ASSOCIATION OF FATTY ACIDS WITH GROWTH AND COGNITION OF TANZANIAN CHILDREN

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ABSTRACT

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Background: There is an increasing interest in the effects of n-3 and n-6 fatty acids in human health. Evidence from experimental and epidemiological studies has shown that essential fatty acids are important for growth and cognitive development of children. In most developing countries the availability of fats in food supply is generally low, most times lower than the minimum recommended levels. Among children in Tanzania, 42% below five years of age are stunted, 6% are wasted, and 21% are underweight. This therefore sets high risks of developing essential fatty acid deficiency to all the vulnerable groups and most importantly children. The majority of research and interventions in Tanzanian children child growth and cognition in Tanzania has mostly focused on protein- energy deficiency in growth and the role of iron deficiency in cognition. No studies have assessed the FA status of Tanzanian children, nor have the relationships between fatty acid status and growth and cognition been measured. Therefore, the objective of this research was to measure whole blood levels of FA in these children and to establish the association of their FA levels with their growth and cognitive abilities.

Methods: The study was conducted in Rudewa Mbuyuni village, Kilosa, Tanzania. A total of 335 two-to-six year old children participated in this study. A drop of whole blood was collected on an antioxidant treated card and the resulting dried blood spot was analyzed for FA composition using gas chromatography. Weight and height were measured. These values, along with age,

were used in calculations of weight-for-height, length-for-age, body mass index-for-age and weight-for-length z-scores with the World Health Organization Anthro software suite. Seeds, nuts and oil samples were collected from the study area. Fats were extracted from these foods by acidified methanol. FA composition of the extracts was measured using a DSQII quadruple GC/MS. Dimensional change card sort (DCCS) task was used to assess the executive function.

Results: Whole blood FA levels were measured and the results indicated that a significant proportion of these children had low levels of essential FA. Approximately 23% of these children were EFA deficient as defined by a T/T ratio >0.02. Whole blood levels of linoleic acid were positively associated with height-for-age z score in this population. Thus, those children with lower levels of linoleic acid (an EFA) were also stunted. We found that LA had a strong positive association with executive function. DHA was positively associated with executive functions. Although these results are just associations, they are signaling on the possible role of n-6 FA in cognition. The results also showed that the locally available and crude sunflower oil and pumpkin seeds were high in the n-6 FA, particularly linoleic acid.

Conclusion: This dissertation shows the strong association between whole blood essential fatty acids and both growth and cognitive function. It affirms the importance of n-6 FA in growth as shown by others [1] and highlights the association of n-6 FA in cognitive functions, which has been mainly overshadowed by the emphasis on n-3 FAs. Promoting adequate total dietary intake may increase the amount of essential fatty acid consumed which will eventually provide the long chain fatty acid known to play a critical role in both growth and cognition. These findings provide a foundation for further research on the importance of fatty acids in eradicating malnutrition in developing countries.

To my husband John and daughters
Canicia, Charityrose and Carissa

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KEY TO ABBREVIATIONS

AA	Arachidonic Acid
AI	Adequate Intake
ALA	Alpha linoleic acid
BAZ	BMI for age z-score
BMI	Body mass Index
CMR	Child Mortality Rate
DBS	Dried Blood spot
DCCS	Dimensional change card Sort
DHA	Docosahexanoic acid
EFA	Essential Fatty acids
EFAD	Essential fatty acid deficiency
EPA	Eicosapentanoic acid
FA	Fatty Acid
FADS	Fatty acid Desaturase
FAMEs	Fatty acid methyl esters
FAO	Food and Agriculture Organization
HAZ	Height for age z score
IMR	Infant Mortality rate
IOM	Institute of Medicine
LA	Linoleic acid
LCPUFA	Long chain Fatty acid
MUFA	Monounsaturated fatty acid
PEM	Protein Energy Malnutrition
PUFA	Poly unsaturated fatty acid
WAZ	Weight for age z-score
WHZ	Weight for age z-score
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

Background

Dietary consumption of lipids, mostly in the form of fatty acids, is important for human growth and development. Fatty acids are classified based on the number and type of carbon double bonds in their structure. Fatty acids without double bonds are called saturated, while those with double bonds are referred to as unsaturated fatty acids [2]. Unsaturated fatty acids with more than one double bond are called polyunsaturated fatty acids (PUFA). PUFAs are further classified into various groups based on the location of the first double bond from the methyl carbon end of their structure [3]. The n-3 PUFAs are those with the first double bond between the third and fourth carbon atoms, while the n-6 PUFAs are those with the first double bond between the sixth and seventh carbons[3]. Alpha-linolenic acid (ALA) is an n-3 PUFA mainly found in foods such as flaxseed, walnut, canola and soy. Unfortunately, most of these n-3 PUFA sources are not as widely available and affordable in Tanzania. Linoleic acid (LA) is a n-6 PUFA found in high concentrations in corn, sunflower, and peanut oil. Fish and meat are also good sources of ALA and LA[4].

ALA and LA are recognized as essential fatty acids (EFA) because they have to be supplied by diet and cannot be synthesized from metabolic precursors in the body[3]. LA is metabolized through a series of desaturation and elongation steps to arachidonic acid (AA), whereas ALA utilizes the same enzymes to give rise to its downstream metabolites, eicosapentanoic acid and docosahexaneoic acid (DHA)[3]. Because they can be synthesized within the body from ALA and

LA, there is no general agreement as to whether EPA, DHA and AA are essential in the diet. EPA and DHA are found in foods such as algae, fatty fish and eggs. AA is mostly found in meat, poultry and eggs [4]. Breast milk is also a good source of EPA, DHA and LA, but the specific amounts present are affected by the mother's diet and fat stores [5]. Studies have shown[6] that when children are well breast fed they usually have a good brain development as well as they exhibit a good cognitive ability later in life. Cow's milk has very low amounts of ALA and LA and does not contain EPA or DHA. Since many animal foods are good sources of EPA, DHA and AA, which are downstream metabolites of ALA and LA, populations consuming little or no animal foods depend on the synthesis of EPA, DHA and AA by the human body[1, 7]. This has a more serious impact because, in most cases, these populations are poor and they cannot afford enough food let alone nutritious foods. In that case, the consumed fatty acids are likely to be used to meet energy requirements rather than being converted to form EPA, DHA and AA.

The conversion of ALA to EPA and DHA, and LA to AA in the body is very inefficient, ranging from 1% to 10% [3]. The conversion rate is also affected by a common polymorphism in the fatty acid desaturase (FADS) gene cluster responsible for converting ALA/LA to their metabolites. Depending on the polymorphism present, some individuals form more EPA and DHA from ALA, and AA from LA than others[8]. These downstream molecules are incorporated into cell membranes and regulate cell membranes, cellular signaling cascades, protein functions, gene expression and pro and anti-inflammatory pathways. Since long chain EFA accumulates rapidly in the last trimester of pregnancy, preterm birth puts the baby at a higher risk for deficiency[6, 9].

Both essential fatty acids (ALA and LA) and their derivatives (EPA, DHA and AA) have important roles in growth and development and many other physiological needs of humans. A higher n-3 PUFA intake or supplementation during pregnancy results in improvements in gestational age, birth weight and length. High amounts of DHA accumulate in the fetus during the last trimester of pregnancy and during the first year of the child's life[6]. High concentrations of DHA are also found in the brain, which grows rapidly during this time. This attests to DHA's importance in neurodevelopment[7]. DHA also accumulates in the retina, indicating its role in visual acuity [10]. Similarly, n-6 FA are important for linear and physical growth of the children[11].

EFAs are important for membrane functions and myelination [12]. DHA is an important component in the formation of the myelin sheath. It increases in the brain during development signifying that it plays a crucial role in the formation of the central nervous system. The growth of neural cell membranes requires lipids, which are added to the growing membranes[13]. EFA deficiencies in diets during brain development results in reduced brain DHA, resulting in delayed myelination [7, 9]. EFA deficiency decreases mean cell body size of neurons in the hippocampus, hypothalamus, parietal cortex, thereby delaying the maturation of brain gangliosides and glycoproteins associated with learning impairment [6, 14, 15]. Early nutrition is vital to survival and quality of life. Because infants and children are growing rapidly, they have unique nutritional needs and are at the greatest risk for nutritional deficiency. Better nutrition means a stronger immune system, less illness and better health.

In Tanzania, most families cannot afford to eat animal foods. In populations like these, sufficient intake of ALA and LA from vegetable oils, seeds and nuts is needed. Unfortunately in this population, only small amounts of oils are usually added in the foods during meal preparation. The added fat/oil is usually subjected to very high heat, which exposes the oil to oxidation. Even when the oil is rich in EFAs, the quality of the oil is compromised before consumption. In Tanzania, inadequate intake of food prevails; therefore, energy intake is likely insufficient to meet the energy needs of the growing child, and the low amount of fat consumed will primarily be used for energy. When energy (mostly lipids) intakes are low, the body prefers using ALA and LA for energy expenditure rather than converting them to EPA, DHA or AA [1]. Thus, low intakes of animal foods, fat and energy increase the risk of EFA deficiency in Tanzanian populations.

Unfortunately, data on dietary fat intake and status in Tanzania are very limited; those that do exist show that intake of n-3 fatty acids is very low compared with the intake among children in most developed countries[16]. To date, researchers have emphasized on the role of protein, carbohydrate, vitamin and mineral deficiency in the stunting and wasting of Tanzanian children. Despite the role of EFA in growth, development and health, EFA deficiency has been largely ignored as the cause of stunting and wasting, poor cognitive abilities in Tanzanian children. Neither levels of FA intake nor EFA status has been measured in infants and children in Tanzania[1]. Given the importance of EFA in growth and development there is an urgent need to assess EFA intake and status in this population.

In this study, we collected capillary whole blood from the children and utilized it to assess FA levels. Since it is not practical to harvest RBC from children in rural villages in Tanzania, we used a special DBS cards to collect blood samples. Our long-term goal was to understand to what degree fatty acid deficiency affects growth and development among children between two to six years old in Tanzania. Therefore, the objective of this research was to measure whole blood levels of FA in these children and to establish the association of their FA levels with their growth and cognitive abilities.

Specific Aims

Specific Aim 1. Establish the associations between fatty acid status and weight for height (wasting), weight for age (underweight), height for age (stunting) and BMI for age z-scores in these children.

Specific Aim 2. Assess the relationship between EFA status and cognitive abilities of these children.

Specific Aim 3. Identify and determine FA content of oils, seeds and nuts used in this population.

Significance

The significance of this research is that knowledge of EFA status in Tanzanian children two to six years of age will provide a foundation for future studies to identify and guide diet and health interventions in this community for the benefit of the children. Following the FAO

recommendations on FA intake for infants and young children, which are based on energy requirements, it is important to recognize that these requirements in the developing world are not met. This leads to high prevalence of underweight and stunted children in these communities. Therefore, assessing the EFA status of these children would assist in providing baseline information on these important nutrients. The study also seeks to link the cognitive abilities of these children with this key class of nutrients. Such studies are lacking in Tanzania, especially in this age group. The assessment of fatty acid content of various seeds, nuts and oils locally available in this population would provide detailed information of their fatty acid content. Future intervention studies can use this information when formulating their interventions so as to address EFA deficiency, growth faltering and cognitive delays.

CHAPTER 2: LITERATURE REVIEW

Metabolic Role of Lipids

Lipids are required constituents for intracellular processes. Lipids are concentrated forms of energy, yielding roughly 9 kcal per gram when oxidized. They contain more than twice the energy per gram as protein and carbohydrate, which explains why human beings store fat as their primary reservoir of energy. Fatty acids are the simplest form of lipids. They exist as free fatty acids or as complexes containing other fats, proteins or sugars. In all forms, fatty acids play a number of roles in metabolism [3]. Fatty acids play an important role in providing fuel to the body. In addition to providing energy, lipids are important components of phospholipids and glycolipids, which are essential components of cell membranes [2]. Polyenoic fatty acids are critical for membrane structure and also serve as precursor for eicosanoids, which regulate cellular activity. Dietary lipids also are a source of sterols and precursors of vitamin D.

Fatty acid synthesis

Synthesis of fatty acids is governed by the enzyme acetyl CoA carboxylase, which converts acetyl CoA to malonyl CoA. A series of malonyl CoA units are then added to the growing fatty acid chain to end up in the formation of palmitic acid (C16:0). At this point more complex fatty acids can be formed through elongation and desaturation; however humans do not possess enzymes capable of inserting points of unsaturation below the 7^{th} omega carbon, making n-3 and n-6 fatty acids essential [17]. Diets rich in n-6 fatty acids may result in suppression of the elongation and desaturation of C18: 3 n-3 to C20:5n-3 and C22:6n-3. Diets with high ratio of n-

6 to n-3 have been linked to reduced production of important n-3 polyenoic fatty acids required for the developing nervous system [18]. On the other hand, changing prostaglandin profiles through elevated intakes of n-3 fatty acids such as EPA and DHA from fish and fish oils decreases the clotting capacity of blood[19].

Essential Fatty Acids

The essential fatty acids (EFA), alpha linolenic acid (ALA) and linoleic acid (LA), are fatty acids, which the body cannot synthesize and must therefore be supplied to the body through diet. Synthesis of EPA, DHA from ALA and AA from LA (Figure 1) occurs through desaturation, chain elongation and chain shortening, where an enzyme Δ6 desaturase is the rate-limiting step. Both the LA (to AA), ALA (to DHA via DPA) and OA compete for conversion by this enzyme. This enzyme has an affinity for its substrate in the order ALA>>LA>>OA[20]. The minimum intake of EFA to prevent deficiency symptoms in an individual has been estimated to be 2.5%E LA plus 0.5%E ALA[5]. The adequate intakes (AI) are used for EFA recommendations. AI is the recommended average daily nutrient intake level based on observed or experimentally determined approximations or estimates of nutrient intake by groups of healthy people who are assumed to maintain adequate nutrition state as evidenced by normal growth or normal levels of nutrient in their respective stores in the body. These are usually used when there is not sufficient evidence to formulate RDA for that particular nutrient [21].

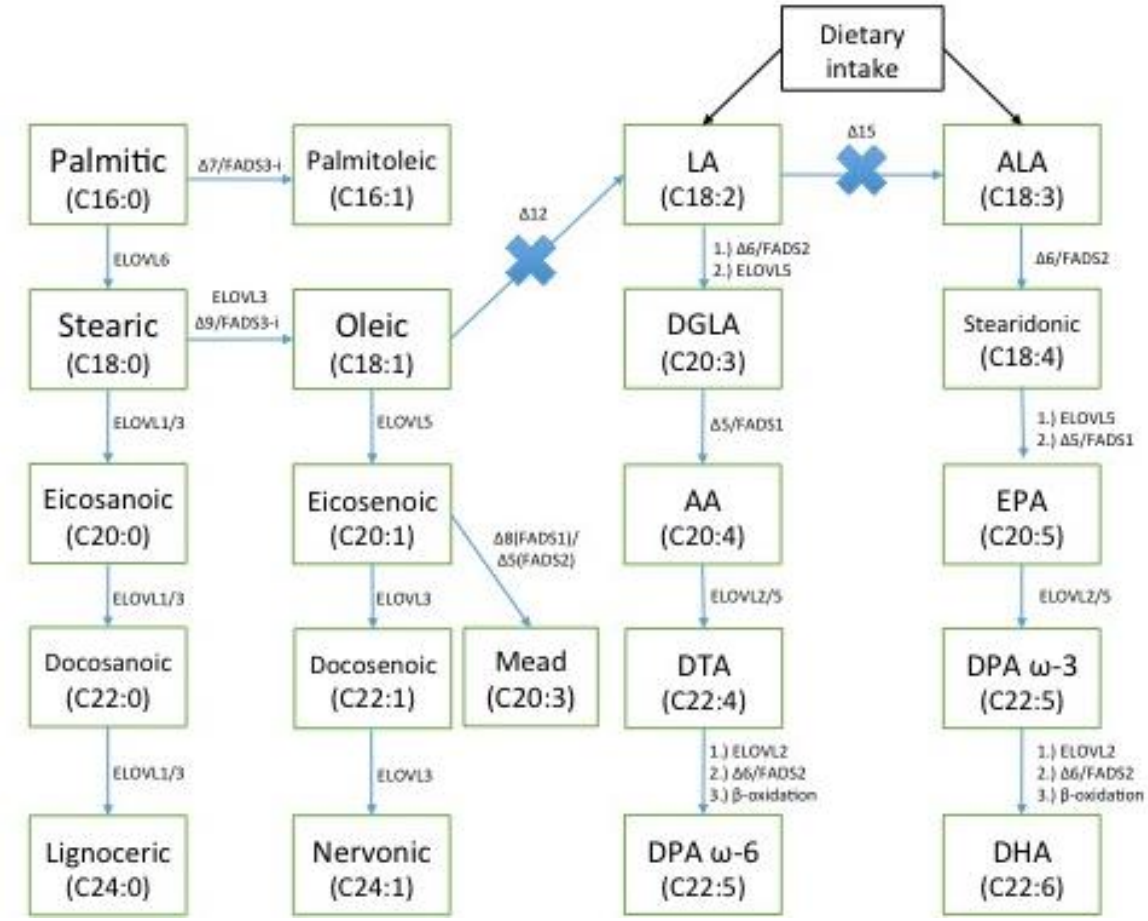

Figure 1: Fatty acid biosynthesis

In ensuring adequate consumption of ALA, EPA and DHA, recommendations have been made for LA, ALA, EPA and DHA for both infants and young children (Table 1). The focus is to ensure that the amount consumed is enough to prevent nutrient deficiency as well as to reduce chronic disease risk. With the increasing prevalence of chronic diseases in the world, it is important that nutrient recommendations levels are set such that they are enough to reduce the risk of chronic disease as well as preventing nutrient inadequacy.

Table 1: Recommended AI for ALA and LA from FAO[5] and IOM (2005)

	Total fat %E	ALA (AI) %E	LA (AI) %E	ALA (AI) mg/d	LA (AI) g/d	DHA (AI)mg/kg	DHA +EPA mg/d
FAO (2010)							
6-24 months	35	0.4-0.6	3.0-4.5			10-12	
2-4years	25-35	≥0.5%E*	2-3%E*				100-150
4-6years		≥0.5%E*	2-3%E*				150-200
IOM (2005)							
1-3years	30-40	0.6-1.2	5-10	700	7		
4-18 years	25-35	0.6-1.2	5-10	900	10		

*FAO recommendations do not show specific recommendation for 2-18years, adult doses are indicated

Essential fatty acid deficiency

Essential fatty acid deficiency (EFAD) combines both inadequate status of both n-3 and n-6 fatty acid families. EFA and LCPUFA form the structural components of all cell membranes and therefore plays a role in modulating membrane fluidity and flexibility. The derivatives of the EFAs have important functions in the body, such as platelet aggregation, constriction and dilation of blood vessels[2] and a lot of chemotaxic processes in the immune system[12]. Some of the clinical features of EFAD are non-specific and usually develop prior to chronic marginal EFA status[20]. Some of these features/symptoms include, but are not limited to, increased susceptibility to infection, impaired growth, reduced learning ability, and impaired vision.

During deficiency Mead acid is endogenously synthesized from oleic acid and it is detected in plasma and tissue. Mead acid replaces other PUFA in the membrane and produces the 5-lipoxygenase, which is a pro inflammatory mediator associated with platelet hyperactivity, altered cell-cell adhesion and vasoconstriction. The conversion of oleic acid (18:1 n-9) to mead acid (20:3 n-9) occurs only when the concentration of linoleic acid and alpha linolenic acid is very low, thus accumulation of mead acid has been used as a functional marker of EFA

deficiency (EFAD). The functional marker reflects a physiological (e.g. cognitive ability) response upon micronutrient deficiency or biochemical (e.g. micronutrient-dependent enzyme activity). As EFAD develops, levels of AA in tissues are usually maintained particularly in brain while mead acid increases. It is still a question whether this is the best way to describe EFAD, especially in terms of n-3 FA, because n-3 deficiency does not necessarily lead to an increase in mead acid accumulation.

