DETERMINATION OF OMEGA-3 LONG CHAIN POLYUNSATURATED FATTY ACID LEVELS USING DRIED BLOOD SPOTS IN ZIMBABWEAN CHILDREN AGED 7 TO 9 YEARS

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Abstract

Background: Blood omega-3 long chain-polyunsaturated fatty acids (LC-PUFAs) have been widely studied in children because of the benefits from optimum physical and mental development. Despite these recognized benefits, the levels of blood omega-3 LC-PUFAs are unknown in Zimbabwean children. Omega-3 LC-PUFA levels were determined and reference intervals were established for the 7-9 year old Zimbabwean children. The association between omega LC-PUFAs and cognitive outcomes was also determined.

Methods: A cross sectional study was conducted from September 2011 to August 2012 on a cohort of peri-urban Zimbabwean children aged 7-9 years born to mothers enrolled at late pregnancy into an HIV prevention program between 2002 and 2004. Whole dried blood spots were sampled and LC-PUFAs were quantified using gas liquid chromatography. Differences in LC-PUFAs between groups were compared using the Kruskal Wallis test. Spearman correlation coefficient was used for the relationship between LC-PUFA levels and cognitive development.

Results: LC-PUFAs levels were determined in 297 Zimbabwean children of whom 170 (52%) were girls. The LC-PUFAs (wt/wt) ranges were; EPA 0.06–0.55%, DPA 0.38–1.98%, DHA 1.13–3.52%, ARA 5.58–14.64% and ARA: EPA ratio 15.47–1633.33. There were no gender differences in omega-3 LC-PUFAs levels (all $p>0.05$). EPA was statistically significantly elevated in the 8 years age group compared to those aged 7 and 9 years (0.20 vs 0.17 vs 0.18, respectively, $p=0.049$). ARA:EPA ratio was statistically significantly elevated in the 7 years age group compared to those aged 8 and 9 years (64.38 vs 56.43 vs 55.87 respectively, $p=0.014$).

Conclusions: In this cohort of children, lower EPA levels and higher ARA:EPA ratios were observed compared to those reported in apparently healthy children elsewhere. The high ARA:EPA ratios make the children vulnerable to inflammatory pathologies. Identification and incorporation into diet of locally available foodstuffs rich in omega-3 LC-PUFAs is recommended as is as omega-3 supplementation.

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List of Abbreviations and Acronyms

ALA	α-Linolenic Acid (18:3n-3)
ARA	Arachidonic Acid (20:4n-6))
ASD	Autistic Spectrum Disorders
BF_3	Boron tri-Fluoride
BHT	Butylated Hydroxytoluene
CDC	Centre of Disease Control
CVD	Cardiovascular Disease
DBS	Dried Blood Spots
DHA	Docosahexaenoic Acid (22:6n-3)
DGF	Gesellschaft für Fettwissenschaft
DPA	Docosapentaenoic Acid (22:5n-3)
EFAs	Essential Fatty Acids
EPA	Eicosapentaenoic Acid (20:5n-3)
FAME	Fatty Acid Methyl Ester
FADS	Fatty Acid Desaturase
FAO	Food and Agricultural Organization
FID	Flame ionization detector
GCI	General Cognitive Index

GLC	Gas Liquid Chromatography
HPLC	High-Performance Liquid Chromatography
IL	Interleukins
IQ	Intelligence Quotient
JREC	Joint Research Ethics Committee approval
LA	cis-Linoleic Acid (C18:2n-6)
LC-PUFAs	Long Chain Polyunsaturated Fatty Acids
LT	Leukotrienes
Lx	Lipoxins
MeOH	Methanol
MUFA	Monounsaturated Fatty Acid
HCl	Hydrochloric Acid
MOHCC	Ministry of Health and Child Care
MRCZ	Medical Research Council of Zimbabwe
MSCA	McCarthy Scales of Children's Abilities
PKU	phenylketonuria
PG	Prostaglandins
PMTCT	Prevention of Mother to Child Transmission
PUFAs	Polyunsaturated Fatty Acids

RCZ	Research Council of Zimbabwe
Rv	Resolvins
SD	Standard Deviation
SPE	Solid Phase Extraction
TNF-α	Tumor Necrosis Factor –α
TXA	Thromboxanes
UK	United Kingdom
% wt/wt	Percent weight per weight

Chapter 1

1.0 Introduction, Background and Literature Review

1.1 Introduction

Omega-3 long chain-polyunsaturated fatty acids (LC-PUFAs) are vital at all stages of life. In children, omega-3 LC-PUFAs are essential in foetal, neonatal, infant and early childhood for growth, development and general health(1). They have also been shown to be critical for optimal cognitive development(2), behavior(2) and visual acuity development(3). However studies demonstrating the importance of omega-3 LC-PUFAs on children have mostly been done in developed countries(1-3) with a limited number of studies done on African children(4-6) In Southern Africa, a region with high prevalence of childhood infectious diseases(7), the levels of omega-3 LC-PUFAs in children at all stages of growth are unknown except in South Africa. Studies from South Africa reported the importance of intervention with omega-3 LC-PUFA supplements in children aged 6-11 years and the positive effect on cognitive development(5, 6). No omega-3 studies have been conducted in Zimbabwean children.

The Ministry of Health and Child Care (MOHCC) of Zimbabwe and other health programs in Zimbabwe are mainly focusing on the general nutritional health of children under 5 years old, with little emphasis on those above 5 years who are at the critical stage of cognitive and academic development. In most sub-Saharan Africa healthcare facilities, omega-3 LC-PUFA levels are not on clinical laboratories test menus. Currently there is no clear policy on blood omega-3 LC-PUFAs assessment in Zimbabwe and laboratories lack the expertise to perform the assays(8).

Despite the benefits of omega-3 LC-PUFAs in children, the levels (wt/wt %) of omega-3 LC-PUFAs are unknown in Zimbabwean children particularly in the 7-9 year old age group in whom

adequate intake of LC-PUFAs should be ensured for brain development(1). The purpose of this study was to determine the levels of omega-3 LC-PUFAs among Zimbabwean children aged between 7 to 9 years using dried blood spots (DBS). The study also aimed to establish the reference intervals for omega-3 LC-PUFAs for the 7 to 9 year olds and to relate the omega-3 LC-PUFA levels to cognitive development assessments done when the participants were between 6 to 8 years old. The findings may provide useful insights to nutritionists, policy makers, MOHCC, health practitioners, non-governmental organizations and the general population. The results from this study can be the basis for future studies on omega-3 LC-PUFAs in Zimbabwe.

1.2 Background

1.2.1 Omega-3 LC-PUFAs Biochemistry

1.2.1.1 LC-PUFA Classification

Fatty acids are hydrocarbon chains carboxylated at one terminus and methylated at the other end(9). They are classified by the number of carbon atoms, double bonds and position of the double bond from the terminal methyl group(9-12). Naturally occurring fatty acids have an even number of 4 to 28 carbon atoms(5) and are classified into saturated, monounsaturated (MUFA) and polyunsaturated (PUFAs) fatty acids(9, 10, 12, 13). PUFAs have at least two double bonds[13]. The LC-PUFAs are straight-chain monocarboxylic acids with at least 20 carbon atoms and at least two carbon-carbon double bonds(9, 10). LC-PUFAs are subdivided into omega-3, omega-6, omega-7 and omega-9 series depending on the position of the terminal double bond relative to the omega carbon(10, 12). The omega-3 and omega-6 series are essential for human nutrition and are only derived from two naturally occurring essential fatty acids (EFAs) freely available in plant oils, α-Linolenic acid (ALA, 18:3n-3) and cis-Linoleic acids (LA, C18:2n-6) (10, 12, 13). Humans cannot synthesize LA or ALA from stearic acid because they lack Δ12 and Δ15-

desaturase enzymes which introduce the carbon-carbon double bonds between the tenth carbon and the methyl end(14).

1.2.1.2 Metabolism of LC-PUFA

The liver is the central site of LC-PUFAs synthesis before uptake by other peripheral cells(15). The human body derives the full benefits of ALA and LA by further anabolising them to their long chain (LC) metabolites via consecutive desaturation and chain elongation(10). ALA is anabolised to eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3) in the endoplasmic reticulum of the hepatocytes and then to docosahexaenoic acid (DHA, 22:6n-3) in the peroxisomes whereas LA is anabolised to arachidonic acid (ARA, 20:4n-6) (9, 10). The reactions are mediated by fatty acid elongase (Elovl2 and E1ov15) and rate limiting Δ5 and Δ6 fatty acid desaturase (FADS) enzymes(12, 16) (**Figure 1**).The synthesis of DHA is mediated by a mitochondrial pathway involving carnitine and α-tocopherol enzymes(17) and peroxisomal β-oxidation in the liver(13). The last step requires a compartmental translocation from the endoplasmic reticulum, which desaturates and elongates, to peroxisomes, the unique place for the β-oxidation of LC-PUFAs(13).

Dihomo-γ-linolenic acid is the precursor of anti-inflammatory 1 series prostaglandins (PGs) (10). ARA forms the precursor of 2 series PG, 2 series thromboxanes (TXA) and the 4 series of leukotrienes (LT) (10) (**Figure 1**). These have pro-inflammatory action and are involved in various pathological processes that include atherosclerosis, bronchial asthma and inflammatory bowel disease(10). EPA forms the precursor of the 3 series PGs, 3 series TXA and 5 series LT anti-inflammatory eicosanoids(10). Eicosanoids, lipoxins (Lx) and resolvins (Rv) function as endogenous anti-inflammatory molecules by suppressing interleukins (IL), IL-1, IL-2, IL-6 and tumor necrosis factor –α (TNF-α) production by T cells(10).

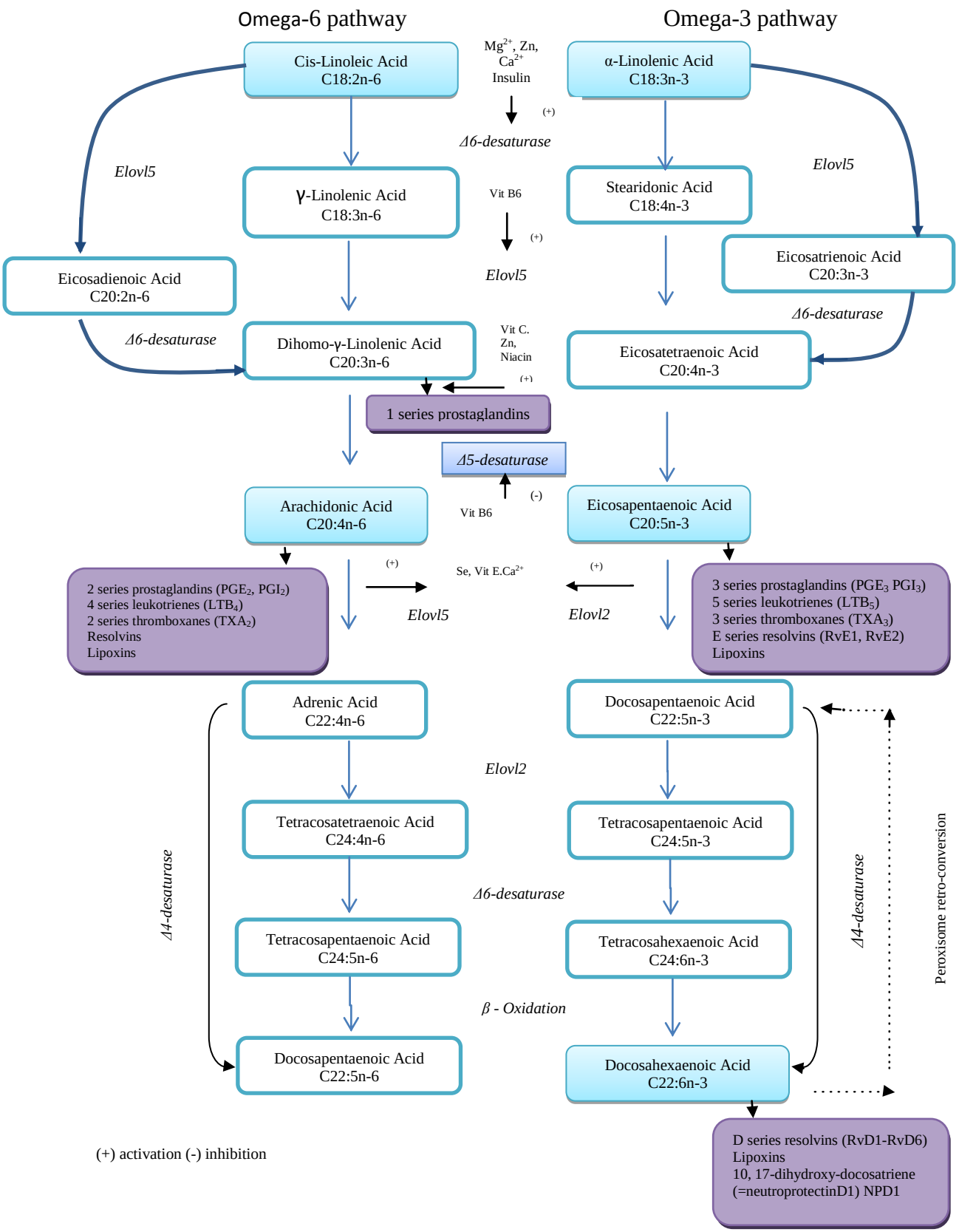


Figure 1: Metabolic Pathways for LC-PUFA synthesis (10, 12, 44)

1.2.2 Physiological Roles of LC-PUFAs

Dietary omega-3 LC-PUFAs are incorporated into cell membranes in all body tissues where they are structurally and functionally integrated via phospholipid molecules(18). Arachidonic acid occurs in high concentration in membrane phospholipids(19). Essential fatty acids and their LC metabolites also function as endogenous angiotensin-converting enzyme and 3-hydroxy-3 methyl-1 glutaryl coenzyme A reductase inhibitors, nitric oxide enhancers, anti-hypertensive and anti-atherosclerosis molecules(10). Docosahexaenoic acid confers cell membranes fluidity and thus determines and influences the behavior of membrane-bound enzymes and receptors(10). LC-PUFAs are important constituents of the phospholipids of all cell membranes(20) where they play critical roles both as structural and functional components(21). Cases in point include regulation of ion channels, modulation of endocytosis, exocytosis and hormonal control, influencing gene expression and immunological effects(3). Docosahexaenoic acid and EPA contribute to energy production by participating in electron transfers in vivo(22).

1.2.3 Factors that Influence the Metabolism of EFAs

The availability of omega-3 LC-PUFAs is affected by protein malnutrition, carnitine and α-tocopherol enzyme deficiency(23), gene mutations in the FADS 1 and FADS 2 genes and excess oxygen free radical production in chronic diseases(12, 23) and defects in the activities of Δ5 and Δ6 fatty acid desaturase(24). Linoleic acid competes with ALA for the endogenous conversion of ALA to the LC derivatives EPA and DHA and also inhibits incorporation of DHA and EPA into tissues(25). Therefore, high levels of LA in the diet result in low ALA and hence low omega-3 LC-PUFA levels(25), this eventually affects the omega-6: omega-3 ratios which are critical in human health outcomes. The rate of endogenous DHA synthesis is low and is unable to achieve desirable physiological levels (omega-6: omega-3 ratio of 1) in individuals devoid of preformed dietary supply(26).The availability of omega-3 LC-PUFAs is also affected by the presence of

high levels of ARA in the cell membranes which competes for the same enzyme for the conversion to EPA(27). Sex hormones, estrogen and testosterone, have been shown to affect EFA metabolism hence availability of LC metabolites, leading to high levels seen in females compared to males(28).

1.2.4 Dietary Sources of LC-PUFAs

Docosahexaenoic acid and EPA are the principal omega-3 LC-PUFAs and occur naturally in fatty fish such as herring, mackerel, cod, salmon, and tuna(13) where they accumulate following the fish's consumption of marine algae(29). Other dietary sources of omega-3 LC-PUFAs include fortified foods (infant and follow-on formula(19), eggs, and yoghurt), nuts, viscera(30), and human milk(19), and nutritional supplements like cod liver oil.

