice, ground nuts, dried beans (two varieties), sorghum flour, maize flour, immature beans, dried cassava leaves, dried amaranth leaves, dried fish (*ndagala*), and smoked fish (*mbuta*) were analyzed by the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories using AOAC Official Method 994.2 for the essential AA profile (plus cysteine) and AOAC Official Method 990.03 for crude protein. For all other foods, the AA composition and digestibility scores were taken from the USDA Food Composition Database and other literature (Escudero, Albarracín, Fernández, de Arellano, & Mucciarelli, 1999; FAO, 1991; Gahlawat & Sehgal, 1998; Graham et al., 1988; Maclean, RomaÑa, Placko, & Graham, 1981; Regnier, Jaguelin, Noblet, & Renaudeau, 2012; Sun, Mu, Zhang, & Arogundade, 2012; USDA, 2017). Rat fecal digestibility scores were used for all foods except cassava leaves and fish-meal where only pig ileal digestibility scores were available(Cervantes-Pahm & Stein, 2014; Regnier et al., 2012)[6].

Samples of raw cassava flour and cooked cassava leaves from the households were analyzed for linamarin using Picrate Kit B2, sourced from Australia University, following Protocol B2 version 1.3 for the cassava flour and a modification of Protocol E version 1.1 for the cooked cassava leaves (CCDN, 2016).

[6] WHO/FAO state that pig ileal digestibility scores are preferable to rat fecal digestibility scores because they reflect absorption of individual amino acids. Pig ileal scores are currently available for a limited range of foods while rat fecal digestibility scores are widely available. For the sake of consistency, rat fecal digestibility scores were used for all foods but cassava leaves and fish meal for which only pig ileal scores were found.

Urine analysis

The evening urine sample was analyzed for thiocyanate using a picrate kit (Kit D) sourced from Australia University following Protocol D1 version 1.3 (CCDN, 2016; Haque & Bradbury, 1999).

Data Analysis

Z-scores for weight-for-age, weight-for-height, and height-for-age were calculated using the WHO Anthro module of commands with STATA 13 SE (WHO, 2006, 2011). Z-scores were calculated based on the WHO Child Growth Standards (WHO, 2006). Children were considered underweight if their weight for age was >2 SD (i.e., a WAZ-score of <-2) below standards, stunted if their weight for length (WFL) Z-score was <-2, and wasted if their weight for height (WFH) Z-score was <-2 or had a MUAC measurement <125mm. Z-scores were calculated using the WHO Anthro module of commands with STATA 13 SE (WHO, 2011). Measurements were dropped by the software as implausible if the z-score was less than -6 or above +6 for height-for-age, less than -6 or above +5 for weight-for-age, or less than -5 or above +5 for weigh-for-height. Pitting edema was measured by pressing a thumb firmly against the skin and holding it for five seconds. If a visible dimple was left, the child was categorized for edema in the place tested. Changes to hair and skin were evaluated visually, sometimes wiping the hair or skin clean with water if the child was especially dusty. Enumerators were shown numerous live examples of children with changes to hair and skin during training to ensure a common understanding.

Percentages of children at risk of inadequacy were calculated as the total number of children whose total estimated intake of a nutrient was below the IOM Estimated Average Requirement (EAR) divided by the total number of children with diet recalls (WHO et al., 2007). The Protein Energy Ratio (PE Ratio) was calculated using the formula: (crude protein grams x 4)/total kilocalorie intake. The requirements for energy intake used the FAO guidelines, accounting for age, weight and sex (FAO, 2001).

The reference protein mg/g of protein used for cysteine and methionine were extrapolated from the ratio of cysteine and methionine recommended for adults by the FAO/WHO/UNU Expert Consultation (WHO et al., 2007). WHO recommends 14.5mg/g for SAAs, rounded up to 15mg/g in the tables. Within the 14.5mg/g, they recommend that at least 10.1mg/g come from methionine, with the remaining 4.5mg/g coming from cysteine. This methionine:cysteine ratio was then extrapolated to the requirement of 17mg/g for children three to ten years old to arrive at 12.19mg/g for methionine and 4.81mg/g for cysteine.

Stata 12 IC was used for all statistical analyses other than the Anthro calculations when Stata 13 CE was used. Chi square and Fisher's Exact tests were used to compare binomial factors. The Wilcoxon-Mann Whitney non-parametric test was used to compare medians. Student's t-test was used to compare means.

Results

Table 2 presents the characteristics of the study participants and their households. A total of 358 children 36 to 59 months old within 301 households

were registered in July 2017 and all data gathered in July and August 2017. From these, caregivers representing 284 households and 338 children completed interviews. Mean household size was significantly larger in the Low-Prevalence Population (LPP) than in the High-Prevalence Population (HPP). Mean age of children in the LPP was about two months older than in the HPP, $p<0.05$.

Table 2: Description of the Study Population

	Low Prevalence Population	High Prevalence Population
Registered Households (301)	149	152
mean household size (SE)	7.1(0.20)**	6.3(0.17)**
Registered Children (358)	181	177
male (186) % (n)	51% (92)	53% (94)
female (172) % (n)	49% (89)	47% (83)
mean age in months (SE)	47.7(0.63)*	45.7 (0.56)*
Measures of the Child		
mean weight/age Z score (SE)	-1.14 (0.09)*	-1.42 (0.08)*
WAZ <-2 %(n)	22.1% (40)	24.3% (43)
mean height/age Z score (SE)	-2.24 (0.13)*	-2.61 (0.10)*
HAZ <-2 % (n)	55.2% (100)*	67.2%(119)*
mean weight/height Z score (SE)	0.393 (0.07)	0.237 (0.08)
WHZ <-2 % (n)	0.6%(1)	1.7%(3)
mean MUAC mm (SE)	148.3 (0.9)*	145.4 (0.9)*
MUAC <125mm % (n)	3.3% (6)	5.1% (9)
Edema in feet % (n)	1.7% (3)	4.5% (8)
Edema in face % (n)	14.4% (26)*	24.9% (44)*
Hair changes % (n)	9.4% (17)***	24.3% (43)***
Health and Environment Characteristics	% (n)	% (n)
Household had case in past 5 years n=279	34.04% (48)***	14.5% (20)***
Child had no illness in past 30 days n=337	26.8% (45)**	13.6% (23)**
Child attends health days at clinic n=335	49.1% (83)*	62.0% (103)*
Child uses a latrine n=338	37.3% (63)	30.8% (52)
Child has thiocyanate in urine n=308	29.4% (48)**	44.8% (65)**

[1]some HH had more than one malnourished member in the past 5 years
*p<=0.05, **p<=0.01, ***p<=0.001 using students t-test for differences in means, Wilcoxon-ranksum (Mann-Whitney) test for differences in medians, chi squared for differences in proportions.

Anthropometric measurements showed that while children in the HPP had a lower mean weight for age Z-score (WAZ), they did not have significantly more children below the -2SD threshold for low WAZ. The lower WAZ is primarily attributable to significantly more stunting in the HPP. Though both populations clearly exceeded the WHO "critical" benchmark of 40% stunting, few children in either population were wasted (WFP, 2009b).

More children in the LPP escaped the previous 30 days without illness as reported by the caregiver, but children in the HPP were more likely to attend growth monitoring and vaccination days at the local health clinic or post. Latrine use among children was higher in the LPP, but the difference was not significant.

Cassava flour samples did not have detectable concentrations of linamarin, but cooked leaves from the HPP had almost three times the mean concentration of linamarin as the LPP (107.5 ppm versus 37 ppm, $p=0.0159$), and while all samples from the HPP had detectable linamarin, only 60% of the LPP samples had detectable concentrations. Children in the HPP ate cassava leaves on average 31.4% more often than those in the LPP ($p<0.01$). Similarly, children in the HPP were more likely to have measureable thiocyanate (the urinary metabolite of linamarin and cyanide) in their urine and had a higher mean urinary concentration (0.88 ppm versus 0.53 ppm, $p<0.05$).

Table 3 shows a comparison of dietary protein and energy intake. The children from the LPP consumed significantly more energy than those in the HPP, though the difference in the proportion of children below their individual requirements is not significant. Protein intake was significantly lower in the HPP

though well above the WHO/FAO/UNU requirement of 0.73 g of protein / kg of body weight / day (FAO, 2001). Significantly more children in the HPP were below this protein intake requirement and their protein:energy ratio was lower than the LPP, though still just above requirement.