It is known that the long chain PUFA utilize the same enzyme in forming their derivatives. For mead acid to be formed, one elongation and two desaturases are needed for it to be formed. The two pathways involved are as shown in figure 1 and 2 [8]. To diagnose EFAD, assessment of clinical imbalances of n-3, n-6, n-7 or n-9 and the saturated FA families is conducted. Cut off points used do not necessarily mean that symptoms/signs will be present, rather they are biochemical markers that there is deficiency.

Erythrocyte (RBC) FA content provides a reliable estimate of cellular EFA status. It reflects bone marrow FA availability and plasma RBC phospholipid exchange processes of the previous 2-3 months. Whole plasma is not the preferred compartment for EFA status assessment because it derives FA profiles from at least four different lipid classes from different lipoproteins. FA derived from RBC and plasma membrane phospholipid contains the full range of LC PUFA [22], these relates to the FA composition of brain and are described well with respect to their dietary dependence. A cut off value for RBC mead acid as a functional parameter of biochemical EFAD has been set to ≥ 0.4%[11, 20]. The RBC DHA/AA cut off values to categorize omega 3

sufficient/omega 3 deficient is set to 0.22%. The ratio of mead acid /AA greater than 0.02 has been used for many years to as a gold standard to indicate EFA deficiency[23]. In addressing n-3 deficiency then a number of parameters have been suggested. These include, but are not limited to decreased 22:6n3, increased 22:5n3/22:6n3 and 20:4 n6/22:6n3[20].Other ratio which can be used are ratio of n3/n6 and ratio of palmitoleic acid to LA. Low content of LA in plasma indicates an increased content of MA in plasma. During EFAD there is enhanced conversion of n3, n6, n9 to their derivatives (in order of decreasing efficiency) and an increase MUFA and its derivatives to help maintain membrane fluidity[24]. These methods of assessing the FA levels are slightly affected by fasting/fed state especially when whole blood is used, with the amount being lower during the fasting state as compared to the fed state, no difference in FA levels is however seen when RBC is used[25]. It is, however, important to recognize that, whole blood can also be used to assess FA levels in blood and may therefore used to indicate the FA status. Whole blood contains other cells, however the majority of the cells in whole blood is RBCs thus whole blood cells can be used to assess FA levels.

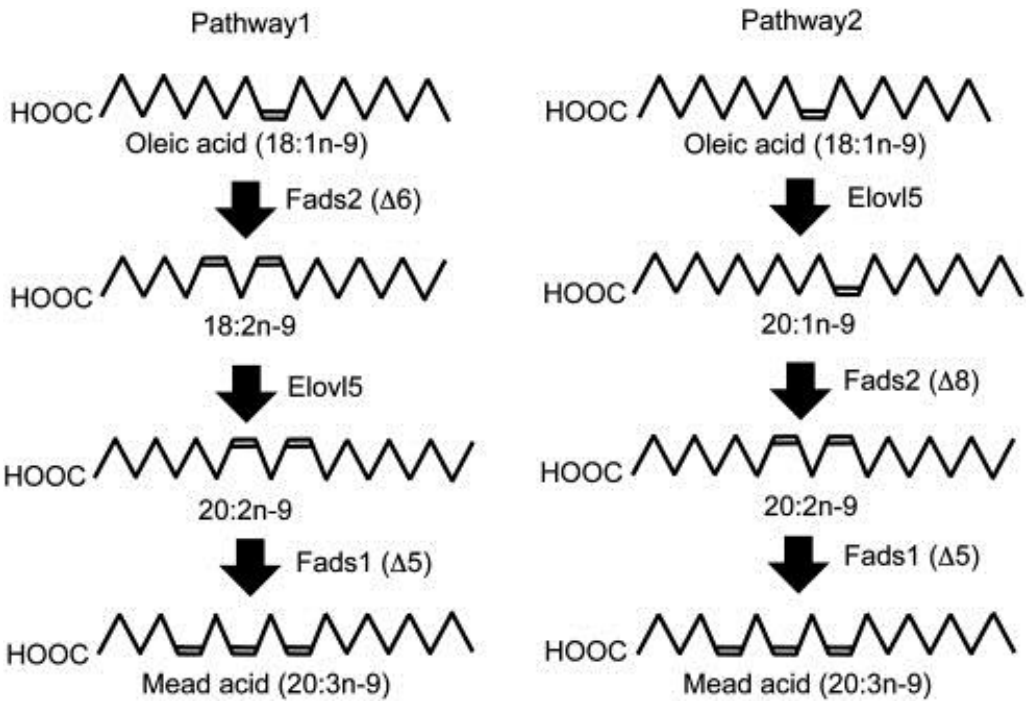

Figure 2: Proposed pathways for synthesis of mead acid from oleic acid (Figure adapted from Ichi et al., [8])

EFA's and growth and development

In many African populations, intakes of lipids are very low. Thus body weight and health are maintained by high intakes of carbohydrates, which makes the major staple in their diet[5]. In most of the developing countries, where food insecurity is chronic, the majority of children are highly affected by malnutrition. In Tanzania, 42% of children below five years of age are stunted due to protein energy malnutrition [26]. PEM usually presents with multiple deficiencies mostly being protein and energy deficiency, however, it is important that we realize that fat being a major source of energy, if not present may as well contribute to PEM. In malnutrition, EFA supply is reduced, EFA desaturation is also reduced and thus there is an increase in EFA expenditure. Thus, EFA status is also compromised during malnutrition, likewise, EFA deficiency will lead to impairment in nutrient absorption and dietary calorie utilization.

Lipids are important in the infant's diet due to their important role in growth and development and physical activity. Several years ago it was revealed that there are specific fat components necessary for growth and development in humans and other species. Despite this early finding, it was up to 1960's when children who were fed skimmed milk developed clinical signs of deficiency [27], researchers investigated more on the role of EFA. The mechanism on how LA, AA and DHA or a combination of these FA affects growth was not fully understood. However, the PGE_2 a cyclooxygenase metabolite of AA, has been implicated through its growth promoting effects and its effects on calcium metabolism or its role on growth-related early gene expression[28]. Also during EFAD there is inefficient use of dietary calories and that may have an additional role in growth [29]. Since PEM has a number of features, experimental designs could provide the biochemical evidence of EFAD in PEM. However, such studies are lacking. There are some animal studies, which provide information on the effects of macronutrients as well as micronutrients on EFA status. No effect of n-3 FA was seen during EFAD, where as there is a decrease in LA and its metabolites during PEM. The amount of n-9 FA in malnourished children was found to be elevated compared to children who had normal nutrition status[29].

Many studies conducted in developed countries have shown that the long chain polyunsaturated fatty acids (LCPUFA) have a major role in growth and development[1, 2]. Results from different supplementation studies on infants and children showed increased length at term[30, 31] increased weight at two month[31], and a reduction in weight z-

scores[32, 33] at 12 months. On the other hand, omega-3 supplementation has shown no significant effects on o growth in other studies[34]. A study [35] assessed intake of marine fish and seafood intake among pregnant women in the US, found that an increase in n-3 LCPUFAs, DHA and EPA unexpectedly led to a modest decrease in fetal growth, and no association was observed with length of gestation [36]. On the other hand, a cohort study [37] in Norway assessed maternal fish intake and infant birth weight, length and head circumference. They found that a positive association between birth size and fish intake existed, while a negative association for head circumference was seen[37].

Similarly, the limited data available from developing countries show that a higher EPA/DHA intake or ALA supplementation during pregnancy may improve growth in terms of birth weight and length as well as gestational duration. These different studies [38]assessed growth at four, six, twelve months, two years and three years. Some studies used z-scores while other studies assessed physical measurements such as weight[39] [40], length(cm) and head circumference(cm) [38]. From their findings [1]there is no clear and consistent benefit of supplementing LCPUFA on neurodevelopmental outcomes and physical growth in term infants. Both studies using DHA alone or DHA and AA have produced inconsistent findings. A review of studies conducted in developing countries on n-3 and growth showed that DHA during pregnancy, lactation and early life is associated with significant benefits in infant growth (birth weight and length) and development [1]. Similarly, limited data from developing countries shows that ALA/DHA supplementation of malnourished infants improves growth and development. Studies involving children above 3 years did not show significant difference

between children in the supplemented and the control group[41]. These findings are seen in children below two years of age and reports on studies conducted to children above two years of age have not shown the same results[1].

Therefore, even the limited number of studies[1] in developing countries, it is widely known and documented that the EFAs are essential for normal childhood growth. Following that, European Food Safety Authority has set recommendations for the amount of DHA and EPA to be consumed by different groups in the population[2]. Generally, human beings require the EFA for growth and development as well as maintaining several metabolic pathways, which are essential to one's life. It is however, important to maintain a balance between these EFAs, as their downstream metabolites tend to work in opposite direction.

EFA and cognitive function/development

Long chain polyunsaturated fatty acids play an important role in the central nervous system. LCPUFA makes about 15-30% of the brain dry weight [42]. The lipid part of the brain is mainly phosphoglycerides and cholesterol, with about 22% of cerebral cortex and 24% of white matter consisting phospholipids[9]. The variation in the supply of both essential and non-essential FA affects the structural composition of the brain and of myelin sheath. Fatty acids comprise all neuronal phospholipid membranes. They also modulate cognition by influencing brain membrane ionic permeability, synaptic transmission and cognitive abilities. Brain phospholipids are rich in AA and DHA [43]. Thus, the function of LCPUFA in the structure and architecture of the brain is denoted by the dependence of the LCPUFA in different stages of life, as well as their

abundance in the brain. Studies in animals have shown that when animals are fed diets without alpha linolenic acid, the amount of DHA in retina and brain was replaced by docosapentanoic acid, indicating that there is a compensation mechanism [44]. Also other studies have shown that low LA and ALA intake have resulted in low brain phospholipids AA and DHA, increased in brain n-9 and n-7 MUFAs and PUFAs in rats [45]. DHA affects the neurotransmission systems, such as acetyl cholinergic systems, norepinephrinergic and dopaminergic systems. Studies also show that DHA affects neuronal membrane fluidity and blood brain barrier functions[46].

During the perinatal period n-3 fatty acids are required for the genesis of the synapses and photoreceptor membrane, they are also needed for normal functioning of the tissue and finally the response to injury to the nervous system. A significant and rapid development of neurons occur during fetal growth, studies have shown that about 20-60mg of n-3LCPUFA per kg bodyweight/day is needed for the fetus during the last trimester of the pregnancy[47]. After birth and up until the first five years of life, there is a significant development of the brain. At this time, nutrient intake and other factors shape the development of the brain. It is for this reason that LCPUFA are recommended to form the large portion of the infant's diet[47], to enhance the development of a mature brain. Several studies have shown the benefit of PUFA in neural development[48, 49]. In addition, various studies have shown that the retinal tissue also accumulates large amounts of DHA[27].

On the other hand, dietary intake plays an important role in fat composition in the brain. High intake of n-6 PUFA reduces the synthesis of n-3 PUFA, and as mentioned earlier the intake of

the 20 or 22 Carbon PUFA increases the composition of long chain fatty acids concentration. The ratio of n-6 to n-3 is important because the pathways share enzymes such that the ratio of fatty acids consumed may direct the availability of the desaturase and elongase enzymes down the metabolic pathways. This could therefore lead to an imbalance in the formation of the various metabolic compounds produced. The enzyme Δ6 desaturase is required twice in DHA metabolism and is the rate-limiting enzyme for this pathway. Thus if concentration of n3 is lower to n6 then the amount of DHA produced will be affected[50]. An impairment in the activity of Δ6 and Δ5 desaturase activity will lead to formation of γ-linolenic acid from linoleic acid and AA from γ-linoleic acid and this has an effect on brain lipid composition. DHA and AA are predominantly found in the structural lipids of cell membrane especially in the central nervous system.

In the brain, the n-3 fatty acids are mainly important for cognition. Studies[15] have shown that the balance of n-3 and n-6 is also important for cognition. Executive functions develop when children are between 3-10 years old[51]. Temporal and frontal lobes of the brain promote these functions. During this period there is a rapid increase in the cortical gray matter in the frontal areas [52], the hippocampus and prefrontal cortex continue to develop beyond the first two years of life[53]. The hippocampus has a very high concentration of AA and DHA, thus attesting to the importance of AA and DHA in synaptic plasticity used in memory processing. A study by Sheppard and Cheatham[15] assessed the relationship between the n-6 to n-3 ratio and the executive function planning and working memory in children between 7-9 years old. They found that children with a lower n-6 to n-3 ratio had shorter processing time on

the spatial working memory task and shorter mean planning times on the planning task. In this study, children who had higher consumption of n-3 and a lower n-6: n-3 ratio had a shorter planning time. This attests to the importance of maintaining an optimal balance of n-6 to n-3 for brain functions.

In a study [49] where women were supplemented with LCPUFA during pregnancy, their children showed a higher intelligence quotient (IQ) at 4yrs as compared to those born from mothers who were not supplemented[49]. No significant results were seen at 7 years of age in children's cognitive abilities, as assessed by Kauffman Assessment Battery for Children, among children whose mothers took cod liver oil (LCPUFA) or corn oil (n-6 rich PUFA). In another study[54] children were randomized to receive formula for one year, one contained DHA while the other formula contained AA and DHA, another group were exclusively breastfed for three months. The researchers evaluated the visual acuity, IQ, receptive and expressive vocabulary and visual-motor function. At 39 months, there were no differences between the groups in all of the evaluated parameters [55]. Further, results from a review of studies[1] conducted to establish the association between EPA/DHA and cognitive abilities of the children are inconsistent with some indicating enhanced cognitive abilities and others not showing any benefits.

From these studies and many others [56], which are not covered in this literature review, indicate that there is biochemical and behavioral evidence that EFAs are important predictors of cognitive functions and specifically executive function in children. There is limited literature

on EFA and childhood executive function in developing countries but the available literature mainly in developed countries associates sufficient EFA levels with good cognitive abilities[9].

EFA deficiency in African diets

African diets are mainly made up of a large portion of carbohydrate rich staple served with relishes (usually vegetables, pulses and nuts, legumes, and fish and meat if available) and sauces, which are usually prepared from a variety of foodstuffs. This means most of the dietary energy in African countries comes from the staple, such as maize, sorghum, millets and rice [5]. The relish usually provides oil, protein, vitamins and minerals. In this regard, many of the staples in African communities are constant with variations on relishes in terms of flavor, household resources, season of the year and a number of dietary habits. Many traditional diets in Africa are usually added with oils from plants such as red palm oil, groundnut oil, coconut oil, sunflower oil and sesame oil (Figure 3). However, it is important we acknowledge that the amount of oil added and the method of meal preparation may sometimes affect the fatty acid content remaining in the meal. In this population, the content of EFA in their diet is usually low [5]. Additionally, dietary intake of fats and oils in many African meals is typically low because refined oils and animal foods are expensive and many families cannot afford to purchase them. Thus, in many African populations, intakes of lipids are very low, thus body weight and health are maintained by high intakes of carbohydrates, which makes the major staple in their diet[5]. Despite having staples in the diet, the meals are usually not enough due to chronic food insecurity. The majority of children are highly affected with malnutrition, mainly PEM. PEM usually presents with multiple deficiencies mostly being protein and energy deficiency, however

it is important that we appreciate that fat being a major source of energy, if not present may as well contribute to PEM. As we are aware PEM may lead to EFA deficiency through its effect in reduced EFA supply and reduced EFA desaturation which will therefore promote EFA utilization for energy expenditure[29].

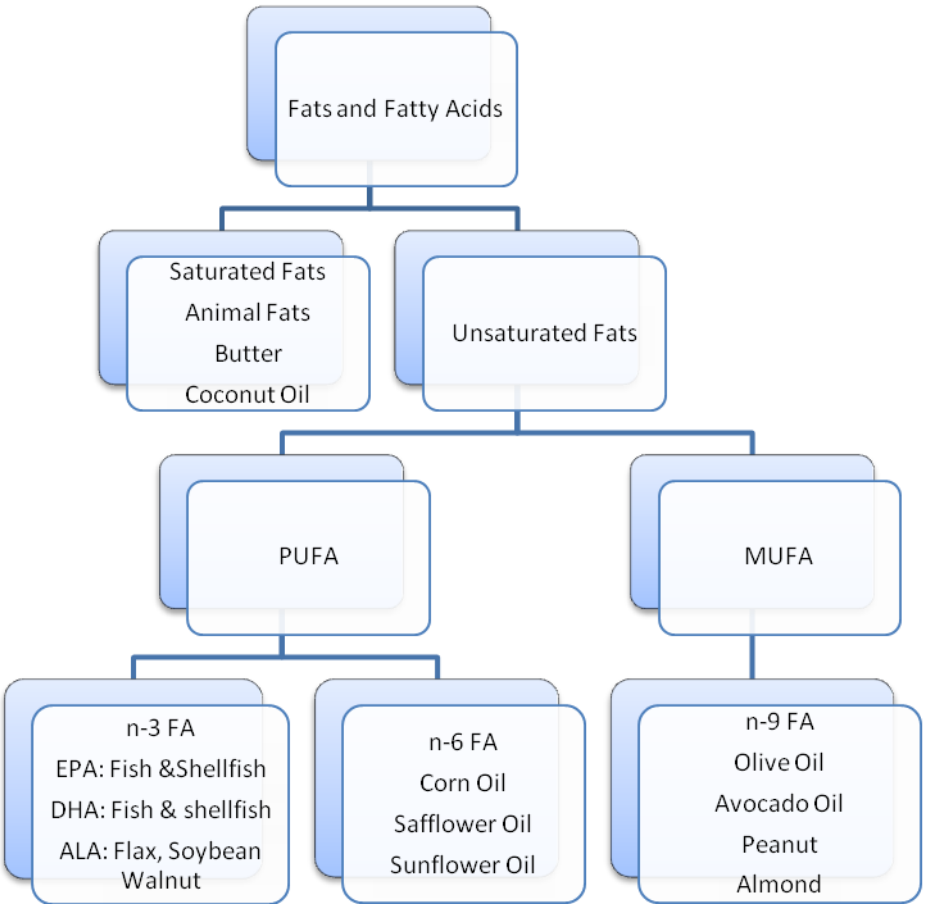

Figure 3: Different sources of fatty acids

GAP in literature

As discussed earlier on, the ALA and LA are essential fatty acids because the body cannot synthesize them, and therefore they need to be supplied through the diet. A recommendation ranging from 25-40% energy as total fat intake has been established[4] [5]. Information on many developing countries indicate that intake of dietary fat and fatty acids are lower than the recommended amount of 35%E. In many developing countries, the total fat intake is very low as compared to the recommended amount. Evidence from these studies[39] shows that the intake was between 24.5% to 29.6%E in Guatemala and South Africa and about 19.5% E in Bangladesh among children between 2-10 years of age. The intake of the specific LA and ALA were also assessed in these studies and levels were below the recommendation. In some of these studies intakes of LA and ALA were lower than half of what is recommended[29, 57, 58]. The fatty acid status is difficult to assess in most developing countries and the few studies conducted have reported low fatty acid status of children in these countries[59]. There have been no FA or LCPUFA trials in Tanzania, so this represents an important research gap.