1.2.5 Omega-6: Omega-3 ratios

The ratio of omega-6 LC-PUFA:omega-3 LC-PUFA is more important diagnostically than concentrations alone(31). This ratio is of particular interest because these LC-PUFAs compete for the same desaturase and elongase enzyme systems. The ratio of omega-6: omega-3 fatty acid in human ancestry diet was about 1(24). However, with the introduction and exposure to refined foods in diets rich in omega-6 fatty acids, this ratio has increased to 20:1 in most modern populations(32). A dietary omega-6: omega-3 ratio of 1-4:1 is optimal(33). Deficient intake of omega-3 fatty acids or excessive intake of omega-6 fatty acids favors omega-6 fatty acids metabolism(32).

1.3 Literature Review

1.3.1 Importance of Omega-3LC-PUFA in Children

1.3.1.1 Early Life

Adequate supply of maternal omega-3 LC-PUFAs during foetal and neonatal life depends on maternal intake before conception, during pregnancy, and lactation stages(19). The ample supply and metabolism of omega-3 LC-PUFAs during pregnancy minimizes the risk of adverse pregnancy outcomes(34) and promotes optimal growth, development and health(1). Maternal intake and foetal supply of DHA and ARA is critical to the function and optimal development of the human body systems – optimum neurological(35), brain(11, 36) nervous(11), retina(3) respiratory(37) and immune system(38) function – and positively influences their function throughout life. These benefits have been attributed to the cell membrane fluidity conferred by DHA(10).

The recommended daily maternal intake during pregnancy and lactation is at least 300mg of DHA per day(27, 39). Deficiencies and imbalances of DHA and ARA during the developmental phase and throughout life have significant negative effects on brain function(21). Omega-3 LC-PUFAs intake may be inadequate in the foetus and neonate whose mothers have omega-3 LC-PUFA dietary deficiency. For children aged 2 to 10 years, the Food and Agricultural Organization (FAO) set age dependent recommendations for adequate EPA and DHA intake of 200 to 350 mg per day(27). This recommendation targets early prevention of chronic diseases like cardiovascular disease (CVD), obesity and diabetes mellitus(27).

1.3.1.2 Inflammatory Pathologies

Omega-3 LC-PUFAs eicosanoids have been reported to mediate resolution of symptoms of different types of inflammation caused by allergies(33). These include hay fever, asthma,

eczema, atopy and food allergies. Although much is known about the prevalence of food allergy in the developed world little is known about the prevalence rates of food allergies in developing countries(40) particularly the Central Africa region(41). Food allergies are reported to affect up to 6% children in developed countries(40). In Zimbabwe 19% of children born from 1990 to 1999 and 12% of the children born between 2000 and 2011 were reactive to a variety of food allergens(41). Optimal dietary EPA and DHA would therefore be a salient silent intervention in the children with food allergy pathologies.

1.3.1.3 CVD and Diabetes Mellitus

Omega-3 LC-PUFA supplementations have been shown to restore endothelial function in children with familial hypercholesterolemia thus reducing the risk of early coronary atherosclerosis(42). Diets rich in fish oil have been reported as reducing cardiovascular risk in diabetics by inhibiting platelet activation and aggregation, and improving lipid profiles thereby reducing cardiovascular mortality(43). Omega-3 LC-PUFA sufficiency has also been associated with reduced risk of islet autoimmunity in children at increased genetic risk for type 1 diabetes(31).

In addition adequate omega-3 LC-PUFAs nutrition has also been reported to be beneficial in metabolic syndrome(44), haematological pathologies(4, 45) and inborn errors of metabolism(30, 46).

1.3.1.4 Inborn Errors of Metabolism

In born errors of metabolism (IEM) requiring dietary restrictions pose a risk of omega-3 LC-PUFA deficiency because foods with high omega-3 LC-PUFA are also high in protein(30). LC-PUFA supplementation of protein-restricted phenylketonuria (PKU) children normalizes

decreased DHA concentrations and has a beneficial effect on their nutritional status and neurological(30) and motor skills outcomes(46).

1.3.1.5 Hypertension

Supplementing infants with infant formula fortified with DHA and ARA during the first four months of life has been reported to be associated with lower blood pressure at six years compared to the children who were not supplemented(45). Blood pressure tends to track from childhood into adult life and consequently early adequate dietary LC-PUFA intake might have lasting effects on reduced blood pressure and cardiovascular risk(19).

1.3.1.6 Sickle Cell Anemia

A study in Sudanese children with sickle cell anemia (HbSS) reported that supplementation with DHA and EPA was effective at reducing the frequency and severity of vaso-occlusive episodes, severe anemia and blood transfusion rates(4). Omega-3 LC-PUFAs have been suggested to be an effective, safe and affordable treatment for sickle cell anemia(44).

1.3.1.7 Brain Development

Omega-3 LC-PUFA-deficiency affects the development of the frontal lobes of the brain(46). The brain develops very rapidly during the last trimester of foetal life and the first two years of childhood (‘brain spurt”) (47). However, it has been reported that by the age of 2 years, the frontal lobes that are responsible for executive functions are not yet fully developed(48). The spurts continue between 7 to 9 years, in mid-teenage years(48) and thereafter develop at constant rate until at the age of 45 years(48).

1.3.1.8 Childhood Neurodevelopmental Disorders

Development of brain tissue in children from the perinatal stage depends on the availability of adequate DHA and ARA(19) and other growth factors(10). Decreases, deficiencies or

imbalances in LC-PUFAs intake during this critical period of growth may impair brain growth and the development of appropriate synaptic junction thereby leading to childhood neurodevelopmental disorders' and autistic spectrum disorders (ASD) (49). Individuals with these conditions have been reported to improve with fish oil supplementation(50). According to Centre for Disease Control (CDC) the prevalence of ASD is 1:88 in the USA (51). However, in Zimbabwe autism prevalence is unknown, but it is speculated to be high (personal communication). In Zimbabwe, ASD has been neglected though the number of children suffering from autism is on the increase (personal communication). Since some studies have reported that LC-PUFAs are beneficial in children with autism(50), it can be of importance to carry out intervention studies in Zimbabwean children with autism.

1.3.1.9 Cognitive Development

Access to omega-3 LC-PUFAs early in life is important for intellectual development during the first years of life, and has an impact on intelligence quotient (IQ), and academic performance up to the age of 18(52). This is critical for early primary school performance, which is the baseline for academic life. Most of the research studies on the association between omega-3 LC-PUFA intake and cognitive development have focussed on preterm and low birth weight infants(5). Since very little is known about the effect of DHA intake and supplementation on the cognitive development of school-age children, Dalton *et al.* (2009) studied the effect of an experimental fish-flour spread rich in Omega-3 LC-PUFA on cognition of South African children (7-9 years) compared with those on a placebo over a six-month period and reported an improvement of verbal learning ability and memory(5). This was also previously suggested in another South African study in children aged 6-11 years supplemented with Omega-3 LC-PUFA rich oil for 9 months(6). Learning difficulties have been observed in 6-12 year old Indian children with low

omega-3 LC-PUFA concentrations in plasma phospholipids(53). A recent study on South African children showed improved verbal and nonverbal learning memory in children with poor iron and omega-3 LC-PUFA status after supplementing them with iron but no benefits on cognition and impaired working memory were found in anaemic children supplementation with DHA or EPA(54).

1.3.2. Omega-3 LC-PUFA Reference Intervals

Monitoring of fatty acid levels in individual patients or in populations requires availability of reference intervals obtained from apparently healthy individuals to allow interpretation of the results(55). Fatty acids reference intervals have been established for glycerophospholipids in children aged 2 and 6 years(55) and in apparently healthy children who were on a normal diet for their age elsewhere(30). However, scanty studies have been done in low income settings particularly in African children and none in Zimbabwe. Hence there are no currently available reference intervals for LC-PUFAs in these settings.

1.3.3 Local Studies in LC-PUFAs

Despite these well documented benefits of omega-3 LC-PUFAs in children, in Zimbabwe no studies have been done to determine the nutritional status of omega-3 LC-PUFAs in children. However, Mohamed K *et al.* (8)(2007) in 1995-1996 studied the relationship between omega-3, omega-6 and trans-fatty acids levels and risk of preeclampsia among women delivering at Harare Maternity Hospital. They reported that omega-3 LC-PUFA levels in Zimbabwean pregnant women were lower compared to those of the USA pregnant women(8). The results provided little support for the hypothesized inverse relation between omega-3 LC-PUFA and risk of preeclampsia(8). However, blood sample analysis was done in the USA due to lack of testing facilities and expertise to measure omega-3 LC-PUFAs locally(8).

1.4 Study Justification

Despite the documented benefits of adequate omega-3 LC-PUFAs intake in children, (1-6, 11, 19, 27, 34-43, 45-50, 52-55) the levels of omega-3 LC-PUFAs in Zimbabwean children are unknown. Further, there are no laboratories measuring omega-3LC-PUFA levels and no deliberate government policy to promote their adequate intake. It is therefore essential to establish omega-3 LC-PUFA nutritional status in Zimbabwean children and to assess if there is need for intervention. The study determined levels of omega-LC-PUFAs in apparently healthy children aged 7 to 9 years. The findings may provide useful insights to nutritionists, policy makers, MOHCC, health practitioners and non-governmental organizations. The findings can be the basis for future studies on omega-3 LC-PUFAs and establishment of a testing laboratory in Zimbabwe.

1.5 Research Questions

1. What are the levels of omega-3 LC-PUFA in 7-9 year old Zimbabwean children?
2. Do omega-3 LC-PUFA levels among 7-9 year old Zimbabwean children vary by age and gender?
3. Are omega-3 LC PUFA levels in 7-9 year old Zimbabwean children correlated with cognitive development outcomes?

1.6 Primary Objectives

1. To determine levels of omega-3 LC-PUFA in Zimbabwean children aged 7-9 years
2. To determine if omega-3 LC-PUFAs levels vary by age and gender among 7-9 year old Zimbabwean children

3. To establish reference intervals for omega-3 LC-PUFAs among Zimbabwean children aged 7-9 years

1.7 Secondary Objectives

1. To determine the relationship between omega-3 LC-PUFA levels and cognitive development outcomes among Zimbabwean children aged 7-9 years

1.8 Null Hypotheses

1. H_0: There is no difference in the omega-3 LC-PUFA levels in children under study when stratified by age and gender.

2. $H_{0:}$ Cognitive development is not correlated to omega-3 LC-PUFA levels in 7-9 year old Zimbabwean children.

Chapter 2

2.0 Materials and Methods

2.1 Participants

2.1.1 Study Design

This was a cross-sectional study in which 319 Zimbabwean children aged 7-9 years were enrolled. A DBS sample was collected from each participant for the determination of omega-3 LC-PUFA levels between September 2011 and August 2012. 228 of the children who participated in this current study had their cognitive development assessed at 6 to 8 years using McCarthy Scales of Children's Abilities (MSCA) and the data so obtained were correlated to levels of omega-3 LC-PUFA.

2.1.2 Study Setting

The study was conducted at three peri-urban primary health care clinics around Harare, (Epworth, St Mary's and Seke North), offering maternal and child health services. The catchment populations for these centres were about 161 840 for Epworth, and about 354 472 for Chitungwiza (St Mary's and Seke North) (56). Epworth is about 15km due east of Harare while St Marys and Seke North are about 25km south-east of Harare.

2.1.3 Study Participants

Children aged 7 to 9 years whose mothers were recruited from the national Prevention of Mother to Child Transmission (PMTCT) program were invited.

2.1.4 The Study Cohort

Only 319 out of the 452 children in the original PMTCT study were available to participate in the study. These fell short of the desired sample size of 385.

2.1.4.1 Reference population: The reference population is 7-9 years old Zimbabwean children.

2.1.4.2 Source Population: The source population were children aged 7-9 years old in peri-urban areas around Harare (Epworth, St Mary's and Seke North).

2.1.4.3 Study Population: The study population was 319 consented and assenting children born to mothers who were recruited from a national PMTCT program in 2002 and were aged between 7 and 9 years at the time of the study.

2.1.4.4 Sampling Frame

Purposive sampling was used to select children born to mothers recruited from a national PMTCT program and who met the inclusion criteria.

2.1.5 Inclusion and Exclusion Criteria

2.1.5.1 Inclusion Criteria

Only children born to mothers who took part in the National PMTCT program in Zimbabwe from 2002-2004 were included. Both boys and girls aged 7-9 years were eligible.

2.1.5.2 Exclusion Criteria

Children who were not born to the specified cohort, or who were siblings to the original cohort or whose care givers declined to take part in the study were excluded. HIV exposed and infected participants were excluded.

2.1.6 Sample Size

The sample size was calculated using the formula for estimating a single mean as outlined below:

$$n = [Z\delta/\Delta]^2 = \left(\frac{1.96 \times 0.10}{0.01}\right)^2 = 385$$

Where Z =1.96

δ = (variance of measurements)

Δ = range within which you want to estimate the true mean (Δ=0.01)

Purposive sampling was used to come up with the population study size of 385.

2.1.7 Outcome Measures

The outcome measures were the levels of omega 3 LC-PUFAs in the 7-9 year old children

2.1.8 Study Factors

The study factors were children's age and gender

2.2 Ethical Considerations

2.2.2 IRB Approval

The study protocol complied with the Declaration of Helsinki and was approved by the Joint Research Ethics Committee approval: (JREC) (JREC/170/12) and the Medical Research Council of Zimbabwe (MRCZ) approval: (MRCZ/B/359). Permission to ship the samples to Stirling University was granted by the Research Council of Zimbabwe (RCZ).

2.2.1 Informed Consent

Written informed consent was sought and granted by the parents or legal guardians of the participants. Written assent was also sought from the targeted children. The written informed participation consent and assent including permission to conduct analyses on the collected and stored DBS sample were given after the study had been explained to them orally as well as in

writing. The Medical Research Council of Zimbabwe (MRCZ) approved informed consent and assent forms from the Nutrition Protocol are shown in **Appendix 4**.

2.3 Methodology of Omega-3 LCPUFA

The method below used for analysis of DBS samples in this study was based on a rapid method developed by Bell *et al*. (2011) (57), which is a modification of a method previous developed by Marangoni *et al*. (2004) (58). Blood sample analysis was done at the Aquaculture Institute – University of Stirling Scotland United Kingdom (UK).

2.3.1 Principle of the Omega-3 LC-PUFA Method - Assay Principle

Capillary blood samples were spotted on to filter paper treated with BHT to minimize oxidative degradation of fatty acids. The fatty acids from fatty acid-containing compounds were eluted and extracted from the DBS and then transesterified with methanol-hydrochloric acid MeOH-HCl to form fatty acid methyl esters (FAMEs). FAMEs and each individual omega 3 LC-PUFA were quantitatively measured by capillary gas liquid chromatography (GLC) and compared to a known quantity of internal standard (the Supelco 37) and the respective omega-3 LC-PUFA standards(59, 60).

2.3.2 Principle of the Gas Liquid Chromatography

The GLC is used to quantify vapourisable materials. The GLC depends on the partition of volatile substance between a gas phase and a liquid (stationary) phase. The relative time the substance spends in the two phases determines the elution time. Gas liquid chromatography equipment consists of four components: a column, an injector, a detector coupled to a data acquisition system and an oven. Chromatography involves the separation of a mixture of compounds (solutes) into separate components. The sample is vaporized by injection into a

heated system, eluted through a column by an inert gaseous mobile phase and detected by Flame ionization detection (FID) (61).

2.3.3 Reagents, Working Solutions, Materials, Instruments and Equipment

2.3.3.1Reagents

High-performance liquid chromatography (HPLC) grade methanol, iso-hexane and diethyl ether, and analytical grade potassium chloride (KCl) and hydrochloric acid (HCl) were purchased from Fisher Scientific, Loughborough UK. Butylated hydroxytoluene (BHT) and FAME mixtures (Supelco™ 37 FAME mix) were purchased from Sigma Aldrich, Dorset/Irvine UK. Nitrogen (oxygen free) was purchased from BOC Gases, Guildford UK.

2.3.3.2 Working Solutions

1. **Saturated KCl solution** was made by preparing a 4M solution of KCl (149.12 g in 500 ml distilled water). The solution was stirred and heat was applied. More KCl was added until the solution was saturated. The saturated solution was then allowed to cool at room temperature. The saturated solution was diluted 1:1 with water to make a 50 % saturated solution, which was stored in a clear reagent bottle and was stable for one year.

2. **Transesterification reagent (1.25 M HCl in methanol):** 10.7 ml concentrated HCl was added into 80 ml methanol and the final volume was made up to 100 ml with methanol, which was stored in a clear reagent bottle and was stable for one month.

3. **0.01% (w/v) BHT standard solution:** 0.1 g BHT was dissolved in diethyl ether and made up to 100 ml.