Table 3: Protein and energy intake comparison of the two populations

		Kcalories (n=333)	
		LPP (n=165)	HPP (n=168)
Kcal	med	1174[b]	1015[b]
	IQR	763	692
% of children below requirement for energy	%	43.6%	53.6%
	(n)	(72)	(90)

		LPP (n=165)	HPP (n=168)	EAR
		g/kg		
Protein	med	1.78[ac]	1.47[ac]	0.73
	IQR	1.36	1.41	
Digestibility-corrected protein	med	1.36[ac]	1.17[ac]	0.73
	IQR	1.08	1.15	
Children with protein intake below requirement 0.73mg/kg/day	%	13.9%[c]	28.0%[c]	
	(n)	(23)	(47)	
Protein:Energy Ratio	med	7.3%[ac]	6.3%[ac]	5.0%
	IQR	4.3%	3.9%	

[a] significantly different from the requirement using one-sample Wilcoxon signed rank test, $p<0.001$
[b]=$p<0.05$, [c]=$p<0.01$, HPP and LPP significantly different, chi squared test for differences in proportions and Mann-Whitney test for differences in medians

Table 4 examines the quality of the protein in the diets, comparing it against the scoring pattern of amino acids requirements, a type of reference protein. SAAs are the limiting amino acids in the diets of both populations, specifically methionine. Table 5 compares the amino acid intake of the diets, and their sufficiency. The median intake for all AAs (presented as mg/kg/day) exceeded the WHO minimum requirements. Intake for all amino acids was

significantly higher in the LPP. A higher proportion of children in the HPP had intake below the Estimated Average Requirement (EAR) for all AAs except isoleucine and the Aromatic Amino Acids. While the profile in Table 4 shows methionine to be less limiting for the HPP, lower total protein intake in

Table 4: Comparison of Median Protein Quality by Population

		LPP (n=165)	HPP (n=168)	reference	LPP	HPP
		mg/g prot			AA / reference	
Tryptophan	med	11.5	11.5	6.6	1.75	1.74
	IQR	1.8	1.7			
Histidine	med	26.4	25.3	16	1.65	1.58
	IQR	2.9	5.1			
Threonine	med	39.1	37.1	25	1.56	1.48
	IQR	3.4	6.3			
Isoleucine	med	46.0	44.6	30	1.53	1.49
	IQR	3.7	6.7			
Leucine	med	75.8	72.4	61	1.24	1.19
	IQR	11.6	18.4			
Lysine	med	66.0	65.3	48	1.37	1.36
	IQR	10.1	11.9			
Sulfur Amino Acids	med	23.1	25.5	23	1.00	1.11
	IQR	5.5	6.7			
Cysteine	*med*	*10.2*	*10.4*	*6.50*[1]	*1.57*	*1.60*
	IQR	*1.5*	*2.0*			
Methionine	*med*	*12.7*[2]	*14.0*[2]	*16.50*[1]	*0.77*	*0.85*
	IQR	*3.8*	*6.5*			
Aromatic Amino Acids[3]	med	82.5	77.8	41	2.01	1.90
	IQR	20.3	22.5			
Valine	med	52.0	48.5	40	1.30	1.21
	IQR	7.7	13.2			

[1]The reference mg/g of protein used for Cysteine and Methionine were extrapolated from the ratio of Cysteine and Methionine recommended in the WHO 2007 "Protein and amino acid requirements in human nutrition: report of a joint FAO/WHO/UNU expert consultation", Methionine:Cysteine ratio of 2.54:1. (about 70% Met)(WHO et al., 2007).

[2]Significantly different from the requirement, using one-sample Wilcoxon signed rank test, $p<0.001$

[3]Tyrosine and phenylalanine

combination with protein that is very low in methionine results in low total methionine intake in the HPP and a significantly higher proportion of children in the HPP with methionine intake below the EAR.

Table 5: Comparison of Individual Amino Acid Intakes

		LPP (n=165)	HPP (n=168)	EAR		LPP (n=165)	HPP (n=168)
		mg/kg				population below EAR % (n)	
Tryptophan	med	16.4[b]	13.8[b]	4.8	%	3.64[d]	8.93[d]
	IQR	12.4	13.5		n	6	15
Histidine	med	36.0[b]	29.2[b]	12	%	10.3[d]	17.86[d]
	IQR	31.2	33.0		n	17	30
Threonine	med	53.7[b]	42.8[b]	18	%	7.27[d]	14.88[d]
	IQR	45.8	48.2		n	12	25
Isoleucine	med	61.8[b]	51.6[b]	22	%	9.7	15.48
	IQR	49.1	56.6		n	16	26
Leucine	med	106.6[c]	82.5[c]	44	%	12.12[e]	25.6[e]
	IQR	86.9	104.0		n	20	43
Lysine	med	88.2[b]	73.1[b]	35	%	10.91[e]	21.43[e]
	IQR	71.2	77.4		n	18	36
Sulfur Amino Acids	med	32.4[a]	29.6[a]	17	%	13.33[d]	23.21[d]
	IQR	26.7	26.2		n	22	39
Cysteine	*med*	*14.4*[b]	*13.1*[b]	*4.81*[1]	*%*	*6.06*[d]	*12.5*[d]
	IQR	*12.0*	*13.0*		*n*	*10*	*21*
Methionine	*med*	*17.3*[a]	*16.6*[a]	*12.19*[1]	*%*	*23.64*[d]	*35.12*[d]
	IQR	*14.8*	*15.6*		*n*	*39*	*59*
Aromatic Amino Acids	med	119.2[b]	92.5[b]	30	%	6.06	11.31
	IQR	107.9	114.4		n	10	19
Valine	med	68.6[b]	56.1[b]	29	%	9.7[f]	25.0[f]
	IQR	58.4	68.6		n	16	42

[a] - p<0.05, [b] - p<0.01, [c] - p<0.001, Significantly different between populations using the Mann Whitney test
[d] - p<0.05, [e] - p<0.01, [f] - p<0.001, Significantly different between populations using the Chi square test
[1]The reference mg/g of protein used for cysteine and methionine were extrapolated from the ratio of cysteine and methionine recommended in the WHO 2007 "Protein and amino acid requirements in human nutrition : report of a joint FAO/WHO/UNU expert consultation", Table 22, page 147 - methionine:cysteine of 2.54:1 (or 70% methionine).

Discussion

The results of this study support the hypothesis that children in a population with a high prevalence of kwashiorkor in eastern DRC have lower SAA intake than a neighboring population with low prevalence of kwashiorkor. Methionine in particular, was the first limiting amino acid. The HPP had a higher proportion of children with low protein intake, but the proportions with low energy intake in the two populations were not significantly different.

While children in the HPP are clearly more likely to have inadequate SAA intake, the situation is likely worse than it appears when using the current requirements. The WHO estimated average intakes were set based largely on studies of replete infants and adults living in clean environments with the caveat that children who have parasitic loads, are stunted, have recently been ill, or who live in an environment with poor sanitation, will have higher requirements (WHO et al., 2007). Children in the HPP were more likely to defecate in the open, had more illness and were more stunted than the LPP; hence, likely had higher requirements than either the WHO reference population, or the LPP. Children in the HPP were also exposed to more cyanide in their food, as seen in our analyses of cassava leaves and urine. This is important since detoxification of cyanide requires sulfur from the SAAs (Banea-Mayambu et al., 2000; Tor-Agbidye et al., 1999). The children in the HPP therefore had lower intakes of SAAs, methionine in particular, with likely higher demand.

There are many areas of the world with ill, stunted children living in highly infectious environments, yet only a fraction of these areas have endemic kwashiorkor (Courtney-Martin et al., 2012; Dewey, Meaton, Fjeld, Lönnerdal, & Reeds, 1996; Kurpad et al., 2003; Powanda, 1977; WHO et al., 2007; Wilmore, 1999). This study focused on intake of SAAs below requirement as a potential factor unique to populations with high kwashiorkor prevalence, finding that SAAs, methionine in particular, were limiting in the study diets and a higher proportion of the HPP had inadequate intake than the LPP. As early as 1952, Brock and Autret estimated that methionine was more limiting in Nigerian populations with higher prevalence of kwashiorkor (Brock & Autret, 1952). Researchers in the 1960s found that children with kwashiorkor consistently have low circulating levels of SAAs (Badaloo et al., 2002; Jahoor et al., 2006b, 2006c). Roediger first proposed plausible metabolic pathways linking SAA deficiency with signs characteristic of kwashiorkor more than 20 years ago, and others have added extensively to this body of knowledge (Roediger & Waterlow, 1995).

One of the riddles of kwashiorkor is the wide variety of symptoms commonly seen together and the lack of a common nutritionally related biological mechanism to link them all. Recent advances in the understanding of amino acid metabolism now provide additional insights. Methionine and cysteine are both necessary for all human proteins, with hair and skin proteins generally considered the lowest priority proteins and among the first tissues to show depletion when SAAs are inadequate. Epithelial cells in the mucosa have very high turnover, requiring relatively large amounts of both SAAs, a shortage of

which compromises the integrity of the mucosa, such as seen in kwashiorkor, reducing its absorptive capacity (Brunser et al., 1966; Jahoor et al., 2006c).

Cysteine and methionine are usually combined in a single requirement, though each serves very different metabolic functions. Methionine is primarily a methyl donor, interlinking with choline, betaine, folate and B12 cycles and forms a key substrate for carnitine (Nimni et al., 2007). While there are multiple dietary sources of labile methyl groups, all must link with methionine to maintain their cycles. A shortage of labile methyl groups interrupts the production of proteins, fatty acid oxidation, and mitochondrial function, among other effects (Zhu, Wu, Tang, Leng, & Cai, 2014). Some of the signs associated with these interrupted processes are low protein production and turnover, cellular apoptosis leading to increased oxidative stress, lethargy and a fatty liver, all signs associated with kwashiorkor.

