Long-chain omega-3 polyunsaturated fatty acids in relation to gut integrity, growth and cognitive development of rural African children
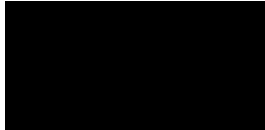
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Thesis submitted for the degree of Doctor of Philosophy at the London School of Hygiene and Tropical Medicine

AUTHOR'S DECLARATION

I have read and understood the School's definition of plagiarism and cheating given in the Research Degrees Handbook. I declare that this thesis is my own work, and that I have acknowledged all results and quotations from the published or unpublished work of other people.

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Date: 15/02/2010

Full name: Liandré Frances van der Merwe

For Mabinta and Daboe

and their children

ABSTRACT

Background and rationale: Weaning foods fed to infants in rural Gambia are often contaminated, resulting in infections which contribute to initiating a persistent inflammation of the gut. This enteropathy, which causes intestinal damage and malabsorption, is strongly associated with the high degree of growth faltering seen in Gambian infants. There is evidence that supplementary omega-3 long-chain polyunsaturated fatty acids (n-3 LCPs) might ameliorate this damage by reducing gastro-intestinal inflammation. Additionally, n-3 LCPs have been shown to benefit mental development and problem-solving ability in infants, but this has not yet been tested in an African population.

Methods: A randomised, double-blind, controlled trial (500mg combined n-3 LCPs per day for six months) was conducted in a population of rural African infants aged 3 months – 9 months. The primary outcomes were infant anthropometric indicators and gut integrity (measured by urinary lactulose-mannitol ratios). Plasma fatty acid status (plasma fatty acid profiles), cognitive development (Willatts Test and an attention assessment at 12 months of age), intestinal mucosal inflammation (faecal calprotectin), and daily morbidities were the secondary outcome measures.

Results: One-hundred and seventy-two Gambian infants completed the trial. Except for an increase in mid-upper-arm circumference z-scores in the intervention group (95% CI: 0.06,0.56; p=0.017), no significant differences between treatment groups were detected for growth and lactulose-mannitol ratios at 9 months. At 12 months mid-upper-arm circumference remained greater in the intervention group, and significant increases in skinfold thicknesses were detected (p≤0.022 for all). Supplementation resulted in a significant increase in plasma n-3 LCP levels (p<0.001) and decrease in n-6 LCP:n-3 LCP ratios (p<0.001). Plasma n-6 fatty acid levels were not affected. No difference was detected for the other secondary outcomes.

Conclusion: Fish oil supplementation proved safe and successfully increased plasma n-3 fatty acid status, but the results of this trial do not support the use of supplementary n-3 LCPs in young, breast-fed, rural Gambian infants for improving overall growth performance, intestinal integrity, and cognitive development, or reducing intestinal and systemic inflammation.

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ACKNOWLDEGEMENTS

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GLOSSARY OF ABBREVIATIONS

AA	Arachidonic acid
AGP	α1-Acid glycoprotein
CRP	C-reactive protein
CV	Coefficient of variation
DHA	Docosahexaenoic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FA	Fatty acid
GC	Gas chromatography
GCP	Good clinical practice
HC	Head circumference
HDN	Haemorrhagic disease of the newborn
IBD	Inflammatory bowel disease
PI	Principal investigator
LCP	Long-chain polyunsaturated fatty acid
PUFA	Polyunsaturated fatty acid
LMR	Lactulose:mannitol ratio
mo	month
MRC	Medical Research Council
MUAC	Mid-upper arm circumference
n	Omega; sample size; number
SAE	Serious adverse event
SD	Standard deviation
TSM	Trial safety monitor

CHAPTER 1:   BACKGROUND AND INTRODUCTION

1.1   Growth faltering and intestinal inflammation in rural Gambian infants

1.1.1 Background

1.1.1.1   The Gambia

The Republic of The Gambia is a long, narrow country in West Africa with an area of 10 380km^2. It is bordered in the North, East and South by Senegal and on the West by the Atlantic Ocean. Its long narrow shape, seen in Figure 1, corresponds to the meandering of the River Gambia, around which the country is formed. River flood plains, flanked by low hills, form the predominant terrain. The climate is tropical with two main seasons: a hot rainy season (June to October) and a cooler, dry season (November to May).

The nation achieved independence from the United Kingdom in 1962, and became a republic within the Commonwealth five years later. According to a United Nations 2007 estimate, the country has 1.7 million inhabitants, most of who belong to the ethnic tribes of Mandinka (42%), Fula (18%), Wolof (16%), Jola (10%), and Sarahule (9%).

Sixty-three percent of inhabitants live in rural areas, where 23% of people have no access to safe drinking water. Life expectancy at birth is 57 years, and the infant mortality rate is 97/1000 live births (2005 est. (1)). Seventy percent of the labour force is employed by the agricultural sector, which contributes around 30% to the (nominal) gross domestic profit of $808 million. Groundnut production, followed by other crops, livestock farming, fishing and forestry, make up the Agricultural Sector (1).

Further country details are provided in Box 1.
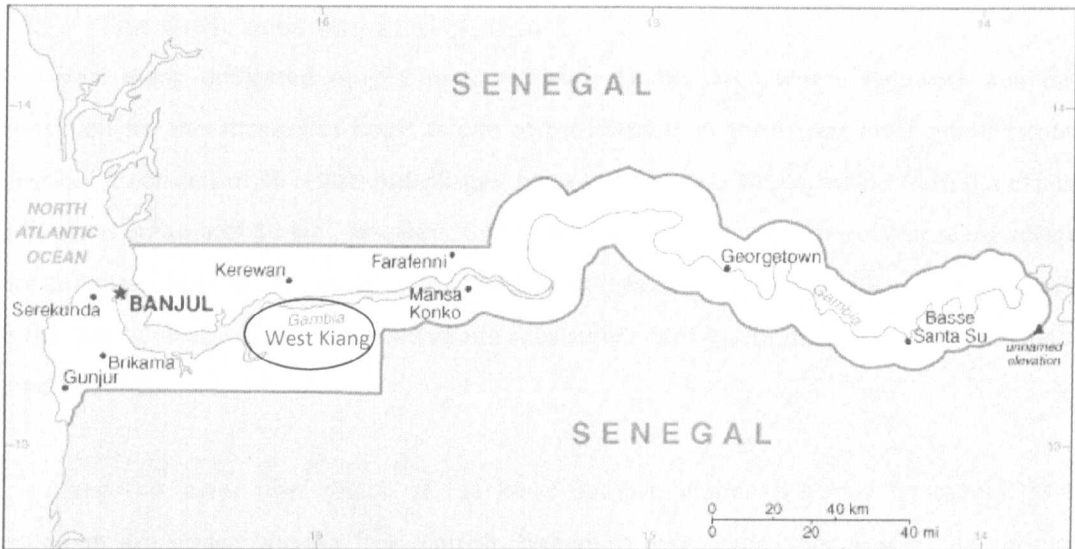
Figure 1: Map of The Gambia, showing the West Kiang area and the River Gambia

Box 1: Country profile

Government:	Democratic
Capital:	Banjul
Area:	10 380; 11.5% of which water
Population density:	153.5/km^2
Religion:	90% Muslim
GDP (nominal) per capita:	$495
GDP (purchasing power parity):	$1 389
Population growth rate:	2.4%
Life expectancy at birth:	57 years
Total fertility rate:	4.76 children born/woman
Birth rate:	34.9 births/1,000 population
Death rate:	11.3 deaths/1,000 population
Net migration rate:	2.97 migrant(s)/1,000 population
Percent living below the international poverty line:	roughly 30%
Adult literacy rate:	37.8%.

1.1.2 The study area and field station

West Kiang (indicated on the map in Figure 1), the area where fieldwork and data collection for this thesis was done, is one of the districts of the Lower River administrative division. It consists of 36 registered villages, lies approximately 145km inland from the capital, and spans an area of 80 km^2. Roughly 16 000 individuals of the registered West Kiang villages are surveyed regularly by means of the Kiang West Demographic Surveillance System. Keneba is the largest village in this rural area where subsistence farming (primarily rice and groundnut) predominates.

Along the lower river region of the River Gambia, marine fish may be caught. Most abundant are bonga (an oily fish), catfish, threadfins, barracuda, tongue-sole and shrimps. Most of the shrimp and sole, as well as large fish such as barracuda, are taken further down to the coast where they are purchased by industrial fishing companies. The smaller fish are sold in the villages, often dried and salted. These are frequently added as an ingredient to sauces, or sometimes served fried in small amounts.

The staple food is rice. It is eaten with sauces made of groundnut, leaves, and small quantities of vegetables (when available), meats and fish.

The Medical Research Council (MRC) has been involved with work in the area since the 1950s. The Nutrition Group of the MRC Laboratories is based in Keneba. The MRC clinic provides medical care focussing on maternal and child health. It also provides emergency, midwifery and nursing care in collaboration with the local Government Divisional Health services. Supported by its reputation for improving community health and providing employment, the MRC enjoys an excellent relationship with the locals living in the area. The station houses its own entire infrastructure in this isolated area, consisting of laboratories, clinic, vehicles, electricity and water, in order to adequately support the body of research projects it runs. Some photos of the station can be viewed in Figure 2.
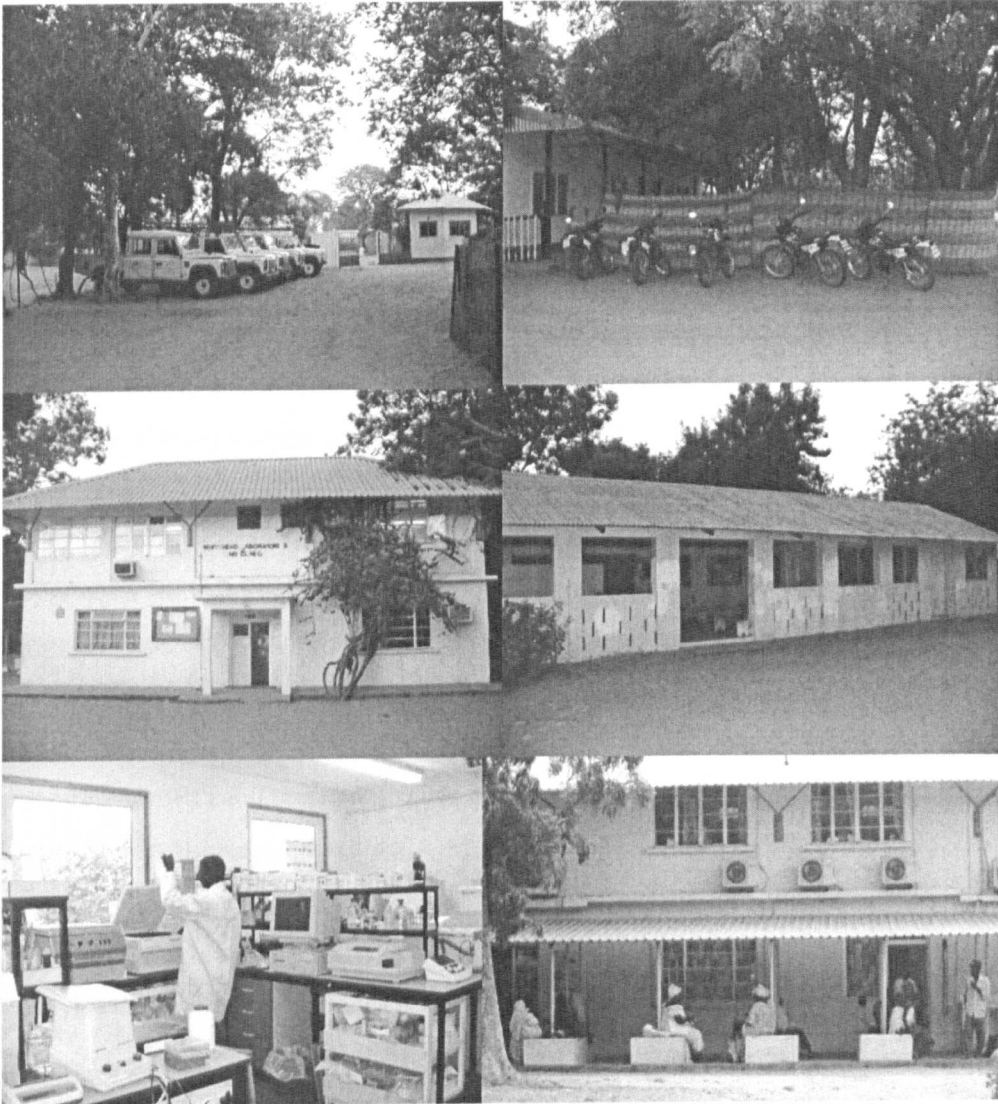
Figure 2: Photographs of the MRC Keneba field station showing, clockwise, from top right: the motorbike fleet; gate clinic; consultation room, bleeding laboratory and data office; laboratory; library, records office, research room and administrative office; some of the station vehicles.

1.1.3 Growth faltering

Growth faltering and under-nutrition are important underliers of infant morbidity and mortality in sub-Saharan Africa, as in many other developing countries (2-4). Malnutrition increases the risk of disease and early death, and, according to the latest Lancet Maternal and Child Undernutrition Series (2008), plays a major role in over a third of under-five deaths each year (2).

It is well-known that malnutrition delays growth. Acute starvation, provided it is not too severe or enduring, may leave almost no imprint on a child's eventual attainment in growth (5). Chronic malnutrition, in contrast, has lasting effects. As explained by Tanner (1989), in some populations most members grow to be smaller adults than they should because of chronic undernourishment during most or all of their childhood (5). Such chronic malnutrition is a feature of many countries in the developing world (6).

The growth velocity (an important indicator of nutritional status (5)) of Gambian infants falls severely from around 3-4 months (mo) onwards compared with reference standards (7-9). Before this age, infants are only slightly below their expected weight-for-age, with evidence of catch-up growth taking place quite successfully from a moderately low birth-weight. This catch-up growth, and the deterioration in mean Z-scores for weight- and length-for-age, head circumference (HC) and body mass index around weaning age, is demonstrated in Figure 3 by a sample of infants measured against Western reference norms (10, 11) recently (12). Although annual increments in growth between 1 and 7 years of age are similar to those in Europe, Gambian children remain shorter and lighter than their UK counterparts, never fully regaining the losses incurred in early infancy (7).