4. **Iso-hexane/diethyl ether 95:5 (v/v):** 95 ml of iso-hexane was made up to 100ml by adding diethyl ether, which was stored in a clear reagent bottle and was stable for three months.

The working solution were prepared and stored in brown bottles and were stable for 1 week

2.3.3.3 Materials

Blood collection filter paper cards (Whatman 903, lot number (LN): 6833909/82), air tight zip lock foil bags and desiccant (Whatman item number: 10534321, 10548234 and LN: 47152, 9194013 respectively) were purchased from Whatman, Maidstone/Banbury UK. Automatic lancing device equipped with a lancet (Accu-Check®, Safe-T-Pro Plus) was purchased from Roche Diagnostics, Mannheim Germany. Pyrex tubes (10 ml) which had Teflon-lined screw caps and autosampler vials were purchased from Chromacol, Herts UK. Measuring cylinders (100 and 500 ml), 1 litre conical flask, 25 ml beaker, micro pipette tips (200µl), glass test tubes and glass Pasteur pipettes were purchased from Fisher Scientific, Loughborough, UK. A micropipette was purchased from Gilson Microman, Middleton UK. Solid Phase Extraction (SPE) and normal phase columns (500 mg/3 ml) were purchased from Clean-Up Extraction Columns (UCT, Bristol USA).

2.3.3.4 Instruments and Equipment

Thermo Finnigan Model Trace GLC equipped with an auto sampler and an FID was purchased from Thermo Scientific, Thermo Fisher Trace, Hertfordshire UK. An analytical balance was purchased from European Instruments Oxford Balances, Oxford UK. A vortex mixer was purchased from IMLAB, Labworld yellowline, Boutersem Belgium. A CTC-PAL the liquid handling autosampler and Ultra High Throughput System was purchased from CTC Analytics

AG, Zwingen Switzerland and was equipped with a 70^{o}C dry heating block and a stirrer. The nitrogen evaporator was purchased from (N-EVAP™ 111, Organomation Associates, Berlin USA). The capillary column was purchased from ZB Wax; Phenomenex, Macclesfield, Cheshire, UK.

2.3.4 Whole Blood Sample Collection and DBS Preparation

Whole blood samples were collected by finger prick technique using a sterile automatic lancing device equipped with a lancet. Blood was spotted directly and absorbed onto each of four marked circles on a BHT treated and participant identification number labeled filter paper card prepared according to the methods previously described by Ichihara *et al.* (2002)(62). The filter paper cards were air dried for three hours, on a filter paper card rack at room temperature, and were transported to the laboratory for packing and storage. The DBS samples were packed in individual air tight zip lock foil bags with a desiccant and stored at -25^{o}C until shipment and analysis. These were the recommended storage conditions to maintain omega-3 LC-PUFA stability according to stability studies by Bell *et al.*(57). The DBS were shipped on blue ice for analysis to the Aquaculture Institute – University of Stirling Scotland UK, with permission to ship from RCZ.

2.3.5 Extraction and Detection Procedure

2.3.5.1 Fatty Acid Direct Transesterification and Extraction -Lepage and Roy Method

Sample processing (direct transesterification, extraction and purification) was performed using the CTC-PAL automated system (**Figure 2**) which extracts lipid from the DBS and prepares and purifies FAMEs. Direct transesterification was done using the one-step transesterification

modified and optimized original Lepage and Roy method(63). The automated method was programmed using the software package called Cronus (Axel Samrau, Sprockhövel Germany).The automated system allows for a much greater throughput of samples, by greatly reducing sample preparation times.

2.3.5.1.1 Automated Method(64)

One DBS circle (per sample), one DBS circle (internal standard), and one DBS circle (control) were cut out from the main DBS filter paper cards using a pair of scissors and forceps and were placed each in a pre-labeled 10 ml screw cap vial which was loaded on the CTC-PAL machine carousel. The CTC-PAL then automatically carried out the steps outlined below for preparation of FAMEs by direct acid-catalysed transesterification of total lipid and extraction to determine fatty acid composition by GLC.

One milliliter of 1.25 M analytical grade HCl in HPLC grade methanol was added to the DBS in the 10ml Teflon cap liner screw cap sample tubes which were placed on a heating block and heated at 70^{o}C for 1 hour. The tubes were returned to the original position on the machine carousel and cooled to room temperature. Three milliliters iso-hexane containing 0.01% (w/v) BHT and 4 ml 50% saturated KCl (1:1) solution were added to the tubes which were shaken on an agitator for 4 min and the mixture were allowed to settle for 2 minutes. Two and half milliliters of the top organic phase containing FAMEs were harvested and passed through a pre-conditioned SPE cartridge (Clean-Up Extraction Columns) pre-washed with 5 ml iso-hexane (Fisher Scientific, Loughborough, UK). The FAMEs were eluted with 5 ml iso-hexane/diethyl ether (95:5 (v/v)) into corresponding pre-labeled 10 ml test tubes.

2.3.5.1.2 Manual Steps(64)

The 10 ml test tubes were removed from the carousel and the contents (FAMEs) were dried by evaporation under nitrogen, using the nitrogen evaporator (N-EVAP™ 111) (**Figure 3**), at room temperature in a chemical fumehood. The FAMEs in the test tube were redissolved in 200µl iso-hexane and transferred to corresponding pre-labeled autosampler vials using a glass Pasteur pipette for GLC analysis.

Figure 2: The CTC-PAL the Ultra High Throughput System (CTC Analytics AG, Zwingen Switzerland)

Figure 3: The N-EVAP™ 111 (Organomation Associates, Berlin USA)

2.3.6 Detection Method

The GLC FAME detection method(65) was based on a rapid method by Bell *et al*. (2011)(57) which was a modification of a previous method developed by Marangoni *et al*. (2004) (58).

The total GLC analysis of FAMEs consisted of several steps that included the sample injection, separation, identification and quantification of FAMEs(66). FAMEs were injected (50µl), separated and quantified by GLC using a 60 m x 0.32 mm x 0.25 µm film thick capillary

column(57). Hydrogen gas (H_2) was used as a carrier gas at a flow rate of 4.0 ml/min and the temperature program was from 50 to 150^{o}C at 40^{o}C/min then 195^{o}C at 2^{o}C/min and finally to 215^{o}C at 0.5^{o}C/min, as shown in **Appendix 1**. The FAMEs were analysed by flame ionization detector fitted on a GLC. Individual FAME peaks were identified for full fatty acid profiles ranging from C14:0 to C22:6 carbons by the use of pure reference compounds, well-characterized in-house standards as well as commercial FAME mixtures. Total run time per analysis was around 1 hour. The fatty acids data were collected from the GLC and processed using the Chromcard software for Windows (version 2.1) computer package (Thermoquest Italia S. p. A., Milan, Italy). The individual FAME results were expressed as a relative percentage of the total fatty acids. The total omega-6 LC-PUFAs included DGLA ARA and DPA (n-6) whereas the total omega-3 LC-PUFAs included EPA, DPA (n-3) and DHA. The results of ALA, EPA, DPA, DHA, ARA, and the calculated total omega-3 PUFAs, total omega-3 LC-PUFA: total omega LC-PUFAs, ARA: EPA, total omega-6 LC-PUFA: total omega-3 LC-PUFA ratios were selected for data analysis. A picture of the GLC is shown in **Figure 4**.

Figure 4: The Thermo Finnigan Model Trace Gas Liquid Chromatography (Thermo Scientific, Hertfordshire UK)

2.3.7 LC-PUFA Analysis

The FAMEs were identified by comparison of their retention times with those of individual purified standards. The individual fatty acids were quantified as a percentage weight of the total fatty acids measured using the peak areas of each individual FAMEs as shown in **Figure 5**.

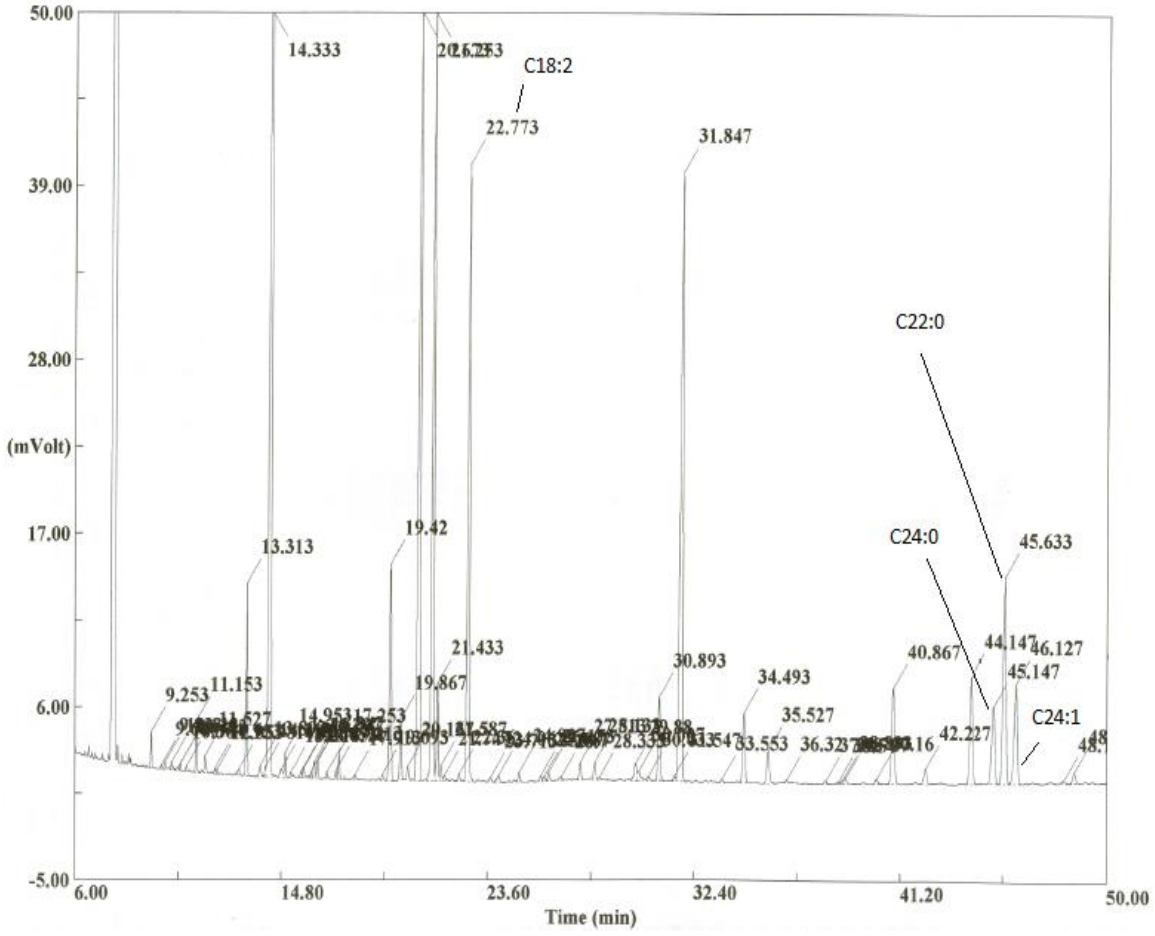


Figure 5: The GLC FAME Chromatogram Example

2.3.8 Calibration of the GLC

The GLC was calibrated using duplicate injections of standard mixtures of known composition (Supelco standards). The Institute of Aquaculture laboratory participates in the Deutsche Gesellschaft für Fettwissenschaft (DGF) LVU inter laboratory ring test (organized by the German Society for Fat Science (DGF) and the European Lipid Federation). Calibration was stable for up to three months(65).

2.3.9 System Suitability Test using Secondary Reference Material

At the beginning and end of each batch of samples or once a week, a marinol FAME secondary reference material was analysed. A total sample loading of 20,000,000 to 35,000,000 area units with about 50 - 80 peaks and smallest components of 0.1% was optimal. The system suitability test was also used to check retention times(65).

2.3.10 Data acquisition and storage

A hard copy of each analysis was obtained at the time of complete analysis and data was stored on the computer hard drive and backed onto a memory stick. The retention times of samples were compared with those of the standards as well as the peak areas, thereby identifying the different FAMEs in the sample. Data (peak areas) was transcribed manually from the hard copy to an excel spreadsheet for further processing(64) as shown in **Appendix 2-3**.

2.3.11 Results Calculation

Individual fatty acid data were presented as relative levels percentage of total fatty acids, (wt/wt). The area % for all the fatty acids, were entered on an excel spreadsheet giving a total % of identified fatty acids (crude %) as shown in **Table 1**. This was normalized to 100 % of the identified fatty acids by highlighting the first individual FAME crude % and using the following

formula (=individual FAME crude%*100/total of crude %), that is X*100/total crude%. The total calculated weight from the GLC was normalized to take care of contamination from any plastic and hydrocarbon artifact factors present in the analysed sample. The ARA: EPA, total omega-6: total omega-3 ratios were calculated for whole blood membrane phospholipids.

Table 1: An Example of Participant Results Calculation –Excel File

	SN 601	
	crude%	**corrected%**
14:0	0.55	0.57
15:0	0.1	0.10
16:0	18.19	18.85
18:0	13.49	13.98
20:0	0.25	0.26
22:0	0.82	0.85
24:0	1.45	1.50
Total saturated		**36.12**
16:1n-9	0.23	0.24
16:1n-7	0.31	0.32
18:1n-9	10.43	10.81
18:1n-7	1.15	1.19
20:1n-9	0.17	0.18
24:1n-9	0.9	0.93
Total monounsaturated		**13.67**
18:2n-6	24.19	25.07
18:3n-6	0.12	0.12
20:2n-6	0.41	0.42
20:3n-6	0.9	0.93
20:4n-6	12.78	13.24
22:4n-6	1.54	1.60
22:5n-6	0.56	0.58
Total n-6 PUFA		**41.97**
18:3n-3	0.3	0.31
20:5n-3	0.12	0.12
22:5n-3	0.69	0.72
22:6n-3	2.63	2.73
Total n-3 PUFA		**3.88**
16:0DMA	1.44	1.49
18:0DMA	2.14	2.22
18:1DMA	0.63	0.65
Total DMA		**4.36**
Total	96.49	100.00
20:4n-6/20:5n-3		**106.50**
% n-3 LC-PUFA/Total LC- PUFA		**17.90**
Total n-3 LC-PUFA		**3.57**
Total n-6 PUFA/Total n-3 PUFA		**10.83**

2.3.12 Quality Control

An internal quality control sample (the Supelco 37standard which is a reference standard), with certified and known values was run with each batch of samples, from sample preparation to FAME detection. Peaks, peak areas and retention times were checked and if the total peak area was below 15 000 000 and had an area % below 0.06 on one of the FAMEs, the sample was reported as under loaded and a repeat injection was done. If the total peak area was above 35 000 000, the sample was reported as overloaded and a repeat injection or FAME sample dilution was done(64).

2.3.13 Quality Assurance

The Supelco 37 controls were run simultaneously with each run/batch of participant to ensure reproducibility of the known control values. The results of quality control material are presented in **Table 2**. All the results obtained for the Supelco 37 quality control materials were within the range cited by the manufacturer.

Table 2: Supelco 37 Standard – Quality Control

LC-PUFAs (% wt/wt)	**Quality Controls**	
	Supelco 37 standard QC replication Results mean (SD) 6 runs	**Supelco 37 Standard QC range**
EPA	2.514 (0.004)	2.498-2.529
DHA	2.071 (0.042)	1.901-2.241
ARA	2.567 (0.003)	2.555-2.579
ARA: EPA	1.021 (0.002)	1.015-1.028

2.4 MSCA Cognitive Development Assessment

A culturally modified validated short form MSCA that was used served as a screening instrument for potential learning disorder and was administered by a qualified local pediatrician(67). The MSCA is an individually administered assessment of cognitive development of children aged 2½ to 8½ years(68, 69). MSCA assesses the child's level of general intelligence function and motor ability aiming to identify possible developmental delay in different skill areas using different scales(68, 69). It consists of 18 items which are summed to generate 5 domains: 1) verbal, which refers to those cognitive abilities related to information processing; 2) quantitative, which relates to numerical abilities; 3) memory assesses short term retention of information (verbal, perceptive or numerical); 4) perceptive–performance, which refers to tasks related to perceptive information processing and 5) motor abilities(69). Scores from the verbal, perceptual-performance, and quantitative domains which were content oriented were computed to generate the General Cognitive Index (GCI) (69). The mean for the General Cognitive Index (GCI) is set at 100, with a standard deviation (SD) of 16 according to the MSCA administration manual(69). The Verbal Scale consists of five subsets and assesses comprehension and use of language. The Quantitative Scale consists of three subsets and measures mathematical abilities. The Perceptual-Performance Scale consists of seven subsets and evaluates a child's ability to conceptualize and reason without words. Cut off index values for these three scales were 30. The Verbal, Quantitative and Perceptual-Performance Scales were combined to produce the General Cognitive Index score with a cut off index value of 68.