The unique properties of cysteine derive from the position of its sulfur atom, donating the sulfur-containing moiety during synthesis reactions, or stabilizing the secondary structure of proteins through sulfur-sulfur bonds and can also be linked to many of the signs of kwashiorkor (Nimni et al., 2007). Glutathione (GSH) is produced from cysteine, but is converted back to cysteine when levels of cysteine are insufficient for other priority uses like *de novo* protein synthesis during an immune response or the detoxification of cyanide (Grimble, 2006). Studies on GSH and cysteine in kwashiorkor are not conclusive, but indicate that low levels of GSH observed in kwashiorkor may be due to a combination of increased demand for GSH due to heightened oxidative stress,

and to the limited availability of cysteine for its production (Badaloo et al., 2002; Golden, 1998; Grimble, 2006). Children with kwashiorkor showed marked increases in GSH production during recovery after five days of supplementation with N-acetylcysteine (Badaloo et al., 2002). The glycocalyx, which forms a gel-like coating around epithelial cells, is primarily composed of sulfated glycosaminoglycans. These bind a large amount of water in the interstitial space that is released when sulfur is short and this release of water is a proposed mechanism for the pitting edema characteristic of kwashiorkor (Amadi et al., 2009; Golden, 2015).

This study was able to show an association between high prevalence of kwashiorkor and low intakes of SAAs, in a diet where methionine was the first limiting amino acid, and where requirement was likely heightened due to increased incidence of illness, exposure to an infectious environment and exposure to cyanide. We describe the multiple potential metabolic pathways by which inadequate SAAs might contribute to the signs characteristic of kwashiorkor. This was an observational study and does not provide evidence on causation. Although a previous study that supplemented cysteine without methionine did not reduce incidence of kwashiorkor, trials providing a balance of methionine with cysteine and glycine are necessary to explore the causative role of SAAs in the etiology of kwashiorkor (Ciliberto et al., 2005).

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Chapter 4

The strong association of low dietary sulfur amino acid and recent illness with risk of kwashiorkor

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Abstract

Background: Though kwashiorkor was identified as a syndrome more than 80 years ago, its etiology is still unknown. It is found only in areas where sanitation and high-quality dietary protein intake are poor. Recent research indicates a potential link to sulfur amino acids (SAAs), but their relative role in the etiology of kwashiorkor remains unclear.

Objective: To determine the relative contribution of low dietary intake of SAAs, including that mediated through other factors, to the risk of kwashiorkor among rural children in DRC.

Methods: Among a population of 338 children 36 to 59 months old and a 3.3% prevalence of kwashiorkor, two non-exclusive high-risk outcomes were identified: 90 with hair changes and facial edema (HE), and 82 whose families had a history of a child with kwashiorkor in the previous 5 years (FH). Caregivers were interviewed, a child 24-hour quantitative dietary recall administered and urine analyzed for thiocyanate. Direct comparisons and path analysis provided the relative associations of multiple factors to HE and FH.

Results: Path analysis with FH as the outcome indicated low dietary intake of SAAs had the strongest total effect, increasing risk of FH (0.18 SE 0.08), followed by lower FCS (-0.15 SE 0.05) and the child not using a latrine (-0.15 SE 0.05). Path analysis with HE as the outcome indicated FH as predictive factor had the strongest total effect (0.26 SE 0.07), followed by recent illness (0.18 SE 0.04) and low Food Consumption Scores (-0.16 SE 0.00). Low intake of SAAs did not have a significant effect on risk of HE.

Conclusions: A low-quality diet, low intake of SAAs in particular, coupled with poor sanitation may predispose children to kwashiorkor. The increased demand for nutrients to mount an immune response during infection may create a gap between intake and requirement for SAAs, potentially leading to the onset of kwashiorkor.

Keywords: kwashiorkor, edematous malnutrition, etiology, diet, sulfur amino acid, cysteine, methionine, Congo, conflict

Introduction

Kwashiorkor is a classification of severe acute malnutrition (SAM) characterized by edema, loss of hair pigment and quality, lethargy, fatty liver, skin lesions and metabolic changes, among other signs, though currently only bipedal pitting edema is used for a clinical diagnosis (Bahwere et al., 2006; Lancet, 1970; Scrimshaw & Béhar, 1961; WHO, 2013). Prior to the establishment of this criterion, kwashiorkor research often considered other signs that generally precede the bipedal edema (Scrimshaw & Béhar, 1961). Facial edema and pale, brittle hair were considered signs of "pre-kwashiorkor" or "subclinical kwashiorkor" as these children had similar metabolic anomalies as those with edema and were considered at very high risk of progressing into the more advanced stages of kwashiorkor (Gopalan, 1968; Whitehead & Dean, 1964).

Though kwashiorkor was recognized as a syndrome more than 80 years ago, its etiology remains a puzzle and no single causal factor has been isolated (Alvarez et al., 2016; Briend et al., 2013; Frison et al., 2015; Kismul et al., 2015; Rytter et al., 2015). Endemic kwashiorkor is found only in areas where protein intake is typically low and sanitation poor, but the role of diet in the etiology of kwashiorkor is not clear (Annegers, 1973; Courtright & Canner, 1995; Gopalan, 1955, 1968; Kamalu, 1993; Lindtjørn, 1987; Newman & Gulliver, 1979; Scrimshaw & Viteri, 2010; Trowell & Davies, 1952).

Several studies using food frequency measures found few differences in the frequency of protein-rich food consumption between households with and

without cases of kwashiorkor. On the other hand, two studies using quantitative 24-hour recalls found significant differences in amounts of protein consumed (Gupte, 1975; Gupte & Mehta, 1971; Kismul et al., 2014; Lin et al., 2007; Sullivan et al., 2006). Research into potential shortages of individual amino acids rather than total protein has led to investigation of the potential role of low dietary intake of sulfur amino acids (SAAs) (Roediger & Waterlow, 1995, p. p. 130). Children with kwashiorkor consistently have low circulating (i.e., serum and erythrocyte) concentrations of SAAs, and there are plausible metabolic pathways linking inadequate circulating SAAs with the many signs of kwashiorkor (Alvarez et al., 2016; Arroyave et al., 1962; Badaloo et al., 2002; Borum, 1983; Courtney-Martin et al., 2012; Di Giovanni et al., 2016; Fukagawa, 2006; Golden, 2015; Holt et al., 1963; Ittyerah et al., 1965; Jahoor, 2012; Jahoor et al., 2005, 2006b, 2006c; Manary et al., 1998; van de Poll, Dejong, & Soeters, 2006; Zeisel, 2000; Zhu et al., 2014).

Animal-source proteins are rich in SAAs, while cheaper vegetable proteins like beans and soy are particularly low in SAAs (USDA, 2017). Cassava and white sweet potatoes are limiting in SAA content, and are staples in many kwashiorkor-endemic regions (Newman, 1995). Among the poorest, these tubers are often eaten in combination with beans, providing a diet with SAAs as the first limiting amino acid (Newman, 1995; Ngudi, Kuo, & Lambein, 2002). Cassava roots and leaves also naturally contain cyanide (Banea-Mayambu et al., 1992). Sulfur from cysteine is required to detoxify cyanide (CN), converting it to thiocyanate (SCN) which is then excreted in the urine (Zottola, 2009). Cassava,

therefore, not only contributes very little SAA to the diet, but may also increase requirement for SAAs (Cardoso et al., 2004; Ngudi et al., 2002).

Malnutrition results from a combination of diet and factors that inhibit utilization or increase demand for key nutrients. The UNICEF conceptual framework of the determinants of child undernutrition proposes that a combination of food insecurity, poor childcare and healthcare, and high exposure to an infectious environment lead to disease and a poor diet, all contributing to malnutrition (UNICEF, 2015). According to a WHO/FAO/UNU expert panel, children who have recently been ill, are infected with parasites, living in an infectious environment, or are stunted, have higher protein and amino acid requirements; however, these conditions are not accounted for in setting the current requirements (WHO et al., 2007). Adequacy of dietary nutrients must therefore consider the relative roles of factors that influence true requirements as well as intake.

The underlying causes of malnutrition highlighted by the UNICEF framework are by and large household characteristics: food security, maternal and childcare practices, and sanitation and healthcare (UNICEF, 2015). These are characteristics that are very difficult to change and vary little from year to year unless influenced by a major event like conflict, construction of a clinic or nutrition-specific interventions (Devereux & Waidler, 2017). Therefore, controlling for such events, a household with a history of malnutrition likely has a combination of long-term characteristics that will continue to put its most vulnerable members at high risk of that type of malnutrition.

This study examines 338 children from one Health Area in DRC with a 3.6% prevalence of kwashiorkor, to test the hypothesis that low SAA intake has a stronger association than other measured factors to risk of kwashiorkor, operationalized as having a family history of kwashiorkor (FH) and as having hair changes or facial edema (HE).