Many suitably-targeted macro- and micronutrient intervention trials have been undertaken in The Gambia and other developing countries in an attempt to reduce malnutrition and hence its consequences for the children involved. Effects on long-term growth faltering, however, have been small or nil (13-17). Even when a considerably large amount of high-quality food was supplemented, growth remained poor and stunting ensued (18). Growth faltering could thus not, to any significant degree, be attributed simply to micronutrient or energy inadequacies.
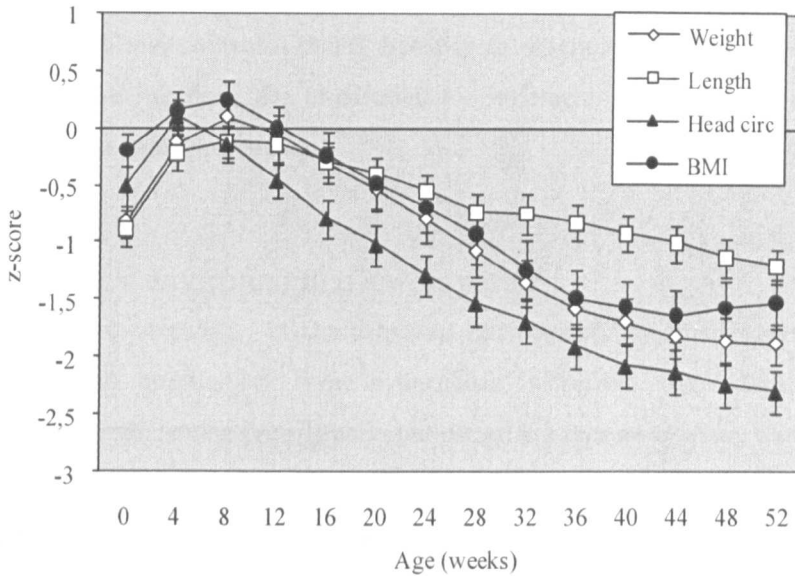
Reprinted from Collinson *et al.* (2005) (12)

Figure 3: Growth performance of Gambian infants as measured against UK standards, expressed as weight, length, HC and body mass index Z-scores, from birth to 52 weeks

A search for other factors involved in the growth-failure sequelae led to investigations around the role of possible nutrition-disease relationships (19). Infections are well known important contributors of poor growth (20-22). Due to the unhygienic and unsanitary environments surrounding them, infants in developing countries are often chronically exposed to infections. Gastrointestinal infection, in particular, is strongly associated with wasting (22, 23). However, although acute episodes of illness often result in short-term weight-loss, efforts to relate long-term growth, especially in height, to overt clinical diseases have produced little or no association (24, 25). Furthermore, since there was no improvement in growth despite a decline in diarrhoea prevalence over a 15 year period (26), investigations in The Gambia were shifted towards other factors, such as sub-clinical disease states, which were likely retarding growth.

The repeated immunostimulation experienced by infants of developing countries, even in the absence of overt clinical disease symptoms, has been discussed as a significant contributor to growth faltering (27-29). The role of the gastrointestinal tract and, notably, the small intestinal mucosa, has received particular interest because it represents an extensive area of

interface between the body and the environment, and thus it not only functions as a barrier to pathogens and environmental toxins, but also as an absorptive surface for nutrients (19, 30, 31). Both these functions are imperative for satisfactory growth performance, as shall be discussed in the following section.

1.1.4 Chronic environmental enteropathy

Persistent enteropathy, as characterised histologically by small-intestine mucosal villous shortening and broadening, crypt hyperplasia, increased crypt depth, and lymphocyte infiltration into the lamina propria and epithelium, is a feature of many Gambian children. (The basic anatomy of the intestinal mucosa can be viewed in Figure 4, and the pathologies mentioned above, studied by small bowel biopsies, in Figure 5). First described in 1962 (32), persistent enteropathy was found to affect individuals throughout the tropics in Africa, Asia, South America and the Caribbean (33). For this reason it acquired the name "tropical enteropthy". The condition was only observed in those living in less developed or more contaminated environments of the tropics. It was later found that people living in temperate areas may also develop similar histological and functional changes if living in environments with similarly high levels of microbiological pathogens (34, 35). For these reasons the expression "chronic environmental enteropathy" is now accepted as a more accurate description of the condition than "tropical enteropathy".

Associated functional changes include subclinical malabsorption of fat and an increased mucosal permeability (36). The latter is demonstrated by markedly and consistently raised lactulose-mannitol ratios (LMRs) in the dual-sugar permeability test (30). Raised LMRs have also been described in children in several other parts of the developing world (37, 38).

The dual-sugar permeability test assesses both gut integrity and absorptive capacity. By measuring absorption of the indigestible disaccharide, lactulose (MW 342), through paracellular tight junctions, gut leakiness may be evaluated. Absorptive capacity, on the other hand, is measured by the passive absorption of the monosaccharide mannitol (MW 182) through the transcellular route. Higher LMR ratios are therefore indicative of poorer gut integrity (39, 40). This concept and test will be further explained in the Methods section.
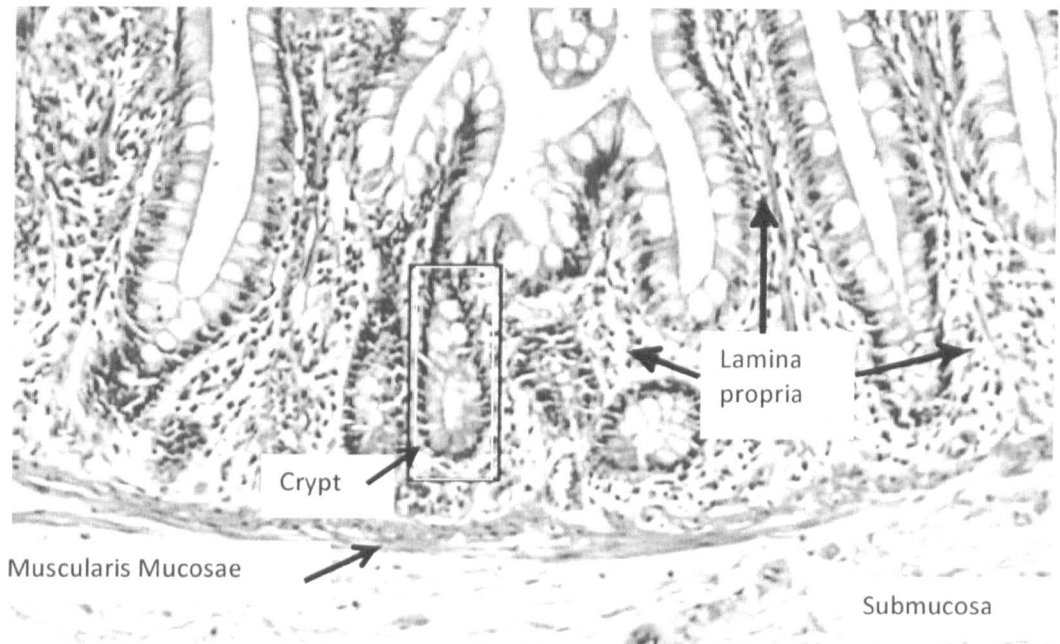
Figure 4: Intestinal mucosa showing the crypt and lamina propria
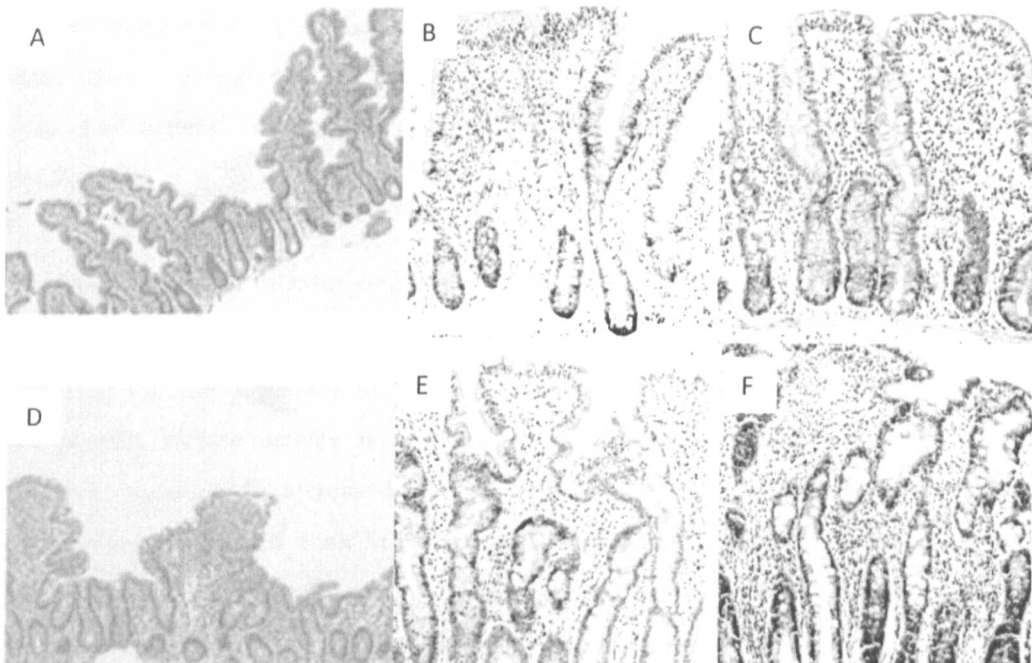
Reprited from Schenk (1972) (41) and Kelly (2004) (42)

Figure 5: Histological intestinal mucosal villi changes found in tropical enteropathy. A & B: Normal; C: Initial signs of cell infiltrate, crypt hyperplasia & villous shortening. D, E & F: Advanced signs of villous broadening & shortening with increased crypt depth, crypt hyperplasia, & cell infiltrate

1.1.4.1   Causes and consequences of environmental enteropathy

The adverse and unhygienic environmental conditions to which infants in developing countries are exposed are thought to be a major cause of enteropathy. It is thought that the passage of bacteria, viruses, and parasites along the gastrointestinal tract with contaminated weaning foods and water leads to infections causing gut injury which, for various reasons (43), is never fully repaired. However, no specific organism or mechanism has yet been recognised as a major cause of enteropathy, and bacteria probably do not require enteropathogenic capabilities to induce these effects (8). Instead, chronic exposure to a wide variety of pathogenic organisms and/or food allergens, causing repeated episodes of often asymptomatic enteropathy, is its generally accepted aetiology (8, 44). The degree of allergic responses contributing to gut inflammation in this disorder is likely to be small, however, as the children susceptible to environmental enteropathy are rarely atopic (45).

Mucosal injuries resulting from inflammatory responses appear to be slow or resistant to healing and repair, and once injured, the gut is vulnerable to still further damage. Even if further injury is not triggered, intestinal mucosal damage - by setting off bacterial overgrowth, absorption of foreign proteins and toxins, or malabsorption of nutrients - may delay healing (43). Two general enteropathic mechanisms are described by Lunn (8), as summarised in Figure 6.

Firstly, a loss of mucosal enzyme function, resulting from partial villous atrophy, causes maldigestion and malabsorption of food. This is especially pertinent in the case of lactase. Essential for the digestion of lactose, a major carbohydrate and source of energy in breast-milk, lactase activity is particularly affected by villous damage. Second, mucosal damage, resulting in increased gut permeability, allows the translocation of antigenic macromolecules which then stimulate and perpetuate local and systemic immune and inflammatory responses. Both of these enteropathic responses, and this inflammatory condition overall, are strongly associated with growth failure (9, 30).

In a study investigating the relationship between enteropathy and growth performance, Lunn et al. (30) showed that abnormalities of the mucosa of the small intestine, i.e. increased LMRs, accounted for up to 43% of growth faltering in a group of rural Gambian children. It is currently accepted that most of this growth faltering (55-60%) is related to increased lactulose uptake, and 40 – 45 % to reduced mannitol updake ((8).

Taken from Lunn (2000) (8)

Figure 6: Diagrammatic representation of the mechanisms leading to poor growth in Gambian children

Growth-depressing intestinal permeability values appear to occur in approximately 75% of rural Gambian children between the ages of 3 and 15 mo (30, 39, 46). As already mentioned, contaminated weaning foods fed to infants are likely to be a major initiator of this persistent inflammation of the gut (8).

Although tropical enteropathy may occur in apparently healthy individuals, it could potentially result in subclinical malabsorption due to the disturbances of digestive and absorptive function causing considerable losses of nutrients (36, 47). Infants affected by enteropathy might maintain satisfactory nutritional status when healthy, but in states of disease or food shortage, they have a greater predisposition towards malnutrition than those without enteropathy (35, 36).

1.1.4.2 Other enteropathies explored: Crohn's Disease and ulcerative colitis

The literature focussing on other inflammatory enteropathic disease states, particularly in regard to intervention studies, is far richer than the literature focussing on environmental enteropathy. In order to make informed inferences about the results of these studies, a brief description will be provided of the inflammatory bowel diseases (IBDs).

Although the consequence of different aetiologies and pathogenic mechanisms (e.g. gene-environmental interactions strongly underlie the pathogenesis of inflammatory IBD (48)), and a feature of both children and adults in the developed but not developing world, these diseases share some similarities with environmental enteropathy in so far as they are characterised by a persistent inflammation of the gastrointestinal tract with an underlying immunologic mechanism. Additionally, the LMRs seen in malnourished Gambian children are similar to those in children with IBD and coelic disease in the UK.

Crohn's disease and ulcerative colitis are the best known forms of IBD and can involve either or both the small and large bowel (48). "Active" IBD is characterised by acute inflammation. "Chronic" IBD is characterised by architectural changes such as crypt distortion and scarring. Crypt abscesses, consisting of neutrophils in crypt lumens, may occur in many forms of IBD (49).

Ulcerative colitis involves the colon, producing a continuous mucosal inflammation always involving the rectum. Its aetiology is unknown. Crohn's disease, on the other hand, can involve any part of the gastrointestinal tract, but most frequently involves the distal small bowel and colon. Inflammation is typically transmural and patchy, and can range from small ulcers to chronic inflammation (48). The aetiology is unknown, although infectious and immunologic

mechanisms are thought to play a part (49, 50). Both conditions are more common in Caucasians and in women.

The distinguishing features of IBD are summarised in a table produced by Fiochhi (1998) (50), where ulcerative colitis is contrasted against Crohn's disease. This summary is given in Table 1.

1.2   Long-chain polyunsaturated fatty acids

The essential polyunsaturated fatty acids (PUFAs), linoleic acid (C18:2 omega-6) and α-linolenic acid (C18:3 omega-3), are vital structural components of cell membrane phospholipids. Unlike cellular proteins which are genetically determined, the fatty acid (FA) composition of cell membranes is largely dependent on dietary intake (51). Linoleic and α-linolenic acid can be converted to their long-chain (≥20 carbon chain length) derivatives via a series of elongation and desaturation reactions. The most important omega-6 (n-6) long-chain polyunsaturated fatty acid (LCP) is arachidonic acid (AA; C20:4n-6), for which linoleic acid serves as parent FA. α-Linolenic acid may be converted to the n-3 LCPs eicosapentaenoic acid (EPA; C20:5n-3) and further to docosahexaenoic acid (DHA; C22:6n-3); these are the major n-3 LCPs (Figure 7). However, these conversions, illustrated in Figure 8, take place inefficiently and in a constricted manner (52-54). Particularly in infants, conversions are slow and studies suggest that pre-formed LCPs are needed to sufficiently meet infants' accretion needs, especially of DHA (55-57).

The essential n-3 and n-6 PUFAs can be synthesised *de novo* only in plants. The four main vegetable oils (palm, soybean, rapeseed and sunflower) are rich in linoleic acid. Various other oils, as well as poultry and certain grains and cereals are further dietary sources of n-6 PUFAs. Fish, flax seeds, some nuts, and milk from grass-fed cows are sources of α-linolenic acid. The n-6 LCP, AA, can be obtained from milk, meat and eggs, while n-3 LCPs are less widely distributed in the food chain and are obtained almost exclusively from marine foods. Organ meats and some egg yolks are its only other dietary sources.