2.5 Statistical Analysis

Statistical Package for Social Scientists (SPSS version 16.0, New York, USA) software package was used for data entry and analysis. The SPSS statistical analyses were double checked by

STATA version 10.0, Texas, USA. Categorical variables were summarized using percentages and continuous non-normal variables were summarized using median and inter-quartile ranges (IQR). Normality of the continuous data was tested using the Shapiro-Wilk and Kolmogorov-Smirnov tests and histograms showing that some of the data were non normal. SPSS Cross tabulations were used to produce multiway tables showing the distribution or association of categorical variables in the demographic data table. Kruskal-Wallis H test was used to compare non-normally distributed continuous outcomes. The reference intervals were calculated using the 2.5 and 97.5 percentiles. Spearman rank correlation coefficient was used to determine the relationship between omega-3LC-PUFA and cognitive development. A p-value of less than 0.05 was considered statistically significant.

Chapter 3

3.0 Results

3.1 Study Population Demographics

A total of 319 of the 384 targeted of the 452 available children from the previous cohort (followed up from birth to between 7-9 years old) assented and consented to participate in the study. Of the 319 children, one had a DBS collected on a non BHT treated filter paper, therefore the child was excluded from testing as were 21 HIV exposed and infected children. Thus, results on 297 children who were apparently healthy and had DBS samples are presented (**Figure 6**). Of the 297 children, 170 (57.2%) were female and 228 had cognitive assessment when the children were 6-8 years. The median age (range) of the 297 children was 9 (7-9) years. **Table 3** presents the age and gender stratification of the children in the study.

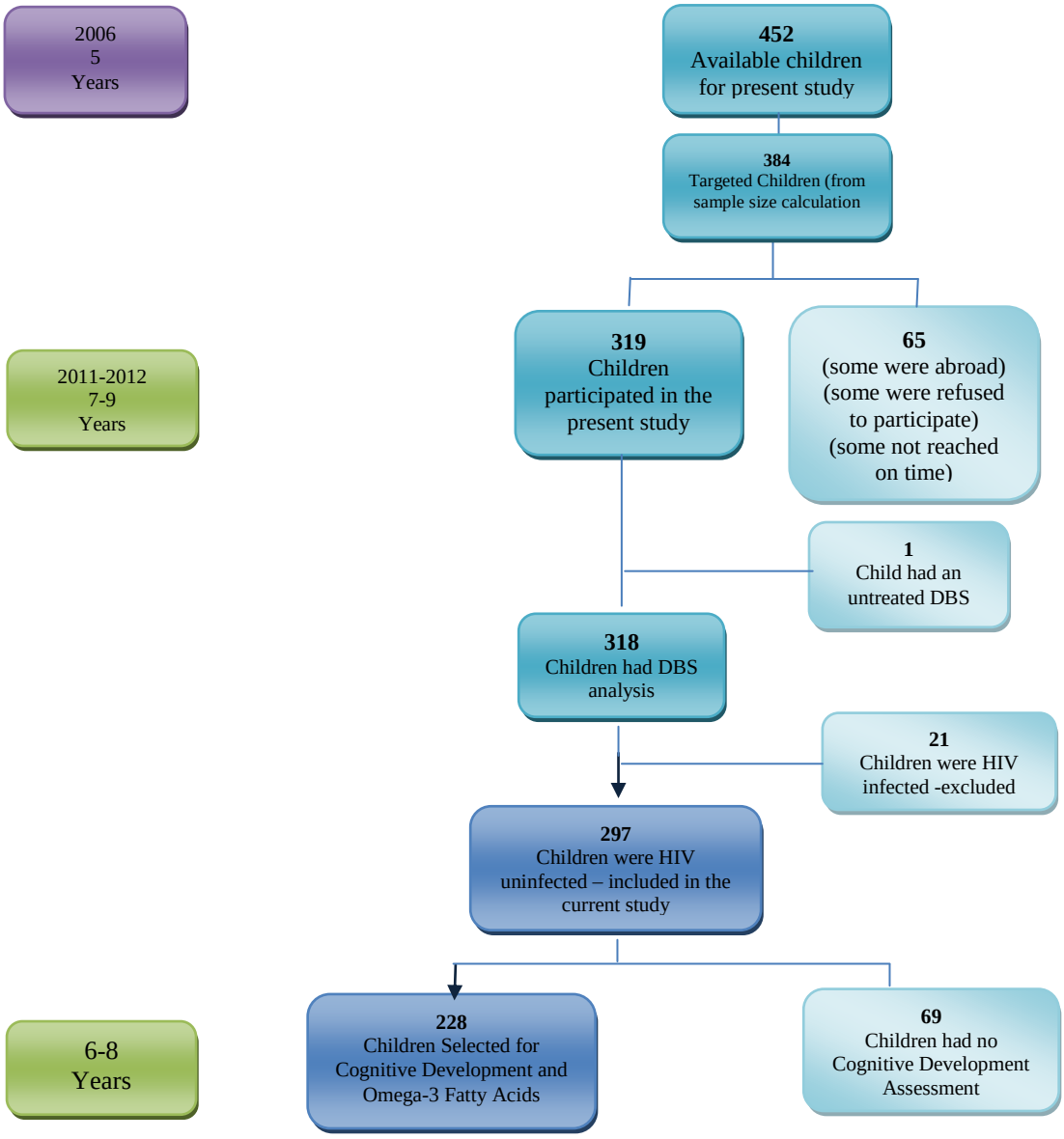


Figure 6: Flow diagram showing paediatric cross sectional selection from maternal cohort study

Table 3: Age and gender stratification of the children

Variable	Frequency n = 297
Children Age Group (years)	
(7)	21 (7.1%)
(8)	93 (31.3%)
(9)	183 (61.6%)
Gender of the children	
Male	127 (42.8%)
Female	170 (57.2%)

3.2 Distribution of Omega-3 LC-PUFA

The data were not normally distributed as shown in **Table 4**.

Distribution of LC-PUFAs in DBS samples obtained from 297children aged between 7 and 9 years

3.2.1 Kolmogorov-Smirnov and Shapiro-Wilk Test of Normality

Two tests of normality, namely the Kolmogorov-Smirnov Test and the Shapiro-Wilk Test were used to test for normality of the LC-PUFA variables. DHA and ARA LC-PUFA values had significant values of the Shapiro-Wilk Test greater the 0.05 indicating that the data was normally distributed, while EPA and DPA LC-PUFAs were not normally distributed.

Table 4: Normality Tables

Tests of Normality[b]

LC-PUFAs	Children	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	gender	Statistic	Df	Sig.	Statistic	Df	Sig.
20:5n-3 (eicosapentaenoic) EPA	1	0.122	126	0.000	0.879	126	0.000
	2	0.124	171	0.000	0.943	171	0.000
22:5n-3 (docosapentaenoic) DPA	1	0.115	126	0.000	0.841	126	0.000
	2	0.138	171	0.000	0.696	171	0.000
22:6n-3 (docosahexaenoic) DHA	1	0.063	126	0.200*	0.988	126	0.342
	2	0.038	171	0.200*	0.997	171	0.988
20:4n-6 (arachidonic) ARA	1	0.071	126	0.196	0.968	126	0.005
	2	0.075	171	0.019	0.979	171	0.012
Total n-3 PUFA	1	0.092	126	0.011	0.943	126	0.000
	2	0.043	171	0.200*	0.979	171	0.010
% n-3 PUFA/Total PUFA	1	0.091	126	0.013	0.955	126	0.000
	2	0.060	171	0.200*	0.963	171	0.000
20:4n-6/20:5n-3	1	0.098	126	0.005	0.922	126	0.000
	2	0.101	171	0.000	0.948	171	0.000

a. Lilliefors Significance Correction *. This is a lower bound of the true significance.

3.3 Distribution of Omega-3 LC-PUFA Results

3.3.1 Overall Range for all Participants

The LC-PUFA and LC-PUFA ratio ranges are shown in descriptive statistics **Table 5**. Of note are the low values found in EPA, DPA, DHA and the high values in ARA and high ARA: EPA and total omega-6 PUFA: total omega-3 PUFA ratios in 44 children.

Table 5**:** Descriptive Statistics Tables for LC-PUFAs

LC-PUFA (% wt/wt)	N	Minimum	Maximum	Mean	Std. Deviation
EPA	297	0.06	0.55	0.1962	0.07051
DPA	297	0.38	1.98	0.8096	0.16959
DHA	297	1.13	3.52	2.1470	0.39437
ARA	297	5.58	14.64	10.5575	1.25152
Total n-3 PUFA	297	2.32	6.25	3.5466	0.52849
% n-3 PUFA/Total PUFA	297	13.34	28.11	18.6550	2.33748
20:4n-6/20:5n-3	297	15.47	163.33	61.1061	23.52959
Total Omega-3 LC-PUFA	297	1.73	5.95	3.1537	0.53422
Total n-6 PUFA/Total n-3 PUFA	297	5.94	16.03	10.8993	1.62528

3.3.2 Distribution and Comparison of LC-PUFA Levels by Participant Variables

3.3.2.1 LC-PUFA Distribution by Gender

The distribution of LC-PUFA was stratified by gender and the medians were compared. The results are presented in **Table 6**. The LC-PUFA levels for males and females were not statistically significantly different (all $p>0.05$). Median DPA levels were however marginally

higher in the males compared to females even though this was not statistically significant (p=0.104).

3.3.2.2 LC-PUFA Distribution by Age

The children were stratified by age into 7, 8, 9 years age groups and the LC-PUFAs levels were compared across the age groups as shown in **Table 6**. The median for DPA, DHA, ARA, total omega-3 PUFA and % omega-3 LC-PUFA: total LC-PUFA were not statistically significantly different across the age groups (all p>0.05). The median EPA was statistically significantly lower in the 7 years age group and statistically significantly elevated in the 8 years age group as compared to the 9 age group (p=0.049). The median ARA: EPA inflammatory ratio was statistically significantly elevated in the 7 years age group compared to the other age groups (p=0.014). The median DHA and ARA levels were however marginally higher in the 7 age group but did not achieve statistical significance (p=0.063 and p=0.075 respectively) when compared to the other age groups.

Table 6: Distribution and comparison of median (IQR) LC-PUFA levels by children's gender and age variables

Variable	LC-PUFAs (% wt/wt)						
	EPA	**DPA**	**DHA**	**ARA**	**Total Omega-3 LC-PUFA**	**% Omega LC-PUFA: Total LC-PUFA**	**ARA: EPA**
				Median (IQR) *p- value**			
Children's Gender							
Male	0.19 (0.15-0.23)	0.81 (0.72-0.91)	2.11 (1.91-2.46)	10.55 (9.80-11.32)	3.25 (2.62-3.42)	18.38 (17.32-19.94)	57.16 (45.24-71.82)
Female	0.18 (0.15-0.24)	0.78 (0.70-0.89)	2.15 (1.84-2.36)	10.67 (9.73-11.46)	3.10 (2.82-3.47)	18.44 (16.94-19.89)	57.72 (44.09-72.91)
	P = .809	**P = .104**	**P = .457**	**P =.801**	**P =.420**	**P = .472**	**P = .961**
Children's Age Group							
(7)	0.17 (0.13-0.18)	0.78 (0.74-0.86)	2.27 (2.03 -2.48)	10.89 (10.68-11.75)	3.18 (2.92-3.53)	18.20 (16.69-19.52)	64.38 (59.71-91.04)
(8)	0.20 (0.16-0.24)	0.81 (0.70-0.93)	2.16 (1.93-2.51)	10.63 (9.61-11.39)	3.18 (2.87-3.57)	18.64 (17.35-20.18)	56.43 (41.39-72.02)
(9)	0.18 (0.15-0.23)	0.79 (0.70-0.89)	2.10 (1.83-2.35)	10.48 (9.76-11.35)	3.09 (2.80-3.40)	18.34 (16.94-19.88)	55.87 (44.90- 70.64)
	P = .049**	**P = .831**	**P = .063**	**P = .075**	**P = .227**	**P = .329**	**P = .014****

* *p-values* calculated using Kruskal Wallis Test * * Statistically Significance (p<0.005) (2-tailed)

3.4 Reference intervals of LC-PUFAs in Zimbabwean Children aged 7-9 Years

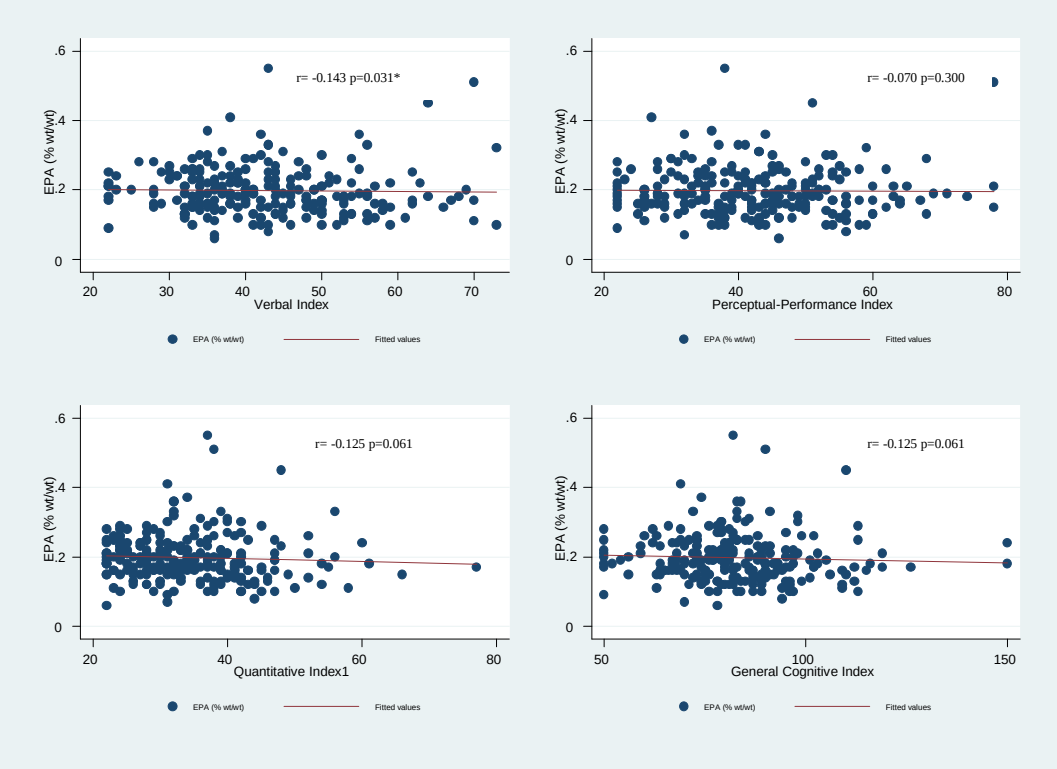
The reference intervals were established using LC-PUFA results for the 297 apparently healthy participants. These reference intervals were calculated using the central 95% of the data after cutting off the extreme 2.5% tails from both ends (2.5-97.5 percentiles) since the data were not normally distributed. The data for the three omega-3 LC-PUFAs and one omega-6 LC-PUFA are summarized and shown in **Table 7**.