Subjects and Methods

This was a cross-sectional study of all children 36 to 59 months old within a section of the Malehe/Murambi Health Area, Kirotshe Health Zone, North Kivu Province, Democratic Republic of the Congo. This Health Area was selected because it had a high prevalence of kwashiorkor, was easily accessible by motorcycle and had relatively good security. The diet in this tropical, mountainous equatorial region is heavily dependent on low--nutrient starchy tubers and beans, supplemented with small amounts of fish.

Figure 1: Location of the study area marked by the star

Ethical Reviews and Consent

The study was conducted following a protocol approved by the Tufts University Social, Behavioral, and Educational Research Internal Review Board in Medford, Massachusetts, and the *Université Catholique de Bukavu (UCB) Commission Institutionnelle d'Ethique* in Bukavu, DRC. Caregivers of all registered children provided verbal informed consent.

Recruitment and Sample Selection

Having an age 36 to 59 months to the nearest month, as reported by the caregiver, was the sole inclusion criteria among all children living in the Murambi/Malehe Health Area. The sole exclusion criteria was a current illness, such as tuberculosis, that had required treatment for more than six months, as reported by the caregiver. All eligible children in a household were registered.

A sample size of at least 145 children per comparison group (n=290) were necessary to detect a difference in urinary thiocyanate (SCN) of 26 uMol/L (+/- SEM 80), alpha 0.05, power 0.8, similar to differences found in studies on similar cassava-consuming populations (Banea-Mayambu et al., 2000; Banea et al., 2012; Cliff et al., 2011; Okafor et al., 2002). To cover a potential significant refusal rate in providing urine specimens, 25% was added, bringing the target sample size to 180 children per comparison group.

Household Data Collection

To ensure maximum enrollment of eligible children, enumerators went door-to-door throughout the Murambi/Malehe Health Area. Upon receiving the caregiver's verbal consent, the enumerator registered the caregiver's name, and the child's name, sex and birth date to the nearest one month as reported by the caregiver, measured each child's height to the nearest one millimeter using standard UNICEF height/length boards, weight without shoes or heavy clothing to the nearest 100 grams using digital scales (model TIAN SHAN -2003B), and Middle Upper Arm Circumference (MUAC) to the nearest one millimeter. Weight was measured three times and MUAC twice, with the average used for analysis.

Enumerators repeated the measurements if there was a difference of more than 200 g (for weight) or 2 mm (height or MUAC) between them.

During both enrollment and later during the interview, enumerators evaluated the children for light-colored unkinked hair, facial edema, rough darkened skin, visible lethargy, or bilateral pitting edema in the feet (Brock & Autret, 1952). Signs noted during the interview were used in the analysis as being nearer to the time of the diet recall and urine sampling. Enumerators checked for pitting edema by pressing a thumb firmly against the skin and holding it for five seconds before releasing. If a visible dimple was left, the child was categorized as edematous in the place tested. Changes to hair and skin were evaluated visually. Enumerators were shown numerous live examples of children with all signs of kwashiorkor during training to ensure a common frame of reference.

Quantitative 24-hour Diet Recall and Caregiver interview

The caregiver interview included questions about both the household and child, including a 24-hour quantitative diet recall of the child. The diet recall used the USDA Multi-Pass 24-hour recall method, modified to the local context (Blanton et al., 2006). The caregiver used her own pots and plates, standard local measures and models to demonstrate volumes. Enumerators recorded the reported volume of each ingredient used, the volume of each dish after it had been cooked and the volume of cooked food served on the child's plate as well as the volume of food left uneaten on the child's plate. Where the child shared a plate with others, adult male equivalents based on energy requirement by age

and sex of each person eating from the plate (following the procedures used by Smith and Subandoro) were used to estimate the registered child's portion of that serving (L. C. Smith & Subandoro, 2007). Enumerators also probed for food given to the child outside of meals, sometimes by neighbors.

Household data included: the age and sex of all household members (defined as those who normally eat and sleep in the house), cases of kwashiorkor in the household during the previous five years, the structure and condition of the house (narrowed to ownership of a good tin roof as a proxy for wealth since few other durable goods were widely owned), a seven-day food frequency and food security. Household food security used two validated measures: the Food Consumption Score (FCS) and the Coping Strategies Index (CSI) (Maxwell & Caldwell, 2008; WFP, 2009a; Wiesmann, Bassett, Benson, & Hoddinott, 2009). The FCS is a weighted food frequency measure and was used as an indication of diet quality. Child data included: all current signs of kwashiorkor, history of conflict-related displacement, illnesses experienced in the previous 30 days, previous history of malnutrition, defecation habits (open defecation versus use of a latrine), healthcare access (attendance at routine growth monitoring and vaccination days), and whether or not the child was fed between breakfast and supper.

Urine Sample Collection

Within seven days of the diet recall, caregivers were requested to provide an evening urine sample from each child. To best capture traces of cyanide from consumption of cassava roots and leaves, one sample was requested to be

collected after the main evening meal (the meal when focus group participants reported cassava was most commonly eaten) (Haque & Bradbury, 1999).

To minimize rejection of urine sampling, the study team conducted an intense sensitization campaign. On the afternoon of the sample collection, MoH staff supporting the study instructed the mothers how to collect the urine samples as they delivered a receptacle with the child's unique identifier on it. Caregivers were instructed to take each sample, as soon as it was collected, to a nearby collection point where ice in coolers had been prepositioned. The research team retrieved the urine samples from the village collection points in the morning and kept them on ice until they were placed in a freezer later in the morning.

Food Sample Collection

To verify sources of cyanide exposure, enumerators collected samples of raw cassava flour (about 10-15g) from 55 randomly selected households. Twenty households provided samples of cooked cassava leaves (about 10-20g).

Samples of the most commonly eaten foods were purchased in the local market where both study populations buy and sell produce. Samples of fresh, immature beans were purchased from a producer in the study area because they were not available in the market.

Nutrient Analysis and Calculations

All foods sampled except the cooked cassava leaves were analyzed by the University of Missouri-Columbia Agricultural Experiment Station Chemical Laboratories using AOAC Official Method 994.2 for the essential AA profile (plus cysteine) and AOAC Official Method 990.03 for crude protein. Digestibility

scores for all foods and the AA composition for foods not tested by this study were obtained from the USDA Food Composition Database and other literature (Escudero et al., 1999; FAO, 1991; Gahlawat & Sehgal, 1998; Graham et al., 1988; Maclean et al., 1981; Regnier et al., 2012; Sun et al., 2012; USDA, 2017). Rat fecal digestibility scores were used for all foods except cassava leaves and fish-meal (Cervantes-Pahm & Stein, 2014; Regnier et al., 2012)[7].

Picrate Kit B2 sourced from Australia University, following Protocol B2 version 1.3, was used to assess the concentration of linamarin in the cassava flour and a modification of Protocol E version 1.1 for the cooked cassava leaves (CCDN, 2016).

Urine analysis

Picrate Kit D sourced from Australia University, following Protocol D1 version 1.3, was used to analyze the evening urine sample for thiocyanate (CCDN, 2016; Haque & Bradbury, 1999).

Data Analysis

WHO Anthro module with STATA 13 SE, using the WHO Child Growth Standards calculated Z-scores for weight-for-age, weight-for-height, and height-for-age (WHO, 2006, 2011). Measurements were dropped by the software as implausible if the Z-score was below -6 or above +6 for height-for-age, below -6 or above +5 for weight-for-age, or below -5 or above +5 for weigh-for-height. Children were considered underweight, stunted or wasted if less than -2 Z-scores

[7] Pig ileal digestibility scores are preferable to rat fecal digestibility scores because they are considered more reflective of the actual absorption of individual amino acids. Unfortunately, pig ileal scores are available for only a few foods while rat fecal digestibility scores are much more widely available. To allow for a more consistent approach, rat digestibility scores were used for all foods but cassava leaves and fish meal for which only pig ileal scores were available.

(more than 2 SD below WHO standards) for weight for age, height for age, and weight for height respectively. MUAC less than 125mm was also recorded as wasted.

The proportion of children at risk of dietary inadequacy was the number of children with an estimated intake of a nutrient below the WHO Estimated Average Requirement (EAR) divided by the number of children with diet recalls x 100 (WHO et al., 2007). Requirements for energy intake followed the FAO guidelines, accounting for age, weight and sex (FAO, 2001). We used the formula (crude protein grams x 4)/total energy intake to calculate the Protein Energy Ratio.

Two binary outcome variables were created for the analysis: having hair changes or facial edema (HE), and having a family that had had a case of kwashiorkor in the previous five years (FH). These were not exclusive, i.e., a child could qualify for both FH and HE. Two separate sets of analyses were conducted, one using FH as the outcome and the other HE.

Stata 12 IC was used for all statistical analysis other than the Anthro calculations. The Chi Square test was used to compare binary factors. The Wilcoxon-Mann Whitney non-parametric test was used to compare medians. Student's t-test was used to compare means.