Table 1: Distinguishing features of ulcerative colitis and Crohn's disease pathogenesis

Taken from Fiocchi (1998) (50)

Component	Ulcerative colitis	Crohn's disease
Environmental factors	Beneficial effect of smoking	Detrimental effect of smoking
	No beneficial effect of diet	Symptoms improved by selected diets
	Normal intestinal permeability in healthy relatives	Increased intestinal permeability in healthy relatives
Genetic associations	Largely different from Crohn's disease	Largely different from ulcerative colitis
Microbial agents	Limited role of bacterial flora	Important role of bacterial flora
	No association with *M. Paratuberculosis*	Association with *M. Paratuberculosis*
	No association with measles virus	Some association with measles virus
Humoral immunity	Prominent antibody secretion	Moderate antibody secretion
	Evidence for autoimmunity	Limited evidence for autoimmunity
	Strong association with antineutrophil cytoplasmic antibodies	Weak association with antineutrophil cytoplasmic antibodies
Cell-mediated immunity	Prominent neutrophil infiltration in the mucosa	Prominent T-cell infiltration in the mucosa
	Normal/hyporeactive T cells	Hyperreactive T cells
	Normal T-cell apoptosis (?)	Resistance of T cells to apoptosis (?)
Cytokines and mediators	Prominent production of eicosanoids	Moderate production of eicosanoids
	Th2-like profile	Th1-like profile
	Increased cytokine production limited to involved mucosa	Increased cytokine production in involved and uninvolved mucosa
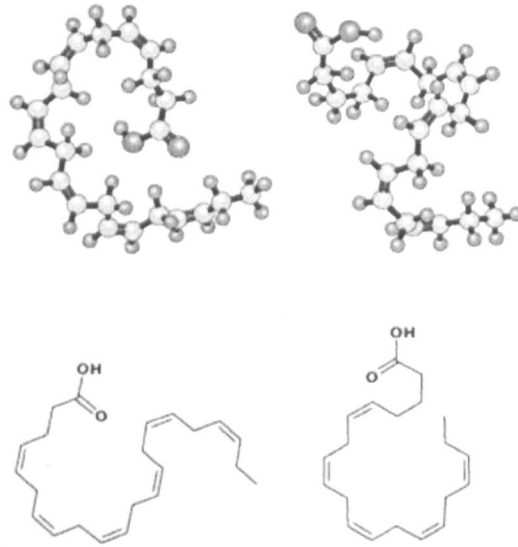
Figure 7: Structure of the n-3 LCPs DHA (left) and EPA (right)
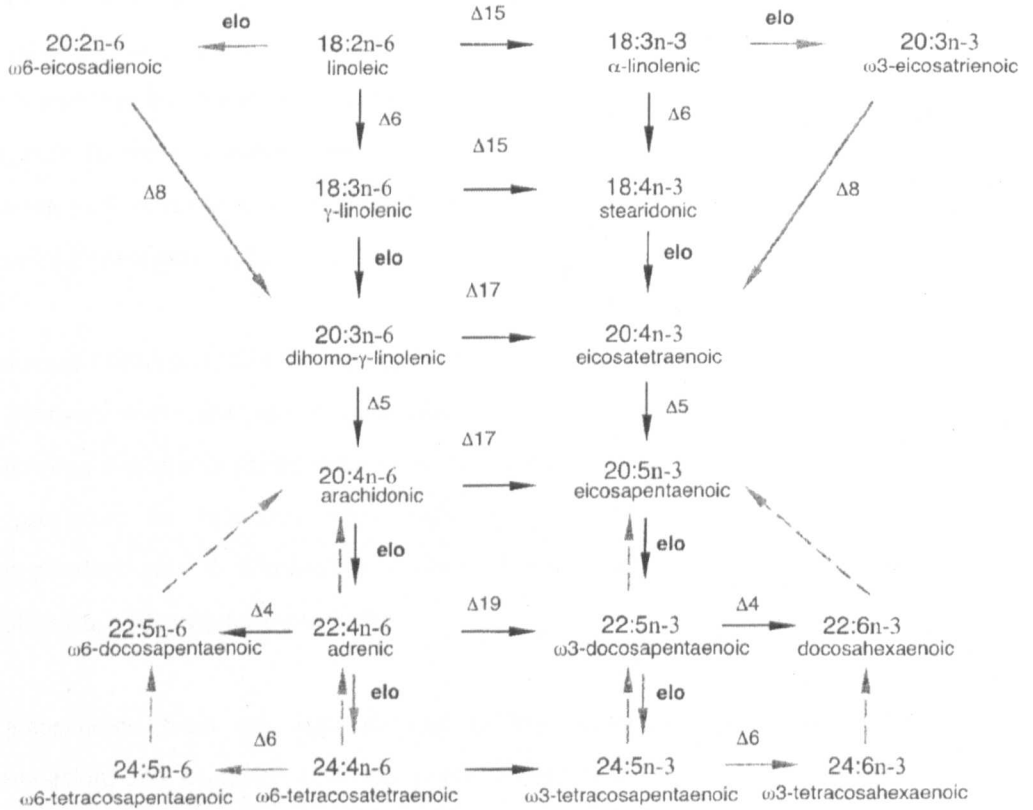
Reprinted from Leonard (2000) (58)

Figure 8: Metabolism of fatty acids to form LCPs

By observing that symptoms such as growth retardation, dermatitis and reproductive failure (resorption of foetuses or perinatal death) that occurred in mice fed fat-free diets for several months could be reversed by supplementation with vegetable oils, Burr and Burr proposed linoleic and α-linolenic acids as essential for growth and development as early as 1930 (59, 60). Later, Interest in the health benefits of n-3 FAs was generated when, in the 1970s, Dyerberg and Bang (61-63) discovered that the Inuit of Greenland had low rates of coronary heart disease and cancer although surviving on remarkably high-fat diets. Due to its antithrombotic and serum cholesterol lowering effects, EPA received particular interest for its potential use in the prevention of heart attacks (64, 65). Subsequently, and up to the present day, n-3 LCPs have, by virtue of their potential benefits in health and disease, remained a subject of rigorous investigation.

As part of their functions in cell phospholipids, chiefly in the central nervous system where they serve as structural components of neuronal phospholipids, PUFAs are important determinants of lipid-protein interactions in membrane domains which affect receptor, ion channel, enzyme, and cellular uptake and excretion activities. LCPs, specifically, influence membrane stability, membrane fluidity, cell mobility, the formation of receptors, and binding of ligands to their receptors. Besides these, they influence the activation of intracellular signalling pathways either directly or through the formation of eicosanoids, gene expression and cell differentiation (51).

Parent PUFAs and their derivatives may serve as substrate for energy generation, but LCPs are relatively protected from β-oxidation. When in free form, LCPs serve as mediators for thromboregulation and varied inflammatory and pathogenic responses, primarily by acting as the precursor for bioactive eicosanoids (66, 67). These signalling molecules include prostaglandins and thromboxanes - both of which are cyclo-oxygenase products, and leukotrienes, a lipoxygenase product.

Eicosanoids from AA are involved in vasoconstriction and platelet aggregation, inflammation, and leukocyte chemotaxis and adhesion (68, 69). In contrast, the less-potent eicosanoids from EPA are involved in attenuation of platelet aggregation and vasoconstriction (70). Apart from their effects on eicosanoid production, n-3 LCPs have further potent inflammation lowering effects via influences on the modulation of inflammation and immunity

by affecting, amongst others, intracellular signalling pathways, transcription factor activity and gene expression (71). Metabolites of n-3 FAs are thus significantly less inflammatory than those of n-6 FAs, and assist in inflammation attenuation.

The two families of n-3 and n-6 PUFAs compete for the same metabolic enzymes, influencing subsequent physiological effects and conversion to eicosanoids, making the n-6 to n-3 FA ratio an important feature in PUFA status (72, 73). It is believed that the ratio which the evolutionary (hunter-gatherer) human diet most likely provided - an n-6:n-3 ratio of 1:1 to 4:1 – is more beneficial to human health than the ratios provided by the typical modern day Western diet of between 10:1 and 30:1 (74-76). The conversion of C18 precursors to LCPs, it has recently been argued, is, however, less influenced by the n-6:n-3 ratio than the absolute amount of n-3 FA consumed (77).

Roughly 2.2 g of PUFAs are deposited in maternal and foetal tissues daily during pregnancy (78). LCPs are required in high concentration by developing organs of the foetus and are therefore vital for growth. Marked increases in AA and DHA concentrations in the brain and retina arise, accompanying central nervous system maturation (79-81). LCP plasma and tissue pools also accumulate (82), predetermining the infant's FA status at birth. The foetus relies heavily upon its mother's diet for its own LCP supply, making maternal LCP status during gestation a major determinant of the FA status of the infant at birth (83, 84).

1.2.1   Dietary n-3 LCPs in rural Gambian infants

In many rural areas of the developing world diets are low in n-3 LCPs (85-88). Breastfed babies are probably relatively protected from deficiency as human milk provides preformed LCPs which are well absorbed and readily used. Most local weaning diets, however, (introduced at 3 to 4 mo of age), have very low n-3 LCP content (88). Thus the period of transition from being fully breastfed, to partial breastfeeding and then weaning is important for the n-3 LCP status of infants.

Gambian infants' n-3 FAs intakes per kilogram of body weight drop to profound inadequacy shortly after weaning, when compared to the Food and Agriculture Organisation (1994) recommended intakes (78), as demonstrated in Figure 9. The earliest breast-milk supplements are paps made of rice or sometimes other grains. At around 8 or 9 mo infants are fed by the mother with small quantities of her own portion during meals. Breast-feeding stops

by roughly 20 mo, by which time the infant receives much the same dietary composition as the adult (7).

Growth and stresses such as those brought by pregnancy, and excessive loss and replacement of tissue, increase the requirement for PUFAs and, if the requirement is not met by the diet, deficiency occurs (89). The growth of new tissue during pregnancy, as mentioned above, raises the requirement for essential FAs, and also for DHA. A Dutch research group found that amounts of DHA in the plasma phospholipids in women who were pregnant for the first time were significantly higher than in women who had been pregnant at least once before, and that a significant negative correlation existed between gravida number and the DHA content in maternal plasma samples (90). Furthermore, measures of DHA status gave significantly better values in the umbilical cord plasma and vessel walls of infants born to women after their first pregnancy than to those after their second or higher pregnancy, and significant associations were observed between birth order and the relative amounts of DHA in cord tissues. Although DHA levels probably have time to normalise between pregnancies (91), the high fertility rate of Gambian women (4.8 births/woman) could make them susceptible to a reduction and eventual depletion of DHA stores after consecutive pregnancies, which could then be reflected in a lower neonatal DHA status.

Aside from the low n-3 LCP intake through weaning foods and possible increased deficiency risks in those born to multigravidae, the gut damage that Gambian infants commonly present with places them at further risk of n-3 LCP deficiencies. Chronic environmental enteropathy, especially when accompanied by diarrhoea, leads to fat malabsorption and fat losses (92). All these factors combined, therefore, likely leave Gambian infants at an increased n-3 LCP requirement.
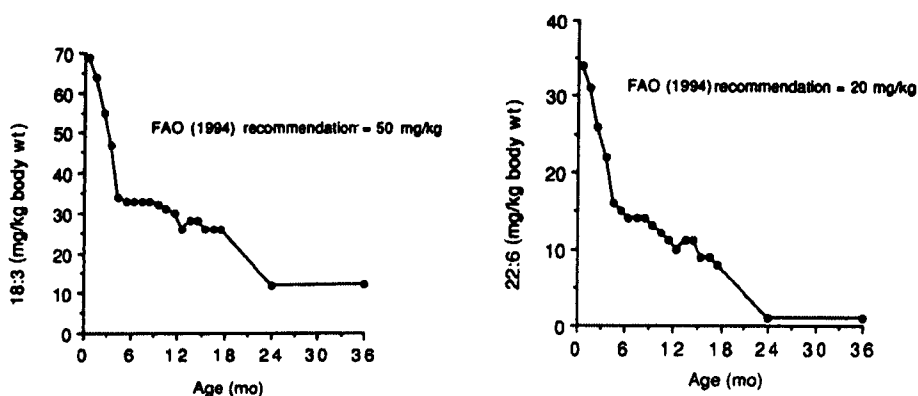
Reprinted from Prentice & Paul (2000) (88)

Figure 9: Intakes of the n-3 fatty acids α-linolenic acid (18:3; left) and DHA (22:6; right) in Gambian children from birth to 36 months

Studies at MRC, Keneba, have identified a range of health problems that might be caused partly by an n-3 LCP-deficient diet, and yet these have not been investigated in any detail to date (88). This has primarily been due to the difficulty of assaying the minor components of the FA spectrum in order to examine possible associations. Measuring the relative proportions of individual FAs requires a significant amount of time and requires particularly careful sample preparation and handling to reduce measurement error (93). These errors are not easy to avoid and are also influenced by the amount of individual fat in the sample (94). Accounting for only a fraction of the total fat in animal cells, this makes LCPs especially challenging to measure. Nonetheless, developments in gas chromatography (GC) methods have now greatly improved their analytical sensitivity and permit accurate analysis in small sample volumes.

Detrimental health outcomes that might be caused, in part, by poor n-3 LCP status include growth failure and persistent inflammation of the gastrointestinal tract. There is evidence that supplementary n-3 LCPs might ameliorate this enteropathy by reducing intestinal inflammation. A reduction in intestinal inflammation and associated gut damage could, in turn, reduce the degree of associated growth faltering. Additionally, n-3 LCPs have been found to have beneficial effects on mental development and problem-solving ability in infants.

In the light of these potential benefits, the next section will concentrate on n-3 LCPs in regard to health and development, especially of infants.

1.3   n-3 LCPs in relation to infant health and development

1.3.1 Growth

As mentioned in the previous section, n-3 FAs remained the subject of intensive investigation after interest in their roles in human health were sparked in the 1970s. The conference on Health Effects of PUFAs in Seafoods held in Washington, DC (95) in 1985, was the first major international meeting to establish the importance of n-3 LCPs in immune and membrane functioning (51). The participants recommended, amongst others, the support of research into the role of n-3 FAs in growth and development.

Consequently, various groups in the scientific community began researching on the essentiality of n-3 LCPs throughout the life cycle (51), using both animal and human models. Studies in rats and rhesus monkeys ascertained that n-3 deficient diets during pregnancy and lactation result in impaired development in the offspring (96, 97). Crawford *et al.* (1973) (98) found that human milk, unlike cow's milk, contained EPA and DHA, and recommended their inclusion in infant formula. It was also confirmed that EPA and DHA concentrations in the erythrocytes of breast-fed infants are higher than in those of bottle-fed infants (99).

LCP supplementation has been extensively investigated in several randomised trials with growth as the endpoint. Two systematic reviews addressing LCP supplementation in infants have been published for the Cochrane Library. The first one, examining effects in term infants (100), included twelve studies which measured growth as outcome (55, 101-115) and concluded that there is no effect of LCPs on term infant growth, based on meta-analysis results and null effects found in all included studies.

The second review, examining effects of LCPs in preterm infants (116), included thirteen studies assessing growth at different postnatal ages. Largely due to differences in formulations and doses, results of the effects of LCPs on growth were mixed: four studies reported positive effects (117-120), two studies reported negative effects using a formula containing only n-3 FAs and no AA (121, 122), one study reported mild negative effects using a formula supplemented with both n-3 LCPs and AA (123), and six studies found no effect (79, 124-128). Meta-analysis of five studies (which were in suitable format) (79, 117, 118, 122, 125) showed benefits on weight and length gain at 2 mo post-term. Meta-analysis at 12 mo (n=271) and 18 mo (n=396) post-term, however, showed no significant effects on weight, length, or HC as a result of LCP supplementation. Overall results led to the conclusion that "no clear long-term

benefits were demonstrated for infants receiving formula supplemented with LCPs. There was no evidence that supplementation of formula with n-3 and n-6 LCP impaired the growth of preterm infants."