Table 7: Reference intervals of LC-PUFAs in Zimbabwean children aged 7 to 9 years

LC-PUFAs (% wt/wt)	**All Participants (n = 297) Median (IQR)**	**Reference Intervals 7-9 years old n=297**	
		2.5th Percentile (90 % CI)	**97.5th Percentile (90 % CI)**
EPA	0.18 (0.15-0.23)	**0.09** (0.08 0.10)	**0.37** (0.33 0.45)
DPA	0.79 (0.70-0.89)	**0.53** (0.49 0.57)	**1.15** (1.07 1.26)
DHA	2.14 (1.87-2.42)	**1.35** (1.26 1.46)	**2.93** (2.86 3.16)
ARA	10.62 (9.77-11.38)	**7.85** (6.86 8.32)	**12.92** (12.57 13.55)
Total Omega-3 PUFA	3.55 (3.22-3.87)	**2.55** (2.39 2.64)	**4.64** (4.45 5.10)
% Omega LC-PUFA: Total LC-PUFA	18.42 (17.10-19.92)	**14.54** (14.06 14.98)	**24.56** (23.00 26.34)
ARA: EPA	57.47 (44.72-72.24)	**26.51** (21.58 28.26)	**110.83** (104.50 144.63)
Total Omega-3 LC-PUFA	3.13 (2.83-3.49)	**2.14** (1.98 2.28)	**4.29** (4.03 4.71)
Total Omega-6 LC-PUFA: Total Omega-3 LC-PUFA	17.83 (16.56-19.38)	**7.91** (7.42 8.48)	**14.48** (14.04 15.31)

3.5 Relationship Between Omega-3 LC-PUFA and Cognitive Development Assessment Outcomes

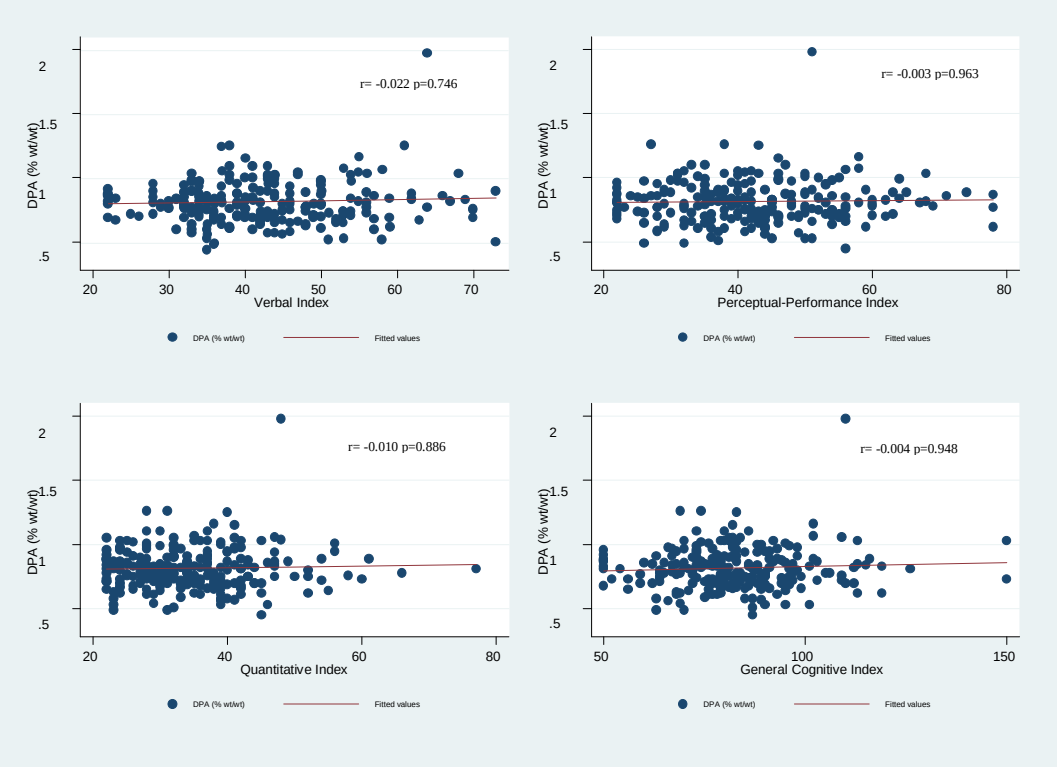
The relationship between omega-3 LC-PUFAs and cognitive development outcomes (MSCA) was assessed for the 228 participants who had complete data (**Figure 6**). The relationship was assessed using scatter plots and Spearman correlation coefficients. The corresponding correlation coefficients and *p-values* are shown in the scatter plots below (**Figure 7-9**).



*. Correlation is significant at the 0.05 level (2-tailed)

Figure 7: Scatter Plots for EPA and cognitive development

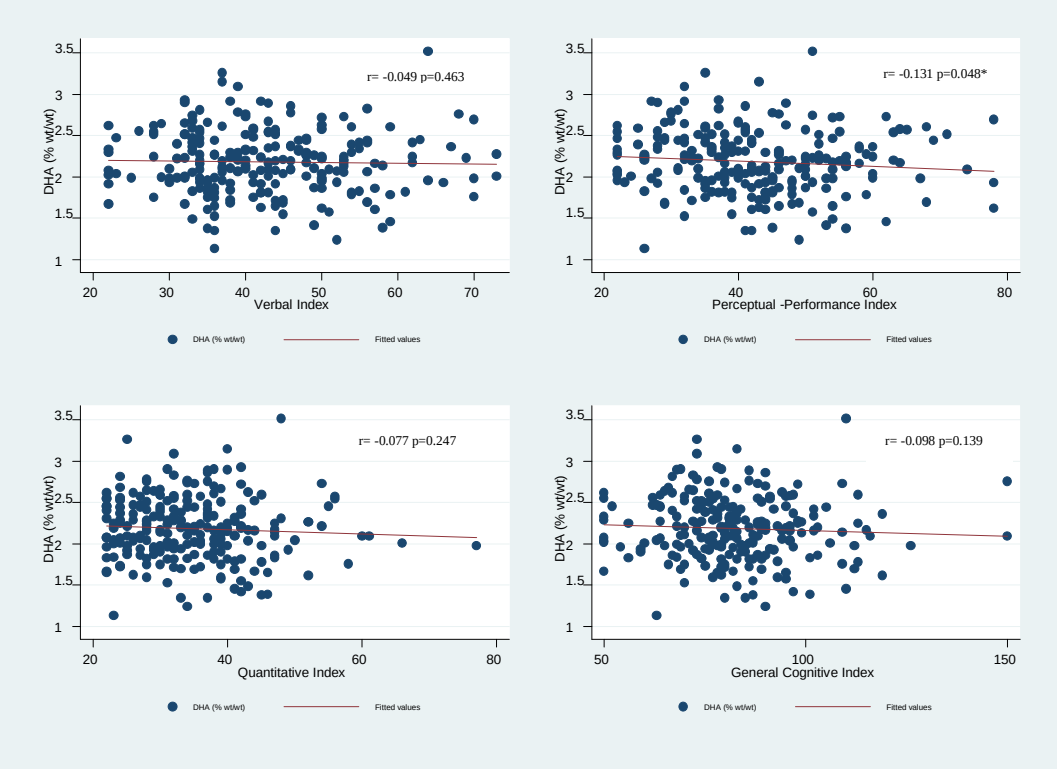
Figure 7, shows weak negative correlations between EPA and cognitive development indices. None of the cognitive development indices were significantly correlated with EPA, except verbal scale index (r= -0.143, p=0.031).



*. Correlation is significant at the 0.05 level (2-tailed)

Figure 8: Scatter Plots for DPA and cognitive development

Figure 8 indicates weak and negative correlations between cognitive development indices and DPA, which are all not statistically significant.



*. Correlation is significant at the 0.05 level (2-tailed)

Figure 9: Scatter Plots for DHA and cognitive development

Figure 9 shows weak and negative correlations between cognitive development indices and DHA. Cognitive development indices; perceptual-performance (r= -0.131 p=0.048) were significantly and negatively (but weakly) correlated with DHA.

Chapter 4

4.0 Discussion and Conclusions

4.1 Discussion

To our knowledge, this is the first study to determine levels of omega-3 LC-PUFAs in 7-9 year old Zimbabwean children and to establish LC-PUFA reference intervals in these 7-9 year old Zimbabwean children. This is also the first study, globally, to determine LC-PUFA reference intervals using DBS.

The levels for omega-3 LC-PUFAs (EPA, DPA and DHA) in 22 children in this study were strikingly low while those of omega-6 LC-PUFA (ARA) were surprisingly high compared to results obtained from other studies(5, 50, 55, 70). Generally, these children had very low omega-3 PUFAs and very high saturated fats, monounsaturated and omega-6 fatty acids as shown in Table 3. The highest EPA value obtained in this study of 0.55% wt/wt was lower than mean values obtained from the other studies with similar age groups(5, 50, 55, 70). Results of the present study also demonstrated the lowest EPA value of 0.06% wt/wt reported in apparently healthy children in literature compared to other studies(5, 50, 55, 70). This might be a reflection of the different geographical backgrounds, diet, age and genetic make-up of the children in the studies.

The low EPA and high ARA levels are of health concern because they lead to very high ARA:EPA inflammatory ratios and total omega-6 PUFA:total omega-3 PUFA dietary ratios. The high ratios observed in this study reflect possible imbalances in the dietary intake of omega-6 and omega-3 providing foods. The imbalances could be as a result of contemporary changes in human nutrition caused by increased consumption of diets rich in saturated fats, monounsaturated and omega-6 fatty acids including use of cooking oils, vegetable oils and bread

spreads rich in omega-6 PUFAs together with a decrease in omega-3 PUFA-rich foods(16). The possible explanations for the obtained levels include lack of omega-3 rich foods, urbanization, lifestyle and dietary changes in this peri-urban population.The other reason is that Zimbabwe went through economic hardships between 2007 and 2008 and this could have led to dietary intake imbalances and being a landlocked country, dietary fish intake for omega-3 LC-PUFA supply is generally low.

Another possible consequence of the low EPA values and very high ARA:EPA ratios is increased activity of the ARA metabolic pathway which would have deleterious effects such as neurological and neurodevelopmental disorders(49, 50). High concentrations of ARA compete with EPA for incorporation into cell membrane phospholipid. ARA gives rise to pro-inflammatory eicosanoids while EPA gives rise to anti-inflammatory eicosanoids(12). The low EPA levels could also have been due to deficiencies and defects in the Δ6 or Δ5 desaturase enzyme(71) or mutations in the fatty acid desaturase (FADS) gene(13). Deficiencies in EPA, which is a precursor of anti-inflammatory eicosanoids, results in children being susceptible to inflammatory pathologies due to the presence of pro-inflammatory ARA eicosanoids. Deficiencies in DHA results in children predisposes them to impaired brain development during 7-9 year old “brain spurt”(48), leading to compromised intellectual development, academic performance, low verbal learning ability and memory and learning difficulties(5, 6).

The LC-PUFA levels of all parameters except DHA were lower in the present study compared with expected values from the Stirling Aquaculture laboratory(57) which used the same method and sample type (DBS) as the present study. Our median EPA in the present study were similar to those obtained by Mohammed *et al.* for pregnant Zimbabwean women(8), indicating the general view of the dietary intake of foods low in omega-3 LC-PUFAs and ALA.

Our findings of no gender difference in median LC-PUFAs levels were in agreement with those of Glaser *et al.* on a paediatric population(55). However, another paediatric study reported a more pronounced low omega-3 LC-PUFA status in girls than boys(70). Depending on the ages, sex hormones (testosterone and oestrogen) influence the enzymatic synthesis of LC-PUFAs, leading to gender related differences in LC-PUFA status with higher levels occurring in adult females(28). The conversion of essential fatty acids (EFA) into their LC metabolites is stimulated by oestrogen and inhibited by testosterone(28). The reason for the lack of gender differences in LC-PUFA levels observed in this study was perhaps due to the young age of the participants. The onset of puberty, which defines differences in hormonal levels, might be different between black Zimbabwean children and children from other settings.

The observed differences in median EPA and ARA:EPA ratio across the children's age groups is probably due to the diet to which these children are exposed to, with 7 years old children having low EPA and high ARA values leading to high ARA:EPA ratio. Being their first year in school the feeding patterns may be altered and the type of food may be different from what they were used to before going to school.

The study established DBS LC-PUFA reference intervals for the apparently healthy 7-9 year old Zimbabwean children who participated in the study. However, these LC-PUFA reference intervals cannot be generalised to the rest of the population since the LC-PUFA results were from children from a select group born to a cohort residing in a peri-urban setting which did not include children residing in rural and urban settings. The established LC-PUFA reference could be compared to the other two studies(30, 55) which established LC-PUFA reference intervals due to methodological differences used.

From the above discussion the results showed that there were no statistically significant parameter ($p>0.05$) in omega-3 LC-PUFAs by gender hence the null hypothesis that "there was no difference in omega 3 levels among the children when stratified by age and gender was accepted for gender. However, statistically significant parameter ($p<0.05$) were observed in some of the omega-3 LC-PUFAs when children were stratified by age.

Relationship between Omega-3 LC-PUFA and Cognitive Development Assessment Outcomes

There was a weak negative correlation between children's LC-PUFA levels at 7-9 years and the cognitive outcomes at 6-8 years. EPA was significantly negatively (weak) correlated with verbal scale index ($r= -0.143$, $p=0.031$), indicating that verbal scale index increased with low EPA. Perceptual-performance were significantly and negatively (but weakly) correlated with DHA ($r= -0.131$ $p=0.048$), indicating that the perceptual-performance index increased with low DHA.

Thirty two participants had very low EPA and DHA levels and 12 had very high ARA levels of which when given in balance would have a positive effect on the cognitive development of children(50) and brain development (21). The implication of the obtained results might be seen in the children's academic performance, since low levels of LC-PUFAs have been shown to affect learning abilities(53). Maybe the other reason for the negative relationship is that the cognitive assessments were done at different time points from DBS samples collection. Comparisons between umbilical venous plasma and red blood cell phospholipid DHA and ARA levels at birth and cognitive function at seven years of age and were unable to demonstrate an association between neonatal fatty acid level and cognitive development(72). However a study by Campoy et al. suggested that higher DHA in maternal erythrocyte may be related to children's later cognition function(73).

In the current study, maternal supplementation with omega-3 done from 37 week gestation and from six months postpartum seemed not to influence the omega-3 levels in participants at 7-9 years and were not correlated with cognitive development at 6-8 years.

From the above discussion the results showed that there were no statistically significant parameter ($p>0.05$) and negative correlation in omega-3 LC-PUFAs by cognitive development hence the null hypothesis that "Cognitive development is not correlated to omega-3 LC-PUFA levels in 7-9 year old Zimbabwean children" was accepted. However, statistically significant parameters ($p<0.05$) were observed in EPA levels and verbal scale index and DHA levels and perceptual-performance index.

Recommendations

Our results showed generalized lower values across the omega-3 LC-PUFA range. The levels of these could be improved by, identification and encouragement of intake of locally available omega-3 LC-PUFA rich foods. There is need for a public awareness of the benefits of omega-3 LC-PUFAs throughout life and food sources rich in omega-3 LC-PUFAs. The fortification of foodstuffs with omega-3 LC-PUFAs (DHA and EPA) and ARA is becoming more common in some parts of the world(74). This could also be implemented in Zimbabwe to improve omega-3 levels. Docosahexaenoic acid and ARA fortified infant and follow-on formulae have however been developed to counter the negative effects of these LC-PUFA deficiencies on infant growth and development and these formulae been available in Zimbabwe since 2007. Omega-3 LC-PUFA-enriched ready-to-eat baby foods and children foods can also be made to supply the needed omega-3 LC-PUFA levels in Zimbabwean children. Supplementation of EPA and DHA omega-3 fish oils and algae based oils to balance ARA is recommended since low levels are recognized confounders of general health. Limited intake of ARA-rich foods is also

recommended if the desirable total omega-6 PUFA:total omega-3 PUFA ratio of 1-4:1[33] is to be achieved. The findings can be the basis for future omega-3 LC-PUFA intervention studies. The acquired techniques for LC-PUFA analysis may be used as the basis of technology transfer to Zimbabwe. Further studies comparing the LC-PUFA levels in children with cognitive impairment to those without could be beneficial to ascertain the real effect of LC-PUFAs and cognitive development in the children.

The study has a number of limitations. Firstly no dietary intake assessment was done during specimen collection hence causes of low omega-3 LC-PUFA levels are assumption based. Secondly, the established reference intervals are limited to the children born to the specified cohort and a limited age group was used for this study. The study population was also restricted to children in a peri-urban setting that may not be truly reflective of the Zimbabwean population. The established reference intervals could also not be compared to those from other populations because of analytical methodology differences(30, 55).

4.2 Conclusion

Nevertheless, this is an important study which observed very low EPA levels and very high ARA:EPA and total omega-6 PUFA:total omega-3 PUFA ratio ever reported in apparently healthy children. The levels of omega-3 LC-PUFAs in Zimbabwean children can be improved by exposing the children to foods with higher omega-3 LC-PUFAs and lower omega-6 LC-PUFAs. The findings may provide useful insights to nutritionists, policy makers, Ministry of Health and Child Care, health practitioners, non-governmental organizations, parents and legal guardians of the participants and the general population. The cognitive development indices were negatively correlated to omega-3 LC-PUFA levels, indicating the possible need to do the LC-PUFA analysis at the same time with the cognitive assessment and using other statistical analysis.