The effects of more general factors on the outcomes of malnutrition are often partly direct and partly mediated by more immediate causes, an aspect obscured in regression models. Path analysis was used to assess the relative contributions of each factor to risk of the outcomes, taking into account this

mediation. Two separate path models, one with FH and one with HE as outcome variables, used the Stata 12 Structural Equation Model (SEM) commands, adjusted for clustering at the household level. Initial path models were based on the UNICEF framework, with basic causes at the broad, societal level, underlying causes at the household level, and immediate causes at the level of the child. Through an iterative process, those effects that were not significant were eliminated, leaving only the significant effects.

Results

Data were collected during July and August 2016. A lack of seasonality in kwashiorkor treatment admissions indicates timing is unlikely to have affected dietary intake or prevalence of signs of kwashiorkor (PRONANUT, 2013). A total of 338 children were registered, with 16 caregivers not giving consent for their children, and no children fit the exclusion criterion, providing 95.3% coverage. Ninety children had changes to hair or facial edema (HE) (Table 6). Eighty-two children lived in households in which a member had been diagnosed with kwashiorkor in the previous five years (FH). Table 6 provides a description of the entire sample, as well as those with FH and those with HE. It does not compare children with FH to those with HE as there are 39 children with both of these outcomes.

Children with FH were not more likely than the rest of the sample to be stunted, wasted, or to have bipedal edema, but were more likely to have hair changes and facial edema (Table 6). The FH children had lower household diet quality (FCS) and worse food security (CSI). Similarly, they were more likely to

have low protein and SAA intake, but they did not consume less energy. These children were less likely to live in a house with a good tin roof (a sign of wealth), less likely to use a latrine, more likely to have had to flee from conflict ("displaced"), more likely to have been ill recently, but more likely to have attended health days at the clinic.

Children with HE were more stunted and wasted than the rest of the sample (Table 6). All but one of the cases with bipedal edema also had HE. The HE children were more likely than the rest of the sample to have a family history of kwashiorkor, were less likely to use a latrine and were more likely to have been ill recently, but were also more likely to have attended the health days at the clinic. Although they had lower household diet quality (FCS) and worse food security (CSI), the children did not consume significantly less energy and were not more likely to have low protein or SAA intake.

Fewer children with either HE or FH generally used a latrine to defecate than rest of the sample, and more had been ill. All of the children with both FH and HE had been ill recently while just 85% (36) of those with only FH had been ill ($p=0.014$). Of those with only HE, 94% (48) had been ill.

The overlap between HE and FH is itself an interesting observation. A child in the sample with FH had a 1.0:2.1 chance of developing HE. A child without FH had a 1:5.0 chance of developing HE. In other words, having a family with a history of kwashiorkor more than doubled the odds of developing hair changes and facial edema.

Table 6: Characteristics of the Sample Population

Variable	Units	n	Total Sample	Family history of kwashiorkor	Hair changes or facial edema
Households Interviewed			284	68	73
Adult Equivalents in household	mean (SD)	278	4.4 (1.8)	4.1 (1.7)	4.1 (1.7)
Children with Interviews			338	82	90
Female	% (n)	338	48.2 (163)	50.0 (41)	48.9 (44)
age in months	mean (SD)	338	46.7 (8.1)	46.3 (7.6)	45.5 (8.0)
Measures of the Child					
WAZ <-2	% (n)	338	23.7 (80)	22.0 (18)	26.7 (24)
HAZ <-2	% (n)	338	61.2 (207)	63.4 (52)	72.2 (65)[b]
WHZ <-2	% (n)	338	1.2 (4)	0 (0)	3.3 (3)[a]
MUAC <125mm	% (n)	338	4.1% (14)	4.9 (4)	13.3 (12)[c]
Edema in feet	% (n)	338	3.3 (11)	6.1 (5)	11.1 (10)[c]
Edema in face % (n)	% (n)	338	20.4 (69)	37.8 (31)[c]	76.7 (69)[c]
Hair changes % (n)	% (n)	338	17.7 (60)	30.5 (25)[c]	66.7 (60)[c]
Child's Diet					
kcal / kg bodyweight	median (IQR)	333	78.1 (54.4)	72.8 (63.4)	78.4 (64.2)
% children below requirement for energy	% (n)	333	48.6 (162)	52.4 (43)	46.1 (41)
% children below requirement for protein	% (n)	333	21.0 (70)	32.9 (27)[b]	21.3 (19)
Protein Energy Ratio	mean (SD)	333	8.7 (3.5)	7.6 (3.3)[b]	8.3 (3.3)
% children below requirement for SAA	% (n)	333	18.3 (61)	31.7 (26)[c]	20.2 (18)
Childcare and Environment Characteristics					
Household has 5 year history of kwashiorkor	% (n)	277	23.8 (66)	100 (66)[c]	44.9 (31)[c]
Household Food Consumption Score[2]	mean (SD)	275	53.9 (17.5)	48.1 (16.2)[b]	47.2 (13.9)[c]
Household Coping Strategies Index[2]	median (IQR)	276	28 (23)	33 (21)[b]	35 (25)[c]
House has good tin roof	% (n)	282	25.5 (72)	17.7 (12)[a]	19.4 (14)
Child was illness in past 30 days	% (n)	337	79.8 (269)	92.6 (75)[c]	96.7 (87)[c]
Child was fed between breakfast and supper	% (n)	338	47.0 (159)	40.2 (33)	43.3 (39)
Child attends health days at clinic	% (n)	335	55.5 (186)	64.6 (53)[a]	66.7 (60)[b]
Child uses a latrine	% (n)	338	34.0 (115)	17.1 (14)[c]	20.0 (18)[c]
Child has thiocyanate in urine	% (n)	295	37.3 (110)	43.3 (29)	41.2 (33)
Child was previously displaced	% (n)	338	59.8 (202)	73.2 (60)[b]	66.7 (60)

[1]some HH had more than one malnourished member in the past 5 years
[2]note that a higher Food Consumption Score and lower Coping Strategies Index indicates better food security
using Students t-test for differences in means, Wilcoxon-ranksum (Mann-Whitney) test for differences in medians, chi squared and Stata Immediate Test of Proportions for differences in proportions
a, b, c - p<0.05, 0.01, 0.001 respectively - comparison of those with the outcome against those without the outcome, not one outcome against the other outcome

Only 36.7% (113) of the 308 children submitting urine samples had detectable levels of thiocyanate. Though a higher percentage of children with FH or HE had detectable urinary thiocyanate, the differences were not significant. The population as a whole had a urinary SCN concentration of 11.9 umol/L (22.3). Children with and without FH had a mean (SD) urinary SCN concentrations of 15.2 (3.2) and 11.3 (1.4) umol/L, p=0.22. Children with and without HE had means of 16.2 (3.2), and 10.6 (1.3) umol/L, p=0.06.

Raw cassava flour was well processed, with a mean (SD) of only 0.71 (1.7) ppm of linamarin (the precursor of cyanide), ranging from 0 to 5 ppm (Codex upper limit is 10ppm (FAO & WFP, 2006)). Cooked cassava leaves, on the other hand, had a mean of 72.2 (85.8) ppm linamarin, ranging from 0 to 300 ppm. Those with FH did not consume cassava leaves more often than the rest of the population, but those with HE ate them on average of 1.44 (0.15) days per week, while the rest of the population ate them 1.14 (0.07) days per week, p=0.035.

Path Analysis

Separate path models were developed for each major outcome (i.e., HE and FH). Thiocyanate in the urine and attendance at clinic health days did not have a significant total effect path coefficient for either path model, and inclusion of the thiocyanate reduced the total sample size, so neither were included in the models. To incorporate both quality and quantity of protein in the analysis, low intake of SAAs, the limiting amino acid, was used instead of low protein intake or

the protein energy ratio. Measures in Table 7 indicate the final models fit the data well (Hooper, Coughlan, & Mullen, 2008).