The rates of malnutrition in the country are higher, with about 42% of all children below five years being stunted[26]. Stunting is an indication of chronic malnutrition due to inadequate dietary intake as well as illnesses. Many families in this country cannot meet their energy requirements and that makes the children less than five more at risk of having lower energy intake. ALA and LA and their derivatives are found in seeds, oils and animal sources such as fish and meat, most of these foods are not part of the daily diet of most of Tanzanian population, hence increasing the risk for EFA deficiency[1, 16, 60]. In Tanzania, the amount and quality of

fats and oils which are used by the majority of its population may compromise the amount and quality of fatty acids obtained by the body. Most of these families are using the oils for cooking and shallow frying is one of the preferred methods of cooking especially when oil is available, which exposes the oil to oxidation. Similarly the amount used is very low and cannot meet the recommended amounts. Majority of the oils available to most of the population in this country include sunflower oil, Korie (a commercial vegetable oil), red palm oil, which have higher amounts of saturated fats as well as n-6 FA.

In this research, we assessed the FA levels and related them to growth and cognitive status of children above two years and below six years for a number of reasons. Firstly, there are no studies in Tanzania, which have assessed FA levels of children of this age group. Secondly, after two years of age a majority of these children stop breastfeeding and start consuming family meals, which are usually low in fats and oils and therefore increases the risks of having lower fat intake and hence low FA status. Thirdly, this is the age when there is rapid growth and development; at the age of six years this country enrolls children in preprimary education and therefore a very important time to assess their cognitive abilities. Thus these could be critical periods at which interventions are most likely to be effective.

CHAPTER 3: WHOLE BLOOD LEVELS OF THE N-6 ESSENTIAL FATTY ACID LINOLEIC ACID ARE INVERSELY ASSOCIATED WITH STUNTING IN 2-TO-6 YEAR OLD TANZANIAN CHILDREN: A CROSS-SECTIONAL STUDY

Data in this chapter is under review in PLoSOne

Abstract

Background: In Tanzania, 35% of all children below five years of age are stunted. Dietary fatty acids (FA) are critical for growth and development. However, whole blood FA levels in Tanzanian children are poorly described. **Objective:** The objectives of this cross-sectional study were to assess 1) whole blood levels of essential fatty acids and 2) the association between whole blood FA levels and growth parameters in Tanzanian children 2-6 years of age. **Methods:** A drop of blood was collected on an antioxidant treated card and analyzed for FA composition. Weight and height were measured and *z*-scores calculated. Relationships between FAs and growth parameters were analyzed by linear regression. **Results:** Of the 334 children that participated, 30.3% were stunted. The average whole blood level of Mead acid was 0.15%. The anthropometric *z*-score height-for-age (HAZ) was inversely associated with Mead acid, the Mead acid to arachidonic acid (T/T) ratio, and total n-9 FA. Additionally, HAZ was positively associated with linoleic and total n-6 FA. BMI-for-age was positively associated with oleic acid, total n-9 FA and T/T ratio but inversely associated with arachidonic and total n-6 FA. Weight-for-height was inversely associated with arachidonic acid and total n-6 FAs and positively associated with oleic and total n-9 FA. Weight-for-age was not associated with any FA tested. Total n-3 FAs were not associated with any growth parameters measured. **Conclusions:** The EFA linoleic acid and the markers of FA deficiency were associated with HAZ, an indicator for stunting in 2-6 year old Tanzanian children. Total n-6, total n-9, and a number of individual FAs

were associated with growth. Increasing dietary intake of EFA and n-6 FAs may be a strategy to combat stunting and chronic malnutrition.

Introduction

Stunting, an inability to achieve height growth potential, is one of the major nutritional challenges in Tanzania. Approximately 35% of children in Tanzania are stunted [61]. Only recently, supplementation strategies incorporating fatty acids (FAs) have become common in nutritional efforts to prevent stunting in some countries [62]. Results from trials of nutrient supplements containing FAs suggest that provision of FAs improves growth of infants and children [63, 64]. However, these lipid supplementation strategies are currently uncommon in Tanzania.

FAs are pivotal for the growth and development of humans. In addition to providing energy, FAs have several physiological functions. FAs have important structural and signaling roles in cell membranes as well as being important for vision, skin integrity, wound healing, heart health, cognition, and immune responses [65]. The essential FAs (EFAs), linoleic (LA), and alpha-linolenic acid (ALA), must be consumed in the diet. Some oils and seeds contain large amounts of LA and ALA. Sunflower, corn, peanut, and soybean oil are good sources of LA. Canola, flaxseed, walnut, and soy are rich sources of ALA [66]. These foods are not typically consumed by infants and young children in Tanzania. In rural areas in Tanzania where malnutrition is highly prevalent, total fat intake is low [5]. This low fat intake increases the likelihood that the EFAs in the diets of infants and young children are insufficient. Thus, inadequate EFA intake

may be partially responsible for the prevalence of growth stunting in Tanzanian infants and children.

Although there are FA supplementation studies in other developing countries, few report blood or plasma FA levels [1]. Additionally, levels of EFA intake have not been measured in infants and children in Tanzania. Furthermore, whole blood FA levels in populations of children from developing countries, specifically Tanzania, are unknown [1]. Given the importance of EFAs, there is a need to assess whole blood FA levels and their relationship to growth in developing countries such as Tanzania [1, 67]. Therefore, we assessed FA levels in 2-to-6 year old Tanzanian children to determine if some FA levels are associated with growth. We hypothesized that whole blood EFA levels in Tanzanian children would positively correlate with the growth parameters: weight-for-age (WAZ), weight-for-height (WHZ), height-for-age (HAZ) and BMI-for-age [44] *z*-scores.

Subjects and methods

Study setting [68]

The study was conducted in Rudewa Mbuyuni village, Kilosa district of Tanzania. Kilosa district covers a total area of 14,245 km^2 of which, 5367 km^2 are suitable for agriculture. Kilosa district is characterized by a dry tropical climate of semi-arid type. Rudewa Mbuyuni consists of five hamlets and about 820 households. The major economic activities in the village are crop farming and livestock keeping. Most villagers are subsistence farmers growing sisal, cotton, paddy, maize, sorghum, pearl millet, sunflower, simsim, cowpeas, pigeon peas, bananas,

coconuts, tomatoes, pumpkins, and sweet potatoes. Increasing numbers of villagers are micro-scale traders. Most households keep domestic animals such as cattle, goats, sheep, donkeys, chickens, and ducks. About 98% of the households in Rudewa Mbuyuni have access to drinkable water sources. About 40% of houses in the village are roofed with galvanized iron sheets, while the rest are roofed with thatched grasses. Only half of the adult population has attained primary education and can read and write. Common diseases in the district include malaria, acute respiratory infections, diarrhea, anemia, parasitemia, and skin infections. In the village there is a dispensary, which offers health services. As per the national guideline, antenatal services are given to pregnant mothers. Children below five years old attend a growth-monitoring clinic once a month, and the children are de-wormed with albendazole and given vitamin A supplements twice a year (June and December). Most young children eat uji, a cornmeal-based porridge, in addition to consuming family meals.

Sample size and subjects

Children (n=334) between 2-6 years of age residing in Rudewa Mbuyuni village participated in the study. Power analysis based on an estimated medium effect size of 0.4 indicated that 242 participants would yield a power of 80%. We enrolled 334 participants, which raised our power to 90%. All households with children between the ages of 2-6 years were identified and invited to participate in the study. Children who were sick or hospitalized at the time of data collection as well as those who were legally declared intellectually disabled were excluded from the study. Consent was given by the parent or caregiver of the participating child. This consent was verbal because the majority of adults in this village are illiterate. A script of the written consent form

that explained the objective of the study was read to the parent/caregiver. They were assured that participation was voluntary and confidential, and that their information would remain anonymous. Parents or caregivers then gave their verbal assent in Swahili. Swahili is the official national language and is used for instruction in primary school education in Tanzania. This study observed all the ethical standards of and was approved by the Institutional Review Board at Michigan State University (IRB#13-700) and the Tanzanian National Institute for Medical Research (NIMR/HQ/R.8a/Vol. IX/1189). Data were collected from December 2013 to August 2014. Although this data was collected throughout multiple seasons, there was no significant difference in whole blood fatty acid levels from one season to another (data not shown).

Anthropometric measurements

Height was measured to the nearest 0.1cm with a stadiometer (Shorr Productions, Perspective Enterprises, Portage, Missouri). Weight was measured using a digital bathroom scale to the nearest 0.1kg (A SECA, Vogel & Haike, Hamburg, Germany). Measurements were repeated, and the average of the two measurements was used (see appendix 1 for detailed method). Date of birth of the child was recorded from the reproductive and child health clinic (RCH) card, and mother's recall was used for those who did not have the RCH card. Sex of the child was also recorded.

Blood measurements

A drop of blood was obtained by puncturing the tip of the middle finger using sterile, single-use lancets. This procedure was relatively painless. The first drop of blood was wiped away with a dry pad. Then, approximately 30µl of capillary blood was collected and applied to a dried blood spot (DBS) card that had been pre-treated with an antioxidant cocktail. The cards were then stored in a dry and cool environment. The DBS cards were shipped to the USA for FA analysis at OmegaQuant Analytics, LLC (Sioux Falls, SD). The average time between sample collection and arrival at the US lab was 8 ± 5 days. After arrival in the US lab, a punch from the DBS card was combined with the derivatizing reagent [boron trifluoride in methanol (14%), toluene, and methanol (35:30:35 parts)], shaken and heated at 100°C for 45 minutes. After cooling, 40 parts of both hexane and distilled water were added. After briefly vortexing, the samples were spun to separate layers and an aliquot of the hexane layer that contained the FA methyl esters was extracted. FA analysis was performed as previously described [69]. Whole blood FA proportions are expressed as a percent of total identified FAs. Additional drops of blood from the same puncture site were used to assess hemoglobin (Hb) concentration and malaria status. An HemoCue photometer (HemoCue AB, Angelholm, Sweden) was used to measure hemoglobin (Hb) concentration. Malaria status was measured using a rapid test kit (Premier Medical Co. Ltd., India) and confirmed by blood smear (see appendix 1 for detailed method).

Data reduction and statistical analyses

EFA are converted into longer-chain metabolites by desaturases and elongases. The substrate-specificity for these enzymes is n-3>n-6>n-9 [29]. Thus, in situations where individuals do not consume sufficient amounts of the EFAs, the non-essential n-9 FA oleic acid (C18:1 n9) is converted to Mead acid (C20:3 n9) [20]. The triene-to-tetraene (T/T) ratio is the ratio of Mead acid to AA.

The weight, height, date of birth and sex data were entered into WHO Anthro [70] and WHO AnthroPlus [71] to calculate *z*-scores. WHZ are missing for the 63 children who were older than 5 years of age. Data from those participants were excluded from analyses including WHZ. The WHO standard population and definitions of moderate and severe stunting, wasting, and underweight were applied to the data [72]. Each *z*-score indicates how many standard deviations from expected, as described by the WHO standard population, the individual in question falls on the basis of her anthropometric measurements. Stunted children have low HAZ. Wasted children have low WHZ. Underweight children have low WAZ. Malnourished children have low BAZ.

Basic descriptive analyses were conducted to obtain means and frequencies. Pearson correlations were calculated for all continuous predictors and covariates. Since there was high collinearity between the various FAs, individual linear regression models were employed for each FA of interest. Regression models included the FA of interest, Hb concentration, and malaria status. T tests were conducted to identify differences in blood levels of FAs between

boys and girls. SPSS version 22 (IBM Corporation, Armonk, NY) was used for these statistical analyses.

FA patterns were generated by principal components analysis (PCA). PCA reduces the number of variables and allows correlated variables to be assessed simultaneously. A linear transformation was performed to enable interpretation. In this case, varimax rotation was performed. Three factors were retained as determined by eigenvalues >1.2. The procedure assigns each person a score for each of the three factors that emerged from the data. Multiple linear regressions using these factor scores as predictors, were used to determine the relationships between these factors and the growth parameters. SAS version 9.4 (Cary, NC) was used for these statistical analyses.

Results

Subject Characteristics

In this study, the mean age of children was 44.9 ± 14.8 months. There were more females (53.3%) than males (46.7%) (**Table** 2). The average height of participants was 94.3 $\pm$ 9.58 cm, and the average weight of participants was 13.9 $\pm$ 2.60 kg. Hb levels ranged from 6 g/dl to 15 g/dl, and 17% of the children tested positive for malaria. The mean HAZ was -1.52. The mean WAZ was -0.97. The mean WHZ was -0.086, and the mean BAZ was 0.03. No WHZ were calculated for the 63 children who were older than 5 years of age at the time of the study. The standard deviations of the HAZ, WAZ, and WHZ distributions were relatively constant and close

to the expected value of 1.0 (range: 0.87 – 1.16). About a third (30.3%) of these children were stunted and 13% were underweight according to WHO criteria [72] (**Table 3**). Approximately, 1% were wasted or had a low BMI for their age.

Table 2: Demographic Characteristics of the Participants[1]

	Mean ± SD			
Age groups, mos	18 - 36 (n = 108)	36.1 - 48 (n = 96)	48.1 - 60 (n = 58)	> 60 (n = 72)
Age, mos	28.9 ± 3.95	41.2 ± 3.26	54.2 ± 3.75	66.6 ± 4.45
Sex, *n* (%) male	56 (51.9)	41 (42.7)	30 (51.7)	28 (38.9)
Height, cm	84.6 ± 4.10	93.1 ± 4.90	100 ± 5.35	106 ± 6.02
Weight, kg	11.5 ± 1.51	13.5 ± 1.67	15.2 ± 1.59	16.7 ± 2.18
HAZ	-1.71 ± 1.26	-1.48 ± 1.15	-1.40 ± 1.10	-1.52 ± 1.07
Hb, g/dL	9.91 ± 1.49	10.3 ± 1.43	10.7 ± 1.23	10.4 ± 1.62
+ malarial RDT, *n* (%)	17 (15.7)	11 (11.5)	14 (24.1)	14 (19.4)

[1]HAZ, height-for-age *z*-score; Hb, hemoglobin; mos, months; RDT, rapid diagnostic test

Table 3: Nutrition and Growth Status of Children[1]

	Based On	Severe (< -3 SD)	Moderate (< -2 SD)	Unaffected
Stunting	HAZ	9.9%	20.3%	69.9%
Malnutrition	BAZ	0%	1.2%	98.8%
Underweight	WAZ	2.1%	11%	86.9%
Wasting	WHZ	0%	1.5%	98.5%

[1]BAZ, BMI-for-age *z* score; HAZ, height-for-age *z* score; WAZ, weight-for-age *z* score; WHZ, weight-for-height *z* score. The WHO definitions of moderate and severe stunting, wasting, underweight and malnutrition were applied to the data [72].

Fatty Acid Levels in Whole Blood

The mean proportions of selected FAs are shown in **Table 4**. Boys and girls had similar whole blood FA levels. Over one-fifth (23.1%) of these children had a whole blood T/T ratio greater than 0.02 while 16% of these children had whole blood Mead acid levels above 0.21%. The

mean T/T ratio in this population was 0.016 $\pm$ 0.008. There was no significant difference between the T/T ratios for boys and girls (p=0.137). Girls and boys had similar AA levels, but boys had significantly higher Mead acid levels than girls (p=0.04).

Table 4: Whole Blood Fatty Acid Proportions[1]

Fatty Acid	Mean ± SD	Range
Oleic	21.7 ± 3.08	14.30 - 33.6
Linoleic	17.9 ± 2.78	11.60 - 17.90
α-Linolenic	0.37 ± 0.17	0.10 - 1.32
Mead	0.15 ± 0.07	0.02 - 0.43
Arachidonic	9.92 ± 1.57	4.03 - 14.02
DHA	2.79 ± 0.76	1.04 - 5.01
EPA	0.41 ± 0.21	0.06 - 1.71
Total n-3[2]	4.50 ± 1.01	2.09 - 7.05
Total n-6[3]	32.5 ± 3.55	22.80 - 42.40
Total n-9[4]	22.7 ± 3.01	15.10 - 33.90
Total Saturated Fat[5]	38.4 ± 1.79	33.10 - 43.60
T/T ratio	0.016 ± 0.008	0.002 - 0.055

[1]Percent of total fatty acids. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; T/T, triene-to-tetraene
[2]Total n-3 includes alpha-linolenic, EPA, docosapentaenoic n-3, and DHA.
[3]Total n-6 includes linoleic, linoelaidic, gamma-linolenic, eicosadienoic, di-homo-gamma-linolenic, arachidonic, docosatetraenoic, docosapentaenoic n-6.
[4]Total n-9 includes oleic, elaidic, eicosanoic, Mead, nervonic.
[5]Total saturated fat includes myristic, palmitic, stearic, arachidic, behenic, lignoceric.

Correlations between Fatty Acids and Growth Parameters

Pearson correlations were calculated for all continuous predictors and covariates (**Table 5**). HAZ was inversely correlated with T/T ratio (-0.154, p=0.005) and whole blood Mead acid levels (-0.114, p=0.037). Additionally, total n-9 and oleic acid levels were also inversely correlated with HAZ (p=0.007). Whole blood LA levels were positively correlated with HAZ (0.157, p=0.004). Also, total n-6 levels were positively associated with HAZ (0.204, p<0.001). The BAZ and T/T ratio were positively correlated (0.123, p=0.025) whereas an inverse correlation was observed between arachidonic acid (AA) (-0.13, p=0.018), total n-6 (-0.127, p=0.02) and BAZ. No significant associations were observed between WHZ and the blood FA levels except for AA, which was negatively correlated WHZ (-0.159, p=0.009). None of the fatty acids correlated with WAZ.

Regressions between Fatty Acids and Growth Parameters

Regression results for HAZ and some selected FAs are shown in **Figure 4** and **Table 6**. A strong negative relationship was observed between HAZ and the T/T ratio (p=0.006). A similar strong negative relationship was observed for Mead acid, oleic acid and the total n-9 FA with HAZ. A positive relationship was observed between HAZ and total n-6 FAs (p=0.001) as well as LA (p=0.008). On the other hand, Oleic acid, total n-9 FAs and the T/T ratio were positively associated with BAZ (**Table 7**).