A meta-analysis published in 2005 (101), including fourteen studies investigating the effects of LCP supplementation on the weight, length and HC of term infants in North America (55, 104), Europe (107, 111-114, 129-131) and Australia (81, 103), found no evidence to support the hypothesis that LCP supplementation, with or without added AA, influences the growth of term infants at any assessment age, in either a positive or negative way.

It is important to note, however, that these trials have been focused on generally well-nourished infants in affluent societies, who were already growing near optimum rate. It is likely to be expected that the impact of LCP supplementation on growth would be insignificant in these conditions. Infants living in less advantaged areas of the developing world, in addition to already displaying poor overall growth, might have intakes of these fats that fall below recommendations. Such children may, in contrast, respond differently to LCP supplementation. Yet, apart from scattered observational data, this question is not featured in the literature. Furthermore, because n-3 LCPs may improve infant intestinal mucosal integrity (by their immunomodulatory effects, discussed further in the next section), the rationale exists that they may have their effect via this pathway in a population such as infants in rural Gambia suffering from high rates of enteropathy.

As summarised above, two trials found that preterm infants who received (exclusively) formula milk supplemented with n-3 LCPs but no AA suffered a negatively affected growth (121, 122). This was most likely brought about by reduced circulating AA levels in these infants who were consuming no AA and were thus dependent entirely on metabolic conversions from precursors in order to meet their AA supply (130). However, in breast-fed infants, this risk of AA inadequacy is minimised and several trials have found no danger of DHA supplementation-induced AA deficiency, considering that a rich source of preformed AA is ensured via the breast-milk.

1.3.2 Immune function, inflammation, and gut integrity

Figure 10 demonstrates how EPA affects eicosanoid production by serving as precursor to non-inflammatory eicosanoids and reducing the production of the proinflammatory series-2 eicosanoids.

Apart from their roles in modulating eicosanoid generation from AA and acting as precursor to alternative eicosanoids, n-3 LCPs have more recently been found to give rise to a novel group of mediators termed D and E-series resolvins, derived from DHA and EPA, respectively. These compounds, formed by cyclooxygenase-2 and lipoxygenase, appear to assist in anti-inflammatory and inflammation resolving actions (132-136). It is thought that resolvins reduce inflammation by inhibiting not only the production of inflammatory products, but also their transport (133).

Furthermore, EPA, together with DHA, may inhibit inflammatory cytokine production (137), and so influence immune function by diminishing the production of chronic inflammatory factors such as interleukins 1β, 2 and 6, and tumour necrosis factor (138) from AA. EPA and DHA may also act as modulators of gene expression, and reduce pro-inflammatory signal transduction in pathways such as nuclear factor kappa B and peroxisome proliferator-activated receptor gamma (PPAR-γ) expression (139). By modulating the activation of nuclear factor kappa B, e.g. the transcription of genes regulating the inflammatory response (cytokines, chemokines, cell adhesion molecules and acute phase proteins) is in turn influenced (140).

Numerous diseases and conditions in humans, such as rheumatoid arthritis, type-1 diabetes, cystic fibrosis, psoriasis, systemic inflammatory response to surgery, and IBD, are characterised by the excessive or inappropriate production of inflammatory mediators. The anti-inflammatory effects of n-3 LCPs led to the hypothesis that deficiencies of these FAs may result in increased inflammation, and that n-3 LCP supplementation in those with inflammatory diseases may bring about therapeutic benefits. The mechanisms by which n-3 LCPs might work to reduce intestinal inflammation, as summarised by Teitelbaum (2001) (141), are shown diagrammatically in Figure 11. Accordingly, many n-3 LCP supplementation trials have been conducted to test this hypothesis.
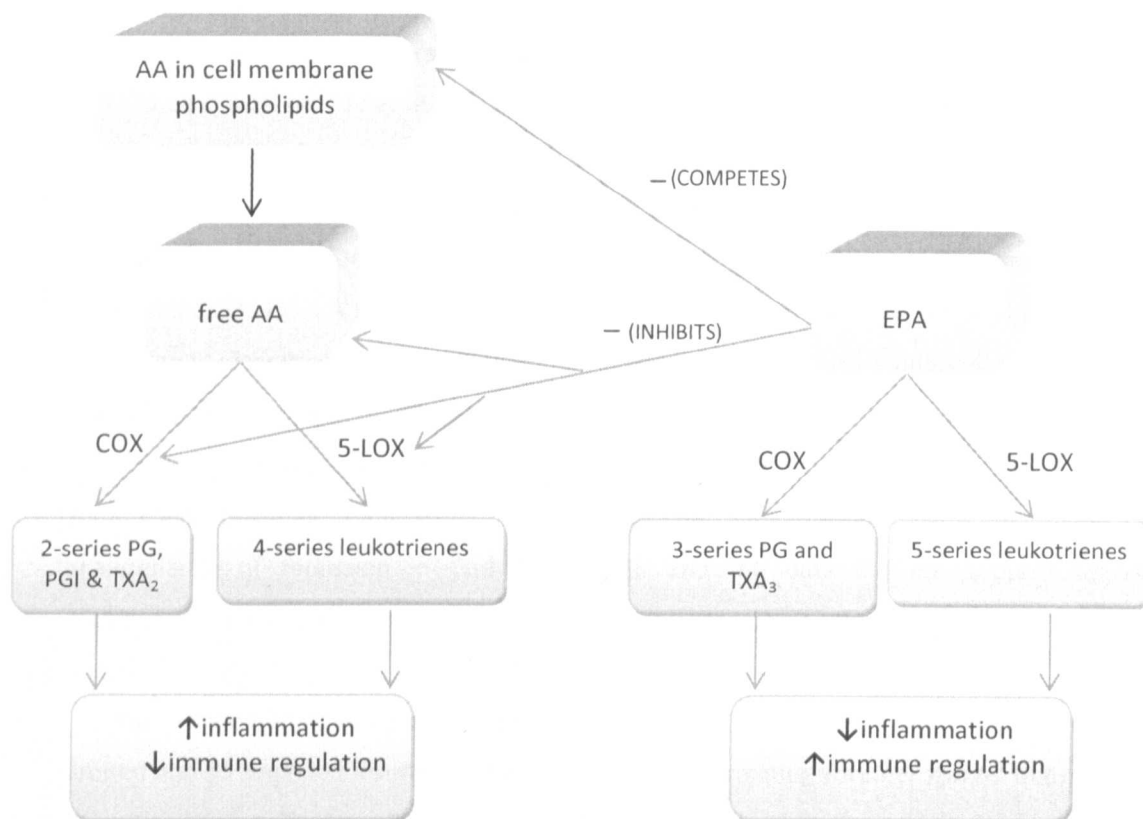
Figure 10: Synthesis of eicosanoids from arachidonic acid and eicosapentaenoic acid, and sites of inhibition by eicosapentaenoic acid. PG: prostaglandin; PGI: prostacyclin; TXA: thromboxane; COX: cyclooxygenase; LOX: lipoxygenase

Most cases have demonstrated anti-inflammatory effects although, except in rheumatoid arthritis, evidence for clinical benefits is still lacking. Several reviews have been published on the topics of n-3 LCPs/fish oil and rheumatoid arthritis (142-145), cystic fibrosis (146), psoriasis (147), patients critically ill or undergoing surgery (148), and IBD (149, 150). In a general review on PUFAs and inflammation, Calder (2006, citing the aforementioned reviews) concluded that the anti-inflammatory actions of n-3 LCP-induced effects may be of therapeutic use in conditions with an acute or chronic inflammatory component (151).

The several clinical trials conducted to test the effects of n-3 PUFA supplements on clinical outcomes in IBD, which have been included in published systematic reviews (152-155), have

been summarised in Table 1. Overall, the studies have reported mixed findings. The results of a comprehensive systematic review on IBD suggested that the available data were insufficient to draw conclusions about the effects of n-3 FAs on clinical, endoscopic, or histologic scores or induced remission or relapse rates, and that future studies are required to assess the effects of n-3 FAs on clinical outcomes in IBD (152).

Since 2007 (i.e. after the start of the trial described in this thesis) two Cochrane reviews on omega-3 FAs in relation to remission in ulcerative colitis and Crohn's Disease have been published (153, 155). A similar Cochrane review addressing fish oil in relation to induction of remission in ulcerative colitis was published in 2007 (154). Only three studies were included on the subject of remission in ulcerative colitis. A pooled meta-analysis showed no benefit of n-3 FAs. However, it was stated that further studies using enteric coated capsules (which release their contents in the small bowel) may be justified. Similarly, no benefit was found for maintenance of remission in Crohn's Disease, and the data did not support routine maintenance treatment with n-3 FAs.

The data on trials using fish oil for the induction of remission in ulcerative colitis were limited and so provided insufficient information for determining whether fish oil treatment is effective. All three analyses found fish oil/n-3 FA treatment to be safe.

Thus, despite several favourable studies as shown in Table 2 – particularly that of Belluzzi (1996) which, in a well-designed trial with a substantial sample size (n=78), found n-3 LCPs effective in significantly improving remission in Crohn's Disease – the most recent view is that n-3 LPS have either no clinical benefits in IBDs, or that the data are inconclusive.

Despite the bulk of investigations surrounding the role and therapeutic potential of n-3 LCPs in IBD and other inflammatory conditions, studies investigating the physiological functions of early dietary n-3 LCPs in relation to environmental enteropathy and its related growth faltering in infants are lacking, leaving it a largely under-researched area.
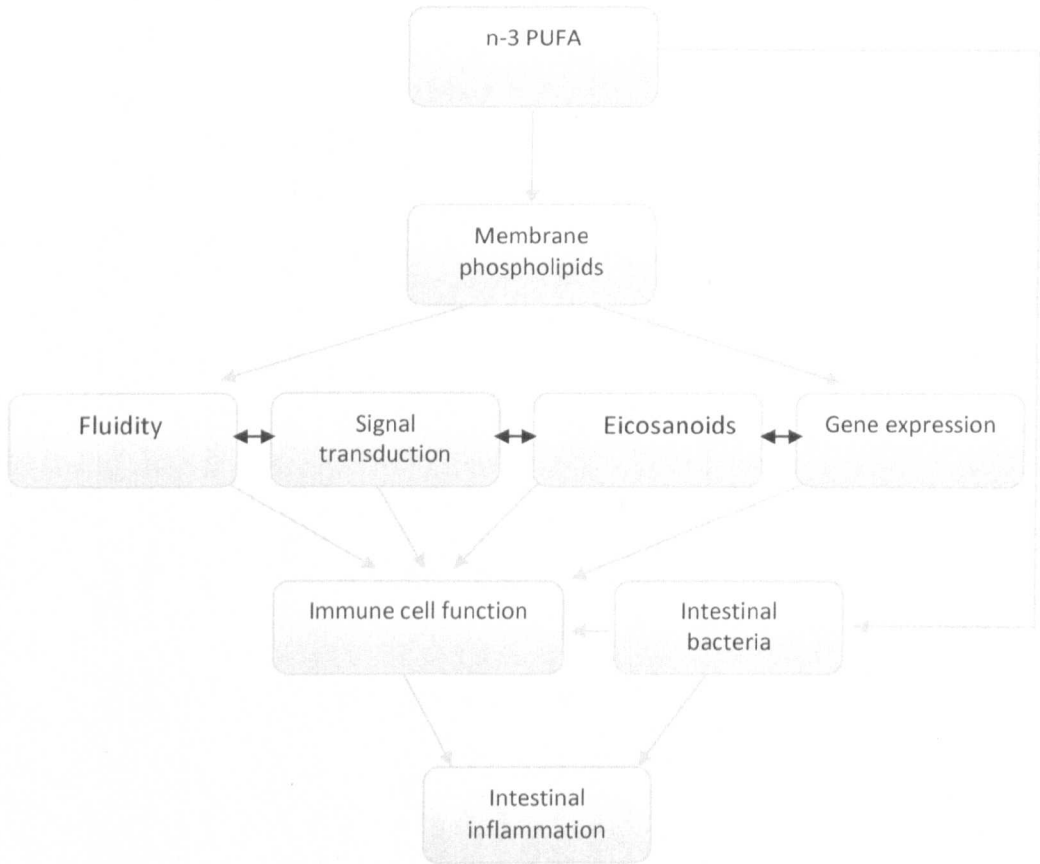
Reproduced from Teitelbaum (2001) (141)

Figure 11: Mechanism by which n-3 LCPs might exert immunomodulatory and anti-inflammatory effects

Table 2: Summary of effects of n-3 fatty acids on clinical scores, endoscopic scores, histologic scores, relapse, remission, and corticosteroid requirements in IBD

Year, sample size (reference)	Design	mo	FA source & dose	Control	Blind-ing	Clinical score	Endoscopic score	Histologic score	Relapse	Remission	Steroid require-ment
1998, n=18 (156)	RCT[1]	6	Fish oil, 15ml/d	Sunflower oil, 15ml/d	Yes	NSE[2]	Improved*	Improved*	NR[2]	NSE	NSE
1992, n=11 (157)	RXT[1]	3	Max EPA[3], 15capsules/d	Oleic/palmitic/linoleic acids, 15caps/d	NR	Improved*	NR	NSE	NR	NR	NR
1996, n=78 (158)	RCT	12	Fish oil, enteric coated, 15g/d	Migloyol,[4] 15g/d	NR	NR	NR	NR	Reduced**	NR	NR
2000, n=10 (159)	RXT	2	Fish oil 5.4g/d, then sulfasalazine 2g/d	Sulfasalazine 2g/d, then fish oil 5.4g/d	NR	NSE	NR	NSE	NR	NR	NR
2008, n=363 (160)	RCT	12	4.4g/d EPA+1.6g/d DHA	4g medium-chain triglycerides	NR	NR	NR	NR	NSE	NR	NR
1993, n=43 (161)	RCT	9	Max EPA, 12g/d then 6g/d	Olive oil, 12g/d then 6g/d; evening primrose oil, 3g/d then 1.5g/d	NR	NR	NR	NSE	NSE	NR	NR
1992, n=96 (162)	RCT	12	Hi-EPA fish oil, 20ml/d	Olive oil, 20ml/d	Yes	NR	NR	NR	NSE	NSE	Improved***

Year, n (ref)	Type	Duration	Intervention	Comparator						
1996, n=64 (163)	RCT	24	Fish oil, 5.1g/d	Corn oil, 5.1g/d	Improved	NR	NR	NSE	NR	NR
1989, n=39 (164)	RXT	7	Max-EPA, 11ml/d	Olive oil, 11 mL/d	Yes	NSE	Improved*	NR	NR	NR
1996, n=204 (165)	RCT	12	Fish oil, 6 g/d	Corn oil, 6 g/d	NR	NR	NR	NSE	NR	NR
1996; n=50 (166)	RCT	12	Max-EPA 10 capsules/d; mesalizine	Olive oil, 10 capsules/d; mesalazine	NR	NR	NR	NSE	Improved high-risk subgroup **	NR
2002, n=63 (167)	RCT	12	[4]GLA+EPA+DHA, 6 capsules/d	Sunflower oil, 6 capsules/d	NR	NR	NR	NSE	NR	NR
2005, n=38 (168)	RCT	12	Salicylate + 1.2g EPA/d + 0/6g DHA/d	Salicylate + olive oil	NR	NR	NR	Reduced ***	Improved ***	NR
1992, n=24 (169)	RXT	4	Max=EPA, 18 capsules/d	Vegetable oil, 18 capsules/d	NR	NR	NR	NR	NR	NSE

[1]RCT: Randomised controlled trial. RXT: Randomised cross-over trial

[2]NSE: no significant effect; NR: not reported

[3]Max-EPA: fish oil supplement containing EPA:DHA ratio 3:2. One capsule/1ml supplies roughly 300mg combined DHA & EPA

[4]Mygliol: neutral coconut and palmkernel oil-derived caprylic/capric triglyceride, GLA: gamma-linolenic acid

*p<0.05

**p<0.01

***p<0.001

1.3.3 Cognitive development

DHA and AA accrue rapidly in the human brain during the third trimester and the early postnatal period, when the rate of brain growth is maximal and therefore vulnerable to the effects of nutritional deficiencies. Epidemiological evidence reports associations between infant DHA status and neurodevelopmental outcomes as shown in several studies (170-174).