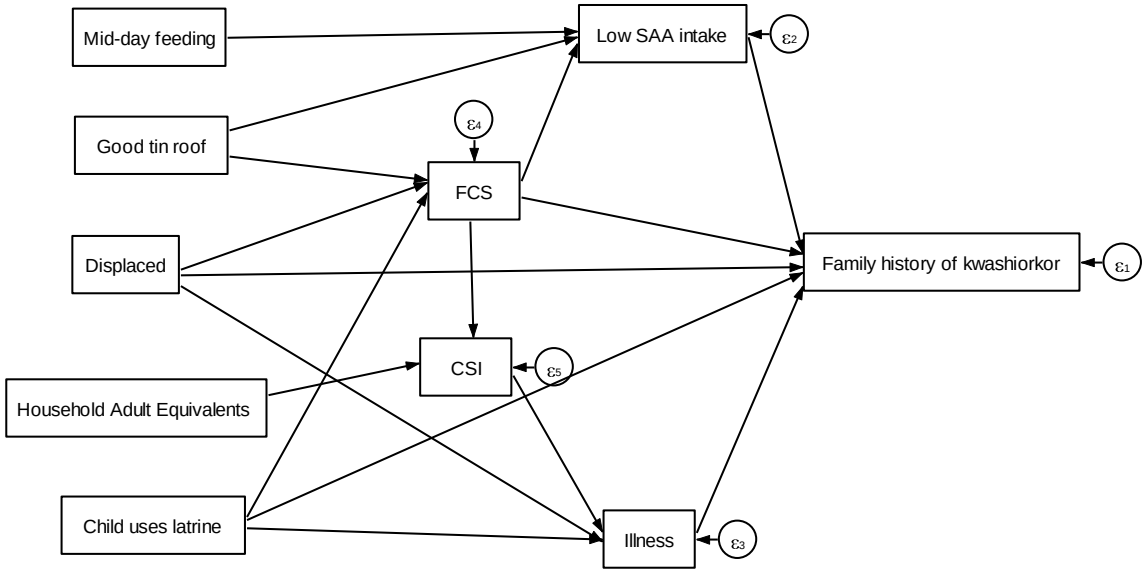
Table 7: Goodness of Fit Measures for the Path Analysis Models

	Family History (FH)	Hair changes or facial edema (HE)	Cut-off for good model fit (Hooper et al., 2008)
Chi2 MS	18.7	18.3	
df, p-value	19, 0.48	18, 0.44	p > 0.05
RMSEA	0.000	0.007	<0.05
RMSEA CI	0.000, 0.048	0.000, 0.051	lower near 0, upper <0.08
CFI	1.000	0.998	>0.95
TLI	1.004	0.997	>0.95
SRMR	0.029	0.028	<0.05
CD	0.228	0.289	<0.05

Chi2 MS (Chi2 test of model versus saturated), RMSEA (Root mean squared error of approximation), CFI (Comparative Fit Index), TLI (Tucker-Lewis Index), SRMR (Standardized root mean squared residual), CD(Coefficient of Determination)

The Family History (FH) model

The FH model framework (figure 4) shows not only the pathways by which the more immediate causes mediate the effect of the more basic causes, but also some of the interaction among the underlying causes. FCS and CSI measure different aspects of food security, but were strongly associated, and much of the effect of food security and diet quality on nutrition was actually mediated by illness. The effect of latrines was partially mediated by diet quality, which does not seem intuitive. Small children must be escorted to the latrine to prevent them falling into the pit below so use of a latrine may be acting as a proxy for the individual attention available to a child.



Note: FCS - Food Consumption Score, CSI - Coping Strategies Index

Figure 4: Family History path model framework

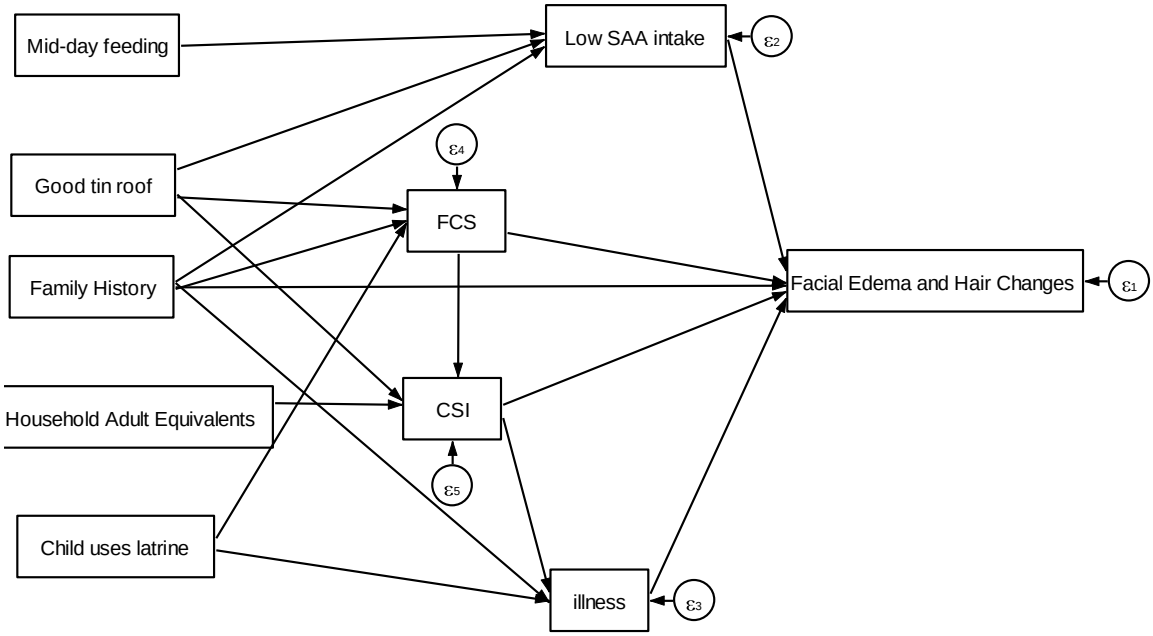
Table 8 gives the total-effect standardized coefficients for each variable in the FH path analyses, adjusted for clustering at the household level. Low SAA intake had the largest total effect on FH followed by FCS and latrine use. The effect of displacement was also relatively large. Midday feeding and household adult equivalents (HH AEs) were not significant in either model but did improve the fit of the models so were retained.

Table 8: Standardized total effects for the Family History (FH) path analysis model (n=319)

	Standardized. Coefficient (SE)	z	P>|z|
Low SAA	0.175 (0.080)	2.48	0.013
Food Consumption Score	-0.155 (0.001)	-2.69	0.007
Child uses latrine	-0.152 (0.054)	-2.58	0.010
Displaced	0.136 (0.054)	2.21	0.027
Illness	0.118 (0.046)	2.71	0.007
Good tin roof	-0.058 (0.019)	-2.92	0.003
Coping Strategies Index	0.025 (0.000)	3.74	0.000
Midday feeding	-0.021 (0.011)	-1.62	0.105
Household Adult Equivalents	-0.004 (0.000)	-1.72	0.086

The Hair Changes and Facial Edema (HE) Model

Figure 5 provides the path model for HE as an outcome. The very large effect of family history on HE is almost entirely direct, with only about 10 percent mediated through SAA intake and FCS. The effect of latrine use on HE is primarily direct. Most of the effect of a good tin roof (locally considered a proxy for wealth) is mediated through FCS and low SAA intake. Use of a latrine had the largest effect on illness, followed by CSI. Displacement was not significant and reduced model fit, so was dropped from the HE model. Midday feeding and HH AE were also not significant, but improved the fit of the model so were retained.



Note: FCS - Food Consumption Score, CSI - Coping Strategies Index

Figure 5: Hair Changes and Facial Edema (HE) path model framework

Table 9 gives the total-effect standardized coefficients for each variable in the path analyses, adjusted for clustering at the household level. Having a family history of kwashiorkor had the largest total effect on HE of all variables. Illness and FCS also had large total effects on HE with CSI close behind, while low SAA

intake was not significant. Midday feeding and HH AEs were not significant but improved the model fit so were retained.

Table 9: Standardized total effects for the Hair Changes or Facial Edema (HE) path analysis model (n=313)

	Standardized Coefficient (SE)	z	P>|z|
Family History	0.258 (0.068)	3.84	0.000
Illness	0.178 (0.044)	4.37	0.000
Food Consumption Score	-0.163 (0.001)	-3.11	0.002
Coping Strategies Index	0.135 (0.002)	2.09	0.036
Child uses latrine	-0.063 (0.017)	-3.38	0.001
Good tin roof	-0.044 (0.018)	-2.40	0.016
Household Adult Equivalents	-0.018 (0.003)	-1.68	0.093
Low SAA	-0.037 (0.075)	-0.56	0.574
Midday feeding	0.004 (0.006)	0.61	0.542

Discussion

Previous studies on the etiology of kwashiorkor have not considered the quality of proteins in the diet, nor the effect of mediation among causal factors (Kismul et al., 2015; Kismul et al., 2014; Lin et al., 2007; Rytter et al., 2015; Sullivan et al., 2006). This study compares the relative association of multiple factors with outcomes that indicate a high risk of kwashiorkor, using two non-exclusive outcome measures: a family history of kwashiorkor (FH), and having either hair changes or facial edema (HE), taking into account protein quality and mediation.

We hypothesized that low intake of SAAs would have a stronger association with the risk of kwashiorkor than any other factor. SAAs were previously established as the limiting amino acids in the diets of this study population and there are metabolically plausible links between a shortage of

SAAs and the signs of kwashiorkor. Path analysis in this study showed SAAs had the strongest standardized total effect coefficient on FH, but were not significantly associated with HE; rather, having had a recent illness had the strongest association with HE.

Children living in a household with a history of kwashiorkor (FH) were more likely to defecate in the open, were more likely to have experienced deprivations during displacement from conflict, were ill more often, had poorer food security (CSI), and consumed a less diverse, lower-quality diet (lower FCS, Protein Energy Ratio and SAA intake) than children in the rest of the sample. More illness and lower diet quality were very strongly associated with both HE and FH, but children with HE were even more likely to have been ill in the previous month and more likely to be stunted and wasted than the rest of the population.