Table 5: Correlations between Variables[1]

	Hb	Oleic	Linoleic	α-Linolenic	Nervonic	Mead	AA	T/T Ratio	Total n-6	Total n-9	HAZ	WAZ	BAZ	WHZ
Age	**0.166**	-0.077	-0.044	0.102	0.038	-0.067	0.081	-0.085	0.038	-0.072	0.097	-0.075	**-0.224**	-0.075
	0.002	0.162	0.422	0.062	0.490	0.224	0.141	0.120	0.490	0.187	0.079	0.170	**<0.001**	0.215
Hb	1.00	**-0.189**	0.103	-0.104	0.084	0.019	**0.216**	-0.054	**0.205**	**-0.190**	0.050	0.088	0.069	0.039
		0.001	0.060	0.058	0.124	0.734	**<0.001**	0.322	**<0.001**	**<0.001**	0.366	0.110	0.206	0.524
Oleic		1.00	**-0.445**	-0.061	**-0.302**	**0.183**	**-0.733**	**0.403**	**-0.780**	**0.992**	**-0.147**	-0.059	0.100	0.117
			<0.001	0.265	**<0.001**	**0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.007**	0.282	0.067	0.054
Linoleic			1.00	**-0.205**	-0.077	**-0.414**	0.100	**-0.414**	**0.810**	**-0.466**	**0.157**	0.102	-0.058	-0.032
				<0.001	0.162	**<0.001**	0.067	**<0.001**	**<0.001**	**<0.001**	**0.004**	0.062	0.291	0.594
α-Linolenic				1.00	**0.165**	-0.004	0.008	0.003	-0.134	-0.038	-0.043	-0.045	-0.004	0.020
					0.002	0.943	0.879	0.950	0.014	0.490	0.431	0.414	0.941	0.742
Nervonic					1.00	-0.065	**0.310**	**-0.155**	0.136	**-0.199**	0.011	-0.017	-0.040	-0.056
						0.239	**0.000**	**0.005**	0.013	**<0.001**	0.837	0.753	0.463	0.359
Mead						1.00	**-0.148**	**0.933**	**-0.371**	**0.197**	**-0.114**	-0.043	0.089	0.071
							0.007	**<0.001**	**<0.001**	**<0.001**	**0.037**	0.435	0.105	0.247
AA							1.00	**-0.441**	**0.641**	**-0.723**	0.107	0.003	**-0.130**	**-0.159**
								<0.001	**<0.001**	**<0.001**	0.052	0.960	**0.018**	**0.009**
T/T Ratio								1.00	**-0.542**	**0.411**	**-0.154**	-0.054	**0.123**	0.109
									<0.001	**<0.001**	**0.005**	0.324	**0.025**	0.072
Total n-6[2]									1.00	**-0.789**	**0.204**	0.093	**-0.127**	-0.112
										<0.001	**<0.001**	0.089	**0.020**	0.066
Total n-9[3]										1.00	**-0.148**	-0.062	0.096	0.115
											0.007	0.255	0.079	0.059
HAZ											1.00	**0.758**	**-0.142**	0.048
												<0.001	**0.009**	0.427
WAZ												1.00	**0.535**	**0.675**
													<0.001	**<0.001**
BAZ													1.00	**0.976**
														<0.001

[1]Top number is the Pearson's correlation coefficient. Bottom number is the p-value. All significant associations (p<0.05) are in bold. AA, arachidonic acid; BAZ, BMI-for-age *z* score; HAZ, height-for-age *z* score; Hb, hemoglobin; T/T, triene-to-tetraene; WAZ, weight-for-age *z* score; WHZ, weight-for-height *z* score. [2]Total n-6 includes LA, linoelaidic, γ-linolenic, eicosadienoic, di-homo-gamma-linolenic, arachidonic, docosatetraenoic, docosapentaenoic n-6. [3]Total n-9 includes oleic, elaidic, eicosanoic, Mead, nervonic.

AA and total n-6 FAs were negatively associated with BAZ (**Table 7**). No significant relationship was observed between any of the selected FAs and WAZ (**Table 8**). A negative relationship was observed between WHZ and AA as well as total n-6 FAs ($p<0.043$) (**Table 9**). There was a positive relationship between WHZ and oleic acid as well as total n-9 FAs ($p<0.044$) (**Table 9**). DHA, EPA, and total n-3 FAs were not associated with any growth parameters.

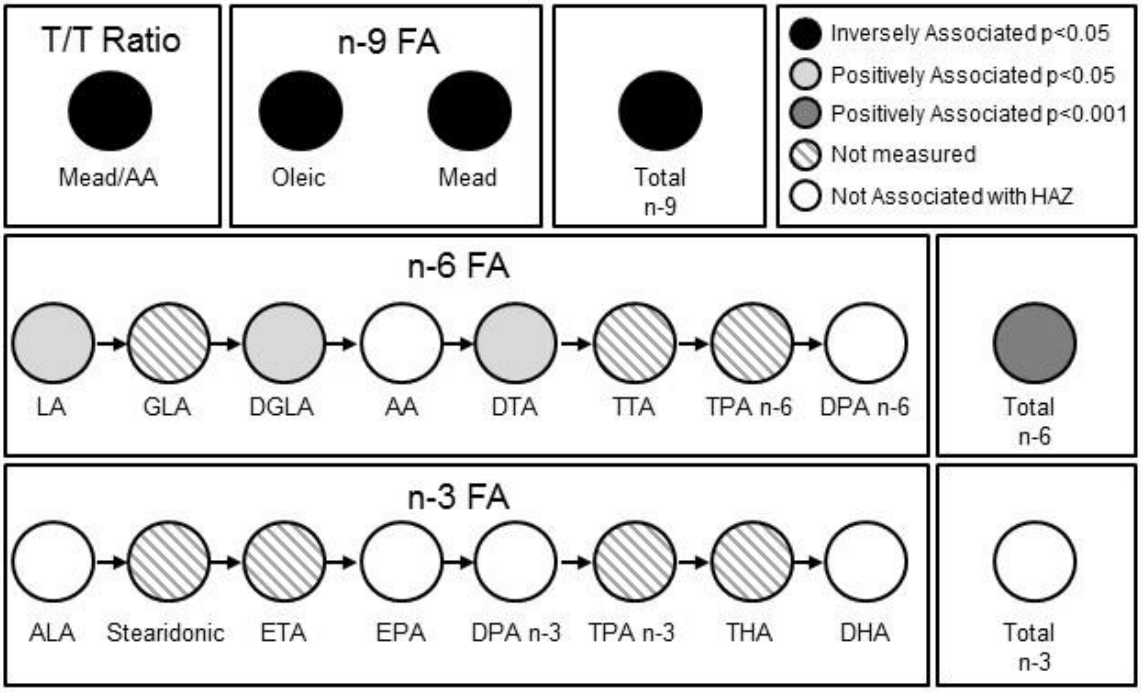

Figure 4: Association between HAZ and selected fatty acids.

Triene/Tetraene ratio (20:3n-9/20:4n-6); Linoleic acid, LA (18:2n-6); Gamma-linoleic, GLA (18:3n-6); Di-homo-gamma-linoleic, DGLA (20:3n-6); Arachidonic, AA (20:4n-6); Docosatetraenoic, DTA (22:4n-6); Tetracosatetraenoic, TTA (24:4n-6); Tetracosapentaenoic, TPAn-6 (24:5n-6); Docosapentaenoic, DPAn-6 (22:5n-6); Alpha-linolenic, ALA (18:3n-3); Eicosatetraenoic, ETA (20:4n-3); Eicosapentaenoic, EPA (20:5n-3), Docosapentaenoic, DPAn-3 (22:5n-3); Tetracosapentaenoic, TPAn-3 (24:5n-3); Tetrahexanoic, THA (24:6n-3); Docosahexanoic, DHA (22:6n-3).

Table 6: Regression[1] Results Between HAZ and Selected Fatty Acids

Fatty Acid	B ± SE	T-value	p-value
Oleic	-0.052 ± .020	-2.503	**0.013**
Linoleic	0.061 ± .020	2.680	**0.008**
α-Linolenic	-0.248 ± .365	-0.680	0.497
Mead	-1.877 ± .888	-2.115	**0.035**
Arachidonic	0.070 ± .041	1.692	0.092
T/T ratio	-20.3 ± 7.37	-2.755	**0.006**
Total n-3[2]	0.058 ± .063	0.907	0.365
Total n-6[3]	0.064 ± .020	3.512	**0.001**
Total n-9[4]	-0.054 ±.020	-2.521	**0.012**
Total Saturated[5]	-0.071 ± 0.04	-1.977	**0.049**

[1]Model: HAZ = fatty acid + malaria status + hemoglobin concentration. HAZ, height-for-age *z* score; T/T, triene-to-tetraene. All significant associations (p<0.05) are in bold

[2]Total n-3 includes alpha-linolenic, eicosapentaenoic, docosapentaenoic n-3, and docosahexaenoic.

[3]Total n-6 includes linoleic, linoelaidic, γ-linolenic, eicosadienoic, di-homo-gamma-linolenic, arachidonic, docosatetraenoic, docosapentaenoic n-6.

[4]Total n-9 includes oleic, elaidic, eicosanoic, Mead, nervonic.

[5]Total saturated fat includes myristic, palmitic, stearic, arachidic, behenic, lignoceric.

Table 7: Regression[1] Results Between BAZ and Selected Fatty Acids

Fatty Acid	B ± SE	T-value	p-value
Oleic	0.035 ± 0.016	2.14	**0.033**
Linoleic	-0.021 ± 0.018	-1.19	0.237
α-Linolenic	0.041 ± 0.284	0.145	0.885
Mead	1.09 ± 0.693	1.57	0.118
Arachidonic	-0.087 ± 0.032	-2.73	**0.007**
T/T ratio	13.26 ± 5.760	2.30	**0.022**
Total n-3[2]	-0.015 ± 0.049	-0.30	0.762
Total n-6[3]	-0.038 ± 0.014	-2.67	**0.008**
Total n-9[4]	0.053 ± 0.020	2.07	**0.039**
Total Saturated[5]	0.072 ± 0.028	1.32	0.189

[1]Model: BAZ = fatty acid + malaria status + hemoglobin concentration. BAZ, BMI-for-age *z*- score; T/T, triene-to-tetraene. All significant associations (p<0.05) are in bold

[2]Total n-3 includes alpha-linolenic, eicosapentaenoic, docosapentaenoic n-3, and docosahexaenoic.

[3]Total n-6 includes linoleic, linoelaidic, gamma-linolenic, eicosadienoic, di-homo-gamma-linolenic, arachidonic, docosatetraenoic, docosapentaenoic n-6.

[4]Total n-9 includes oleic, elaidic, eicosanoic, Mead, nervonic.

[5]Total saturated fat includes myristic, palmitic, stearic, arachidic, behenic, lignoceric.

Table 8: Regression[1] Results Between WAZ and Selected Fatty Acids

Fatty Acid	B ± SE	T-value	p-value
Oleic	-0.012 ± 0.017	-0.716	0.475
Linoleic	0.030 ± 0.018	1.622	0.235
α-Linolenic	-0.172 ± 0.292	-0.591	0.555
Mead	-0.595 ± 0.713	-0.835	0.405
Arachidonic	-0.013 ± 0.033	-0.392	0.695
T/T ratio	-5.304 ± 5.950	-0.891	0.374
Total n-3[2]	0.025 ± 0.051	0.496	0.620
Total n-6[3]	0.019 ± 0.015	1.283	0.200
Total n-9[4]	-0.013 ± 0.017	-0.772	0.441
Total Saturated[5]	-0.025 ± 0.029	-0.865	0.387

[1]Model: WAZ = fatty acid + malaria status + hemoglobin concentration. WAZ, weight-for-age *z* score; T/T, triene-to-tetraene

[2]Total n-3 includes alpha-linolenic, eicosapentaenoic, docosapentaenoic n-3, and docosahexaenoic.

[3]Total n-6 includes linoleic, linoelaidic, γ-linolenic, eicosadienoic, di-homo-gamma-linolenic, arachidonic, docosatetraenoic, docosapentaenoic n-6.

[4]Total n-9 includes oleic, elaidic, eicosanoic, Mead, nervonic.

[5]Total saturated fat includes myristic, palmitic, stearic, arachidic, behenic, lignoceric.

Table 9: Regression[1] Results Between WHZ and Selected Fatty Acids

Fatty Acid	B ± SE	T-value	p-value
Oleic	0.037 ± 0.018	2.081	**0.038**
Linoleic	-0.012 ± 0.019	-0.648	0.518
α-Linolenic	0.137 ± 0.302	0.453	0.651
Mead	0.801 ± 0.709	1.129	0.260
Arachidonic	-0.101 ± 0.036	-2.847	**0.005**
T/T ratio	10.86 ± 5.95	1.826	0.069
Total n-3[2]	0.006 ± 0.050	0.106	0.916
Total n-6[3]	-0.035 ± 0.015	-2.044	**0.042**
Total n-9[4]	0.037 ± 0.018	2.036	**0.043**
Total Saturated[5]	0.003 ± 0.030	0.107	0.915

[1]Model: WHZ = fatty acid + malaria status + hemoglobin concentration. WHZ, weight-for-height *z* score; T/T, triene-to-tetraene. All significant associations ($p<0.05$) are in bold

[2]Total n-3 includes ALA, EPA, DPA n-3, and DHA.

[3]Total n-6 includes LA, linoelaidic, γ-linolenic, eicosadienoic, DGLA, AA, docosatetraenoic, DPA n-6.

[4]Total n-9 includes oleic, elaidic, eicosanoic, Mead, nervonic.

[5]Total saturated fat includes myristic, palmitic, stearic, arachidic, behenic, lignoceric.

Principal Component Analysis

When PCA was used to determine how combinations of these variables might be associated with growth, three factors emerged. The factor loading matrix is shown in **Table 10**. Because

Factor 1 contains AA, LA and total n-6, it is labeled n-6 FA. Factor 2 is labeled n-3 FA because it contains DHA, EPA and total n-3. Factor 3 is labeled n-9 and saturated fats. Multiple linear regression between these three factors and HAZ (p=0.0128) revealed that n-6 FA (Factor 1, p=0.010) and n-9 and saturated fats (Factor 3, p=0.010) were significantly associated with stunting. Specifically, the n-6 FA factor was positively associated with HAZ. The n-9 and saturated FA factor was inversely associated with HAZ. In further multiple linear regression analyses, the overall models were not significant (WAZ (p=0.572), WHZ (p=0.381) or BAZ (p=0.160)). However, the n-6 FA factor was negatively associated with BAZ (p=0.038) and WHZ (p=0.031).

Table 10: Factor Loading Matrix for Fatty Acids in the Whole Blood of 2-6 year old Tanzanian Children[1]

	Factor 1: n-6 FA	Factor 2: n-3 FA	Factor 3: n-9 and saturated FA
Arachidonic	**0.84**	0.25	-0.07
Total n-6[4]	**0.78**	-0.16	-0.57
Linoleic	**0.38**	-0.38	-0.73
Total n-3[2]	0.41	**0.88**	-0.03
DHA	0.46	**0.71**	-0.14
EPA	0.10	**0.62**	0.19
α-Linolenic	-0.23	**0.61**	-0.07
Total Saturated Fat[3]	-0.07	-0.03	**0.81**
Mead Acid	-0.05	-0.11	**0.73**
Oleic Acid	-0.92	-0.14	**0.10**

[1]The factor loading value indicates the correlation between the fatty acid and the factor. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.
[2]Total n-3 includes alpha-linolenic, EPA, docosapentaenoic n-3, and DHA.
[3]Total saturated fat includes myristic, palmitic, stearic, arachidic, behenic, lignoceric.
[4]Total n-6 includes linoleic, linoelaidic, gamma-linolenic, eicosadienoic, di-homo-gamma-linolenic, arachidonic, docosatetraenoic, docosapentaenoic n-6.

Discussion

Whole blood FA levels in 2-to-6 year old Tanzanian children have not previously been measured. In fact, this is one of the few studies [73, 74] to assess the relationship between growth parameters and whole blood FA levels directly instead of using dietary intake or supplementation regimens to determine fat intake. We used an oxidation-inhibiting collection card (Omega Quant Analytics, LLC (Sioux Falls, SD)) and shipped samples to the United States to enable direct measurements of whole blood FA levels. The system has previously been used and fatty acids were determined to be stable when collected with these methods [75].

Tanzanian children in this study had higher whole blood Mead acid levels compared to those previously reported for European children [76]. Some blood FA levels in Tanzanian children were associated with growth parameters. Specifically, the n-6 FAs were inversely associated with stunting and positively associated with wasting. The n-9 FAs were positively associated with stunting and negatively associated with underweight. LA was inversely associated with stunting. However, ALA and total n-3 were not associated with any growth parameters. Further, about 16% of all participants had Mead acid levels above 0.21%, and 23% had a T/T ratio greater than 0.02 indicating potential deficiencies in EFA intake. Children with high whole blood levels of Mead acid and elevated T/T ratios were at risk of being stunted. This was evidenced by the inverse association of the T/T ratio and Mead acid with HAZ.

These data suggest that n-6 FAs are important for linear growth whereas n-9 FAs can participate in the accumulation of overall body mass but may not play a role in linear growth. The linear growth-restriction observed in children with higher whole blood Mead acid and T/T ratios may explain the positive relationship between BAZ and Mead acid as well as the positive relationship between BAZ and T/T ratio. It is plausible that a shorter child consuming the same number of calories as a taller child and gaining the same amount of body weight as a taller child would have a larger BMI. This could also explain why n-6 FAs were negatively associated with WHZ and BAZ despite being positively associated with HAZ whereas n-9 FAs were negatively associated with HAZ but positively associated with WHZ and BAZ (**Table 11**).

Table 11: Summary of Significant Associations Between Fatty Acids and Growth Parameters.

	GROWTH PARAMETERS		
	HAZ	BAZ	WHZ
Oleic	⬇	⬆	⬆
Mead	⬇	---	---
AA	---	⬇	⬇
Total n-9[1]	⬇	⬆	⬆
Total n-6[2]	⬆	⬇	⬇
T/T ratio	⬇	⬆	---

Triene/Tetraene ratio (20:3n-9/20:4n-6)

[1]Total n-9 includes oleic, elaidic, eicosanoic, Mead, nervonic.

[2]Total n-6 includes linoleic, linoelaidic, gamma-linolenic, eicosadienoic, di-homo-gamma-linolenic, arachidonic, docosatetraenoic, docosapentaenoic n-6.

Although it has not previously been shown in Tanzania, research conducted in other populations suggests that an adequate supply of EFAs and n-6 FAs are required for normal in

utero and childhood growth [1, 11, 30-33, 77]. We observed significant positive associations between n-6 FA levels, LA and HAZ. Causal relationships cannot be determined from cross-sectional studies, but supplementation studies have demonstrated that n-6 FAs enable optimal growth [1]. However, the mechanism underlying the role of n-6 FAs in growth is not well understood. Additionally, LA and AA deficiency have previously been associated with poor growth. FAs not only provide energy and serve as building blocks for the membranes of cells and cellular compartments, but also are signaling molecules. FA can bind directly to receptors or stimulate cell signaling after they have been converted from their original structure to new metabolites [78]. Thus, the positive associations between n-6 FAs and growth may be related to the activity of n-6 FA metabolites. For instance, prostaglandin E_2 (PGE2), a cyclooxygenase metabolite of AA [79] increases insulin-like growth factor-1 expression and increases calcium accretion in humans [80]. In our study of 2-to-6 year old Tanzanian children we observed no association between n-3 FAs and growth parameters, consistent with some [34, 81] but not all [81, 82] previous reports. There are limited data available in developing countries to support higher intakes of EPA/DHA or ALA in pregnancy to improve birth weight and length. Recently, genetic variation in FA desaturases, whose activity is important in both the n-6 and n-3 FA metabolic pathways, was determined to be associated with height [83]. Taken together, it is likely that FAs are important in growth, and the role of EFA in growth of children in developing countries needs to be further studied.

Two to six years of age is a period of rapid growth, and thus a time of increased need for nutrients such as FA. Therefore, in environments where dietary quality is poor, children of

these ages are likely to develop deficiencies of many nutrients, including FAs. Breastfed children typically consume sufficient EFA due to the presence of EFA in human milk [84]. In Rudewa Mbuyuni the majority of children are breastfed up to two years (70.4%) as is the custom in most areas of Tanzania [26]. Most breastfed children are also given cereal-based porridge that is high in carbohydrates and energy but is not nutrient dense [85]. After cessation of breastfeeding, children consume mainly the cereal-based porridge and other family foods. These foods usually do not contain sufficient amounts of fat, nor sufficient amounts of EFAs. Additionally, poor feeding practices [86] and exposure to infectious diseases likely affect digestion and absorption leading to malabsorption and predisposing these children to poor FA status.