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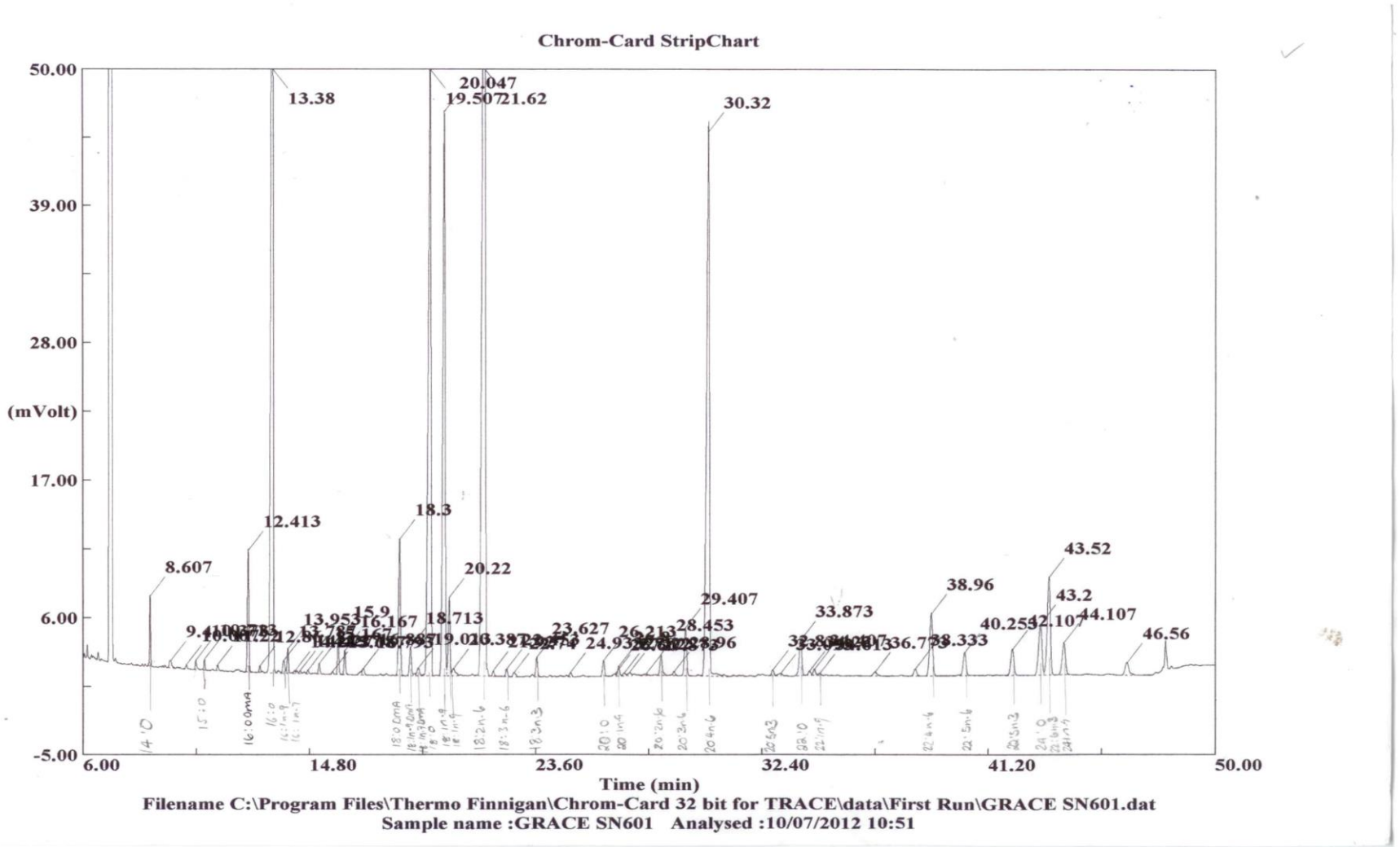
Appendices

Appendix 1: GLC Operating Conditions

```
TRACE GC Method: C:\Program Files\Thermo Finnigan\Chrom-Card 32 bit for TRACE\data\First Run\GLC
-------------------------------------------------
Acquisition Time
   Use Oven Run Time:                    Yes
Oven Method
   Initial Temperature (C):              50
   Initial Time (min):                   0.00
   Number of Ramps:                      4
   Rate #1 (deg/min):                    40.0
   Final Temperature #1 (C):             150
   Hold Time #1 (min):                   0.00
   Rate #2 (deg/min):                    2.0
   Final Temperature #2 (C):             200
   Hold Time #2 (min):                   0.00
   Rate #3 (deg/min):                    1.0
   Final Temperature #3 (C):             220
   Hold Time #3 (min):                   0.00
   Rate #4 (deg/min):                    40.0
   Final Temperature #4 (C):             240
   Hold Time #4 (min):                   5.00
   Post Run Temperature:                 Off
   Enable Cryogenics:                    Off
   Maximum Temperature (C):              260
   Prep Run Timeout (min):               10.00
   Equilibration Time (min):             0.50
-------------------------------------------------------------
Right SSL Method
   Base Temperature:                     On
   Base Temperature (C):                 250
   Mode:                                 Splitless
   Split Flow:                           Off
   Split Flow Flow (ml/min):             10
   Splitless Time (min):                 1.00
   Surge Pressure:                       Off
   Surge Pressure (kPa):                 3.00
   Surge Duration (min):                 0.00
   Constant Purge:                       On
   Stop Purge At: (min):                 0.00
Right Carrier Method
   Mode:                                 Constant Flow
   Initial Value:                        On
   Initial Value (ml/min):               1.50
   Initial Time:                         1.00
   Gas Saver:                            Off
   Gas Saver Flow (ml/min):              20
   Gas Saver Time:                       2.00
   Vacuum Compensation:                  Off
-------------------------------------------------------------
No Left Inlet
-------------------------------------------------------------
Right FID Method
   Base Temperature:                     On
   Base Temperature (C):                 250
   Flame:                                On
   Flameout Retry:                       On
   Ignition Threshold (mA):              1.0
   H2:                                   On
   H2 Flow (ml/min):                     35
   Air:                                  On
   Air Flow (ml/min):                    350
   Makeup Gas:                           Off
   Makeup Gas Flow (ml/min):             30
Right Signal Method
   Offset:                               Off
   Offset Value:                         0
   Autozero:                             Off
   Range:                                1
   Gain:                                 1
   Negative Polarity:                    Off
   Analog Filter:                        Off
-------------------------------------------------------------
No Left Detector
-------------------------------------------------------------
No Aux Detector
```

Appendix 2: An Example of Participant FAME Chromatogram



Appendix 3: An Example of Participant Result file with Retention Time and % Area

Chrom-Card Report

Page: 1 Sample: GRACE SN601 (GRACE SN601)

Method Name : FAME GLC 3
Method File : C:\...\Thermo Finnigan\Chrom-Card 32 bit for TRACE\data\First
Chromatogram : GRACE SN601
GC Method : GLC 3 FAME CURRENT Sampler Method: FAME GLC 3 Vial # 1
Operator ID : James Company Name : Nutrition Group
Analysed : 10/07/2012 10:51 Printed : 10/07/2012 11:44
Sample ID : GRACE SN601 (# 1) Channel : (FID)
Analysis Type : UnkNown (Area) Calc. Method : Area %

Peak Number (#)	Retention Time (min)	Area (.1*uV*sec)	Area % (%)	Component Name
1	8.607	142840	0.545	14:0
2	9.400	40713	0.155	
3	10.007	15652	0.060	
4	10.373	31612	0.121	~~15:0~~
5	10.713	27090	0.103	15:0
6	11.220	21536	0.082	
7	12.413	377413	1.441	16:0 DMA
8	12.860	18982	0.072	
9	13.380	4762842	18.187	16:0
10	13.787	59475	0.227	16:1n-7 9
11	13.953	80074	0.306	16:1n-7
12	14.213	11025	0.042	
13	14.427	12264	0.047	
14	14.727	13909	0.053	
15	15.167	38808	0.148	
16	15.687	11823	0.045	
17	15.900	105038	0.401	
18	16.167	82505	0.315	~~16:2~~
19	16.793	18586	0.071	
20	16.887	30878	0.118	~~16:3~~
21	18.300	560562	2.141	18:0 DMA
22	18.713	121962	0.466	~~16:4~~ 18:1n-9 DMA
23	19.013	43521	0.166	18:1n-7 DMA
24	19.507	3532464	13.489	18:0
25	20.047	2732460	10.434	18:1n-9
26	20.220	299989	1.146	18:1n-9 7
27	20.387	55719	0.213	~~18:1n-7~~
28	21.620	6335811	24.194	18:2n-6
29	21.927	20425	0.078	
30	22.453	30672	0.117	18:3n-6
31	22.740	24492	0.094	~~18:3n-6~~
32	23.627	78243	0.299	18:3n-3
33	24.933	20381	0.078	~~18:4n-3~~
34	26.213	64867	0.248	20:0
35	26.660	12565	0.048	
36	26.800	43760	0.167	20:1n-11 9
37	27.020	15633	0.060	~~20:1n-7~~
38	27.220	22759	0.087	
39	27.873	14771	0.056	

Appendix 3 continued

Page: 2 Sample: 1 GRACE SN601 (GRACE SN601)

Peak Number (#)	Retention Time (min)	Area (.1*uV*sec)	Area % (%)	Component Name
40	28.453	108129	0.413	20:2n-6
41	28.960	21661	0.083	
42	29.407	234285	0.895	20:3n-6
43	30.320	3346527	12.779	20:4n-6
44	32.800	30253	0.116	20:5n-3
45	33.093	14617	0.056	~~20:5n-3~~
46	33.873	213522	0.815	22:0
47	34.220	22410	0.086	~~22:0~~
48	34.407	32410	0.124	
49	34.613	11236	0.043	22:1n-11 9
50	36.773	31978	0.122	
51	38.333	42670	0.163	
52	38.960	404275	1.544	22:4n-6
53	40.253	146784	0.561	22:5n-6
54	42.107	180074	0.688	22:5n-3
55	43.200	379455	1.449	24:0
56	43.520	688708	2.630	~~24:0~~ 22:6n-3
57	44.107	234615	0.896	24:1n-9
58	46.560	110091	0.420	
		26187820		

Appendix 4: Informed Consent Forms

STUDY ID NUMBER:.....

The burden of malnutrition from birth in a cohort of children 7 to 9 years born to HIV negative and positive mothers recruited in a national PMTCT program.

Principal Investigator: Patience Kuona [MBChB, MMED Paediatrics (UZ)]
Co-Investigator(s): Marshall Munjoma
Phone number(s): 0772396029 / 0734612649

What you should know about this research study:

We give you this consent so that you may read about the purpose, risks, and benefits of this research study.

Routine care is based upon the best known treatment and is provided with the main goal of helping the individual patient. The main goal of research studies is to gain knowledge that may help future patients.

We cannot promise that this research will benefit your child. Just like regular care, this research can have side effects that can be serious or minor.

You have the right to refuse to allow your child to take part, or agree for your child to take part now and change your mind later.

Whatever you decide, it will not affect your child's regular care.

Please review this consent form carefully. Ask any questions before you make a decision.

Your choice to allow your child to participate is voluntary.

If you have questions concerning this study , you can contact: Dr Patience Kuona at 0772396029

PURPOSE:

Malnutrition and iron deficiency are major problems in our children. These problems affect children in the short and long term with a big effect on the involved child and the society. The levels of omega 3 fatty acids and selenium in our children are unknown. You are being asked to participate in a research study to find out how common malnutrition and iron deficiency are in a cohort of children who were born in a national PMTCT program in the Better Health for African Mothers and Children study. This research is also going to find out the levels of selenium and omega 3 fatty acids in the children who are now of school going age. The results of this study could assist us in knowing how children born in a national PMTCT program grow after birth and improve their care. Your child was selected because they were born in the BHAMAC study cohort. The research team is made up of doctors, nurses and scientists. The research is funded by The Letten Foundation.

PROCEDURES:

If you allow your child to take part in this research, a few questions will be asked about your child and their health. A doctor will examine your child. We will collect a few drops of blood from your child by a needle prick on the thumb and blot it on a filter paper. We will also draw 5ml of blood for measuring selenium levels. This will only be done once. We also request your permission to store unused blood from your child. Omega 3 test and selenium are not available in the country currently. We also request your permission to take some of the blood outside the country so these tests can be done. If your child is wasted, you will be asked to give them a therapeutic food known as plumpy nut. We will then follow up your child for at most 4 months to see if their nutritional status recovers. We will measure their weekly weight gain.

RISK AND DISCOMFORTS:

The blood testing may cause discomfort or a small bruise as with any other blood test. Plumpy nut is peanut based and may cause allergy to susceptible individuals.

BENEFITS AND/OR COMPENSATION:

The study result will help us understand the growth pattern and nutritional state of the children born in a national PMTCT program. It will define if there is significant iron deficiency in school going children and levels of omega 3 fatty acid levels and selenium. This could influence policy on supplementation of these nutrients. This will assist us in improving care to children born in PMTCT programs. By participating in this research you will have the advantage of knowing

your child's nutritional status. Illness that occur during the study period will be treated free of charge. Taking part in this study will not cost you. All tests will be done free of charge. We will not pay you to take part in the study but we will refund transport cost for the study visits.

CONFIDENTIALITY:

Information about you will be stored using a study number in safe paper and computer files. No-one will be able to access the information about you except the research team. No-one will be able to identify your child from the information we will collect. Dr Patience Kuona will be responsible for keeping your personal information confidential.

VOLUNTARY PARTICIPATION AND WITHDRAWAL:

Participation in this research is voluntary. If you decide not to participate, your decision will not affect your future relationship with this clinic, and University of Oslo. If you decide to participate you are free to withdraw your consent and assent at any time and discontinue participation without penalty.

In the event of injury, contact Dr Kuona at 0772396029

Before you sign this form, please ask any questions on any aspect of this study that is unclear to you. You may take as much time as necessary to think it over.

AUTHORIZATION

YOU ARE MAKING A DECISION WHETHER OR NOT TO ALLOW YOUR CHILD TO PARTICIPATE IN THIS STUDY. YOUR SIGNATURE INDICATES THAT YOU HAVE READ AND UNDERSTOOD THE INFORMATION PROVIDED ABOVE, HAVE HAD ALL YOUR QUESTIONS ANSWERED, AND HAVE DECIDED TO ALLOW YOUR CHILD TO PARTICIPATE.

The date you sign this document to enroll your child in this study, that is, today's date, MUST fall between the dates indicated on the approval stamp affixed to each page. These dates indicate that this form is valid when you enroll your child in the study but do not reflect how long your child may participate in the study. Each page of this Informed Consent Form is stamped to indicate the form's validity as approved by the MRCZ.

_____ _____

Name of Parent (please print) Date

_____ _____ AM

Signature of Parent or legally authorized representative Time PM

_____

Relationship to the Subject

_____

Signature of Witness Signature of Research Staff

(*Optional*)

YOU WILL BE GIVEN A COPY OF THIS CONSENT FORM TO KEEP.

If you have any questions concerning this study or consent form beyond those answered by the investigator, including questions about the research, your rights as a research subject or research-related injuries; or if you feel that you have been treated unfairly and would like to talk to someone other than a member of the research team, please feel free to contact the Medical Research Council of Zimbabwe on telephone 791792 or 791193.

Zimbabwe
MRCZ/B/222
EXPIRY DATE: 21/07/2012

The burden of malnutrition from birth in a cohort of children 7 to 9 years born to HIV negative and positive mothers recruited in a national PMTCT program.

Muongorori Mukuru: Patience Kuona [MBChB, MMED Paediatrics (UZ)]
Vabatsiri Paongororo: Marshall Munjoma
Runhare rwemuongorori: 0772396029 / 0734612649

ZVAMUNOFANIRA KUZIVA MAERERANO NEONGORORO INO:

Tinokupai gwaro iri rine tsananguro pamusoro peongororo ino kuti muverenge chinangwa, zvimhingamupini uye zvamunogona kuwana muongororo ino.