Open defecation (i.e., not using a latrine) had the largest effect of any factor on illness, but open defecation's greatest effect on FH was direct rather than mediated by illness. On the other hand, open defecation's only effect on HE was mediated through illness. The direct effect of open defecation on FH could be explained by environmental enteric dysfunction, something that would not have been reported as an illness in the survey, but which could both reduce absorption and increase requirement for key nutrients, especially SAAs (Bickler et al., 2011; Humphrey, 2009).

This suggests that in the FH group, constant exposure to poor sanitation and frequent illness is reducing absorption and increasing requirements of

nutrients that are not met by a poor-quality diet, so the FH child is never truly replete, but perhaps marginally adequate. The very strong effect of FH on HE, even when controlling for all other measured cofactors, may be interpreted as a constant background state of vulnerability to kwashiorkor that by itself does not necessarily lead to kwashiorkor. When illness of sufficient severity strikes, as seen in the HE group, a larger gap may develop between the child's requirements and stores of nutrients that cannot be met through further adaptation.

It has long been noted that illness very often precedes the onset of kwashiorkor and that kwashiorkor is found only in areas with poor diets and poor sanitation, and during conflict (Gopalan, 1968; Scrimshaw & Viteri, 2010; Trowell & Davies, 1952). Data from this study provides further insight into these dynamics, highlighting the relative contributions of sanitation and illness in combination with chronic poor-quality diets. The role of SAAs in this etiology remains unclear, though the low SAA intake and potentially higher requirements among children in the FH appears to provide an underlying vulnerability that only becomes apparent when the child must mount an immune response to an acute infection.

SAAs are a focus of kwashiorkor research primarily because children with kwashiorkor consistently have low circulating levels of SAAs and their metabolites (Arroyave et al., 1962; Badaloo et al., 2002; Holt et al., 1963; Jahoor et al., 2006b, 2006c). The levels of circulating essential AAs such as methionine are determined by a combination of dietary intake, protein and non-protein

demands, and mobilization from endogenous sources (Reid et al., 2000). Protein mobilization is inhibited in children with kwashiorkor, but it remains unclear why (Jahoor et al., 2005; Manary et al., 1998). The majority of AAs in circulation come from endogenous sources and low levels seen in kwashiorkor may be as much a reflection of this weak protein breakdown as diet or illness (Reeds & Fuller, 1983). This study attempted to measure factors that indicated relative levels of dietary intake and demands related to infection, but there is currently no feasible way to incorporate the child's ability to mobilize SAAs from endogenous sources into observational studies such as this. This is a promising avenue of future research that warrants further attention.

As a cross-sectional study, these results report only associations and cannot show causation. Experimental research to test causation by lowering incidence of kwashiorkor would do well to improve sanitation habits and address other causes of acute illness. Diet should be improved generally, but with particular attention to increased intake of SAAs rather than total calories.

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Chapter 5: Summary and Discussion

Kwashiorkor is a type of severe acute malnutrition (SAM) found only in the poorest regions of the world with inadequate sanitation and diets generally low in quality protein (Autret & Behar, 1954). Though kwashiorkor has long been identified as a syndrome, its etiology remains unclear and there are no effective preventive interventions (Briend, 2014). Without effective preventive strategies, hundreds of thousands of children each year will continue to be affected, many of whom will die (Briend et al., 2013).

A better understanding of the environment and diets associated with high kwashiorkor prevalence is key to designing effective prevention strategies. Reviews of previously prevalent nutrition deficiency diseases like scurvy, beri-beri and pellagra, recall how detailed epidemiological studies provided the clues upon which the first successful preventive interventions were designed (Carpenter, 1986, 2000; Jarrow, 2014). It was the honing of these successful preventive interventions which gave the vital information that elucidated the biological mechanisms leading to the signs characterizing the diseases. In other words, successful strategies to prevent and even treat these diseases, based on evidence from epidemiological research, informed our understanding of the etiology of these nutrition deficiency diseases (Carpenter, 1986, 2000; Jarrow, 2014).

The primary aim of this research was to provide evidence upon which effective interventions to prevent kwashiorkor can be based. Clarifying the association of multiple potential environmental and dietary factors with risk of kwashiorkor, giving special attention to sulfur amino acids (SAAs), is a key step toward designing preventive interventions.

The first step in the study was to identify two populations that were as similar as possible, but with very different prevalence of kwashiorkor. Discussions with healthcare staff in Masisi Territory led the researcher to the Murambi/Malehe Health Area. An anthropometric survey of children 12 to 59 months old in this Health Area identified two adjacent populations, one with no cases of kwashiorkor and another with a 9.6% prevalence of kwashiorkor. Their physical proximity generally controlled for many potential confounders including climate, water sources, market access, and quality of health care.

The research team then conducted a detailed 24-hour quantitative diet recall of the children 36 to 59 months old in these two populations and analyzed the energy, protein and amino acid content of these diets. Along with the diet recalls, the interview included household and child-level factors that the literature has linked with risk for kwashiorkor (Chapter 1). The median dietary intakes of energy were not significantly different between the two populations, but median intakes of protein were significantly lower for the high-prevalence population. Methionine was the limiting indispensable amino acid (IAA) in the diets of both populations, and more children in the high-prevalence population were at increased risk of inadequate methionine intake than the low-prevalence population (hypothesis 1).

Though the 3.3% prevalence of kwashiorkor in the total population for whom the study had full diet recalls is higher than in most epidemiological kwashiorkor study populations, this was only 11 cases among 338 children, too small a sample to provide strong evidence of association. Instead, the researcher operationalized risk using two outcomes from the kwashiorkor and nutrition literature which indicated a high risk of

developing kwashiorkor: living in a family with a history of kwashiorkor (FH - having had a case in the household in the previous five years) and having pale, brittle hair and facial edema (HE - signs that are characteristic of kwashiorkor and which generally precede the bipedal pitting edema diagnostic criterion) (Gopalan, 1968; Maxwell et al., 2008).

As indicated by the UNICEF framework for malnutrition, the effect of general poverty as well as societal and political causes of malnutrition are partially mediated by underlying causes at the household and community level which, in turn, are partially mediated by the immediate causes of malnutrition (Levitt, Pelletier, & Pell, 2009). The researcher used path analysis to capture the total effects (direct plus indirect effects) of the factors measured, developing two separate models, one for each of the risk outcomes, in order to account for this mediation (Iacobucci, 2012). Neither frequency nor quantity of cassava consumption were significant in either model (hypothesis #3).

In the first model, a family history of kwashiorkor (FH) was the outcome variable. Low intake of SAAs had the strongest total effect coefficient increasing risk of FH, followed by diet quality as measured by the food consumption score (FCS). In the path analysis, low intake of SAAs had the strongest association with a family history of kwashiorkor, but it was not significantly associated with hair changes and facial edema (hypothesis #2). Similarly, the models indicated that having been displaced from their homes by conflict was significantly associated with a family history of kwashiorkor, but not with current hair changes or facial edema.

In the second model, hair changes and facial edema (HE) was the outcome variable and family history of kwashiorkor (FH) was used as a potential causal factor. In this

second model FH had by far the strongest total effect coefficient increasing risk of HE. Having had a recent illness and lower food consumption score also had strong total effect increasing risk of HE, though not nearly as strong a total effect coefficient as FH. Indeed, when we examined the characteristics of the children directly, the study found that a child with a family history of kwashiorkor was more than twice as likely to develop hair changes and facial edema than children without that family history. Interestingly, all children with both FH and HE had been ill in the previous month.

Discussion

Previous studies evaluating the association of kwashiorkor and diet did not consider the quality of protein in the diet (Kismul et al., 2014; Lin et al., 2007; Sullivan et al., 2006). Additionally, recent advances in statistics and access to those advances through commercially available statistical software allowed this study to use path analysis to account for the effect of mediation when comparing the association of multiple factors with risk of kwashiorkor. This study therefore fills a critical gap in evidence on how diet and the environment may increase risk of kwashiorkor.

The detailed anthropometric survey was able to show how uneven the distribution of cases of kwashiorkor is, at a level never before documented. Discussions with healthcare staff across multiple Health Areas in two different provinces in eastern DRC indicated that this is a common trend throughout the region. Further research is necessary to confirm if this trend holds true in other regions where kwashiorkor is endemic. If so, it could provide an important opportunity to improve the design of future research, whether observational studies or clinical trials. It could also help to improve treatment programming strategies, allowing treatment and prevention resources to be

focused on areas with the highest prevalence, improving both the efficiency and effectiveness of these programs.

Analysis of the path models concluded that a chronic poor-quality diet and poor sanitation, but most particularly low SAA intake, keeps children marginally adequate in nutrients, and vulnerable to kwashiorkor. But it often takes the increase in nutrient requirement caused by an illness to create a large enough gap between intake and requirement to push the children into overt kwashiorkor (hypothesis #2).