During the perinatal period, n-3 FAs are required by the membranes of photoreceptor cells for membrane biogenesis, and by synapses for synaptogenesis. They are also required during response to injury to the nervous system and retinal stimulation, both of which trigger the release of DHA from membrane phospholipids (51, 175). After 30 weeks gestation a preferential desaturation of n-3 PUFAs occurs in the brain, DHA increases quadratically, and AA decreases linearly in phosphatidylethanolamine (176). In the retina and forebrain, the proportion of n-3 FAs increases while n-6 FAs decrease throughout development, as demonstrated by a sharp increase in the ratio DHA:AA (51).

Evidence around the importance of essential FAs in early cognitive development first emerged when infants receiving breast-milk displayed cognitive advantages over those receiving formula milk (177-180). Although the mechanisms underlying neurodevelopment related to DHA intake in humans remain to be elucidated, it has been suggested that the effects on central nervous system development are likely mediated by the role of essential FAs on gene expression, membrane structures, and electrophysiologic responses (181). Since breast-milk and the standard formula sold at the time differed in respect to their LCP contents - with formula milk containing no preformed LCPs - trials testing the effect of LCP-enriched formulas on infant visual and neurodevelopment were undertaken to test for any benefits derived.

Visual development, or acuity, is typically tested using electrophysiological measures such as visual evoked potential, or forced choice preferential looking. Cognitive development may be measured using either generalised or more specific developmental tests. Commonly used standard generalised tests are the Bayley, Griffith, Catell and Gesell scales of infant development, or the Brunet-Lezine test of psychomotor development, which predominantly measure perceptual and motor abilities. More specific, non-standardised evaluations, testing for example problem solving (e.g. the Willatts Test), information processing, language development, or more abstract cognitive abilities each measuring different components of

brain development, are also used. The Willatts Test is based on the original thoughts and theories of the prominent Swiss philosopher-scientist Jean Piaget, which were developed in the early-mid 20th century. The procedure and scoring were later designed and developed by Willatts (182, 183). It was found that the ability of young children to solve simple problems develops rapidly during a limited period after 6 mo of age. At 9-12 mo this problem-solving ability enables the child to solve more complex problems, such as combined pulling and searching to retrieve a toy.

At least nine formula milk supplementation trials conducted in preterm (79, 80, 121, 122, 124, 127, 184-187) and thirteen trials in term infants (55, 81, 102, 105, 108, 113, 188-197) have been described and summarised in a thorough review on PUFAs in brain and visual development by Uauy in 2001 (181). The review concluded that evidence for a beneficial effect of AA and DHA supplementation on central nervous system functioning is strong, and that the preliminary information on cognitive development is insufficient to fully establish a claim for an LCP effect on mental development.

A Cochrane review, also published in 2001 (198), included seven studies assessing LCP-supplemented formula milk in relation to the intellectual development of term infants. Three studies, using the Bayley Scales of infant development, showed no effect of supplementation on development (81, 113, 190, 199, 200), while one study reported a positive effect of LCP supplementation at 18 mo (108). Using the Brunet-Lezine developmental quotient, one study reported a positive effect of supplementation at 4 mo (192) but not at follow up at 1 and 2 years (191). Another study (201) found beneficial effects of LCPs on novelty preference measured by the Fagan Infant Test at 9 mo, and Willatts *et al.* (1998) reported better problem solving at 10 mo with supplementation (195). The reviewer concluded that "At present there is little evidence from randomised trials of LCP supplementation to support the hypothesis that LCP supplementation confers a benefit for visual or general development of term infants...A beneficial effect on information processing is possible but larger studies over longer periods are required to conclude that LCPUFA supplementation provides a benefit when compared with standard formula."

Since 2001 this Cochrane review has been updated and re-published in 2008 (100) (review also cited under Section 1.3.1) to include four further studies, all of which measured infant development using the Bayley Scales and found no effect of the LCP-supplemented formula

(104-106, 110, 115). A Cochrane review investigating the subject of LCP-supplemented formula in preterm infants found similar results.

Considering that investigators used different developmental tests, measuring different components of brain development, conflicting results are not surprising. Studies examining the role of LCPs in infant development using standard generalised tests have rarely shown any benefit. Studies using more specific components such as look duration, attention, or problem-solving abilities (Willatts Test), however, mostly found benefits of LCP supplementation. Indeed, DHA is important to cognitive processes supported by the frontal regions of the brain (202-207), where functions which integrate and control attention and response components with long-term and working memory take place. A number of studies on look duration in human as well as primate infants have found associations between n-3 or DHA levels/intakes and accelerated development in attention maturity (122, 131, 208, 209). DHA has also been shown to lead to improvements in synaptic efficiency (210) and transmission speed (211), theoretically aiding in the efficiency with which information is processed. For this reason, tests which measure frontal region processes, such as attention and problem solving, may be more valuable and appropriate when examining n-3 LCP in relation to cognitive development. Furthermore, it has been suggested that problem-solving measures are more indicative of infant cognition than are the more standardised tests (196, 212).

Overall, no real benefit of LCP supplementation in formula fed to generally healthy infants in developed country settings is suggested by the literature. However, research investigating n-3 LCP supplementation in a developing country setting such as rural Gambia is lacking. Developmental advantages such as those reported by groups using frontal cortex related measures, e.g the Willatts Test and attention assessments, might be detected in children with less adequate nourishment. Furthermore, most of the formula milk LCP doses were designed so as to match concentrations in breast-milk. If a larger dose would be administered, aiming not to just to meet a requirement, but to act as therapeutic agent, effects may become more noticeable. If, then, a DHA-derived cognitive benefit was to be observed in Gambian infants, intervention programmes designed to improve n-3 LCP status in infancy could lead to improvement in cognitive function of children in this and other lower-income communities.

1.4   Introduction to the present study

The effects of supplementing 3 to 9 mo old Gambian infants with fish oil, providing 500mg combined DHA+EPA per day, are described in this thesis. The knowledge base regarding the role of n-3 LCPs in growth, chronic environmental enteropathy, and cognitive outcomes in infants living in rural areas of the developing world is weak. Some evidence of an effect of n-3 LCPs as found in fish oil on reducing chronic inflammation by immune-modulating effects is found in the literature.

Growth and stress increase the requirement for PUFA, and unless intake is equal to increased need, deficiency occurs. Lactation is a time of special mobilisation of PUFA for the synthesis of infant tissue, of which the brain, rich in n-3 LCPs, places an important demand. The requirement for PUFAs during lactation has been estimated at 5-7% of calories (89). In Gambian infants, fat loss due to high rates of chronic environmental enteropathy and diarrhea is likely, and intakes through weaning foods are low. The present study aimed to ensure a rich enough supply of n-3 LCPs to infants in the intervention group to not only fulfil a requirement, and account for any fat losses and malabsorption, but also to have a therapeutic effect.

Gut inflammation and damage first sets in at around 3-4 mo, with the introduction of weaning foods, and appears especially resistant to repair. The aim of the study, therefore, was to start supplementing infants (with fish oil containing a high concentration of n-3 LCPs) at 3 mo, in order to prime the intestinal mucosa and delay or prevent gut damage in the first place.

Fish oil was hypothesised not only to reduce intestinal damage caused by chronic enteropathy (as measured by absorptive surface area and intestinal "leakiness" in the dual-sugar permeability test), but to improve growth also - whether as a consequence of an improved intestinal mucosal integrity or directly. The trial further allowed investigations into the effect of fish oil supplementation on developmental outcomes. Although the mechanism is not fully understood, evidence suggests that cognitive function in children is affected by nutritional status particularly during the rapid growth phase in first two years of life (213, 214), and stunting during infancy has been related to poor cognitive function in late childhood (215, 216). It would not be unlikely, therefore, for Gambian infants to suffer developmental insults in early life, considering the high rates of stunting and malnutrition they endure. A trial providing nutritional intervention which includes a developmental outcome was thought to be of benefit in this area where the knowledge gap in regard to rural Gambians is wide.

Further areas of investigation identified were non-specific markers of inflammation, acute phase proteins, and general morbidities.

Studies have concluded that environmental enteropathy is characterised by cell-mediated inflammation in the lamina propria (217). Calprotectin is a sensitive but non-specific marker of intestinal inflammation and disease activity, which can also be used to monitor the response to treatment (218). Different diseases, including bacterial infections, lead to the activation of neutrophils, consequently increasing the concentration of calprotectin in human biological materials.

Calprotectin in faeces correlates with the number of polymorphonuclear granulocytes entering the gut lumen. Studies show that the marker correlates well with endoscopic and histological grading of disease activity in ulcerative colitis and faecal excretion of indium-111-labelled neutrophils (219, 220). Whereas the dual-sugar permeability test reflects the integrity of only the ileum and jejunum (sugars are broken down by bacteria in the large intestine (221)), faecal calprotectin could potentially arise from cell-mediated inflammation in any part of the gastrointestinal tract.

Acute phase proteins were further used to investigate the effect of the investigational product on systemic markers of inflammation and acute phase responses. General morbidities experienced by infants (diarrhoea, vomiting, fever etc.) were measured for safety/side effects monitoring purposes, and to examine any treatment effects in terms of real clinical wellness.

The well-established demographic surveillance system serving the population where the study was set provides a unique opportunity to follow-up this population for long term observations and for the asking of further questions.

1.4.1 Study rationale

1.4.1.1 Justification

The research question was considered important because, amongst others, -

a) It had the potential to provide causal beneficial impact of supplementation on a vulnerable group of the population;

b) It was the first study that looked for an impact of n-3 LCP supplementation on chronic environmental enteropathy and growth faltering in a developing country setting;

c) It would provide further insight into the physiological mechanisms of n-3 LCPs and add further scientific knowledge to the body of literature on the topic;

d) It would provide ground work for future developmental studies in this population.

The study was justified before its start as follows:

Chronic inflammation of the gastrointestinal tract is common in rural Gambian infants and strongly associated with growth faltering. There is undoubtedly a pressing need for interventions to successfully redress or prevent this chronic environmental enteropathy and growth faltering prevalent in rural Gambia and other developing countries.

Improving n-3 LCP status may be associated with outcomes which reduce chronic inflammation and gut damage. N-3 LCP supplementation may also positively influence brain growth, which, as seen in Figure 3 (by inference from the head circumferences), is far below US National Centre for Health Statistics (NCHS) standards. Outcomes will thus contribute to the understanding of the functions of early dietary FAs and their relationship with infant growth and health in developing countries, and any positive results observed be of scientific and public health importance.

Studies investigating the physiological functions of early dietary FAs in relation to environmental enteropathy in infants are lacking. This is, therefore, an under-researched area necessitating contributions towards understanding the physiologic role of PUFAs, and particularly n-3 LCPs, in relation to infant gut integrity, inflammation, and growth faltering, in a developing country setting.

1.4.1.2 Potential costs

Poor environment causes chronic immunostimulation by environmental antigens that keeps the acute phase response working overtime. Increased gut permeability may be an adaptive response - a modulator of immune functioning/absorption appropriate to the environment. Short stature, for example, sacrifices height for the sake of surviving on less nutrition. Similarly, alterations in intestinal morphology and absorption capacity may serve some benefit to the infant. In IBD, for example, mucosal healing is often not associated with clinical well-being (222). Inflammation is, overall, a protective mechanism against pathogens, toxins and allergens. One question therefore was whether a modulated immune response could result in a weakened communication of a harmful luminal signal, and whether the ensuing reduced inflammation and intestinal permeability would be detrimental to host defence, causing, perhaps, a lack of response for controlling pathogens.

Chronic inflammation, however, is detrimental to tissues by perpetuating damage and, unlike acute responses, may stem from immune-dysregulation or an exaggerated response to non-dangerous signals. n-3 LCPs may therefore restore or improve this regulation, overall, and prevent or delay tissue damage in the first place, and seal "permeability leaks" that allow access to antigens. Animal studies, for example, provide support for the role of n-3 LCPs in providing mucoprotection and maintaining intestinal integrity (223), and have shown that n-3 LCPs reduce mucosal damage associated with ulcerative colitis, promote histological recovery, and lower mucosal inflammatory eicosanoid levels (224).

Therefore, it was believed that, although no emphasis should be removed from the importance of improved sanitation and hygiene in infant health and growth, the randomised controlled trial described below was an important attempt to ameliorate the widespread enteropathy seen in Gambian infants, and hence to improve their health, growth and development.

CHAPTER 2:   HYPOTHESES, AIMS AND OBJECTIVES

2.1   Research hypotheses

2.1.1   Primary hypotheses

1. Dietary n-3 LCP supplementation will improve rural African infants' growth performance.

2. Dietary n-3 LCP supplementation will protect infant mucosal epithelial integrity.

2.1.2   Secondary hypotheses

1. Dietary n-3 LCP supplementation improves infant plasma n-3 FA status.

2. Dietary n-3 LCP supplementation will enhance the cognitive development of rural African infants.

3. Dietary n-3 LCP supplementation will reduce the degree of intestinal inflammation of rural African infants.

4. Dietary n-3 LCP supplementation will reduce infant systemic inflammation.

5. Dietary n-3 LCP supplementation will reduce the incidence of morbidities in rural African infants.

6. n-3 LCP status will predict gut integrity and function of rural African infants.

2.2   Aims

2.2.1   Primary aims

To investigate whether supplementing infants with n-3 LCP s will

1. Improve the growth of rural African children;

2. Reduce, delay, or repair gut damage caused by chronic environmental enteropathy.

2.2.2   Secondary aims

To assess the influence of n-3 LCP supplementation on plasma fatty acid profiles, intestinal and systemic inflammation, and morbidity patterns of rural African infants, and enhance infant problem solving and attention behaviour.

2.3   Objectives

2.3.1   Primary objectives

1. To assess the influence of increasing n-3 LCP status on infant growth performance.

2. To test whether n-3 LCP status predicts, and supplementation improves, gut integrity and function as assessed by the LMR in the dual-sugar permeability test.

2.3.2   Secondary objectives

1. To monitor the influence of supplementation with n-3 LCPs on plasma fatty acid concentrations.

2. To test whether n-3 LCP supplementation enhances infant cognitive development as measured by an infant planning test and attention assessment.

3. To test whether n-3 LCP supplementation is associated with the acute phase response protein expression and systemic inflammation, as assessed by plasma C-reactive protein (CRP), α1-acid glycoprotein (AGP) and albumin concentrations.

4. To test whether n-3 LCP status influences the degree of gut mucosal inflammation by measuring markers of intestinal inflammation.

5. To test whether n-3 LCP supplementation is associated with infant morbidity patterns as assessed by morbidity questionnaires and nurse and doctor visits.