Kurapwa kunoita vanhu kwemazuva ese kunoitwa zvichitevedza nzira dzinozivikanwa kuti ndidzo dzepamusoro uye zvinoitwa nechinangwa chekubatsira murwere umwe neumwe. Chinangwa chikuru cheongororo dzinoitwa ndechekuwana ruzivo runogona kubatsira vana vanozouya munguva inotevera.

Hatikwanisi kuvimbisa kuti pane zvamuchawana kubva kuongororo ino. Sezvinongoitika mukurapwa kwemazuva ose ongororo ino inogona kuva nezvinokanganisika.

Munekodzero yekuramba kupinda muongororo, kana kubvuma kupinda muongororo iye zvino asi muchizogona kuramba pamberi.

Chero zvipi zvazvo zvamunenge masarudza kuita hazvikanganisi kurapwa kwenyu.

Tapota nyatso nzwisisai nhaurwa iyi, bvunzai chero mibvunzo musati maita sarudzo yezvamuri kuda.

Kupinda muongororo hakumanikidzwi, makasununguka kuzvisarudzira moga.

Kana mune mibvunzo pamusoro peongororo ino munogona kutaura na Patience Kuona (Tel: 0772396029 / 0734612649)

CHINANGWA CHEONGORORO:

Kusakura zvakanaka kwevana pamusana pekusawana chikafu chinovaka muviri chakakwana uye ayoni yakakwana mumuviri zvakatekeshera munyika yedu. Izvi zvinopa matambudziko akawanda kuvana vedu uye nyika yedu. Hatizivi kuti vana vedu vanowana chikafu cheOmega 3 fatty acids uye Selenium zvakakwana here muropa ravo. Saka imi murikukumbirwa kuti mwana wenyu apinde muchirongwa chekutarisa vana kuti tione uwandu hwevana vasiri kunyatsokura zvakanaka, kuwanda kweayoni,omega 3 fatty acid ne selenium muvana. Izvi zvichabatsira kuona kuti vana vakazvarwa muchirongwa chePMTCT vanokura zvakanaka here uye kuzivikanwa kwehuwandu hwechikafu cheSelenium, ayoni neOmega 3 fatty acids zvinobatsira pakuchengeteka kwevana vedu vachikura. Mwana wenyu asarudzwa nekuti akazvarwa muchirongwa cheBetter Health for African Mothers and children (BHAMAC). Vaongorori

vanosanganisira machiremba, vakoti uye vana mazvikokota vedzidzo. Chirongwa ichi chirikubatsirwa nemari neveLettern Foundation rinova boka ririkubatsira rezveutano.

ZVICHAITWA UYE NGUVA YAZVICHATORA:

Mukabvuma kuti mwana wenyu apinde muchirongwa muchabvunzwa mibvunzo pamusoro pehutano hwemwana wenyu. Chiremba vachamutarisa uye obva atorwa ropa pachigunwe cheruoko uye nepatsinga yeruoko. Izvi zvichangoitwa kamwe chete. Tinokumbirawo mvumo kuti rimwe ropa riende kunze kwenyika kunotariswa huwandu hweOmega 3 fatty acid neSelenium sezvo munyika yedu musina vanokwanisa kuita izvi. Mwana wenyu akawanikwa asiri kunyatsokura zvakanaka pakurema kwemuviri achapiwa plumpy nut chikafu chinoita kuti vana vakure zvakanaka toona kuti anowedzera uremu hwake here. Izvi zvichaitwa kusvikira awedzera uremu hwake kusvika panotarisirwa pazera rake kana kuti mwedzi mina chete. Anenge achiyerwa pasvondo rega rega.

MATAMBUDZIKO NEKUSAKADZIKANA:

Kutorwa ropa kunogona kukonzera kusagadzikana kwekanguva kadiki diki kana kakudunduvira sezvinongoita kutorwa ropa kwose. Plumpy nut inogadzirwa nedovi. Vana vasingapindirani nedovi vanogona kusapindirana nayo.

ZVAMUNOWANA KUBVA MUCHIRONGWA KANA MUBHADHARO:

Chirongwa chino chichatibatsira kuona kuti vana vakazvarwa muchirongwa chekudzivirira kutapurirwa kwevana chirwere cheHIV kubva kumubereki vanokura zvakanaka here. Tichaonawo huwandu hwevana vasina ayoni yakakwana mumuviri. Tichaonawo zvekare huwandu hwemaOmega 3 fatty acids neSelenium muvana vedu. Izvi zvichabatsira bazi rezveutano pakuisa zvinhanho zvekuchengetedza utano hwevana vedu munyika. Imi muchazivawo kuti mwana wenyu arikukura zvakanaka here. Mwana akarwara nguva yechirongwa acharapwa pachena. Ropa remwana richatariswa pachena. Imi muchapiwa mari yekufambisa kuuya kuchirongwa nekudzokera kumba kwenyu.

ZVAKAVANZIKA:

Zvamunenge matiudza zvose zvichachengetedzwa zvakasimba zvingave zvakanyorwa pamapepa kana mucomputer. Hapana anokwanisa kuwana zvamunenge matiudza kunze kwevaongorori vechirongwa nevabatsiri vavo uye hapana anokwanisa kukuzivai. Dr Kuona ndivo vachaita kuti zvamunenge matiudza zvirambe zvakavanzika.

KUZVISARUDZIRA KUPINDA MUCHIRONGWA KANA KUBUDA:

Munozvisarudzira kupinda muchirongwa chino pasina kumanikidzwa. Mukasarudza kusapinda muchirongwa hazvisokanganisi ukamahwenyu nekiriniki ino, vashandi vayo, zvimwe zvipatara zvainoshanda nazvo uye neUniversity of Oslo. Mukasarudza kupinda muchirongwa makasunungukunga kushandura pfungwa dzenyu muchizoramba kupa mvumo yenyu uye kubuda muchirongwa chero nguva zvayo pasina kuripiswa.

Usati wabvuma nekuisa runyoro rwako pafomu iri, tapota bvunza chero mibvunzo maererano neongororo ino pane zvisina kujeka kwauri. Nyatsotora nguva yakakwana zvakakodzera, kuti ufunge nezvazvo.

Peji rekupa mvumo yako rakadhindwa kuratidza kuti richiri kushanda sezvinobvumirwa neMRCZ rinova bato rinoona nezvekupa mvumo yekuitwa kweongororo dzine chekuita neutano muno muZimbabwe. Zuva raunoisa runyoro rwako pafomu rino kuti upinde muongororo kureva zuva ranhasi rinofanira kuva pakati pamazuva akataridzwa pachidhindo chiri papeji rimwe nerimwe. Mazuva aya anotaridza kuti fomu iri richiri kushanda paunopinda muongororo asi hazviratidzi nguva yamuchange muri muongororo.

Ndaverenga ndikanzwisisa gwaro iri maerarano neongororo uye ndanzwisisa tsanangudzo yandapihwa. Ndanzwisisa zvinotarisirwa kubva kwandiri uye zvichaitika kumwana wangu kana ndikapinda muongororo. Mibvunzo yangu maererano neongororo ino yapindurwa nevarikuita ongororo.

Ndanzwisisa kuti ndinokwanisa kubuda muongororo chero nguva zvayo ndisingapi chikonzero uye zvisingakanganisi marapirwo angu mazuva ose.

_____ _____

Zita Remubereki (please print) Zuva

_____ _____ _____AM

Runyoro rwemubereki/ Muchengeti wemwana Nguva PM

_____

Ukama hwangu nemwana arikupinda muongororo

_____

Runyoro rwemushandi weongororo

MUCHAPIHWA MAGWARO ENYU EKUCHENGETA AMAPA MVUMO KUTI MWANA APINDE MUCHIRONGWA.

Kana muine mibvunzo maererano neongororo ino kana fomu rekupa mvumo yekuita ongororo inodarika yapindurwa neari kuita ongororo kusanganisira mibvunzo maererano neongororo, kodzero dzenyu semunhu ari muongororo, kukuvara kune chekuita neongororo kana kuti muchiona sekuti hamuna kubatwa zvakanaka uye kana muchida kutaura nemumwe munhu asiri kuita ongororo makasununguka kubata veMedical Research Council of Zimbabwe panhare dzinoti 791792 kana 791193.

ASSENT FORM

My name is Patience Kuona. I am doing a research study to describe the growth pattern and nutritional status of children who were born in a national program to prevent mother to child transmission of HIV infection. I am going to ask you and your caregiver some questions, examine you and take some blood from you. You may be given a nutritional supplement called plumpynut depending on your nutritional status. You are allowed to refuse to take part and we will continue treating you as usual without prejudicing you. This research is going to assist in describing the growth, nutritional status, iron status, levels of omega 3 fatty acid levels and selenium in children born in national PMTCT programs.

I have discussed this clinical research study with the child using language which is understandable and appropriate. I believe I have fully informed this participant of the nature of the study and its possible risks and benefits. I believe the participant understood this explanation and assented to participate in this study

Ini ndinonzi Patience Kuona. Ndirikutarisa vana vakazvarwa muchirongwa chePMTCT ndakanangana nekutarisisa kukura kwavo. Izvi zvichabatsira kuti tizive kuti ropa ravo rine maomega 3 fatty acids, selenium uye iron yakakwana here. Zvichabatsira kuchengetwa kwevana ava pakukura kwavo.Ini ndichakubvunza mibvunzo nemubereki wako, ndokutarisa uye nekukutora ropa richanotariswa kulaboratory. Wakasununguka kuramba kutariswa kunyangwe wanga wambobvuma hako isu tinoramba tichikurapa semazuva ose.

_____	_____
Name (Zita rako)	Date (Zuva)
_____	_____
Signature of Research Staff	Date

SPECIMEN STORAGE INFORMED CONSENT FORM

Chitungwiza (St Mary's and Seke North Clinics)

The burden of malnutrition from birth in 7-9 year old children born to mothers recruited from a PMTCT program in Zimbabwe

SPECIMEN STORAGE AND SHIPMENT

Consent Version

(English)

PRINCIPAL INVESTIGATOR: Patience Kuona

PHONE: 0772396029

INTRODUCTION:

You have decided to take part in the investigational research study named above, sponsored by the Lettern Foundation. While in this study, blood will be collected from your child. You are kindly being asked to agree to the storage of these samples for use during the study and after the study has ended. We are also asking to ship these samples to another laboratory outside Zimbabwe. This consent form gives you information about the collection, storage, and use of these samples. These samples may be useful for future research. The study staff will talk to you about this information. Please ask if you have any questions. You will be asked to sign or make your mark on this form to indicate whether you agree to have your child's samples stored and tested. You will be offered a copy of this form to keep.

YOUR PARTICIPATION IS VOLUNTARY:

Allowing your samples to be stored is completely voluntary. You may decide not to have any samples stored other than what is needed to complete this study and still be in this research study or any future study.

Even if you decide now that your samples can be stored for future research, you may change your mind at any time. If this happens, you must tell the study staff that you have changed your mind. If you decide not to have your samples stored or used for future research, they will be destroyed at the end of the study.

PURPOSE:

The specific research to be done on the samples from your child include measuring your haemoglobin, selenium, omega 3 fatty acid, serum ferritin and serum transferrin receptor levels. Your child's samples

will only be used for these tests only. No other kinds of tests will be done by anyone on your child's stored specimens without first explaining the test to you and obtaining your permission.

The study researchers do not plan to contact you or your child's regular doctor with any results from tests done on the stored samples. This is because research tests are often done using experimental procedures, so the results may not help for making decisions on managing your health. In the case that a specific test result gives important information about your health, the researchers will tell the study staff and the study staff will try to contact you. If you wish to be contacted with this type of test result, you must give the study staff any change to your contact information. If your child has a regular doctor and you want the study staff to tell this doctor the test results, you must give the study staff the doctor's contact information.

Your child's samples will not be sold or used directly to produce commercial products.

Research studies using your samples will be reviewed by the Norwegian Ethics Board and a special committee at the Medical Research Council of Zimbabwe.

PROCEDURES:

We will collect a few drops of blood from your child by a needle prick on the thumb and blot it on a filter paper. We will also draw 5ml of blood for measuring selenium levels. This will only be done once. Each time your child's blood is drawn, up to 2mL (which is about half a teaspoon) of the sample may be stored.

Your blood will be stored safely and securely in a storage facility at the University of Zimbabwe. Only the people who work at the facility and approved researchers will have access to your child's samples. The people who work at the facility will not have any information that identifies your child. The approved researchers may be given information about your child such as their age and sex, but they will not be given the child's name or any other information that identifies your child. Your child's samples may be shipped to approved researchers who work outside of Zimbabwe.

There is no time limit on how long your samples will be stored.

RISKS and/or DISCOMFORTS:

There are few risks related to storing your samples. When tests are done on the stored samples there is a rare but possible risk to your privacy. It is possible that if others found out information about your child that is learned from tests (such as information about your genes) it could cause you problems with your family (having a family member learn about a disease that may be passed on in families or learning who the true parent of a child is).

POTENTIAL BENEFITS:

There are no direct benefits to you from having your samples stored. You and others could benefit in the future from research done on your blood.

CONFIDENTIALITY:

To keep your information private, your child's samples will be labelled with a code that can only be traced back to your study clinic. Your child's name, where they live, and other personal information will be protected by the study clinic. When researchers are given your child's stored samples, they will not be given your personal information. The results of future tests will not be included in your child's health records. Every effort will be made to keep your child's personal information confidential, but we cannot guarantee absolute confidentiality. Your child's personal information may be disclosed if required by law.

Efforts will be made to keep your child's study records and test results confidential to the extent permitted by law. However, we cannot guarantee absolute confidentiality. Your child will be identified by a code, and personal information from their records will not be released without your written permission. Any publication of this study will not use your child's name or identify them personally. However, your child's records may be reviewed by the Norwegian Ethics Board, the Medical Research Council of Zimbabwe and the study staff.

In addition to the efforts made by the study staff to keep your child's personal information confidential, an Oath of Confidentiality was signed by all our staff working in this study. This Oath requires study staff not to tell people who are not connected with this study, information about your child or other study participants or any other information related to the study.

PROBLEMS OR QUESTIONS:

For questions about the storage of your samples, contact:

Patience Kuona 0772396029

For questions about your rights as a research subject, contact:

The National Coordinator

Medical Research Council of Zimbabwe

National Institute of Health Research

Cnr Mazoe Street/ Josiah Tongogara Avenue

Harare

Ph: +263 4 791792, 791193

Cell: +263 912 433 166

SIGNATURE PAGE

CONSENT FOR SPECIMEN STORAGE AND SHIPMENT

Please carefully read the statements below (or have them read to you) and think about your choice. No matter what you decide it will not affect whether you can be in the research study, or your routine health care.

_____ I agree to have samples of my child's blood shipped outside the country, stored and used for future testing related to nutrition of children.

_____ I agree to have samples of my child's blood shipped outside the country but do not want it to be stored and used for future testing related to nutrition of children.

_____ I do not agree to have samples of my child's blood shipped outside the country, stored and used for future testing related to nutrition in children.

_____ _____

Participant Caregiver/ Parent Name (print) Participant Caregiver Signature or Mark and Date

_____ _____

Study Staff Conducting Consent Discussion (print) Study Staff Signature and Date

_____ _____

Witness Name (print) (As appropriate) Witness Signature and Date

CHIBVUMIRANO CHEMACHENGETERWO ACHAITWA ROPA RICHATORWA MUMUVIRI

KUCHAITIRWA ONGORORO
Chitungwiza(St Mary's and Seke North Clinics)

The burden of malnutrition from birth in 7-9 year old children born to mothers recruited from a PMTCT program in Zimbabwe

KUCHENGETWA UYE KUTAKURWA KWEROPA ZVINENGE ZVATORWA MUMUVIRI
Mhando yechibvumirano

MUONGORORI MUKURU: Patience Kuona

RUNHARE RWEMUONGORORI: 0772396029 / 0734612649

KUTANGA

Mabvuma kupinda muchirongwa chekuongorora kukura zvakanaka kwevana varipakati pemakore manomwe nemapfumbamwe. Chirongwa ichi chiri kubatsirwa neveLettern foundation. Mwana wenyu achatorwa ropa. Muri kukumbirwa mvumo yekuti ropa rake rinenge rasara muongorooro dzekutarisa huwandu hweropa rake,iyoni(ferritn ne transferring receptor level) selenium uye omega 3 fatty acid richengetwe. Ropa iri rinogona kuzoshandiswa munedzimwe ongororo ramangwana Tinokumbirawo mvumo kuti rimwe reropa rake rigoenda kunotariswa kunze kwenyika sezvo tisati tavanemukana wekugona kuongorora huwandu hweselenium neomega 3 fatty acid. Makasununguka kubvunza mibvunzo yenyu kuvaongorori. Muchakumbirwa kuti muise runyoro rwenyu pabepa kutaridza kuti mabvumirana kana kupokana nekuti ropa remwana wenyu richengetwe uye rinoongororwa kunze kwenyika. Muchapiwa magwaro aya enyu ekuchengeta.