Populations that have low dietary intake of EFAs typically exhibit poor growth and development [1]. Historically, FA levels in plasma are used to determine EFAD. The T/T ratio is the ratio of Mead acid to AA. A T/T ratio > 0.02 in plasma samples defines EFAD [23, 87]. Mead acid [88] levels above 0.4% [20] in red blood cells (RBCs) and 0.21% [87] in plasma have also been used to define EFAD. The dried blood spot method utilized in the current study analyzes whole blood samples in which the FA proportions are roughly derived 50:50 from blood cells and plasma. The precise T/T ratio associated with EFA deficiency in whole blood remains to be determined. Data from this study would suggest that a whole blood T/T ratio of 0.02 might be indicative of deficiency. We have observed a strong correlation between AA in whole blood and AA in plasma (r^2=0.89, n=50; WSH unpublished data), and others have reported the same with most correlation coefficients falling between 0.8 and 0.97 for PUFA [89]. Although we have not

measured Mead acid in whole blood and plasma from the same individuals, it is likely Mead acid detection would be similar to that for AA. Thus, the T/T ratio in plasma and whole blood likely would be similar for any one individual. Therefore, it is feasible that a cut point of >0.02 for the T/T ratio in whole blood could be used as a biomarker of EFAD. Additionally, whole blood levels of certain FAs are reflective of dietary FA intake [90]. Thus, in Rudewa Mbuyuni, it is possible that dietary deficiency of EFAs is one of the factors contributing to poor growth because children with higher levels of Mead acid and higher T/T ratios were more likely to be stunted.

During this study, blood samples were collected throughout the day and no fasting was required. This might be expected to increase variability in the whole blood FA measurements; however, in this setting the differences are likely to be small considering that children from the village consume relatively similar and low-fat meals compared to children in other settings. In this study we also did not assess adequacy of other nutrients, such as zinc or protein, which are potentially inadequate in the diets of the children. Deficiencies of some nutrients may interfere with FA metabolism and thus affect whole blood FA levels. Furthermore, deficiencies of some nutrients can also contribute to poor growth. Since we do not know if children with high Mead acid and T/T ratios also had other nutritional deficiencies, the results should be interpreted with caution.

Our study utilized a large population of children and therefore was well-powered to detect differences in the parameters of interest. Among the strengths of this study were the use of

biomarkers rather than reliance on food intake questionnaires to define FA exposure, assessments by well-trained anthropometrists and the recruitment of nearly all eligible children of the appropriate ages in the village. Since this was a cross-sectional study, all reported associations are correlative rather than causative. However, this study was limited to participants from one village in Tanzania. It has previously been observed that food intake varies depending on location within Tanzania with individuals living in rural and urban areas consuming different foods [44]. Therefore, these results are not generalizable to the entire Tanzanian population of children. Although our study did not collect any socio-economic data from the children's parents/caregivers, the population is relatively homogeneous as explained by Ntwenya et al [68]. In sum, this study sets the foundation for future studies assessing whole blood FA composition in children residing in other areas of Tanzania, ideally over their entire life course.

Conclusion

This study assessed whole blood levels of FAs and the association between whole blood FA levels and growth parameters in children 2-6 years of age. EFA levels in 2-to-6 year old Tanzanian children were low as demonstrated by high levels of Mead acid and T/T ratios, both markers of EFA deficiency. In this population, levels of certain FAs in blood were associated with growth parameters. The inadequate levels of FA in this population may not only be associated with impaired growth, but may also be associated with impaired immunity or poor cognitive development. Thus, future studies of nutritional interventions for Tanzanian children should consider providing FAs in addition to sufficient calories, protein and micronutrients.

Additionally, these studies should encourage the incorporation of locally available FA-rich seeds and oils into meals and snacks for infants and children. Finally, the development of simple instrumentation that can be used in the field to analyze FAs and FA metabolites in whole blood would be useful for future studies.

CHAPTER 4: WHOLE BLOOD PUFA IS POSITIVELY ASSOCIATED AND SATURATED FAT IS INVERSELY ASSOCIATED WITH EXECUTIVE FUNCTION IN TANZANIAN CHILDREN AGED FOUR TO SIX YEARS: A CROSS-SECTIONAL STUDY

Data in this chapter is under review in the British Journal of Nutrition

Abstract

Background: Essential fatty acids (EFA) are poly-unsaturated fatty acids (PUFA) that are important for brain development and cognitive function. The objective of this study was to determine the association between whole blood EFAs, Mead acid and cognitive function in Tanzanian children. **Methods:** A total of 325 two-to-six year old children attempted the dimensional change card sort (DCCS) tasks to assess executive function. Blood was collected for FA analysis by gas chromatography. Associations between executive function and FAs levels were assessed by regression. **Results:** 130 four-to-six year old children successfully completed the DCCS tasks. Whole blood levels of linoleic acid, total n-6 FAs and total PUFAs were positively associated with executive function, whereas palmitic acid, α-linolenic acid, nervonic acid, total n-9 FAs, total saturated FAs, and total mono-unsaturated FAs were inversely associated with executive function. Children who had sufficient whole blood levels of EFA were 3.8 times more likely to successfully complete all DCCS tasks than children with insufficient EFA. **Conclusion:** These results suggest that higher whole blood PUFA levels are associated with cognitive abilities. Intervention trials are required to determine if increased n-6 FA and/or total PUFA intake will improve executive function in Tanzanian children.

Introduction

Long chain polyunsaturated fatty acids (LCPUFA) accumulate in the fetus during pregnancy and during early childhood [2]. These PUFA are concentrated in the central nervous system[9]. Essential fatty acids (EFAs) of both the n-6 and n-3 FA families and their LCPUFA metabolites play a significant role in neuronal growth and differentiation of cells and have been associated with cognitive abilities of children[9, 67, 91]. Thus poor PUFA status may affect brain development as well as the cognitive abilities of children[67].

In most developing countries a significant proportion of the population cannot afford diets rich in animal foods[1, 67], and lack of animal foods may lead to PUFA deficiency[67]. Several PUFA supplementation studies conducted in young children (<2y) demonstrated that children fed foods/milk fortified with alpha-linolenic acid (ALA) or docosahexaenoic acid (DHA) alone or together with other micronutrients had enhanced cognitive development[1]. These studies suggest that high intake of LCPUFAs may improve cognitive abilities later in life (i.e. after two years of age)[1, 15]. Sheppard and Cheatham[15] concluded that LCPUFA influence the cognitive development of children especially with regard to planning and memory processing. Previous research utilized food intake data or supplementation programs to estimate FA status of children. To our knowledge few of these studies have directly measured whole blood FA status as it relates to cognitive development in children between four-to-six years of age.

Executive function (EF), the conscious control of thoughts and actions, develops between the ages of two and ten years[92]. EF involves inhibition, working memory and task switching[92] and is controlled by the frontal and temporal lobes of the brain [15]. These two regions of the brain continue to develop after the second year of life and contain high amounts of arachidonic acid (AA) and DHA[93]. A method commonly used to assess EF in young children is the dimensional change card sorting (DCCS) task[92]. Therefore we used the DCCS task to assess cognitive function in this population of young children.

Additionally, although FAs are widely understood to affect growth and cognition, the relationship between blood levels of specific FAs and these health outcomes are infrequently reported. Herein we assess the relationship between FA status and executive function in Tanzanian children using a culturally-modified DCCS test. We hypothesized that whole blood levels of EPA, DHA, and both EFAs (ALA and linoleic acid) would be positively associated with performance on the DCCS tasks.

Methods

Study Site

The study was conducted in Rudewa Mbuyuni village in Kilosa District, Morogoro Tanzania. Conditions in the village have previously been described[68]. Children in the village begin attending primary school around seven years of age. Currently there are no preschool programs in the village. The ministry of Health in Tanzania requires that all children below five years of

age visit a growth-monitoring clinic every month, receive vaccinations according to a published schedule as well as receive vitamin A drops twice a year (June, December).

Subjects and Ethical Approval

All children in the village were invited to participate. From December 2013 to August 2014, 335 apparently healthy children between two to six years of age were enrolled in this cross-sectional study. All participants and their mothers/caregivers verbally consented to participate in the study. The National Medical Research Board (NIMR) of Tanzania (NIMR/HQ/R.8a/Vol. IX/1189) and the Michigan State University [94] Human Research Protection Program (IRB#13-700) approved the study. Ten children refused to take the test at initial contact and withdrew from the study. Swahili was used as the language of communication throughout the study.

Whole Blood Assessments

A capillary blood sample was taken from the middle finger for malaria rapid test (Premier Medical Co. Ltd., India), measurement of hemoglobin (Hb) concentration using a HemoCue photometer (HemoCue AB, Angelholm, Sweden), and dropped onto OxyStop™ treated dried blood spot cards (DBS). DBS cards were stored in a dry, dark, cool environment and shipped to the US, arriving at OmegaQuant (Sioux Falls, South Dakota) for FA analysis within 14 days of sample collection. DBS cards were punched and combined with derivatizing reagent [boron trifluoride in methanol (14%), toluene, and methanol (35:30:35 parts)], shaken and heated at 100°C for 45 minutes. Allowed to cool, then, 40 parts of both hexane and distilled water were

added and briefly vortexed. FA methyl esters were analyzed using a gas chromatograph as described[69]. Whole blood FA proportions are expressed as a percent of total identified FAs. The triene-to-tetraene (T/T) ratio is the ratio of Mead acid to AA. A T/T ratio > 0.02 defines low levels of EFAs[23, 95].

Cognitive Assessment: Dimensional Change Card Sort (DCCS)

The DCCS[51, 92] is conceptually simplistic in that it requires the child to sort a series of bivalent cards (**Figure 5**) based on one of two instructed dimensions (i.e., color or shape). Following sorting of an initial series of eight cards based upon color; the children were instructed to switch the categorization dimension and sort another series of eight cards based upon shape.

Previous research has demonstrated that children younger than three years of age can complete the pre-switch series,[96] but the dimensional change requires engagement of executive function in order to inhibit the previous rule set to execute the correct sorting behavior[92, 97]. Indeed, children with poor executive function exhibit a tendency to perseverate during the post-switch series by continuing to sort the cards by the first dimension despite being able to verbally express the new sorting rules. A critical limitation of the traditional card sort task, however, is the relatively narrow age range in which it can be utilized with Rennie and colleagues[97] observing that children are unable to successfully complete the post-switch sorting series until about four to five years of age. Accordingly, a modified variant of the DCCS task was used in order to increase the number of participants who could perform

the task[96]. As young children and those with poor executive function appear to have greater attentional inertia — manifesting with increased difficulty separating features of an object[98]; separating the sorting attributes into differentiable objects reduces, but does not remove, the inhibitory demands required to complete the switch[96]. Therefore, participants completed three versions of the DCCS, which progressively increased the executive function requirements by increasing the overlap between the shape and color stimulus on the cards (i.e., no overlap, partial overlap, full overlap). The task was modified to use images and colors familiar to the participating children. The mother or caregiver was present during the test to observe the process and allow the child to feel comfortable and confident.

Each version of the DCCS (no overlap, partial overlap, full overlap) had a pre- and post-switch phase. For a child to pass any phase, s/he needed to obtain six correct responses out of eight. If fewer than six correct responses were made in the pre-switch phase, the post-switch phase of that version was not scored. The child was then asked to continue with the next version of the DCCS. Consistent with two dominant approaches to scoring the DCCS, task performance was summarized using: 1) highest test passed and 2) total passes. Scoring for 1) “highest test passed” was ordered. A child scored “0” if they were unable to pass any post-switch phase, “1” if the child passed the no overlap DCCS post-switch task and the partial overlap post-switch, and “2” if the child passed the full overlap post-switch. Scoring for 2) “total passes” was not ordered. It was based on the total number of post-switch phases passed and ranged from 0-3.

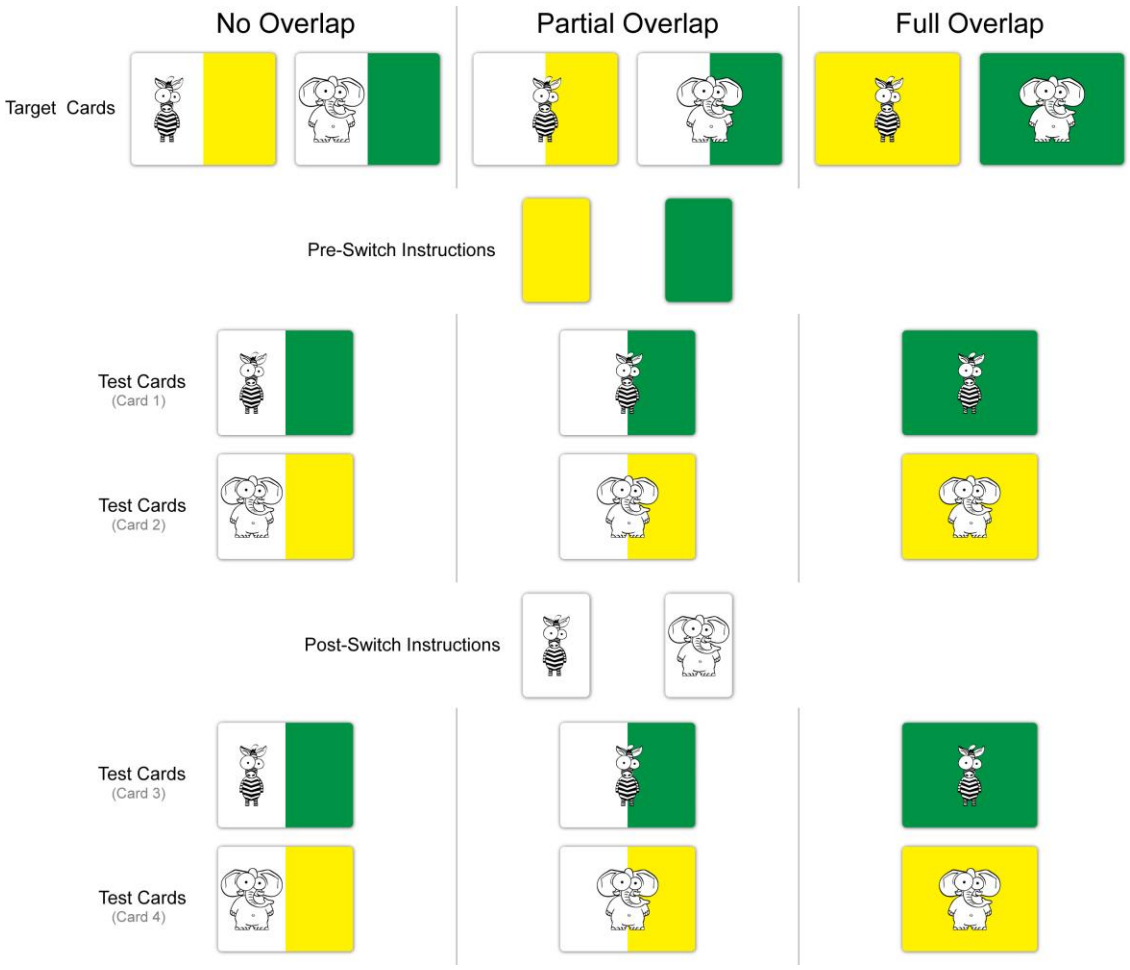

Figure 5: Illustration of the target cards and test cards used during the pre-switch and post-switch phases of the DCCS.

The No Overlap variant (left column), the Partial Overlap variant (middle column), and the Full Overlap variant (right column). See appendix 1 for detailed method.

Data Reduction and Statistical Analyses

An *a priori* power analysis was conducted using the results of previous investigations observing a relationship between nutritional supplementation and performance on the proposed tasks.[99] Assuming a conservative effect size (f^2 = 0.1), a two-sided alpha of 0.05, and a beta of 0.20 (i.e., 80% power) a sample of 81 participants was estimated to provide adequate power.

Because more than half the children less than 48 months of age failed to pass any DCCS task (Figure 6), we excluded them in our analyses. The analyses presented herein are from the 130 children older than 48mos.

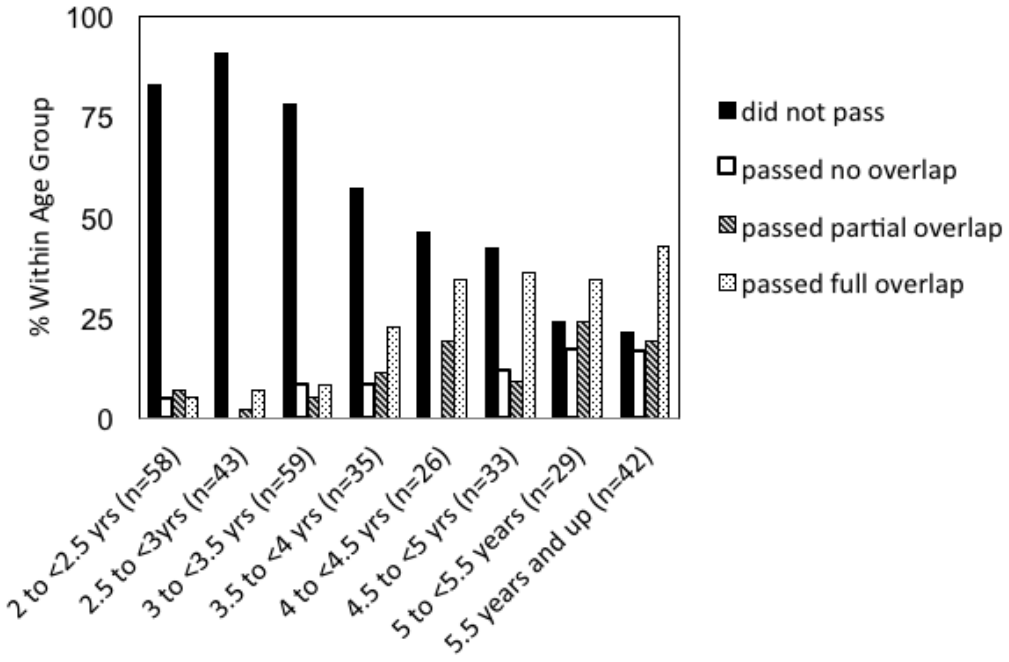

Figure 6: Percentage of children able to pass each stage of the DCCS test.

Pearson's correlation was used to determine correlations between FA and executive function. Linear regression was used to analyze the association between blood FA levels and executive function. Models for linear regression included the FA of interest, hemoglobin levels and malaria status. We included hemoglobin concentrations in our model because it is a known significant predictor of cognitive abilities in our population[100]. None of the children exhibited symptoms of active malaria infection. Positive malaria status indicated subclinical infection and was included as a covariate because it was positively associated with DCCS performance. SPSS

version 22 (Columbia, MD) was used for these statistical analyses. We conducted polytomous logistic regressions for categorical dependent variables using SAS version 9.4 (Cary, NC).

Results

Descriptive statistics of the participants

This study enrolled 335 children between two and six years of age. Of these, 325 attempted the DCCS tasks because 10 refused to complete the DCCS. Basic information about the study participants can be found in Table 12. Fewer than half the children between 24 and 48 months successfully performed any DCCS task (Figure 2). Of the 130 children $\geq$ 48mos who attempted the DCCS tasks, 38% of these children passed the full overlap task, 18% passed the partial overlap task, 13% passed the no overlap task, and 31% failed to successfully complete any of the tasks.