6. To investigate cross-sectional associations of outcome variables with n-3 LCP status.

7. To investigate whether n-3 LCP status at birth predicts later cognitive development.

CHAPTER 3:   METHODS

3.1   Trial design

The study was a parallel-group, randomised, double-blind, placebo controlled two-arm trial designed to evaluate the influence of early n-3 LCP supplementation on infant health and development.

The primary endpoint measures were:

a) Growth

b) Gut integrity

The secondary endpoint measures were:

a) Plasma fatty acid status

b) Infant cognitive development

c) Systemic inflammatory and acute phase markers

d) Intestinal inflammation

e) Infant morbidities

3.2   Study population

The study population comprised infants aged 3-9 mo living in the sixteen larger villages of the West Kiang region of rural Gambia. Initially, all infants were recruited from the database of an ongoing Peri-conceptual Micronutrient Supplementation Trial in West Kiang. This was because the data were available, up-to-date, and relatively reliable, and included most West Kiang mothers likely to give birth starting from a few months before the start of the n-3 LCP supplementation trial onwards.

However, it became apparent that many more births were occurring outside of the periconceptual micronutrient trial than expected. This was because some women who were not likely to give birth and were therefore excluded from the trial (e.g. breast-feeding/not knowing date of last menstrual period/contraceptive use etc.) nevertheless went on to give birth. Additionally, nearly a third of the recruited women did not turn up for an obligatory sonar scan, subsequently not being included in the trial, but possibly giving birth.

Because recruitment was therefore taking longer than expected, potential subjects were later drawn from the West Kiang Demographic Surveillance System rather than the original cohort so as to speed up recruitment. Approximately 135 subjects were already recruited before moving to recruitment from the Demographic Surveillance System database.

3.2.1 Eligibility criteria

All infants born from the Peri-conceptual Micronutrient Supplementation Trial (later this changed to "All infants born" whether from this trial or not) in 16 specified villages of the West Kiang region of The Gambia, not enrolled in any other study, were eligible to take part in the study. Subjects with severe congenital abnormalities that could affect growth and development, those from multiple births, and those with known HIV infection were excluded, as these conditions might have influenced growth in unpredictable ways.

3.2.2 Selection, enrolment, randomisation and allocation

The village selections were made to permit for adequate recruitment numbers, based on projections of village birth rates drawn from 2005 West Kiang Surveillance data. Potential subjects were identified from a central database. They were recruited at 2 to 2.5 mo of age.

A computer-generated permuted block randomisation (block size = 16), ensuring a uniform distribution of treatments across the seasons of birth, was implemented by the trial statistician. After subjects were recruited and their parents had provided written consent, they were enrolled into the study and assigned randomly, using the block-randomisation described above, on an individual basis and according to date of recruitment, to one of four treatment codes. Two of these codes corresponded to the active group, and two to a control group, so that infants were allocated to either the n-3 LCP or control groups in a 1:1 ratio. Infants that remained in the study stayed on the same allocations throughout the duration of treatment.

3.2.3 Sample size

For the purpose of power calculations, "growth" was assumed to be the difference between weight or length at 3 and 9 mo of age and that intervention differences were tested using a t-test. Power and significance levels were set at 90% and 5% respectively.

Below is given the mean and standard deviation (SD) of the difference between measurements at 3 and 9 mo of age (i.e. "growth" rate) for 1621 children born in Keneba since 1989:

Variable	Mean growth	SD growth
weight (kg)	1.94	0.65
length (mm)	100	25

These figures were applied to the usual formula for t-test sample size:

$$n = \frac{(u + v)^2 (\delta_1^2 + \delta_2^2)}{(\mu_1 - \mu_2)^2}$$

where:

n = Sample required; δ_1, δ_2 = Standard deviations; u=1.96, v = 1.28; $\mu_1 - \mu_2$ = Difference between the means

Using this formula values for the minimum detectable differences in growth between intervention groups were obtained. These were expressed both as absolute units (kg and mm) and as percentage of the mean growth. It was concluded that a sample size of 75 per group (see equations below and Table 3) would allow the detection of differences in growth rates of 17.7% and 13.2% for weight and length gain, respectively, between intervention groups.

For growth in weight:

$$\mu_1 - \mu_2 = \sqrt{(1.28 + 1.96)^2 \times (2 \times 0.65)^2} \Big/ 75^2$$

$$= 0.343 \text{kg} \ (17.7\%)$$

For growth in length:

$$\mu_1 - \mu_2 = \sqrt{(1.28 + 1.96)^2 \times (2 \times 25)^2} \Big/ 75^2$$

$$= 13.233 \text{mm} \ (13.2\%)$$

Table 3. Minimum detectable differences in growth between intervention groups in different sample sizes

	Difference in length gain		Difference in weight gain	
n per group	mm	% of mean	Kg	% of mean
25	22.921	22.9%	0.594	30.6%
50	16.208	16.2%	0.420	21.7%
75	**13.233**	**13.2%**	**0.343**	**17.7%**
100	11.460	11.5%	0.297	15.3%
125	10.251	10.3%	0.266	13.7%

A target of n=90 per group was thus set, allowing for a 20% loss to follow-up.

3.2.3.1   Power calculations for gut integrity

No adequately reliable estimates of the variance of LMRs in this population were available before the study. Instead, after approximately 25% of the collected urine samples were analysed, power calculations were made in order to test whether, given the reliability of the test, and the degree of intra and inter-individual variation, it was meaningful to continue with the rest of the sample analysis.

A one-way analysis of variance of LMR on individual study number (using STATA's "loneway" command), gave an intra-class correlation of 0.42 (95% CI: 0.24,0.61). The estimated reliability for the mean of three samples was 66%. Applying the chosen power and significance level, a sample size of n=90 per group would then thus enable the detection of a minimum difference of 0.48 SDs. Adjusting for the reliability estimate of the test then gave a final detectable difference of 0.60 SDs. This adjustment expressed the detectable effect size in terms of between-individual variation, indicating the variance due to genuine differences in individual gut integrity, independent of measurement methods or errors.

3.2.3.2   Sample size and cognitive development

Previous (unpublished) data obtained from Peter Willatts for both the Willatts test and the Attention Assessment were used to calculate how small a difference in cognitive test scores could be detected given our sample size (which had reached n=90). Using β=0.9, α=0.05, a sample size of 90 per group, and SDs of 4.35 and 12.6 for the Willatts Test and mean look duration (Attention Assessment) respectively, the ability to pick up a difference of 2.1 points in the Willatts Test and 6.1 in mean look duration was calculated. These differences are 10% and 16% smaller than the differences detected in the studies reported by Peter Willatts.

3.3   Allocation concealment and blinding

Each of the four treatment codes mentioned in section 3.2.2 was represented by a simple picture (shown in Appendix 1). Infants in the treatment group were therefore allocated to one of two possible pictures, and infants in the placebo group to one of the other two pictures. Four different codes as opposed to two were used to assist in blinding. The wife of a colleague, who was not involved in the study, allocated each picture to a treatment type. This allocation was sent to the company who provided the oils, who labelled the bottled oils accordingly. The trial statistician and another colleague (independent of the trial) then kept the picture-treatment allocation in a sealed envelope until analyses were complete.

The Principal Investigator (PI) remained blinded as to which two codes corresponded to each other as well as to which picture-group each infant-mother pair had been allocated to for the duration of data collection. With the exception of the trial statistician - who required the information for safety monitoring analyses - and the database manager who had access to the information, all researchers remained blinded as to which two treatment codes/images pictures corresponded to each other until all data collection was complete. The PI remained blinded to treatment allocation until analyses were complete.

After mothers had given their consent, and their infants had been recruited, a fieldworker issued them with a card printed with the appropriate picture. The cards were prepared and produced by the data-office staff. One side of the card was printed with the code picture, the other contained the subject details such as DOB, name, sex, and mother's name (Appendix 2).

Mothers were asked to bring their picture-coded cards when their infants were brought to be administered the supplement. This enabled field workers to correctly identify which oil to administer to each infant. Fieldworkers were also given a treatment booklet. It contained details of infants and their corresponding treatment images from which to read off allocations in the event of a mother failing to present her card before supplementation, and from which to cross-check for any card-swapping.

Placebo and treatment oils both contained 1.25% lemon oil with the intention of flavouring the oils similarly and masking the fish-smell/taste of the active oil for blinding purposes, and 0.5% rosemary extract as a natural antioxidant. Field workers who were unaware of which picture codes represented treatment or placebo administered the supplement.

3.4 The study intervention

3.4.1 Description of treatments

The active group received 2ml highly purified fish oil donated by Nordic Naturals Inc, Watsonville, CA. This oil, produced from deep-sea anchovies and sardines, was constituently similar to their commercially available "Omega-3 liquid" (label from commercial product shown in Appendix 3). However, vitamin E (d-alpha-tocopherol) – added to protect against oxidation – was requested to be lowered from a concentration of 30IU/5ml to 5IU/5ml which is more appropriate for infants and was advised by the Independent Trial Monitor. The fish oil contained 10% olive oil to dilute the dose slightly so as to supply 200mg DHA and 300mg EPA per 2ml. The supplement was administered daily for 24-25 weeks. The dosage of 500mg combined DHA+EPA per day was designed to achieve a substantial increase in plasma n-3 PUFA to both eliminate any existing deficiencies and to elicit a therapeutic response.

The placebo group were given the same volume of organic food-grade extra virgin olive oil, also received from Nordic Naturals. This oil predominantly contained oleic acid (67%, a monounsaturated FA), with small amounts of palmitic (14.4%), linoleic (13.2%), and linolenic (0.6%) acids. Both fish and placebo oils were bottled in 237ml blue glass bottles with black, cone-lined caps, and were stored at 4°C.

3.4.2 Administration of treatments

Fieldworkers administering treatments were stationed in each of four study areas. They were assigned the duty to visit the villages that were allocated to them each morning, where they would meet the mothers and subjects at a central meeting point. Mothers were asked to bring their infants to this meeting point every day.

Village assistants appointed in each village were given the task of preparing tea and bread to serve to the mothers when they brought their infants, and of tracing and calling those mothers who were absent. Sterile, graded, 3ml pastettes were used to squeeze the oil into the side of the mouth of each infant (Figure 12). In order to ensure that it had been swallowed, and to enhance absorption, mothers were asked to breast-feed their infants immediately after the oil had been given (Figure 12).

Fieldworkers working in the field were provided with gas-fridges, ice packs and cool boxes for storage and transportation of oils. The supplement was kept cool at all times to prevent heat-induced oxidation and so minimise deterioration and rancidificaiton of oils.

In four cases, the mothers of subjects were required to travel outside of the study area for periods of roughly two to four weeks. In three of these cases, they had access to a fridge. These three mothers were provided with supplement, ice packs and pastettes, and shown how to supplement their infants themselves. While they were away, they were contacted daily by telephone to ask whether they had supplemented their infants, and to ask about the infant's well-being. In the fourth case, the mother was staying in the village of an MRC fieldworker who was on leave for a month. Despite being on leave, this fieldworker visited the mother daily to supplement her infant and complete the morbidity questionnaire.

Towards the end of the study, when it was the dry season and mothers started travelling to the coast more regularly, a fieldworker was stationed at the coast full-time for a month and a half. He went around lodgings supplementing the infants for the duration of their stay at the coast.

The fieldworker administering the dose, using a pre-printed weekly compliance record sheet prepared separately for each infant, recorded daily compliance.

Figure 12: Infant receiving dose, left, and mother breast-feeding immediately afterwards, right

3.5   Measurements and data collection

Primary endpoints and stool measurements were taken in triplicate at baseline and endpoint visits. This was done with the intention of minimising some of the intra-individual variance, and so the standard error, associated with these outcomes and their measurements. By using the median of the three growth measurements, for example, the more inaccurate readings were most likely lost, and by using the median of three urinary-sugar determinations, some of the standard error introduced by day-to-day biological variation was minimised.

Mothers and their infants were brought to MRC Keneba three times at baseline (3 mo of age) and three times at 9 mo of age (alternate days for both), for a five-hour clinic visit. During each of the visits anthropometric measures, urine, and stool samples were collected. Blood was drawn from infants, and breast-milk from the mothers, once at baseline and once at endpoint.

Mothers expressed approximately 5ml breast-milk from both breasts before their infants received their first feed at the clinic, and again immediately after they had fed their infants. The breast-milk samples were pooled by mixing together 1ml of each of the four collected samples, and a 2ml aliquot of this pooled sample stored at -80 °C until analysis.

Human breast-milk lipid concentration increases several fold between the beginning and end of a feed (225-228). In order, therefore, to gain a more accurate indication of the amount of fatty acids consumed by infants during an average feed, it was important to not only sample the fat-rich hind milk, but also the more dilute foremilk. However, this timing could have been a possible source of error in estimating the true average fatty acid concentrations of the breast-milk. Many studies on the topic have used a full breast expression or only a hind-milk sample from women, and the fatty acid concentration of the breast-milk investigated in this way. Sample timing therefore needs to be considered when making comparisons with other literature. Nevertheless, it is known that although the lipid concentration of breast-milk changes across a feed, the fatty acid composition of the lipid stays constant (226, 229, 230). When making comparisons with other studies, the relative fatty acid concentrations of the Gambian mothers' milk were considered, rather than the absolute concentrations of fatty acids, and in this way the errors introduced by sample timing avoided.

A malaria slide was prepared at each blood draw for timely detection of any parasite infection. It also served to protect the investigators, as, were an infant to fall seriously ill shortly after their blood was drawn, the community might have ascribed the cause to the drawing of blood.

Infants were brought for their final follow-up visit involving a cognitive test at 12 mo of age.

Each month a basic weaning foods questionnaire was completed by the mother and fieldworker intending to, for descriptive purposes, gain some indication of the age at which the infants were weaned, and onto which foods.

For safety monitoring purposes only, length, weight, HC and knee-heel length was measured in the field at 5 and 7 mo of age, by trained fieldworkers. These data were used at interim safety monitoring assessments to check for any treatment-group differences in growth.

The various samples and data collected during the clinic visits, and at other time-points, are summarised in Table 4.

3.6   Primary endpoint measurements

3.6.1 Growth

Infant length was measured with a Harpenden Infantometer Measuring Table to within 0.1 cm precision. Weight was measured to within 0.01 kg precision using a Seca (Model 336) electronic baby scale. Knee-heel length, measured with a hand-held Chasmors Knee to Heel Measure sliding calliper (0.1 cm precision), was used as a sensitive measure of lower-leg growth.

Skinfold thickness, measured with a skinfold calliper (Holtain/Tanner/Whitehouse Model) to 2 mm precision at specific sites, always using the left side of the body, was assessed as an indicator of subcutaneous fat accumulation. Mid-upper-arm circumference (MUAC), indicative of muscle mass and subcutaneous fat, was measured on the left arm, using a paper measuring tape specially designed for this purpose, to a precision of 0.1cm. HC was determined as a proxy for brain size. Because it changes rapidly during infancy, HC can also be used to monitor growth. It was measured with a stretch-proof measuring tape (Model CTM08) around the maximum circumference of the head (forehead to occiput) to the nearest 0.1cm.

All equipment was purchased from Chasmors Ltd., London, UK. The PI, assisted by a trained field worker, took anthropometric measurements using regularly calibrated standard equipment.