ZVIRI KWAMURI KUTORA DANHO MUONGORORO INO:

Makasununguka kupa mvumo kana kuramba kuti ropa remwana wenyu rinenge rasara paongororo ino richengetwe. Izvi hazvikutadzisei kuti munge muri muchirongwa ichi. Mukazofunga kechipiri makasununguka kupindura pfungwa dzenyu maererano nekuchengetwa kweropa remwana wenyu kunyange chirongwa chatanga.

DONZVO REONGORORO INO:

Ropa remwana wenyu richaongororwa kutarisa huwandu hwaro, selenium, omega 3 fatty acid, ferritin neserum transferrin receptor. Hapana dzimwe ongororo dzichaitwa pariri imi musina kupa mvumo. Zvatinowana muongororo yeropa remwana hazvizoshambadzirwa kwamuri kana chiremba wemwana wenyu. Kana muchida kuziva zvichawanikwa zvinenge zvinechokuita neutano hwemwana wenyu tinogona kuzivisa chiremba wemwana wenyu. Imi munofanira kutipa runhare kana kero yachiremba wemwana kuti tizovazivisa.

Ropa remwana wenyu haritengesewi kana kushandiswa kugadzira zvingangotengeswa.

Ongororo iyi iri kushandisa ropa remwana wenyu yakaongororwa neNorwergian Ethics Board uye veMedical Research Council yeZimbabwe.

ZVICHAITWA MUONGORORO:

Tichatora ropa shoma shoma kubvapachigunwe chemwana roiswa pakapepa. Tinotora ropa rimwe rinozadza zvipunu zvidiki zviviri rekuzotarisa uwandu hweselenium mumwana. Izvi zvichaitwa kamwechete. Panogona kuzosara ropa ringangosvika chikamu chechipunu chidiki ringangochengetwa.

Ropa richachengetedzwa zvakanaka kuUniversity of Zimbabwe. Vashandi vemuongororo chete ndivovanenge vachiziva nekusvika pakachengetedzwa ropa remwana. Vaongorori vanogona kuziviswa nezvemwana wenyu asi havaziviswi zita rake, kwaanogara uye zvimwe zvingaita kuti vamuzive. Ropa remwana rinogona kuendeswa kunevamwe vaongorori kunze kwenyika yeZimbabwe.

Ropa richachengetwa kwenguva yakareba.

ZVINGANGOITA KUTI MUSAGADZIKANE:

Kuchengetwa kweropa hakuna njodzi dzakawanda Pane zvinhu zvishoma zvingangoita kuti musazogadzikana maererano nemachengeterwo achaitwa ropa remwana. Patinoita ongororo yeropa pangangove nekakusachengetedzeka kezvinobuda muongororo. Zvingangoitika ndezvekuti vamwe vanhu vakaziva zvinenge zvabuda muongororo yeropa renyu (zvakaita sezvinobuda paongororo yemavakirwo emuviri anotevedza dzinza), zvinokwanisa kuunza matambudziko mumhuri(semuenzaniso munhu wemumhuri yenyu anokwanisa kuziva kuti ropa renyu rine hutachiona hunofamba nedzinza kana kuzoziva kuti mubereki chaiye wemwana ndiani).

POTENTIAL BENEFITS:

Hapana zvamunowana pakuchengetwa kweropa asi dzimwe ongororo dzinoitwa pamberi dzinogona kubatsira kuchengetedza utano hwevana.

KUCHENGETA TSINDIDZO:

Ropa remwana richapiwa nhamba inozivikanwa nevaongorori vechirongwa chino chete. Zvinoita kuti mwana azivikanwe sezita rake zvinochengetedzwa zvakabatisisa nevaongorori nekuti hatidi kuti vamwe vanhu vazvizive. Asi kana mutemo wenyika ukati zvizivikanwe ndipopatinozviburitsa chete. Tichaedza patinogonesesa kuti zvichengeteke zvisazivikanwa nevamwe vasiri vashandi vemuongororo. Zvinoita kuti

mwana azivikanwe hazvizoparidzwa pasina mvumo yenyu. Zvichabuda muchirongwa zvichashambadzwa pasina mazita evana. VeNorwergian Ethics Board uye veMedical research council yeZimbabwe vangangoda kutarisa basa redu. Avandivovega vanobvumirwa nemutemo kutarisa zvakanyorwa pamusana pemwana wenyu. Vashandi vemuongororo vakatsidza kuchengetedza zvinenge zvaonekwa pamwana wenyu vasingazvishambadziri.

Kana muine mibvunzo maererano nekuchengetedzwa kweropa richatorwa mwana wenyu munokwanisa kutaura na
Patience Kuona 0772396029

Mibvunzo pamusoro pekodzero dzemwana wenyu muchirongwa inoenda kunevanotevera:
The National Coordinator
Medical Research Council of Zimbabwe
National Institute of Health Research
Cnr Mazoe Street/ Josiah Tongogara Avenue
Harare
Ph: +263 4 791792, 791193
Cel : +263 912 433 166

SIGNATURE PAGE

MVUMO YEKUTI ROPA REMWANA RICHENGETWE NEKUENDESWA KUNZE KWENYIKA

Ndapota nyatsoverengai muteererese nekunzwisisa zvinotevera mugosarudza zvamunoda. Makasununguka kusarudza zvamunoda imi muchiramba muri muchirongwa uye hazvikanganise kurapwa kwenyu mazuva ose.

_____ Ndinobvuma kuti ropa remwana wangu rinoongororwa kunze kwenyika uye kuti richengetwe kuitira ongororo dzemangwana.

_____Ndinobvuma kuti ropa remwana wangu riendeswe kunze kwenyika kunoongororwa asi handidi kuti richengetwe kuitira ongororo dzemangwana.

_____Handibvumi kuti ropa remwana wangu riendeswe kunze kwenyika kana kuchengetwa kuitira ongororo dzemangwana.

_____	_____
Zita remebereki/muchengeti wemwana (PRINT)	Runyoro rwemubereki/muchengeti wemwana nezuva
_____	_____
Muongorori ataura nemubereki (PRINT)	Runyoro rwemuongorori nezuva
_____	_____
Zita remuwitness (PRINT)	Runyoro rwemuwitness nezuva

Appendix 5: Approval Letters

UNIVERSITY OF ZIMBABWE

COLLEGE OF HEALTH SCIENCES

MEMORANDUM

FROM: Chairman, Joint Research Ethics Committee **DATE**: 13 Sept 2012

TO: **Grace Mashavave,** Department of Chemical Pathology **EXT:** 2241/2242

c.c: Chairman, Department of Chemical Pathology

RE: **DETERMINATION OF OMEGA-3 LONG CHAIN POLY UNSATURATED FATTY ACID LEVELS IN CHILDREN AGED 7 TO 9 YEARS IN ZIMBABWE USING DRIED BLOOD SPOTS – JREC/170/12**

Thank you for your application with the above mentioned title seeking approval from the Joint Parirenyatwa Hospital and College of Health Sciences Research Committee (JREC). The Committee has successfully evaluated and discussed the material you supplied.

It was agreed that your application be approved as a research project which is ethically sound.

Wishing you an enjoyable and fruitful research.

Approval Date: 13th September 2012

Expiry Date: 12th September 2013

Professor MM Chidzonga

Telephone: 791792/791193
Telefax: (263) - 4 - 790715
E-mail: mrc.zimbabwe@yahoo.com
Website: http://www.mrcz.org.zw

Medical Research Council of Zimbabwe
Josiah Tongogara / Mazoe Street
P. O. Box CY 573
Causeway
Harare

APPROVAL LETTER

Ref: MRCZ/B/359 **14 January, 2013**

Grace Mashavave
University of Stirling
Scotland

RE:- Determination of OMEGA-3 Long Chain Polyunsaturated Fatty Acid Levels in Children Aged 7 to 9 Years in Zimbabwe Using Dried Blood Spots.

Thank you for the above titled proposal that you submitted to the Medical Research Council of Zimbabwe (MRCZ) for review. Please be advised that the Medical Research Council of Zimbabwe has **reviewed** and **approved** your application to conduct the above titled study. This is based on the following documents that were submitted to the MRCZ for review

a) Research Protocol
b) Research Protocol Summary
c) Questionnaire
d) Assent Forms (English and Shona)
e) Specimen Storage Informed Consent Forms (Engish and Shona)

- **APPROVAL NUMBER : MRCZ/B/359**

This number should be used on all correspondence, consent forms and documents as appropriate.

- **TYPE OF MEETING : Expedited**
- **APPROVAL DATE : 14 January 2013**
- **EXPIRY DATE : 13 January 2014**
- After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the MRCZ Website should be submitted three months before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING**: All serious problems having to do with subject safety must be reported to the Institutional Ethical Review Committee (IERC) as well as the MRCZ within 3 working days using standard forms obtainable from the MRCZ Website.
- **MODIFICATIONS**: Prior MRCZ and IERC approval using standard forms obtainable from the MRCZ Website is required before implementing any changes in the Protocol (including changes in the consent documents).
- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the MRCZ using standard forms obtainable from the MRCZ Website.
- **QUESTIONS**: Please contact the MRCZ on Telephone No. (04) 791792, 791193 or by e-mail on mrc.zimbabwe@yahoo.com or mrcz@mrcz.org.zw
- **Other**
- Please be reminded to send in copies of your research results for our records as well as for Health Research Database.
- You're also encouraged to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study.

Yours Faithfully

.....

MRCZ SECRETARIAT
FOR CHAIRPERSON
MEDICAL RESEARCH COUNCIL OF ZIMBABWE

MEDICAL RESEARCH COUNCIL OF ZIMBABWE
14 JAN 2013
APPROVED
P. O. BOX CY 573 CAUSEWAY, HARARE

PROMOTING THE ETHICAL CONDUCT OF HEALTH RESEARCH

G.P. & S. 42914-V.

FORM RA.3 Research Act, Section 26A.

№ 01202

RESEARCH ACT, 1986

RESEARCH COUNCIL OF ZIMBABWE

CERTIFICATE OF REGISTRATION

Name PATIENCE KUONA

Nationality: ZIMBABWEAN Passport No.: BN583581

Institution of Affiliation in Zimbabwe: UNIVERSITY OF ZIMBABWE
COLLEGE OF HEALTH SCIENCES
AVONDALE, HARARE

Residential Address in Zimbabwe: 2 TROWBRIDGE ROAD,
MABELREIGN
HARARE

The bearer has been registered to conduct research in the field of PAEDIATRICS

in terms of section 26A of the Research Act, 1986.

Expiry date: 15 MARCH 2013

Signature of Bearer

Issuing Officer
Research Council of Zimbabwe

Date: 5 APR 2012

RESEARCH COUNCIL OF ZIMBABWE
CABINET OFFICE
P.O. BOX CY 294
CAUSEWAY, HARARE

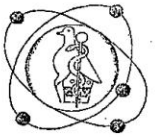
This receipt is not valid unless it is stamped

TITLE: THE BURDEN OF MALNUTRITION FROM BIRTH IN 7-9 YEAR OLD CHILDREN BORN TO MOTHERS RECRUITED FROM A PREVENTION OF MOTHER-TO-CHILD TRANSMISSION OF HIV/AIDS PROGRAM IN ZIMBABWE: MRC2/B/222

BIOLOGICAL SPECIMENS FOR SHIPMENT.

400 DRIED BLOOD SPOTS FOR OMEGA 3 FATTY ACID DETERMINATION.

Telephone: 791792/791193
Telefax: (263) - 4 - 790715
E-mail: mrcz@mrczimshared.co.zw
Website: http://www.mrcz.org.zw



Medical Research Council of Zimbabwe
Josiah Tongogara / Mazoe Street
P. O. Box CY 573
Causeway
Harare

MRCZ APPROVAL LETTER

Ref: MRCZ/B/222 22 July 2011

Dr Patience Kuona
College of Health Sciences
Department of paediatrics and Child Health
University of Zimbabwe
Zimbabwe

RE: The Burden of malnutrition from Birth in 7-9 Year Old Children born to Mothers Recruited from a PMTCT Program in Zimbabwe

Thank you for the above titled proposal that you submitted to the Medical Research Council of Zimbabwe (MRCZ) for review. Please be advised that the Medical Research Council of Zimbabwe has **reviewed** and **approved** your application to conduct the above titled study. This is based on the following documents that were submitted to the MRCZ for review:

a) Study protocol.

- **APPROVAL NUMBER : MRCZ/B/222**

This number should be used on all correspondence, consent forms and documents as appropriate.

- **APPROVAL EFECTIVE DATE : 22 July 2011**
- **EXPIRATION DATE : 21 July 2012**
- **TYPE OF MEETING : Expedited**

After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the MRCZ Offices should be submitted one month before the expiration date for continuing review.

- **SERIOUS ADVERSE EVENT REPORTING:** All serious problems having to do with subject safety must be reported to the Institutional Ethical Review Committee (IERC) as well as the MRCZ within 3 working days using standard forms obtainable from the MRCZ Offices.
- **MODIFICATIONS:** Prior MRCZ and IERC approval using standard forms obtainable from the MRCZ Offices is required before implementing any changes in the Protocol (including changes in the consent documents).
- **TERMINATION OF STUDY:** On termination of a study, a report has to be submitted to the MRCZ using standard forms obtainable from the MRCZ Offices.
- **QUESTIONS:** Please contact the MRCZ on Telephone No. (04) 791792, 791193 or by e-mail on mrcz@mrczimshared.co.zw.
- **Other**
- Please be reminded to send in copies of your research results for our records as well as for Health Research Database.
- You're also encouraged to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study.

Yours Faithfully

.....

MRCZ SECRETARIAT
FOR CHAIRPERSON
MEDICAL RESEARCH COUNCIL OF ZIMBABWE

MEDICAL RESEARCH COUNCIL OF ZIMBABWE
22 JUL 2011
APPROVED
P. O. BOX CY 573 CAUSEWAY, HARARE

PROMOTING THE ETHICAL CONDUCT OF HEALTH RESEARCH
Registered with the USA Office for Human Research Protections (OHRP) as an International IRB (Number IRB00002409 IORG0001913)

Telephone: 24207/8, 24571

Telegraphic Address:
"PROVMED, MARONDERA"
Fax: 23967

ZIMBABWE

Reference:

MINISTRY OF HEALTH AND
CHILD WELFARE
PROVINCIAL MEDICAL DIRECTOR
(MASHONALAND EAST)
P.O.BOX 10
MARONDERA
ZIMBABWE

Ref:

14 December 2011

To whom it may concern

RE: THE BURDEN OF MALNUTRITION FROM BIRTH IN 7-9 YEAR OLD CHILDREN BORN TO MOTHERS RECRUITED FROM A PMTCT PROGRAM IN ZIMBABWE: MRCZ/B/222

Dr. Kuona has been authorised to carry out the above named research at Epworth Poly Clinic.

MINISTRY OF HEALTH
P.M.D. MASHONALAND EAST
2011 -12- 14
P.O. BOX 10
MARONDERA

Dr. S.T Zizhou
PROVINCIAL MEDICAL DIRECTOR – MASHONALAND EAST

/em

2 Trowbridge Road

Mabelreign

Harare

22 March 2011

Director of Health Services

Chitungwiza Local Board

RE: APPLICATION FOR PERMISSION TO CARRY OUT RESEARCH AT SEKE NORTH AND ST MARY'S CLINIC.

Dear sir /madam

I am applying for permission to do a study to measure the prevalence of malnutrition in the children who were born in The Better Health for African Mothers and Children Study (BHAMAC). I am a paediatrician and lecturer at the University of Zimbabwe. I intend to pursue a PhD program with the University of Oslo. Please find attached the copies of my proposal for your perusal.

I hope you will look at my application with favour.

Yours sincerely

Patience

Dr Patience Kuona

MBChB

MMED Paediatrics (UZ)

Approved

CHITUNGWIZA MUNICIPALITY
DIRECTOR OF HEALTH SERVICES
25 MAR 2011
P.O. BOX 7050, ZENGEZA
CHITUNGWIZA, ZIMBABWE