Table 12: Participant characteristics[1]

	Overall (n=325)	Children ≥48 mos (n=130)
Age, mos[1]	45.33 ± 14.70	61.04 ± 7.43
Sex, *n* (%) male	149 (46.5)	59 (45.4)
Hb, (g/dL)[1]	10.29 ± 1.46	10.58 ± 1.45
Malaria, %	16.3	20.0
Duration of breastfeeding, mos[1] (n=312)	22.42 ± 4.06	22.75 ± 4.36

[1]Where appropriate, values are given as mean ± SD

FA levels in whole blood from the 130 children whose data were analyzed are presented in Figure 7. Mean LA levels were 17.6 $\pm$ 2.7 as a percent of whole blood FAs, whereas mean ALA levels were 0.4 $\pm$ 0.2. The mean duration of breastfeeding among children $\geq$ 48mos was 22.8 ± 4.4 mos. Breastfeeding duration was similar between children who failed to complete any DCCS task and those who successfully completed DCCS tasks. In this population, breastfeeding duration closely matched the WHO recommendation to breastfeed up to 24 months[101].

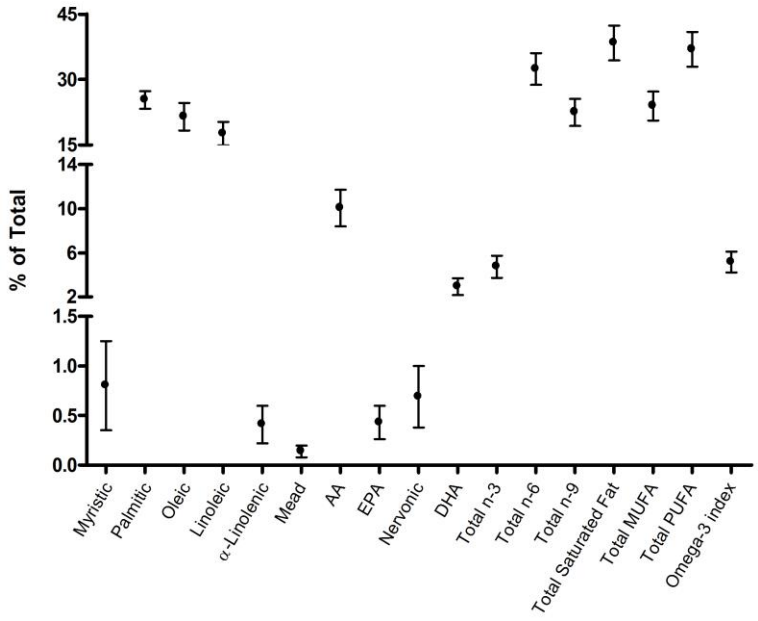

Figure 7: Whole blood fatty acid levels (mean ± SD) of Tanzanian children $\geq$ 48mos of age (n=130).

Correlations between fatty acids and executive functions measures

Palmitic acid, oleic acid, ALA, nervonic acid, total n-9, and total MUFA were inversely correlated with performance on the DCCS tasks (Table 13). LA, DHA, total n-6 and total PUFA were positively correlated with performance on the DCCS tasks. Although the n-3 FA DHA correlated

positively with performance neither EPA nor the omega-3-index[102] were correlated with DCCS performance.

Table 13: Pearson correlations between study parameters

	Malaria	Palmitic	Oleic	LA	ALA	Nervonic	DHA	T/T ratio	Total n-6[1]	Total n-9[2]	Total sat fat[3]	Total MUFA[4]	Total PUFA[5]	Total passes	Highest passed
Age	-0.108	-0.034	-0.138	0.022	-0.116	-0.035	0.040	0.105	0.074	-0.129	0.027	-0.135	0.096	0.144	0.122
	0.223	0.699	0.118	0.800	0.189	0.693	0.650	0.235	0.400	0.142	0.762	0.125	0.278	0.102	0.168
Sex	0.064	0.021	0.095	0.097	-0.050	-0.116	0.119	0.018	-0.033	0.087	-0.067	0.076	-0.032	0.024	0.003
	0.468	0.813	0.282	0.270	0.571	0.190	0.178	0.843	0.711	0.327	0.448	0.393	0.714	0.785	0.971
Hb	**-0.191**	**-0.263**	**-0.281**	**0.228**	**-0.191**	0.021	0.131	**-0.177**	**0.322**	**-0.302**	-0.113	**-0.291**	**0.321**	0.078	0.036
	0.029	**0.002**	**0.001**	**0.009**	**0.030**	0.810	0.138	**0.044**	**<0.001**	**<0.001**	0.199	**0.001**	**<0.001**	0.381	0.686
Malaria	1.0	**0.158**	0.070	**-0.244**	-0.079	-0.020	-0.041	0.099	**-0.188**	0.063	**0.227**	0.096	**-0.170**	**0.252**	**0.258**
		0.072	0.431	**0.005**	0.375	0.826	0.645	0.264	**0.032**	0.479	**0.009**	0.275	**0.053**	**0.004**	**0.003**
Palmitic		1.0	**0.588**	**-0.588**	-0.077	**-0.355**	**-0.518**	**0.608**	**-0.803**	**0.544**	**0.757**	**0.624**	**-0.837**	**-0.192**	**-0.190**
			<0.001	**<0.001**	0.384	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.029**	**0.031**
Oleic			1.0	**-0.448**	-0.018	**-0.277**	**-0.469**	**0.517**	**-0.810**	**0.989**	0.078	**0.983**	**-0.857**	**-0.157**	**-0.180**
				<0.001	0.835	**0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.378	**<0.001**	**<0.001**	**0.074**	**0.040**
Linoleic				1.0	**-0.172**	-0.097	0.107	**-0.456**	**0.799**	**-0.458**	**-0.601**	**-0.533**	**0.715**	**0.224**	**0.236**
					0.050	0.272	0.227	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.010**	**0.007**
ALA					1.0	**0.261**	0.095	-0.122	-0.093	0.020	-0.096	0.039	-0.053	-0.283	-0.253
						0.003	0.280	0.168	0.290	0.822	0.277	0.656	0.549	**0.001**	**0.004**
Nervonic						1.0	**0.319**	**-0.264**	0.094	-0.165	-0.033	**-0.218**	**0.168**	**-0.235**	**-0.236**
							<0.001	**0.002**	0.286	0.060	0.708	**0.013**	**0.056**	**0.007**	**0.007**
DHA							1.0	**-0.489**	**0.338**	**-0.442**	**-0.286**	**-0.487**	**0.523**	**0.168**	**0.166**
								<0.001	**<0.001**	**<0.001**	**0.001**	**<0.001**	**<0.001**	**0.055**	**0.058**
T/T ratio								1.0	**-0.602**	**0.494**	**0.424**	**0.556**	**-0.631**	-0.103	-0.109
									<0.001	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.243	0.217

[1]Total n-6 includes linoleic, linoelaidic, gamma-linolenic, eicosadienoic, di-homo-gamma-linolenic, arachidonic, docosatetraenoic, docosapentaenoic n-6.

[2]Total n-9 includes oleic, elaidic, eicosanoic, Mead, nervonic.

[3]Total saturated fat includes myristic, palmitic, stearic, arachidic, behenic, lignoceric.

[4]Total MUFA includes Palmitoleic, palmitelaidic and nervonic

[5]Total PUFA included Total n-3 and total n-6

Table 13 (cont'd)

	Malaria	Palmitic	Oleic	LA	ALA	Nervonic	DHA	T/T ratio	Total n-6[1]	Total n-9[2]	Total sat fat[3]	Total MUFA[4]	Total PUFA[5]	Total passes	Highest passed
Total n-6[1]									1.0	**-0.817**	**-0.555**	**-0.861**	**0.974**	**0.246**	**0.255**
										<0.001	**<0.001**	**<0.001**	**<0.001**	**0.005**	**0.003**
Total n-9[2]										1.0	0.063	**0.978**	**-0.857**	**-0.184**	**-0.205**
											0.475	**<0.001**	**<0.001**	**0.036**	**0.019**
Total sat fat[3]											1.0	0.142	**-0.544**	-0.161	-0.145
												0.108	**<0.001**	0.068	0.099
Total MUFA[4]												1.0	**-0.901**	**-0.189**	**-0.215**
													<0.001	**0.031**	**0.014**
Total PUFA[5]													1.0	**0.244**	**0.257**
														0.005	**0.003**

Regressions between fatty acids and executive functions measures

Regression results between selected FAs and the ordered assessment (the highest test passed) and the non-ordered assessment (total passes) of DCCS performance are shown in Table 14. A significant inverse relationship was observed between DCCS performance and palmitic acid, ALA, nervonic acid, total n-9, total saturated fat, and total MUFA. Additionally, oleic acid was inversely associated with highest test passed. Linoleic acid, total PUFA, and total n-6 FA were positively associated with DCCS performance. DHA tended to be positively associated with total passes and was positively associated with highest test passed. Finally, there were no significant relationships between EPA, the omega-3 index, Total n-3, mead acid, AA, or the T/T ratio and DCCS performance.

Polytomous logistic regression

Polytomous logistic regression analyses demonstrated that children with low EFA levels (T/T ratio >0.02) tended to perform more poorly on DCCS tasks than children with high EFA levels (T/T ratio $\leq$ 0.02). These models included malaria status and Hb levels as co-variates. For the non-ordered assessment of DCCS performance (total passes), children with higher levels of EFA were seven times more likely to successfully complete all three post-switch DCCS tasks than children with lower levels of EFA (OR: 6.9; 95%CI: 1.4 – 35.3; p=0.02). The overall model p-value was 0.13 when Hb and malaria were included and 0.09 when they were not included. This was also true for the ordered assessment of DCCS performance, where children with higher levels of EFA were four times more likely to successfully complete the full overlap post-switch DCCS task

than children with lower levels of EFA (OR: 3.8; 95%CI: 1.05 – 13.9; p=0.04). The overall model p-value was 0.13 when Hb and malaria were included and 0.09 when they were not included. The inclusion of Hb and malaria in the models did not affect the OR, CI or p-values for the EFA levels comparisons.

Discussion

We assessed the association between the FA levels and executive function of Tanzanian children between four and six years of age. Although Tanzanian children are routinely monitored for growth, there is no formal system to monitor cognitive development. Similar to others, we found that saturated fat[103] and MUFA[104] were inversely associated with cognitive function. In support of our hypothesis that children with higher whole blood levels of EFAs and total PUFAs would be more likely to successfully complete the DCCS tasks, we found that children with higher levels of total PUFA exhibited better executive function. DHA, linoleic acid, and total n-6 FAs were PUFAs that were positively associated with executive function. Furthermore, children with high EFA levels (T/T ratio ≤ 0.02) were four times more likely to pass the full overlap post-switch DCCS task than children with low EFA levels. Thus, whole blood FA levels were associated with executive function in four-to-six year old children in this Tanzanian village.

Table 14: Regression[1] results for the two methods of scoring the DCCS and selected FAs

	Regression results for total passes			Regression results for highest test passed		
Fatty Acid	B ± SE	T-value	p-value	B ± SE	T-value	p-value
Palmitic	-0.139 ± 0.055	-2.519	**0.013**	-0.097 ± 0.037	-2.625	**0.010**
ALA	-1.612 ± 0.548	-2.940	**0.004**	-0.978 ± 0.370	-2.641	**0.009**
Nervonic	-0.911 ± 0.325	-2.804	**0.006**	-0.609 ± 0.218	-2.793	**0.006**
Total n-9[2]	-0.072 ± 0.036	-2.015	**0.046**	-0.058 ± 0.024	-2.454	**0.015**
Total Saturated Fat[3]	-0.154 ± 0.060	-2.580	**0.011**	-0.098 ± 0.04	-2.427	**0.017**
Total MUFA[4]	-0.073 ± 0.033	-2.218	**0.028**	-0.059 ± 0.022	-2.711	**0.008**
Oleic	-0.060 ± 0.035	-1.724	**0.087**	-0.050 ± 0.023	-2.159	**0.033**
Linoleic	0.132 ± 0.039	3.361	**0.001**	0.095 ± 0.026	3.643	**<0.001**
Total PUFA[5]	0.088 ± 0.027	3.227	**0.002**	0.065 ± 0.018	3.586	**<0.001**
Total n-6[6]	0.101 ± 0.030	3.329	**0.001**	0.073 ± 0.020	3.643	**<0.001**
DHA	0.272 ± 0.140	1.949	0.054	0.186 ± 0.094	1.985	**0.049**
EPA	-0.991 ± 0.608	-1.632	0.105	-0.356 ± 0.411	-0.866	0.388
omega-3 index	0.162 ± 0.113	1.432	0.155	0.123 ± 0.076	1.629	0.106
Total n-3[7]	0.034 ± 0.107	0.319	0.750	0.045 ± 0.072	0.623	0.535
Mead	-1.034 ± 1.930	-0.536	0.593	-0.663 ± 1.295	-0.512	0.610
AA	0.091 ± 0.067	1.355	0.178	0.062 ± 0.045	1.387	0.168
T/T ratio	-20.622 ± 16.004	-1.289	0.200	-15.470 ± 10.723	-1.443	0.152

[1]Model: Total Passes = fatty acid + malaria status + hemoglobin concentration. All significant associations ($p<0.05$) are in bold

[2]Total n-9 includes oleic, elaidic, eicosanoic, Mead, nervonic.

[3]Total saturated fat includes myristic, palmitic, stearic, arachidic, behenic, lignoceric.

[4]Total MUFA includes Palmitoleic, palmitelaidic and nervonic

[5]Total PUFA included Total n-3 and total n-6

[6]Total n-6 includes linoleic, linoelaidic, gamma-linolenic, eicosadienoic, di-homo-gamma-linolenic, arachidonic, docosatetraenoic, docosapentaenoic n-6.

[7]Total n-3 includes alpha-linolenic, eicosapentaenoic, docosapentaenoic n-3, and docosahexaenoic.

The DCCS tasks are an excellent tool for assessing executive function in young children from a variety of backgrounds and experiences. This task can be customized to fit the cultural expectations of the population being analyzed. In this case, we customized the DCCS tasks to include animals and colors that were likely to be familiar to the children. Additionally, the test was performed in the local language, Swahili. However, the paper-based format of testing was novel to the children because these children typically do not have access to paper prior to attending school at the age of seven. Consistent with previous investigations conducted in the USA[98] and Scotland[97], in our population more than 50% of those $\geq$ 48mos of age were able to successfully complete at least one DCCS task, regardless of their FA status. This suggests that our application of this modified DCCS in this population is valid. Further, a strength of the present investigation was the use of two common scoring approaches for the DCCS. For each scoring method, performance on the DCCS was similarly related to FA levels.

There is currently debate about the role of dietary PUFA in the cognitive performance of young children. It is well-established that the brain and eyes incorporate these FAs during gestation and childhood[105], and that n-6 and n-3 FAs are important for brain growth and development[9]. Despite the sufficient evidence on the role of PUFA in brain structure, there are still inconsistent effects on cognitive abilities reported [1, 106]. Randomized-controlled supplementation trials, where mothers were supplemented during pregnancy, lactation or both, and the child's later cognitive performance was assessed, report varied conclusions[106]. Some studies have shown positive associations between LCPUFA and executive function, specifically in the domains of planning and working memory [15, 107]. A study by Helland et

al[108] found that the children of mothers consuming LCPUFA supplements during pregnancy had higher IQs at four years of age than the children whose mothers had not been supplemented. However, Ghys et al[109], found no association between cognitive performance at four years of age and phospholipid DHA and AA levels at birth. A recent meta-analysis has shown that PUFA supplementation associated positively with cognition only in PUFA deficient participants[110]. It has been suggested that discrepant results from supplementation studies are due to genetic variation in the *FADS* gene cluster[111]. Additional differences among these studies include timing of supplementation, failure to measure blood levels of FAs, a wide-array of cognitive outcome measures and a focus on n-3 FAs rather than a full analysis of all FAs.

The neurons of the prefrontal cortex continue to be myelinated from childhood into adolescence[93]. However, research to date has focused on FA intake of mothers during pregnancy and lactation, the FA intake of babies through breastmilk or formula, or the FA intake of school-age children. Our research addressed the gap between four-to-seven years of age. Additionally, we report associations between FA blood levels in the children themselves and that child's performance on a test of executive function providing for a more direct link between current FA status and cognition than typically seen in pregnancy, lactation, or child supplementation studies. Furthermore, the direct analysis of blood fatty acids avoids the potential confounding effect of genetic differences that may decrease blood levels of LCPUFA due to altered FA desaturase activity[111]. In our study whole blood total PUFA but not total n-3 FA was positively associated with executive function. Furthermore, children with higher levels of the n-6 FAs performed better on the DCCS tasks than did children with low n-6 levels. Our

research indicates that a broader set of FAs should be considered in studies addressing the effects of FA intake on cognitive function.

There are some limitations to our study. Since this study was cross-sectional, all reported associations are correlative rather than causative. This study was conducted in one village in rural Tanzania, and thus the results are not generalizable to children residing in other areas of Tanzania or other areas of the world. The children in this village have additional nutritional deficiencies, and we have corrected for those factors for which we have data. Although socio-economic data was not collected from the parents/caregivers, the population is relatively homogeneous in this regard[68].

Conclusion

In summary, the results of this study suggest that whole blood PUFA levels are positively associated with cognition. Intervention studies should be implemented to determine if PUFA supplementation or increased dietary intake of PUFA-rich local foods could improve cognitive function in Tanzanian children.

CHAPTER 5: CHARACTERIZATION OF FATTY ACID COMPOSITION OF SELECTED SEEDS AND OILS IN TANZANIA

Abstract

Background: While fats and oils can be obtained from Plant and animal cellular membranes, in most developing countries, the main source of dietary fat is of plant origin. Despite the fact that lipids are major sources of energy in the body, with a number of many other roles including both growth and development, they are usually in short supply in these countries [112]. These nutrients can be found in seeds, nuts and oils. However, Tanzanian diets are mainly starchy and cereal-based with the intake of fats below recommended levels. Additionally, dietary intake of fats and oils in many Tanzanian meals is typically low due to the cost associated with meat and refined oil. Therefore it is prudent to identify potential low-cost dietary alternatives that provide similar nutritional benefits. The objective of this study was to identify fatty acid composition of several seeds, nuts, and oils available in Kilosa, Tanzania, which can be incorporated in children's diets based on their nutrient content. **Method:** Four types of seeds and three oils commonly found in the area were analyzed for their fatty acid composition by gas chromatography (GC-MS) using selected ion monitoring for enhanced sensitivity. Concentrations of fatty acids are reported as mg/g food grade oil or amount of freeze-dried seed material, respectively. **Results:** A substantial amount of linoleic acid (essential fatty acid) was found in sunflower oil (251.50mg/g) and pumpkin seeds (125.60mg/g). Further, red palm oil (296.00mg/g) and Korie a commercial vegetable oil (425.50mg/g) had substantial amounts of oleic acid. Higher values of saturated fatty acids were found in red palm oil (321.05mg/g) and

Korie (326.55mg/g). **Conclusion:** These seeds and oils are thus good sources of the n-6 FA especially linoleic acid as well as other fatty acids, which are important for proper growth and development. Thus recommendations on how they can be incorporated both in the diets and as snacks in this population can be made based on their nutrient content.

Introduction

Lipids are required constituents for intracellular processes. They are necessary for many physiological processes in the body. Despite the fact that lipids are major sources of energy in the body, with a number of other roles including both growth and development, they are usually in short supply in African diets [112]. Most of the dietary energy in African countries comes from carbohydrate rich staples, such as maize, sorghum, millets and rice [5]. Typically, the diet in this population comprises of a maize meal served with a side dish such as vegetables and pulses. If the family can afford, during preparation of these diets, small (~1-2 tbsp.) amounts of oils are added, commonly from plants such as red palm, groundnut, coconut, sunflower and sesame oil. The added seeds, nuts, and oils in these foods are the main sources of fatty acids for this population. Since the majority of their food comes from staples and the amount of fat supplied from these cereals is very low (ranging from 1.5-4%) Tanzanians typically consume diets with only about 6% of total calories from fat. This is less than half the 15% of energy from fat recommended by WHO. As a result of the low fat content of their diet, the content of EFA in their diet is also usually low[113] [5].