Growth data were expressed in absolute units at baseline and endpoint, and growth rate between these two points. Infant weight-for-height, as well as weight/height/head-circumference/MUAC/subscapular and triceps skinfolds-for-age were expressed as z-scores based on the 2006 WHO Growth Reference Data (231, 232). By calculating the z-scores the data were automatically corrected for age and sex.

Table 4: Data collection and measurements by time-point

Time-point	Samples/data
Infants	
Day 1 of 3 mo and 9 mo visits	2ml venous blood
	Weaning foods questionnaire
	Malaria slides
Day 1, 2 and 3 of 3 mo and 9 mo visits	Urine (LMR)
	Stool samples (calprotectin)
	Anthropometry (3 measures)
Throughout for 6 mo	Daily morbidities, nurse/clinic visits
	Compliance monitoring
4 mo	Weaning foods questionnaire
5 mo	Anthropometry in field for safety monitoring
	Weaning foods questionnaire
7 mo	Anthropometry in field for safety monitoring
	Weaning foods questionnaire
8 mo	Weaning foods questionnaire
12 mo	Cognitive test
Mothers	
Day 1 of 3 mo and 9 mo infant visits	4ml breast-milk
12 mo infant visit	Education questionnaire
Other data available	
9 mo	Buffy coats collected for DNA extraction
Birth (from PMMST)	Cord blood serum, maternal serum

3.6.2 Gut integrity

Intestinal surface area and permeability were measured by way of the dual-sugar permeability test. For this, infants were given a 2ml/kg body weight dose of sugar solution, containing 400mg lactulose (Lactulose Solution BP, Sandoz Ltd.) and 100mg mannitol (Sigma-Aldrich Co.) per 2 ml water.

An indication of passive intestinal absorption, the recovery of the monosaccharide, mannitol, is reduced in the presence of villous atrophy. Uptake of the disaccharide, lactulose, is measured as a marker of intestinal leakiness. Whereas lactulose passage across the intestinal

barrier is prevented by healthy enterocytes, in the damaged gut it is more readily absorbed, probably paracellularly. This comparison is illustrated in Figure 13. Intestinal permeability, therefore, is measured as urinary lactulose concentration divided by urinary mannitol concentration. By measuring total urine volume passed, percentage recoveries of the two probes could be calculated as a reflection of the amounts taken up by the passive and the paracellular intestinal routes.

The value of this test is well expressed by Lunn et al. (1991) (30): "Because of its non-invasive nature and simplicity, and because no complicated equipment is required, mucosal status can now be assessed frequently on large numbers of individuals, even under field conditions. It provides a quick and objective way of determining the pathological impact on the gut, and ultimately on growth performance, of the wide range of potentially adverse environmental conditions to which infants in developing countries are constantly exposed."

On the morning that infants were brought into the clinic, they were given the sugar solution by means of a graded syringe approximately 30 minutes after their last feed. Breast-milk was then withheld for a further hour. Total urine volume passed over the next 5 hours was collected using paediatric urine bags (Hollister U-bag, Abbot Labs, Queensborough, Kent, U.K.,). Figure 14 and Figure 15 show photos of the urine collection visits and urine bags.

Urine was drained into collecting bottles (Figure 15) containing 2-3 drops chlorhexidinegluconate (5% weight/volume) as a bacteriostatic agent. After urine volumes were measured, 2 x 2ml aliquots were collected and stored at -80°C for later sugar analysis.

Urinary lactulose and mannitol concentrations were measured in Keneba using a 96-well microplate enzymatic assay. The method was developed by Dr Peter Lunn in Cambridge as an adaptation of a previously described technique for use on the Cobas-Bio centrifugal analyzer (233-236). All samples were measured in duplicate.
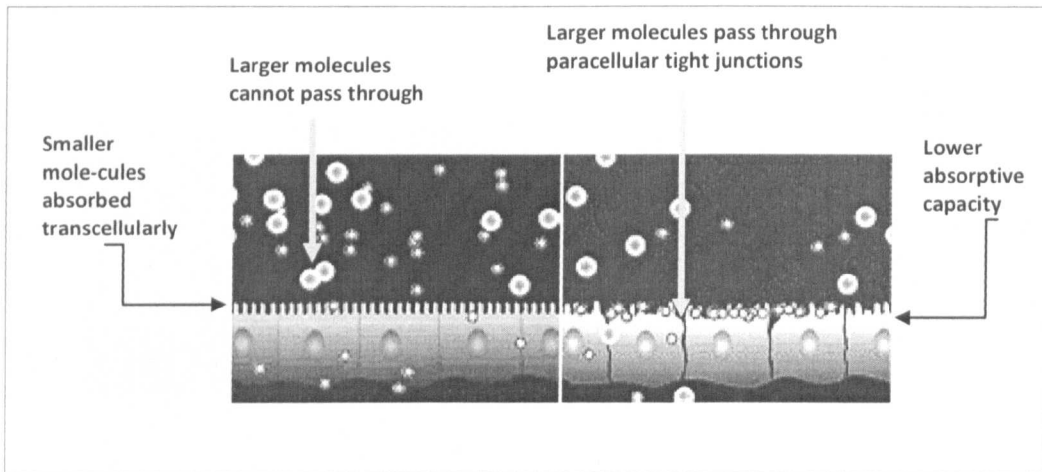
Figure 13: To the left a healthy gut with normal absorptive capacity and healthy tight junctions. To the right intestinal mucosa displaying poor integrity with less absorptive capacity and leaky tight junctions.

Figure 14: Mothers and infants during urine-collection time
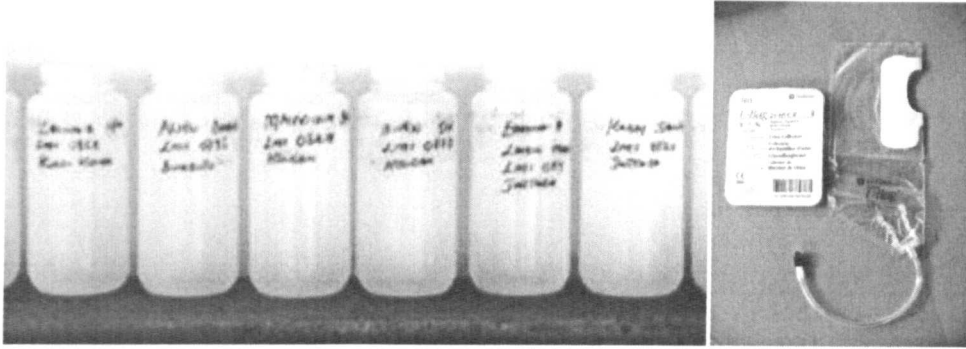
Figure 15: Urine bottles used to drain urine into, and Hollister U-bag used for urine collection

3.6.2.1   Laboratory work in Keneba

Lactulose assay

The assay measures urinary lactulose concentration by measuring the amount of fructose - one of its component monosaccharides - released when enzymatically hydrolysing lactulose with β-galactosidase. Fructose is then phosphorylated by ATP using hexokinase, forming fructose-6-phosphate which is converted to glucose-6-phosphate by phosphoglucose isomerase. Glucose-6-phosphate is further oxidised by glucose-6-phosphate dehydrogenase to form gluconate-6-phosphate, in the presence of NAD. An equimolar amount of NAD becomes reduced to NADH, which absorbs at 340nm. The resulting increase in absorbance is directly proportional to the fructose concentration, and so therefore also the lactulose concentration. This reaction is illustrated in Figure 16.

In order to account for any free fructose which may be present in the urine and augment the lactulose concentration artificially, the concentration of free fructose is also measured (in a similar reaction but without the addition of β-galactosidase) and subtracted from the determined lactulose concentration.

The assay simultaneously allows the measurement of lactose in the sample. Previous data show that in breastfed Gambian infants, poor gut integrity is associated with lactose maldigestion (46). Lactase concentrations are low in a damaged intestine because of the

vulnerable position of lactase bearing cells in the brush border, which become damaged by enteropathy (46). Urinary lactose concentrations were therefore also assessed as an additional observation.
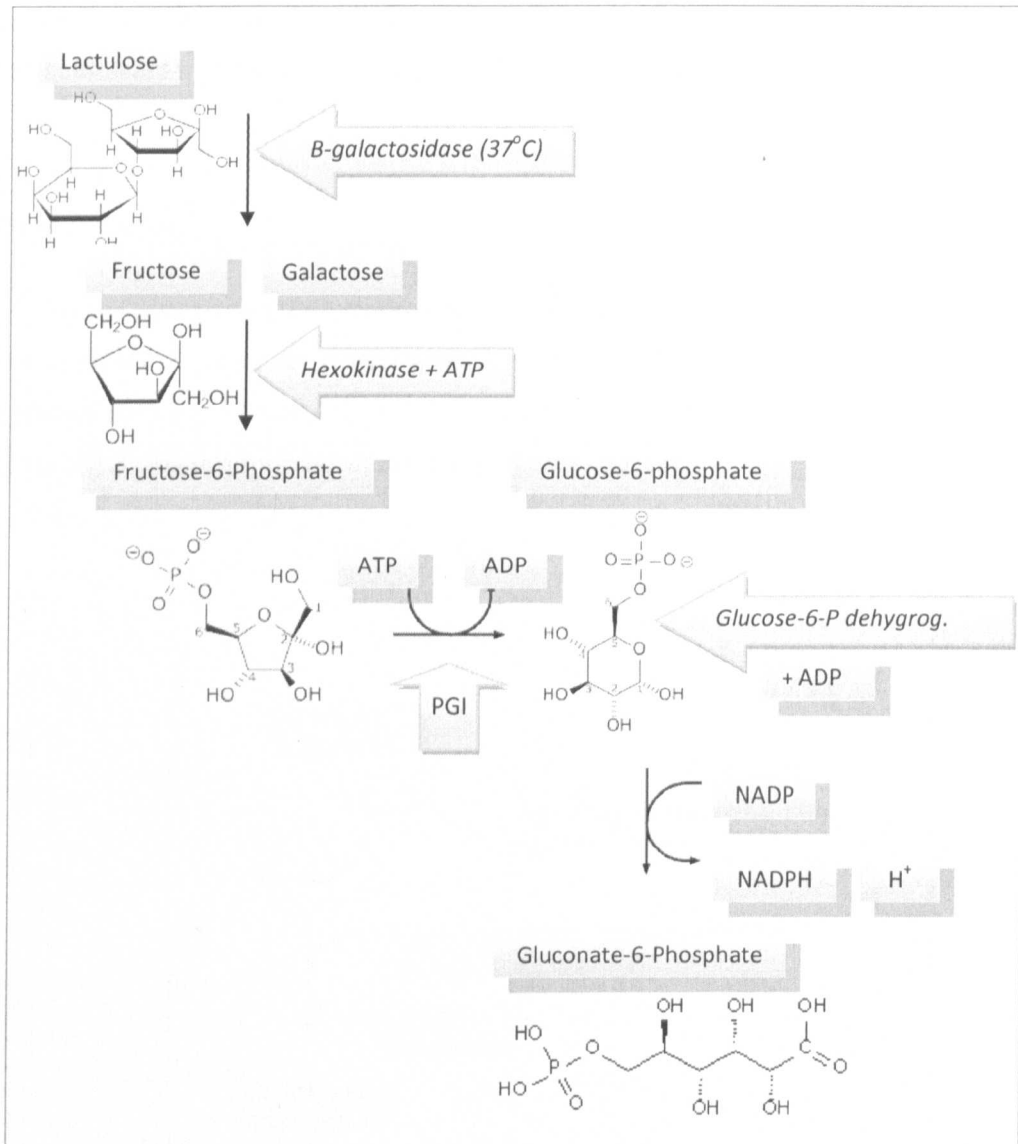
Figure 16: Enzymatic reactions involved in the lactulose assay. PGI – phoglucoseisomerase

When the urine is treated with β-galactosidase, as explained above, lactose is hydrolysed into glucose + galactose. In the reactions that follow, using the same enzymes as for fructose determination, glucose is phosphorylated to glucose-6-phosphate and then oxidised to gluconate-6-phospate in the presence of NAD. During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340nm is directly proportional to glucose (and therefore lactose) concentration. Similar to what is done for the lactulose determination, free glucose is also measured without the addition of β-galactosidase and subtracted from the lactose concentration to give the final urinary lactose value.

Mannitol Assay

For the quantitative, enzymatic determination of mannitol in urine, NAD is added to it in the presence of mannitol dehydrogenase. Mannitol is converted to fructose, while NAD becomes reduced to NADH (Figure 17). The consequent increase in absorbance at 340nm (due to NADH) is directly proportional to mannitol concentration.
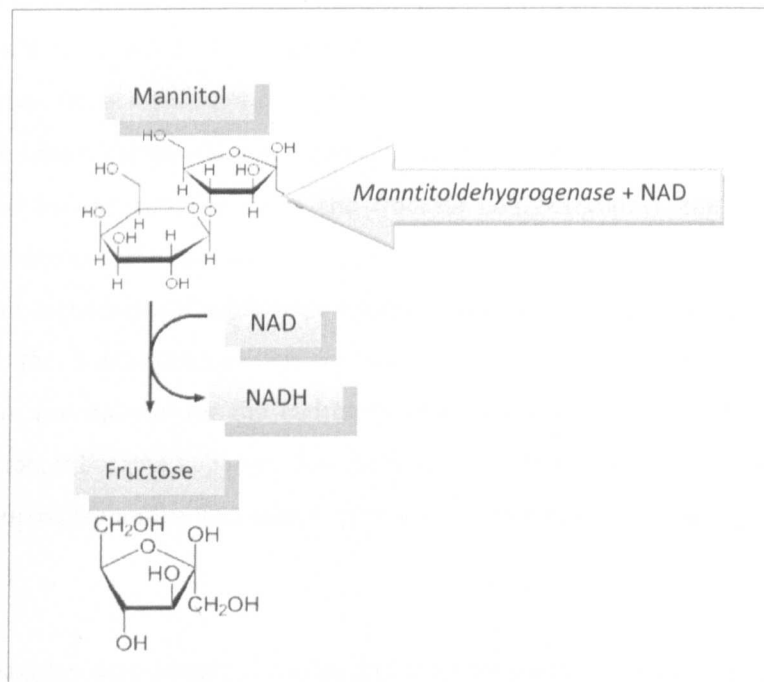
Figure 17: Mannitol assay reaction

All enzymes were purchased from Sigma-Aldrich Co., apart from mannitol dehydrogenase that was bought from Biocatalysts Ltd. Plates were read using an Appliskan plate reader with automated injection feed (Thermo Scientific, part of Thermo Fischer Scientific, Waltham, MA, USA).

Assay workup

Reagents and standards were tested by running a series of standard curve repeats, and later urine-sample repeats.

The mannitol assay responded well during preparatory runs, and the protocol could be followed without the need for any adjustments. The standard curve test produced a good linear fit ($R^2 > 0.99$), with good reproducibility between parallel curves (percent coefficient of variation < 2%). The same held true when testing urine sample replicates.

A great number of challenges were faced with the lactulose assay, however. The first few standard curve series repeats produced intermittent highly erroneous results in inconsistent patterns, with poor reproducibility. After making certain adjustment (e.g. adding small reagent volumes by hand rather than via the automatic injection feed) it appeared as though, on a half-plate containing six sets of standards, the reaction was not going through to completion in all six of the cases. These results are presented in Figure 18. Some curves demonstrated a good linear fit, while others clearly did not. However, when omitting the ß-galactosidase step (for plates intended for measuring glucose and fructose concentrations), the standard curves produced high degrees of reproducibility and goodness of fit. Because the errors were far more prominent in the lactose/lactulose part of the assay, where ß-galactosidase was added, it appeared that the ß-galactosidase enzyme may be at fault, or that the temperature/pH conditions were not optimal for the hydrolysis of lactulose and lactose. Additionally, after further inspection, it became apparent that the hexokinase reaction had not plateaued before the phosphoglucoseisomerase was added, so a longer incubation time for this reaction was also tested.