In the context of eastern DRC, displacement is another potential cause of abrupt reductions in diet quality as households are cut off from their agricultural fields and normal income sources, while also experiencing increased requirements due to physical stress, reduced sanitation and increased exposure to the elements and other diseases like malaria, cholera and measles (Chabwine et al., 2011; Collinson, 2003; Maxwell & Fitzpatrick, 2012). In regions where kwashiorkor is endemic, surges in cases of kwashiorkor are often seen within a very short time after displacement (ACF, 2017; UNICEF, 2017a). For example, in parts of Kasai experiencing conflict in 2017, general kwashiorkor prevalence was estimated to be as high as 3.6% among that part of the population in areas safe enough to evaluate (ACF, 2017). If the uneven distribution patterns seen in Chapter 2 are applied, then there were likely communities where prevalence of kwashiorkor might have been as high as 10%. The results of the FH model indicate that the deprivations and loss of income that accompany such displacement can have a lasting effect on the household.

On the surface, some of these findings are not new. Researchers have long known that prevalence of kwashiorkor is associated with poor sanitation and low-quality diets,

and that an illness often precedes the development of kwashiorkor (Autret & Behar, 1954; Brock & Autret, 1952). We have also known that children with kwashiorkor generally have low circulating levels of SAAs and have difficulty mobilizing endogenous protein (Jahoor et al., 2005, 2006b, 2006c; Manary et al., 1998). This study brings these observations together in the same study population. The evidence from this study suggests that the constant low intake of key AAs such as methionine coupled with poor sanitation make the child vulnerable, or more likely to develop kwashiorkor once an illness or other traumatic event strikes.

The gut mucosa requires a very large amount of cysteine for its constant maintenance and repair (Jahoor et al., 2006c). When the child lives in an environment with poor sanitation, such as indicated by the high rates of open defecation recorded in this study, the child is constantly exposed to infectious agents that increase the requirement of SAAs in the gut even further and which may lead to environmental enteric dysfunction (EED) (Bickler et al., 2011). In a downward spiral, the EED then reduces absorption of these critical nutrients. A child with marginal intake of SAAs and an impaired ability to absorb them would be very vulnerable when demand for SAAs spikes in response to an illness. Although the illness may appear to be the ultimate factor leading to the development of signs of kwashiorkor, as seen in the HE path model, it may be the underlying deficit in SAAs that causes the signs of kwashiorkor.

The majority of the metabolic research relating to kwashiorkor, even that pertaining the SAAs, has focused on cysteine due to the high oxidative damage and low erythrocyte concentrations of both GSH and cysteine observed in children with kwashiorkor (Badaloo et al., 2012; Badaloo et al., 2002; Ciliberto et al., 2005; Jahoor et

al., 2006a; Odigwe et al., 2010; M. I. Smith et al., 2013). Low circulating cysteine (i.e., both serum and erythrocyte) could, in part, be due to a shortage of its precursor, methionine. Though cysteine and methionine are often combined into a single minimum requirement, they serve very different metabolic functions and a shortage of methionine would restrict its transulfuration for cysteine synthesis (Fukagawa, 2006; Stipanuk & Ueki, 2011). Though cysteine and methionine each serve many functions, the primary importance of cysteine derives from the unique position of its sulfur atom and either the donation of that sulfur atom to metabolites or, more often, its ability to bond with other molecules or ions (Mato et al., 2008; Nimni et al., 2007). It was methionine, though, that the dietary comparison of this study showed as the single most limiting amino acid. The primary importance of methionine derives from its centrality in single-carbon (methyl-group) metabolism (Nimni et al., 2007). Although many different nutrients can be methyl donors and receivers, the metabolic cycles of each of these nutrients must necessarily link with methionine and are inhibited when methionine is in short supply (Borum, 1983; Finkelstein, 2003; Zeisel, 2000; Zhu et al., 2014). The signs associated with inadequate cysteine and methionine available for metabolic uses can be plausibly linked to nearly all of the signs of kwashiorkor (Borum, 1983; Finkelstein, 2003; Roediger & Waterlow, 1995; Zeisel, 2000; Zhu et al., 2014). The least understood and investigated of these signs, for example a fatty liver and lethargy, could plausibly be linked with a shortage of labile methyl groups (Borum, 1983; Zeisel, 2000; Zhu et al., 2014).

Coupled with this insight into the diets of high-kwashiorkor-risk populations, the path analysis indicated that chronic exposure to an unsanitary environment and higher

frequency of illness had strong associations with HE, possibly by contributing to EED and increased requirements, increasing the gap between intake and requirement (Bickler et al., 2011). Recent research into the gut microbiome also indicate that the biome is different in children with kwashiorkor and may further alter their ability to absorb SAAs (M. I. Smith et al., 2013). The path analysis indicated the root causes of kwashiorkor in the study area, like most malnutrition, are likely linked to poverty and such political issues as conflict with its attendant deprivations. Long-term solutions to preventing kwashiorkor, and malnutrition in general, need to take these long-term factors into account when designing interventions to reduce incidence of kwashiorkor. Interventions that require access to foods or products that are expensive or not locally available will not be sustainable or accessible to these impoverished populations, especially during conflict when both resources and access to high-quality medical care are the most limited.

Low-nutrient starchy tubers (cassava, white sweet potatoes and taro) and beans, both of which are limiting in SAAs, are the staple foods in the study area because they are the cheapest, most accessible foods. Simply educating women that they should prepare more of the expensive higher-quality proteins in the diet does not take into account the limitations of poverty. The design of interventions needs to be context specific, assisting caregivers to maximize the nutrient density of meals, especially in SAAs, within their limited means. Indeed, mothers in the study often spontaneously mentioned that they knew they should include more animal proteins like fish, milk and meat, in the family diet, but did not have the means to purchase them. Lessons may include advice on how to favor young children, whose relative requirements for SAAs

are much higher during this period of rapid growth, when portioning out their food or providing midday snacks that target this age group.

Interventions should also incorporate elements that reduce requirements for SAAs by reducing their exposure to infection. As with the diet interventions, these more environmental and sanitation interventions will require very low resource, context-specific solutions. Households must be able to implement and propagate proposed interventions with few outside resources; though initially, testing and demonstrating their efficacy to the targeted communities may require additional resources. Target communities should therefore be involved in the design process, not just the more typical tweaking of a design proposed by external researchers.

Implications for the Future

The results of this study have implications for both practitioners and researchers, as well as policymakers. Indeed, the development of effective, context-appropriate interventions to reduce the incidence of kwashiorkor will require practitioners and researchers to work together with each other and the target communities. Although this study provided some insight into the interplay between infection and low SAA dietary intake, as well as the clustering of prevalence at the village level, future research in other contexts is needed to understand how generalizable these findings are.

Because methionine is a precursor of cysteine biosynthesis, research on kwashiorkor should consider both AAs, but individually, taking into account their very different roles in metabolism. The majority of signs of kwashiorkor are also seen in shortages of labile methyl groups or interruptions of methyl group metabolism, and this

study singled out methionine as being particularly at risk of inadequacy in the diet. This link between observed diets and signs of kwashiorkor warrants additional attention.

Although this study focused on dietary intakes and factors that potentially increase requirement, it does not take into account the implications of the reduced ability to mobilize endogenous protein seen in kwashiorkor or the potential effect of an altered gut microbiome. Relatively little is understood about these processes and how they might have become altered in kwashiorkor, and they deserve much more attention in future kwashiorkor research.

Conclusion

In summary, kwashiorkor is a type of severe acute malnutrition that, 80 years after being identified as a syndrome, still affects hundreds of thousands of children each year, with fatality rates that are sometimes three to four times that of marasmus without edema (Alvarez et al., 2016). In North Kivu Province alone, the province where this study was conducted, more than 10,000 children are treated annually (PRONANUT, 2013). This is likely only a fraction of the total number of cases, due to low coverage of treatment programs (ACF, 2011a). The uneven distribution of cases documented by this study means that this enormous burden of kwashiorkor is borne by only a fraction of the total population. Effective, locally sustainable prevention strategies that target these communities are urgently needed to prevent the suffering and loss of life caused by kwashiorkor.

This study provided insights into the potential roles of low SAA intake, methionine in particular, in combination with illness in the development of kwashiorkor. Although low dietary intake of SAAs may be a central factor, sustainably improving intake must

involve addressing the many factors that have pushed intake down while elevating requirements - poverty, poor sanitation, high incidence of illness, and the deprivations associated with displacement. Each of these factors must be considered in the design of sustainable, locally appropriate effective preventive interventions. A previously undocumented phenomena was the very uneven distribution of kwashiorkor at the local level.

Although understanding in detail metabolic mechanisms behind the etiology of kwashiorkor would be helpful in designing prevention strategies, it is not necessary to design effective preventive interventions. As with scurvy, beri-beri, night-blindness and pellagra, epidemiological studies such as this one can still lead to effective prevention strategies. It may be the honing of successful preventive strategies that will provide the key insights needed to finally make kwashiorkor the rare cause of suffering and death that pellagra and scurvy have become.

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