Fatty acids are grouped into essential and non-essential classes. The essential fatty acids are those, which our bodies cannot manufacture and must be supplied through our diets. The dietary essential fatty acids are alpha linolenic acid (ALA), a n-3 fatty acid, and linoleic acid (LA), a n-6 fatty acid. While fats and oils can be obtained from plant and animal cellular membranes, in most developing countries the main source of dietary fat is of plant origin. Some oils, seeds and nuts contain both ALA and LA. Upon consumption, the ALA are then converted to EPA and DHA whereas LA is converted to AA [1]. ALA and LA and their derivatives are also found in animal sources such as fish and meat, however, these foods are not part of the daily diet of most Tanzanians. Hence, this further increases the risk for EFA deficiency [1, 16, 60]. Majority of the oils available to most of the population in Tanzania include sunflower oil, commercial vegetable oils and red palm oil. Also, some seeds and nuts locally available are sometimes not widely consumed, but may provide a source of EFA. The objective of this paper was to characterize the fatty acid composition of selected seeds, nuts and oils widely available in the study area so that their nutritive content can be used to decide on which types of seeds, nuts and oils should be encouraged for consumption for better nutrition and health.

Methods

Procurement and Preparation of Local Tanzanian Oils Seeds and Nuts

All seeds, nuts, and oils were purchased from a local market in Rudewa-Mbuyuni village. The oils were packaged in umber containers to prevent the fatty acids from being oxidized, and seeds and nuts were finely crushed and freeze dried. All samples were shipped to Michigan

State University laboratory where they were purged with high-purity nitrogen and stored at -20°C until analysis of FA content.

Crude Seed Oil Extraction

All glassware used in this analysis was cleaned as followed: soaked in pH < 1 for 24 h, followed by rinsing 3 times in nanopure water, HPLC-grade methanol (Sigma – Aldrich, St. Louis, MO), HPLC-grade acetone (EMD Chemicals Inc., Darmstadt, DE), then HPLC-grade hexane (EMB Millpore Corp., Billerica, MA). The freeze dried grounded seeds were sampled to obtain a total 400 mg freeze dried seed matter, which was transferred to an individual 16×150 mm Teflon-lined screw-capped glass tube. Next, seed samples were incubated at room temperature with 10mL 2:1 v/v HPLC-grade chloroform (Avantor Performance Materials, Inc., Center Valley, PA) / HPLC-grade methanol containing 100μg BHT/mL (Sigma – Aldrich). Samples were placed in a sample rack, and mixed on a low setting for 2 h on a titer plate shaker (Lab-Line Instruments) under dim lighting. Seeds were gravity filtered using lipid free filters (FGE Healthcare UK Limited, Buckinghamshire, UK) into new 16x100mm Teflon-lined screw-capped glass tubes, which contained 2.5mL of 0.88%v/v aqueous KCL (J.T. Baker, Phillipsburg, NJ). This solution was vortexed for 30s, followed by centrifugation at 3000 x g at 10°C for 10 min. After centrifugation, lower organic phase was transferred to clean glass tube, and the aqueous upper phase was washed with 3mL chloroform (Avantor Performance Materials) then centrifuged at 3000x g at 10°C for 10 min. The lower chloroform organic phase was removed and combined with the 2:1 organic phase, then the combined organic phases evaporated at RT under high purity nitrogen. After drying, the total crude seed oil was weighted and calculated.

Methylation of Oils to FAMEs and Neutralization and FAME Isolation

80mg of crude seed oils and food-grade oils were weighed into individual clean 16x100mm glass tubes. Both crude seed oil and food-grade oil samples were re-suspended in chloroform/methanol (2:1 v/v, 100μg BHT/mL) to obtain a final total lipid concentration of 20mg/mL. Resuspended oils were prepared for methylation as previously described by Cequier-Sanchez et al[114]. In brief, an alliqout of 100 μL total lipid extract solution was transferred to a clean 16×100 mm Teflon-lined screw-capped glass tubes. The internal standard nonadecanoic acid (150μg, < 98% purity, Sigma – Aldrich) in HPLC-chloroform was prepared and added to each sample. The samples were then dried under high purity nitrogen at RT. Methylation was performed as previously described by Argen et al[115], but modified as previously described by Pickens et al [116]. FAMEs were neutralized and isolated as described by [117].

FAME Identification, Analysis, and Data Processing

Resuspended FAMEs were transferred to GC vials with glass inserts for analysis. The injection order of resuspended FAME samples was randomized prior to analysis. FAME analysis was performed on a DSQII quadrupole GC/MS (Thermo Scientific) equipped with a DB-23, 30-m column (0.25-mm id, Agilent Technologies, Santa Clara, CA) using helium as a carrier gas. GC temperature profile is as follows: Initial, hold 40°C 1 min; Ramp 1, 100°C/min to 160°C; Ramp 2, 2.8°C/min to 192°C; Ramp 3, 0.5°C/min to 201°C; Ramp 4, 50°C/min to 150°c and hold for 1 min. Selective ion monitoring (SIM) was employed for enhanced sensitivity. Standard FAME mixture (Supelco, Bellefonte, PA) was used to identify and quantify individual FAMEs. Some resuspended FAME samples contained FAME concentrations above the highest standard curve,

so these samples were diluted 1:100 and reanalyzed on the same standard curve as undiluted samples. FAME peak integration and quantification was performed using TargetLynx V4.1 (Waters, Milford, MA) based on the FAME standard's retention time and SIM ions. Limit of quantification was defined as a signal to noise < 10 and the limit of detection (LOD) was defined as signal to noise < 3. FAME concentrations below the LOD and above the LOQ, were assumed to as half the lowest value detectable value for each FAME.

Results

The fatty acid composition of the lipid in the seeds and nuts are presented as mg FA/g seed and for the oils as mg FA/g oil (Tables 15-18). All samples contained saturated fatty acids, Korie and red palm oil had higher amounts of saturated fatty acids than the seeds. Palmitic acid (16:0) was the most abundant saturated FA in the vegetable oil (KRO) and the red palm oil (RPO)(Table 15). The commercial vegetable oil (Korie) had the highest amounts of oleic acid, about a third more oleic acid than in red palm oil (Table 16).

Table 15: Saturated Fats composition of the samples[1]

Sample ID	Sample type	Myristic C14:0	Palmitic C16:0	Stearic C18:0	Arachidic C20:0	Lignoceric C24:0	Total Saturated
Coconut (CCN)	seed	21.48	6.97	1.96	0.06	0.01	30.48
Pumpkin Seeds with shells (PKSY)	seed	0.34	8.7	3.28	0.09	0.02	12.43
Pumpkin Seeds no shells (PKSN)	seed	0.23	22.93	17.15	0.64	0.3	41.25
Oysternut (OYN)	seed	0.05	69.24	17.96	0.04	0.02	87.31
Sunflower Oil (SFO)	oil	1.5	14.5	5.97	4.25	0.45	26.67
Korie Oil (KRO)	oil	3.8	301.75	19.72	0.25	0.03	325.55
Red Palm Oil (RPO)	oil	5.37	290.25	23.27	2.13	0.03	321.05

[1]mg FA/g crude oil or mg FA/g seed

Table 16: Monounsaturated Fatty acid composition of the samples[1]

Sample ID	Sample type	Oleic C18:1	Eicosenoic C20:1	Nervonic C24:1	Total MUFA
Coconut (CCN)	seed	5.11	0.02	0.00	5.13
Pumpkin Seeds with shells (PKSY)	seed	14.54	0.07	0.00	14.61
Pumpkin Seeds no shells (PKSN)	seed	35.53	1.21	0.6	37.34
Oysternut (OYN)	seed	21.76	1.98	0.83	24.57
Sunflower Oil (SFO)	oil	93.75	0.50	0.00	94.25
Korie Oil (KRO)	oil	425.5	0.375	0.00	425.88
Red Palm Oil (RPO)	oil	296.00	0.375	0.00	296.37

[1]mg FA/g crude oil or mg FA/g seed

These samples contained very small amounts of n-3 FA, the fatty acids included, alpha linolenic (ALA, 18:3n-3), eicosapentanoic (EPA, 20:5n-3) and docosahexanoic (DHA, 22:6n-3) as shown in Table 3: Sunflower oil and pumpkin seeds with shells (PKSY) had a better total n-3 amounts compared to all other samples (Table 17). Results of the values of the n-6 FA are shown in Table 18. Linoleic acid was the most abundant n-6 fatty acid in these samples, higher amounts were found in sunflower oil (SFO) and pumpkin seeds without shells (PKSN). On the other hand coconut had very low levels of LA, but contained some small amounts of AA, which were not present in other samples.

Table 17: Total n-3 Fatty acid composition of the samples[1]

Sample ID	Sample type	ALA C18:3	EPA C20:5	DHA C22:6	Total n-3
Coconut (CCN)	seed	0.00	0.09	0.07	0.16
Pumpkin Seeds with shells (PKSY)	seed	0.29	0.00	0.04	0.33
Pumpkin Seeds no shells (PKSN)	seed	0.05	0.00	0.68	0.73
Oysternut (OYN)	seed	0.00	0.12	0.09	0.21
Sunflower Oil (SFO)	oil	0.57	0.00	0.22	0.79
Korie Oil (KRO)	oil	0.10	0.00	0.00	0.10
Red Palm Oil (RPO)	oil	1.37	0.00	0.00	0.00

[1]mg FA/g crude oil or mg FA/g seed ALA, Alpha linolenic acid; EPA, Eicosapentanoic acid; DHA, Docosahexanoic acid

Table 18: Total n-6 Fatty acid composition of the samples[1]

Sample ID	Sample type	LA 18:2	GLA C18:3	Eicosa-dienoic C20:2	Docosa-dienoic C22:2	AA C20:4	Total n-6
Coconut (CCN)	seed	1.21	0.82	0.00	0.05	0.06	2.14
Pumpkin Seeds with shells (PKSY)	seed	41.27	0.00	0.74	0.75	0.00	42.76
Pumpkin Seeds no shells (PKSN)	seed	125.60	0.75	0.49	0.00	0.00	126.84
Oysternut (OYN)	seed	106.31	1.13	0.68	0.78	0.00	108.9
Sunflower Oil (SFO)	oil	251.50	2.88	3.50	0.19	0.00	258.07
Korie Oil (KRO)	oil	101.75	11.75	1.63	1.88	0.00	117.01
Red Palm Oil (RPO)	oil	87.25	0.00	1.63	1.80	0.00	90.68

[1]mg FA/g crude oil or mg FA/g seed. LA, linoleic acid; GLA, γ-linoleic acid

Discussion

The results obtained in this study provide information on the fatty acid contents of some locally available food products in Tanzania. A Tanzania food composition table provides the estimate nutrient contents of nutrients such as protein, carbohydrates, total fat and other vitamins and minerals, but no information of fatty acid content of the foods is shown. Korie is a commercially produced oil, widely used by many Tanzanians, however the fatty acid content of these is not listed anywhere in the label or in the food composition table. Both sunflower and red palm oil samples analyzed in this study were locally processed and thus not as highly refined as Korie oil. Sunflower oil and pumpkin seeds had the highest concentration of linoleic acid in this study. Linoleic acid is important for both growth and development as shown by many other studies as well as in chapter 3 and 4 of this document. Sunflower oil is relatively expensive and although they are cultivated in the area, they are mainly used as cash crops and few families in rural villages save some for household consumption. Pumpkins are found year round and are usually

grown in the backyard of households. Both the pumpkin and pumpkin leaves are consumed daily, but the seeds are mostly saved for seeds and in some cases they are added in sauces. The results here have shown that despite many other nutrients present in the seeds, they contained sufficient amounts of linoleic acid as also shown by other studies [118-120]. Most of the people who consume the pumpkin seeds usually consume them with their outer shell because of convenience. From these results we have seen that pumpkin seeds with shells had lower concentrations of most of the fatty acids compared to those without shells, likely because the shells are high in fiber and low in fat.

All the samples contained only small amounts of n-3 FA, ALA was present in both the pumpkin seeds with shells and without shells. Sunflower oil contained levels of ALA similar or even higher than those observed by others[121] [113]. Sunflower is known to contain very little ALA compared to flaxseed, canola oil and soybean oil, which are rich in ALA. Very small amounts of DHA were found in sunflower oil, pumpkin seeds and the oyster nut. Preformed DHA is mainly found in fish and meat products. Thus in populations where fish and meat is not regularly available, dietary essential fatty acids need to be consumed in sufficient amounts[122]. Further to these observations, from these samples we observed that the ratio of n-6 to n-3 fatty acid is very high, indicating that a better source of n-3 fatty acid is needed to ensure sufficient n-3 intake.

Table 15 shows that the majority of these oils contained a lot of saturated fatty acids particularly palmitic acid. Korie and Red palm oil had the highest amounts of saturated fatty acids as compared to sunflower oil. Generally, oils contained high amounts of oleic acid as the major unsaturated fatty acid in their composition. Our results are similar to those reports by Rui

C et al [121]. Red palm oil and Korie are affordable and readily available to the villagers in Rudewa, and are therefore likely to be included in their daily diets more than the more expensive sunflower oil.

The oil samples are generally higher in saturated fatty acid and linoleic acid, in view of the relationship between growth and cognitive development with the FAs, we have shown that those children with higher levels of saturated FA and MUFA had poor growth and executive functions while those children with higher levels of n-6 FA had good growth and exhibited good executive function (see Chapter 3 and 4). Thus a recommendation for consumption of an oil/seed, which is higher in n-6 FA and lower in saturated FA. From this study, pumpkin seeds without shells and sunflower oil are the better sources of the n-6 FA and also have relatively lower amounts of saturated FA and therefore important for growth and cognition.

A limitation of this study was that the fatty acid content analysis was conducted in raw foods and thus the effect of cooking on these fatty acids has not been considered. It is known that FA content is affected during cooking and therefore the amount that is present in the raw food may not be necessarily the amount available in the cooked food. However some of these seeds, such as the pumpkin seeds and oyster nut are sometimes consumed raw.

Conclusion

Fatty acid content of different foods is generally lacking in Tanzania and in most developing countries. Information on fat content of the food, which is provided in food tables, is on total fat and no specific information about fatty acid is given. Sunflower oil and Pumpkin seeds

contain sufficient levels of the n-6 FAs particularly linoleic acid. Both sunflower oil and pumpkin seeds are available in most of the areas, but pumpkin seeds are more affordable and accessible. There is a need for further studies to assess the fatty acid content of composite meals so as to be able to assess total fatty acid content of a meal from different sources.

CHAPTER 6- SUMMARY

Main findings

Evidence from experimental and epidemiological studies has shown that essential fatty acids are important for growth and cognitive development of children. The deficiency of these nutrients results in impaired growth and delayed cognitive abilities together with delayed brain development. The main goal of this research was to assess associations between fatty acid status and growth and cognitive abilities of children in Tanzania. A review of literature [123] has shown that FA and specifically long-chain PUFA are important for growth and cognitive development. Such studies have been conducted mainly in developed countries and have focused on dietary/supplement intake by pregnant women, lactating women and/or infants. This area is less studied in developing countries, despite the high prevalence of deficiencies leading to delays in both growth and cognitive development. The objective of this thesis was to assess whole blood FA content and establish an association between growth and cognition with FA status. This research was also designed to determine the prevalence of essential fatty acid deficiency (EFAD), to identify local sources of fatty acids, and to determine the FA content of the locally available food sources. This research established the EFAD prevalence at 23% in the children of Rudewa-Mbuyuni, Kilosa, Morogoro. Tanzania. Furthermore, this research demonstrated a positive association between the n-6 FAs and growth of Tanzanian children. Specifically, LA is highly inversely associated with stunting. Children who had insufficient levels of n-6 fatty acids, as measured by the T/T ratio, were more stunted than those with sufficient levels of n-6 fatty acids.

This research also assessed the executive function of children.Utilization of the DCCS task to specifically assess the association between FA status and executive function enabled the identification of the importance of the n-6 FAs in the cognitive development of children. A weak but positive association between the n-3 FA, DHA, with executive function also emerged.

When sources of household dietary fat were assessed, red palm oil and Korie oil were identified as the most commonly used. A small, but measureable, number of households utilized pumpkin seed, sunflower oil, oyster nut and coconut. This research further demonstrates that some of the year-round available seeds, such as pumpkin seeds, are sources of LA. Other locally available oils and nuts are mainly rich in saturated fats as well as n-6 FA. Although sunflower oil also is a locally available source of LA, it is not always affordable to this population. Thus, on the basis of this study, the consumption of pumpkin seeds appears to be an effective way to promote LA intake, which would furthermore support growth and cognitive development of the children in this village.

In conclusion, although previously underappreciated, FAs are pivotal to growth and cognitive development in Tanzanian children. This research further shows that local foods contain these FAs. However, the challenge that remains is finding ways to encourage families to incorporate these foods into the daily diets of their children.

Innovation

This key class of essential nutrients has largely been understudied in developing countries due to technical (FA oxidation) and logistical (storage/shipment/analysis) issues associated with measuring whole blood fatty acids. This study is innovative because we used a unique system to obtain blood samples for FA analysis[25]. This method assures sample integrity at room

temperature for up to two weeks during storage and shipment. These samples were shipped to US for detailed FA analysis, which would be extremely difficult or impossible in Tanzania. Assessing FA levels through testing blood levels is a more validated way of establishing the chronic and recent FA intake rather than relying on other measurements of food intake such as 24-hour dietary recall. Assessing the blood FA profile of children provides foundational knowledge for future intervention studies using locally available and culturally accepted foods to address essential FA deficiency in Tanzania. The study is also innovative because it links the EFA status and cognitive abilities of the children and informs health and nutrition care practitioners on the importance of EFA on growth and cognition.

Limitations

Despite the positive findings of this study, our study does have several limitations. This was a cross sectional study, thus all reported associations are correlative and not causative. The study was conducted in one village in Tanzania so the findings cannot be generalized to the entire Tanzanian population. Also, during this study, blood samples were collected throughout the day and no fasting was required. This could increase variability in the whole blood FA measurements; however, in this setting the differences are likely to be small considering that children from the village consume relatively similar and low-fat meals compared to children in other settings. It has been shown previously that the variation in FA content in the fed and fasting states, particularly following a high fat diet is less than five percent [25]. In this study we also did not assess adequacy of other nutrients, such as zinc or protein, which are potentially inadequate in the diets of the children. Deficiencies of some nutrients may interfere with FA

metabolism and thus affect whole blood FA levels. Furthermore, deficiencies of some nutrients can also contribute to poor growth and poor cognitive abilities.

Also, the fatty acid content analysis of the fatty acid sources used in this population was evaluated in raw foods and thus the effect of cooking on these fatty acids has not been considered. It is known that FA content is affected during cooking and therefore the amount that is present in the raw food may not be necessarily represent the amount available in the cooked food. However some of these seeds, such as the pumpkin seeds and oyster nut are sometimes consumed raw. We also did not account for potential effects of short-term or long-term effects of storage on the FA content of these foods. This could be problematic because fatty acids may be oxidized during storage if storage conditions are not optimal resulting in alteration of the FA composition.

Another limitation of our study is that we assessed cognitive abilities of these children throughout the entire day. The performance of these children might have been influenced by the time of the day the test was administered.

Despite these limitations, through the application of anti-oxidant treated blood spot cards, usage of a test of executive function to assess cognitive development, and determination of local foods as sources of essential fatty acids, this study is a first step towards addressing malnutrition in rural, Tanzanian villages.

Future Directions

Despite the limitations of our studies, the findings of this research contribute to the body of literature supporting the importance of fatty acids in growth and development. The research provides information about the fatty acid status of the children in developing countries, particularly Tanzania. It also is the first to assess executive function as a measure of the cognitive abilities of the malnourished and well-nourished children of rural Tanzania. In regions of the world where diets are low in fats and oils, the baseline information established by this research can be utilized to develop intervention studies to determine the ability of increased intake of n-6 FAs to improve both growth and executive function in young children. In the study population, the whole blood LA levels of children achieving the appropriate height-for-age were 18.7%, whereas those of children moderately or severely stunted were 17.4%, may indicate that dietary intakes of LA that can support blood levels of LA at 18.7% of total fat may be optimal for supporting growth.

Unfortunately, little work has been done to connect dietary intake of fatty acids to blood levels of specific fatty acids in children between the ages of 2 and 6 years. This is area of research that should be further explored Future studies may consider FA supplementation programs while conducting repeated measures of whole blood lipid levels and anthropometrics over time. Studies such as these would be able to establish the link between dietary FA intake and changes in growth or cognitive functions as they relate to whole blood FA status. Also such studies can be planned and implemented at different stages of life so that we could establish the right

window of opportunity for higher efficacy of these foods in improving growth and cognition of the children.

In addition, using the baseline measures of the FA content of locally-available seeds, nuts, and oils to provide direction on how these food components, rather than FA supplements, can be utilized to ensure that children are receiving sufficient amounts of fatty acids, especially EFAs. This research supports the addition of specific FA content to the national food tables in use in Tanzania. Once these numbers are widely accessible for a number of locally available foods, these foods can be used in on-going and future strategies formulating high-energy foods to eliminate malnutrition, thereby improving growth and cognitive development. Some of these local foods are good sources of linoleic acid but of such high economic value that households are unlikely to add them to the diet. For instance, sunflower oil had the highest levels of linoleic acid in this study. It is important to conduct a cost benefit analysis on the use of this oil so that reasonable recommendations can be made.

Lastly, further FA analysis of typical composite diets as well as green leafy vegetables, such as sweet potato leaves or pumpkin leaves (which may be good sources of n-3 FA), normally consumed in these population can be conducted to establish which particular diets are rich in FAs. These studies should carefully consider the effect of cooking on fatty acids particularly PUFA.

APPENDICES

Appendix 1: Methodology

1. Research Design, Sampling and Ethical Approval

1.1. Study Design and Site

A cross sectional study was conducted in Mbuyuni village, Kilosa, Morogoro.Tanzania. The study area was chosen because of my own previous experience working in the village as part of an ongoing research project (with an approved IRB) with the collaborator Dr. Joyce Kinabo, which aims at improving health and nutrition status of the villagers. In the approved IRB, the participants could be involved in the current study. Children from all the five hamlets in this village participated in the study.

1.2. Study Population

Households with children between 2-6 years old were identified through village register supplemented with information from the respective hamlet leaders from each hamlet. This population was chosen, because in this population, almost 95% of the children are breast fed for two years. Since breast milk is one of the rich sources of EFA, the EFA levels in children less than two years is likely to be enough and uniform. The status of EFA in the children after cessation of breastfeeding is likely to differ due to introduction of and complete shift to complementary foods which are usually high in grains and legumes and vegetables and low in animal sources and fats and oils. The purpose of the study was communicated to the village leaders as well as the community members. All eligible children within this age group residing in the village were invited to participate in the study. All healthy children were included in the

study. However, we excluded children who were sick and hospitalized at the time of the data collection as well as those who are legally declared mentally impaired.

1.3. Sample size calculation

Sample size for this study was calculated based on the study design and the estimated prevalence of essential fatty acid deficiency. Although the level of fatty acid deficiency in Tanzania is not known, we do know the range of fatty acid variability in another African country[16]. We utilized that variation to run an A-priori sample size calculation for multiple regression

The calculations are as shown below:

Anticipated effect size (f2): .05

Desired statistical power level: .80

Number of predictors: 4

Probability level: p=0.05

Minimum required sample size=242

1.4. Ethical consideration and approval

An existing IRB for the Tanzanian study led by Dr. Joyce Kinabo from the Sokoine University of Agriculture was used to apply for an IRB approval for this study at Michigan State University. The IRB approval from MSU was obtained (IRB# 13-700). Because the majority of this population is illiterate, a verbal consent script (Appendix 2) was prepared for the participants in

both English and Swahili. The script was read to these participants and mothers/care givers gave their verbal assent.

2. Blood Measurements

2.1. Dried blood spot collection

A capillary blood sample was taken from the middle finger. A light pressure was applied again and about 30 microliters of blood was dropped onto the OxyStop™[25] treated dried blood spot cards (DBS), then placed in a cool and dry place away from direct sunlight and allowed to dry. The DBS cards were then stored in a dry and cool environment away from light. The DBS cards were shipped to the US within 14 days. Fatty acid analysis of the DBS was conducted at Omega Quant (Sioux Falls, South Dakota).

2.1.1. Fatty Acid Analysis

Upon arrival at the lab in US, a punch from the DBS card was combined with the derivatizing reagent [boron trifluoride in methanol (14%), toluene, and methanol (35:30:35 parts)], shaken and heated at 100°C for 45 minutes. After cooling, 40 parts of both hexane and distilled water were added. After briefly vortexing, the samples were spun to separate layers and an aliquot of the hexane layer that contained the FA methyl esters was extracted. FA analysis was performed as previously described [69]. Fatty acids were identified by comparison with a standard mixture of fatty acids. The fatty acid composition was expressed as percentage of total fatty acids. Table 19 shows the fatty acids, which were measured. In addition to these, Total SFA, MUFA, PUFA, n3 FA, n6 FA and the Triene/Tetrane ratio were also calculated.

Table 19: Fatty acids for Analysis

Fatty Acid Name	Chemical formula	Fatty Acid Name	Chemical formula
Myristic	C14:0	Alpha-Linolenic	C18:3
Palmitic	C16:0	Eicosapentaenoic	C20:5
Stearic	C18:0	Docosapentaenoic	C20:5
Arachidic	C20:0	Docosahexaenoic	C22:6
Behenic	C21:0	Linoleic	C18:2
Lignoceric	C24:0	Gamma-Linoleic	C18:3
Palmitoleic	C16:1	Eicosadienoic	C20:2
Oleic	C18:1	DGLA	C20:3
Eicosenoic	C20:1	Arachidonic	C20:4
Nervonic	C24:1	Docosatetraenoic	C22:4
Trans-Palmitoleic	C16:1	Docosapentaenoic	C22:5
Oieic	C18:1	Mead Acid	C20:3
Trans-Linoleic	C18:2		

2.2. Hemoglobin concentration

An additional drop of blood was taken from the first blood prick. Light pressure was applied until there was enough blood to fill the cuvette. The cuvette was then put in the Hemocue (The HemoCue® Hb 201+) and the reading taken after 45 seconds. The Hemocue uses the method as described by Vanzetti[124] based on the principle that, sodium-desoxycholate hemolyses the erythrocytes and hemoglobin is released. Sodium nitrite converts hemoglobin to methemoglobin, which together with sodium azide gives azide-methemoglobin. The absorbance is measured at two wavelengths (570 and 880 nm) in order to compensate for any type of turbidity. Hemoglobin was expressed as g/dl. Based on these results; subjects were categorized into different categories according to their hemoglobin concentration according to WHO classification criteria[125] (Table 20).

Table 20: Hemoglobin cut points as per WHO criteria:

		Anemia		
Population	Non Anemia*	Mild[a]	Moderate	Severe
6-59 months of age	110 or higher	100-109	70-99	< 70
5-11 years of age	115 or higher	110-114	80-109	< 80

*Hemoglobin in gram per litre

[a]Mild is a misnomer: Iron deficiency is already advanced by the time anemia is detected.

2.3. Malaria determination

Using the same finger prick, blood for malaria assessment was drawn and malaria was assessed by two methods. Blood slides with thin blood smears were prepared and stained for malaria parasites. After drying, the thin blood smears were stained with GIEMSA and examined under a microscope (Olympus model CX21FS1) for parasite enumeration per 200 leucocytes. A blood film was declared negative after counting of 200 high power fields. Malaria parasite species confirmation was made on the thin film. The results were reported in terms of either positive or negative. However, the number of trophozites counts for the positive results were also recorded. In addition, a rapid test kit (RDT)(Premier Medical Co. Ltd, India) for malaria was also used to assess the presence of malaria parasites. The two methods were used because 1) blood slide ("gold standard") is the commonly used method and has an ability to show the number of parasites present in the individual, but is subject to instrument as well as individual errors and 2) RDT is a newly introduced method used in most of the hospitals and clinics in Tanzania. However, there have been reports of a number of flaws in this new technology [126]. This way the two methods validated or confirmed the results.

3. Anthropometric Measurements

3.1. Height Measurement

A stadiometer was placed firmly against a wall. When measuring height, the child was asked to stand straight with the head positioned such that the Frankfurt plane is horizontal, feet together, knees straight and heels, buttocks and shoulder blades in contact with the vertical surface of the wall. Hands were hanging loosely with palms facing the thighs. The movable headboard was then lowered until it touched the crown of the head; height was read to the nearest 0.1cm. Two measurements were taken for each child and recorded after each reading. The average height was computed during data processing and used as the actual height of the child.

3.2. Weight Measurement

A Digital bathroom scale (A SECA, Vogel & Haike, Hamburg, Germany) was used to weigh the children with minimum clothing and without shoes. Before taking the measurement, the scales were adjusted to zero and the child was asked to stand on the scale facing the observer as shown in the scale. For children who did not agree to stand on the scale the mother/caregiver was asked to stand on the scale, then the mother/caregiver's weight was tarred and then he/she would carry the child and the weight of the child was taken. Weight was recorded to the nearest 0.1 kg.

3.3. Calculations of the z-score

From the measurements of height and weight, z-scores were calculated using WHO Anthro and WHO Anthro Plus[127]. Z-score or standard deviation [37] unit was defined as the difference between the value for an individual and the median value of the reference population for the same age or height, divided by the standard deviation of the reference population. Values higher than 1 SD indicates normal nutrition status, less than minus 2 SD means moderate undernutrition and less than minus 3 SD indicates severe undernutrition. A z-score more than 3SD indicates overnutrition.

$$\text{Z-score} = \frac{\text{(observed value) - (median reference value)}}{\text{SD of reference population}}$$

The data were categorized and summarized and utilized in further analysis.

4. Dimensional change card sort (DCCS)

A dimensional change card sort (DCCS) technique[92] was employed to assess cognitive abilities of the enrolled children. This method assesses executive function, one measure of the children's cognitive abilities. Further, when the two phases are used (i.e. the pre and post switch phase), the method is hypothesized to depend on the function of specific regions within the prefrontal cortex. The test was administered in Swahili, which is a local language in this community. During administration of the test, mothers or caregivers were present to observe the whole process to allow the child to feel comfortable and confident to perform the test. The test was also modified to use images of animals and colors, which are more likely to be familiar to these children. The pictures of elephants and zebras were used as well as the colors "green"

and “yellow”. Two target cards were shown to the child and then they were asked to sort a series of bivalent test cards according to one dimension e.g. colors (yellow and green) for several trials and then switch sorting the same cards according to a new set of rules. Thus, the test will be switched, and in this post switch test, the child will be asked to sort the same cards from a different dimension e.g. animals (elephant and zebras). A score of zero was given to any incorrect choice, whereas a score of one, was given to all the correct choices. Generally, the score was made categorical and therefore “passing” and ‘failing” at different stages of the test was used for analyzing the data. The detailed procedure on the test are as follows:

- The test cards (Figure 5) were shown to the child and an explanation (with demonstration) on the task to be completed was given
- The test giver sat beside the child so that the view of the cards is the same
- The cards were sorted first by “colors” in the pre switch phase and then by animals in the post test phase
- While showing the cards the test giver explained to the child that they should sort the cards by color first. Using the following dialogue, “We will begin with the color game. In the color game all the yellow cards will be matched (while pointing to the card) with the yellow card I am holding, and all the green cards will be matched (while pointing to the card) with the green color in the card I am holding”. Repeat while pointing, so “if it’s yellow, you point here (while pointing to the card) if it’s green, you point there (while pointing to the card)”. Allow a few minutes and let the child practice and then begin the task. Begin the Pre switch phase, say, “Now, I want you to show me the color in my card, which matches the color in your card”.

- Repeat this for all the eight cards and then switch to the post switch phase, remember to explain the post switch rules; say “now we are going to sort the cards by animals, not by colors anymore” so if it’s an elephant you point here (while pointing to the card) if it’s a zebra you point there (while pointing to the card)”. Allow a few minutes and let the child practise and then begin the task. Let the child do the task for all the eight cards.
- If the child passes the first set of the test proceed to the next test while offering the same instructions.

5. **Other Information collected**

Information on child breastfeeding status, sickness, the different types of complementary foods used and information on dietary sources of lipids (oils, seeds and nuts used during meal preparation) was collected (Appendix 3).

Appendix 2: Script to the Consent Form

Title: Essential Fatty Acids and Growth Stunting In Tanzania

My name is Theresia Jumbe from Sokoine University of Agriculture [22] currently I am a student at Michigan State University (MSU), I cordially invite you to participate in this study. The research is about children's health and nutrition status as it relates to their growth and cognitive abilities. From this study, the researchers hope to establish the status of essential fatty acid in children below five years of age and the role of essential fatty acids in growth, at this a critical age of life. In this research we will take a few measurements, these will include, height, weight, and also we will take a blood sample from your child and measure hemoglobin concentration, fatty acid status as well as presence of malaria parasite. If you agree to participate in this study, your participation in this study will take about 45 minutes to 1 hour of your and your child's time. Before participating in this study it is important you understand the following:

Potential Risks

When taking blood, minor pain might occur, however, every necessary caution will be taken to minimize the pain.

Benefits

There will be no direct benefits to you. The study will contribute to understanding on how essential fatty acids have a role in growth and the high rates of malnutrition specifically stunting in our country. This information will therefore be used to emphasize on importance of these nutrients at an early age.

Privacy and Confidentiality

The information about you will be kept confidential to the maximum extent allowable by law. During data collection, your information will be linked to your name, however once the data is collected it will be coded. Any information with your identity will be kept confidential, and will be treated with high level of privacy. Dr. Joyce Kinabo and Theresia Jumbe will have access to the original information sheet, however they will keep these information for a few years until the closure of the study. The other research members will use coded data to study the relationship between essential fatty acids and growth and cognition. Results from this study may be published or used in professional meetings. The identity of the participants will not be revealed. National institute for medical research (NIMR) review board in Tanzania as well as IRB board in Michigan State University may request to see the data, in this event, your identity will still be kept confidential

Questions and Concerns

If you have concerns or questions about this study, such as scientific issues, how to do any part of it, or to report an injury, please contact the researcher Dr. Jenifer Fenton 208B G.M. Trout Bldg, Michigan State University. (517) 355-8474 ext. 130. Since this study is undertaken in Tanzania then it may be easy for you to contact Dr. Joyce Kinabo, Sokoine University of Agriculture, P.O.Box 3006. Morogoro Tanzania. Tel +255 23 260 3511 - 14 Ext. 4421. You may also contact the graduate student on the project Theresia Jumbe, Sokoine University of Agriculture. P.O. Box 3006. Morogoro Tanzania. +255 754 804010.

Costs and Compensation

You will bear no cost by choosing to participate in this study. You will not pay for any measurement, even for the blood draws and their subsequent analyses. However, a small token will be given to you as an appreciation and compensation for your time and voluntary participation. During measurement taking children will be given sweets as a way to reward them after a finger prick.

I (Print the caregiver/mother's name)……..…have been invited to participate in this research

1. I declare that I have read/ have heard and understood the research objectives
2. Have asked all questions related to the research and I am satisfied with the answers
3. I understand that any information about my household and family members will be treated and kept with required confidentiality
4. I understand that I am participating in this research voluntarily and that I can decide to answer or not answer some of the research questions, and that at any given time I can decide not to continue participating in this research
5. I am ready to continue participating in further research and that if I am required to do so I will receive enough information, and any of my questions will be answered before I choose to participate
6. I allow my child/children to participate in this research, and that blood samples will be collected for various health tests

The signature below means that I voluntarily agree to participate in this research study. As the parent of a child or children under the age of 18, I am consenting for my child/children to participate in this study.

_____	_____
Signature and/or thumbprint of Parent	Date
_____	_____
Witness	Date

Appendix 3: Participant Information Collection Sheet

A **Participants characteristics**

Child's code number
Survey Date (dd/mm/yyyy) ...../...../.....
Village
Hamlet
Name of Child
Name of mother/guardian
Date of Birth (dd/mm/yyyy)
Sex (0=Male, 1 Female)

B **Anthropometric measurements**

Height of child in cm
Weight of child in kg
Head circumference in cm

C **Biochemical measurements**

Hemoglobin (g/dl)
Malaria test (RDT-1= Positive /2= negative)
Dried Blood spots taken (1=Yes, 2=No)

D **Other Information (To be answered by mother/caregiver)**

Did child ever breast feed? 1=Yes, 2=No
Is child still breastfeeding? 1=Yes, 2=No
When did child stop breastfeeding (Age in months)
Number of siblings
Has child been sick in the past two weeks (0=no 1=yes)
Common type of complementary food child is eating
Dietary sources of fats (1=Pumpkin seeds, 2= Oyster nut, 3=groundnuts, 4=coconut,5= sunflower, 6=Korie, 7=redpalm, coconut=8)

Appendix 4: IRB Approval Letter

MICHIGAN STATE UNIVERSITY

Initial IRB Application Approval

To: Jenifer Fenton
208B G.M. Trout Bldg

Re: **IRB#** 13-700 Category: EXPEDITED 2-2
Approval Date: September 11, 2013
Expiration Date: September 10, 2014

Title: Essential fatty acids and growth stunting in Tanzania -RC 100739

The Institutional Review Board has completed their review of your project. I am pleased to advise you that **your project has been approved**.

The committee has found that your research project is appropriate in design, protects the rights and welfare of human subjects, and meets the requirements of MSU's Federal Wide Assurance and the Federal Guidelines (45 CFR 46 and 21 CFR Part 50). The protection of human subjects in research is a partnership between the IRB and the investigators. We look forward to working with you as we both fulfill our responsibilities.

Renewals: IRB approval is valid until the expiration date listed above. If you are continuing your project, you must submit an ***Application for Renewal*** application at least one month before expiration. If the project is completed, please submit an ***Application for Permanent Closure***.

Revisions: The IRB must review any changes in the project, prior to initiation of the change. Please submit an ***Application for Revision*** to have your changes reviewed. If changes are made at the time of renewal, please include an ***Application for Revision*** with the renewal application.

Problems: If issues should arise during the conduct of the research, such as unanticipated problems, adverse events, or any problem that may increase the risk to the human subjects, notify the IRB office promptly. Forms are available to report these issues.

Please use the IRB number listed above on any forms submitted which relate to this project, or on any correspondence with the IRB office.

Good luck in your research. If we can be of further assistance, please contact us at 517-355-2180 or via email at IRB@msu.edu. Thank you for your cooperation.

Sincerely,

H. McGee

Harry McGee, MPH
SIRB Chair

c: THERESIA JUMBE, Sarah Comstock, Matthew Pontifex

Office of Regulatory Affairs
Human Research Protection Programs

Biomedical & Health Institutional Review Board (BIRB)

Community Research Institutional Review Board (CRIRB)

Social Science Behavioral/Education Institutional Review Board (SIRB)

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