After all reagents were prepared freshly and their pH checked, two standard curve series were treated with a four-hour-long ß-galactosidase incubation period (in contrast to the standard two hours), two series with a longer hexokinase reaction incubation, and two series with the standard protocol.

The results of these incubations (Figure 19) were that, as before, the glucose/fructose assay (requiring no B-galactosidase addition) responded well. For lactose/lactulose assays, wells that were allowed to incubate according the standard protocol produced the best curve fits, and wells exposed to a longer hexokinase step incubation were failures. One of the series allowed to incubate for four hours during the B-galactosidase step produced a curve with good fit, whereas the matching second series did not. Hence, it appeared that neither the incubation time nor the enzyme was at fault, but that some other cause was affecting different well columns differently.
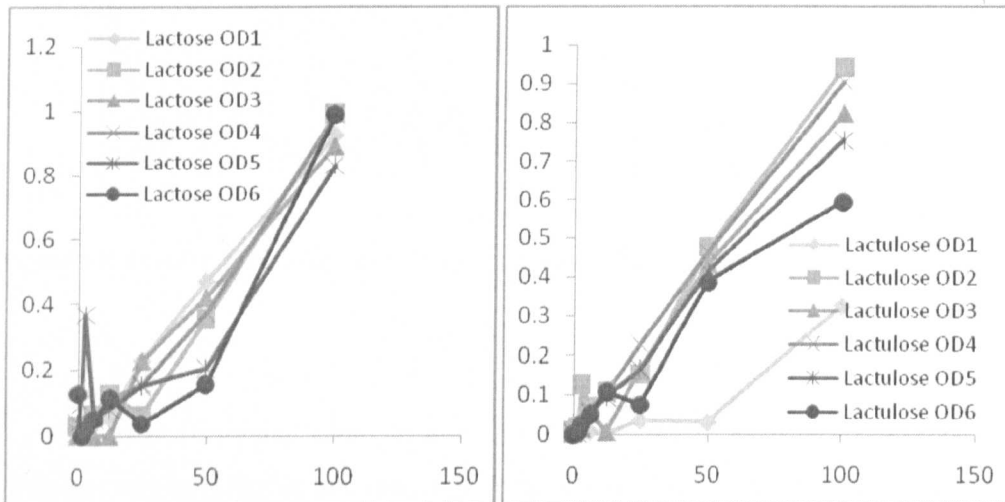
Figure 18: Lactulose assay standard curve test results. "OD"s are "change in optical density." To the left: reaction measuring lactose concentration, and to the right: lactulose concentration.
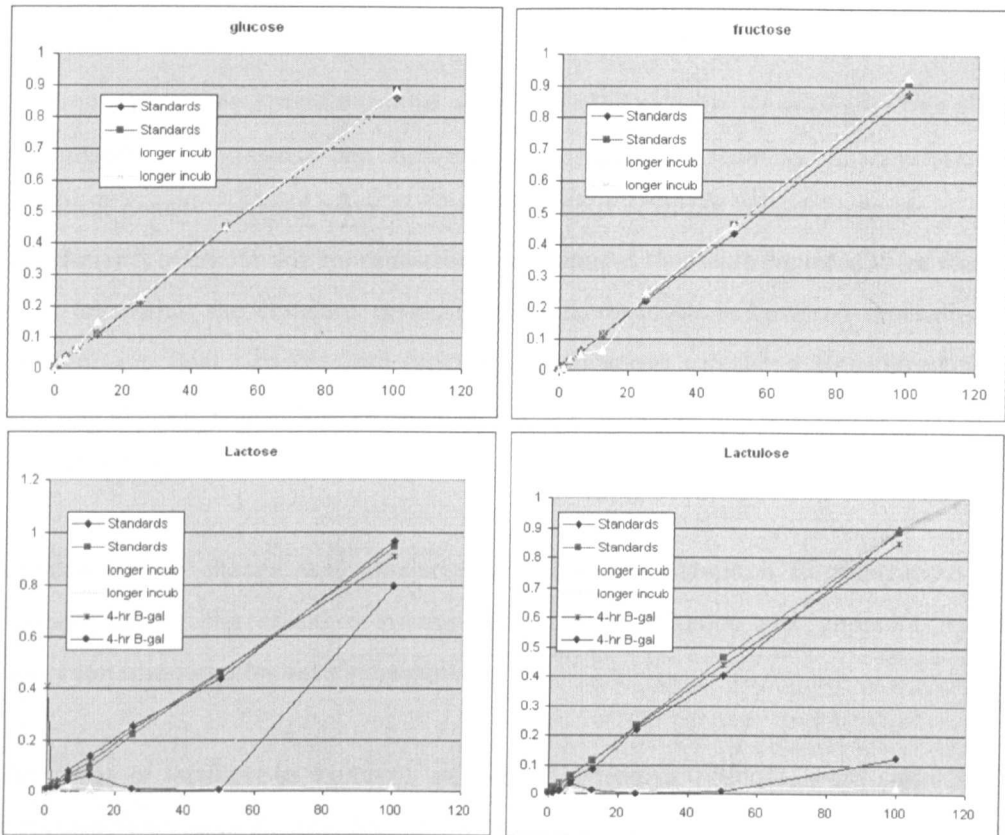
Figure 19: Results of standard curves treated with varying incubation times

Various plate layouts were designed and tested, and so each possible cause for faulty reactions was investigated and ruled out. A big influence appeared to be interferences caused by the plate reader. After seeking advice from Peter Lunn the injection feed jets were cleaned and the machine serviced. This effected a big improvement on assay results. Yet, still at times the optical density readings increased abruptly and unexpectedly half-way through a plate reading and the assay had to be repeated; but this, it was realised, occurred only when a number of plates were read in succession, so the machine was in future turned off and allowed to cool down between runs if many were done in sequence. Although % coefficient of variations (CVs) between replicates were occasionally inexplicably high, overall they were on average acceptable (<10%) and standard curve R^2 always greater than 0.98.

Calculations

Differences between optical densities were calculated to generate values for free glucose, free fructose, lactose+glucose and lactulose+fructose urinary readings. Concentrations for these optical density changes were determined against relevant individual standard curves, and adjustments made for any concentrated urine samples that were required to be diluted in order to fall within the standard curve. Average concentrations between replicates were determined, and final lactulose and lactose concentrations calculated by subtracting free fructose and glucose values from lactulose+fructose and lactose+glucose values, respectively, at baseline and endpoint.

Optical density change was similarly calculated for mannitol. Concentrations were determined against the standard curves, and a mean baseline and endpoint mannitol concentration computed for each individual.

The ratios of lactulose-to-mannitol, as well as lactose-to-lactulose, were calculated for each sample (unaveraged), and the individual median LMRs and lactose:lactulose ratios determined for baseline and endpoint.

Finally, percent recoveries for lactulose and mannitol were calculated by computing the total amount of sugar passed in the urine (concentration of sugar measured by the assay x total urine volume collected) as a percentage of the original dose given.

3.7   Secondary endpoint measurements

3.7.1 Plasma fatty acids

An experienced nurse drew approximately 2ml venous blood from infants at baseline and endpoint. Blood was kept on ice until centrifugation 5 – 20 minutes after collection, and then spun at 3000rpm for 20 minutes at 4 $^{\circ}$C. The plasma supernatants were transferred to microtubes and the headspaces flushed with nitrogen to minimise the oxidation of volatile PUFAs, before storing them in 81-space microtube boxes at -80 $^{\circ}$C.

At baseline blood was drawn into lithium-heparin-coated tubes. As part of a potential collaborative study, buffy coats were collected for DNA extraction purposes at 9 mo. Because

heparin may inhibit PCR reactions, EDTA was used as anticoagulant at 9 mo, after verifying that the change in anticoagulant would not interfere with FA and clinical chemistry assays.

Once filled, boxes were sent to the MRC Collaborative Centre for Human Nutrition Research in Cambridge, UK, by air, on dry ice, for extraction, derivatisation and analysis of FAs by GC with flame ionisation detection. Plasma was sent in four different batches. Once received in Cambridge they were once again stored at -80 °C. In order to avoid batch differences, laboratory analysis commenced only once all samples had been received.

Apart from measuring only the FAs of interest, additional markers were also used to assess PUFA status. Because plasma content of essential fats does not necessarily indicate proper use of these FAs by cells and tissues, additional status markers are useful for determining functional PUFA status. The essential FA status index, for example, was assessed. It measures the ratio of all essential-to-nonessential unsaturated FAs and increases in value as PUFA status improves.

Docosapentaenoic acid (22:5 n-6) is synthesised in conditions of DHA functional shortage. DHA:docosapentaenoic ratio was therefore similarly used as a functional DHA status marker.

The ratio n-6:n-3 FAs was another measure of interest. This ratio has been found to have significant correlations with outcome measures, with lower ratios related to outcomes that are more beneficial.

The FAs and FA relationships of interest are given in Table 5.

Table 5: Fatty acids and fatty acid relationships assessed

Monounsaturated	n-6 PUFAs	n-3 PUFAs	Fatty acid relationships
Oleic acid	C18:2 (linoleic)	C18:3 (α-linolenic)	AA/EPA+DHA
	AA	EPA	DHA/DPA
	Sum of all n-6	DPA	(n-3 + n-6)/(n-7 + n-9)
		DHA	Sum of n-6 ≥ C20/ Sum of n-3 ≥ C20
		Sum of all n-3	

3.7.1.1 Laboratory work at Human Nutrition Research Centre, Cambridge

Preparation of total lipid extract

Total lipid extracts were prepared from 200 µl of thawed plasma, to which 25 µl of internal standard was added to compensate for any losses during extraction and derivatisation, using an adaptation of the method of Folch (237). Plasma was homogenised with chloroform and methanol, and washed with 1M NaCl solution. After vortexing briefly, the mixture was centrifuged at low speed (2000rpm) for ten minutes to separate the phases. The lower phase was then collected and evaporated to near dryness.

Preparation of fatty acid methyl ester (FAME)

FAME's were prepared by acid-catalyzed esterification. Boron triflouride (14%) was added to the reconstituted lipid residue, and the mixture heated for 45 minutes at 75 °C. After cooling, hexane and water were added, and the mixture centrifuged at low speed for ten minutes. The upper hexane layer was then transferred to a glass tube and placed under a vacuum drier. Once dry, the residue was reconstituted with hexane, and this mixture transferred to a GC auto sampler vial, ready for GC analysis.

Analysis of samples by GC

A BPX70 column (70% cyanopropylpolysilphenylene-siloxane, supplied by SGE), with polarity designed specifically for FAME analysis, was used for fractionation. This column can withstand temperatures up to 250 °C and its nominal dimensions are 30m long, 250µl diameter and 0.25 µl film thickness. The carrier gas was helium. The front inlet temperature was set to 240°C and pressure to 13 psi.

Elution profiles for each sample were obtained, consisting of 38 peaks representing relative proportions of the different FAs. Each of the FA components was quantified against a calibration range of external standards of known concentration (ng/µl), using linear regression. Absolute as well as relative percentage values of the different FAs were therefore available.

3.7.2 Cognitive Development

Following the advice of the PI's upgrading panel, this endpoint was included in the trial as an added outcome. The tests were therefore only piloted at a later stage, and once training and piloting was complete, ethical approval was sought and gained later than the approval granted for the main study. For this reason parents were visited a second time to seek informed consent for participating in this component of the study. The information and consent forms used are presented in Appendix 4.

At 12 mo of age (±7 days) infants were brought in for their final follow-up visit to test their cognitive development by means of a 2-step means-end problem-solving test (Willatts Test) and an attention assessment.

The aim of the Willatts Test is to present a challenge to an infant, and then observe whether they are capable of planning and executing a solution in order to solve the challenge. The challenge is to retrieve a toy, which is either concealed or out of reach. The infant needs to manipulate an intermediary object (cloth or cover) in order to retrieve the goal and the intentionality which the child uses to retrieve this goal (toy) is then assessed.

Infant habituation, a decrease in attention to a repeatedly presented stimulus, is considered a basic tool for assessing cognition or processing speed in infancy. As already mentioned in the Introduction, evidence from n-3 and DHA supplementation (and depletion) studies suggest that attention in infancy may be associated with DHA intake.

The Toddler Attention Assessment involves a single-object free-play task, where toddlers' attention to a complex toy is assessed. The toddler is seated at a table, the toy presented for 5 minutes, and the toddler allowed to explore and manipulate it freely. The time from the start to end of each look to the toy, and each look away from the toy, is recorded. A mean duration for looks to the toy and the number of episodes of inattention is then calculated. Procedures for the assessment are described below, and in Colombo *et al.* 2004.

Both the PI and a British graduate student attended a two-day training session with Peter Willatts in Dundee, UK, before the start of the cognitive development pilot study. They received training for conducting both the Willatts Test and the Toddler Attention Assessment, as Peter Willatts had previously worked closely with the developer of the attention test.

The graduate student performed all test scoring. A 30% sample of Willatts problem-solving tests were scored by the PI for test validation purposes. Because this test is open to a high degree of subjective interpretation and inter-observer variability it was important to validate it for quality control purposes.

Due to cultural constraints and distress or curiosity displayed by some of the infants when in contact with white adults, a trained Gambian fieldworker carried out all tests. They were filmed and saved for later viewing and scoring.

To control for any potential stimulatory influences infants may have received in the home were their mother educated as opposed to illiterate, the mother, with the help of the fieldworker, filled out a basic education questionnaire in order to gain an indication of the highest level of education, if any, she had received. This form is shown in Appendix 5.

3.7.2.1   Test procedures

On the morning of the test, mothers were asked to ensure that their infant had been recently fed prior to testing to avoid them being hungry, and therefore distracted, during the 15 minute test.

The mother was asked to sit on a chair with her infant on her lap facing a table placed in front of her. The infant's arms were required to be free to play with the objects in front of them on the table. The mother was asked to make sure her child is secure on her lap, but to not restrict movement by holding him/her. She was also asked not to help, encourage, or prompt her child by either words or actions and not to encourage the infant's eye contact when he/she looks up at her.

For standardisation, the Willatts Test was performed first, followed by the Toddler Attention Assessment. If an infant did not respond during the Willatts Test, the Attention Assessment was completed first, and a second attempt at the Willatts test made thereafter. After completion of both tests, anthropometry measurements of the child were also taken.

3.7.2.2 Willatts Test

The steps of this two step means-end-problem solving test are listed below:

Part 1 – Cover Pretest (carried out twice)

A small toy was handed to the infant so that they showed interest and picked it up. If the infant showed no interest, the toy was replaced with another.

A small towel cloth (20cmx20cm, called the "cover") was presented to the child. He/she was allowed to handle it and become familiar with it.

The toy was placed on a sliding tray (25x30cm), with the cover over it so that it became hidden. It was ensured that the child was watching while this was done.

The tray was pushed towards the child so that the cover with the toy underneath it became within reach.

Thirty seconds were allowed for the child to solve the problem and retrieve the toy by removing the cover (Figure 20, left).

Part 2 – Cloth Pretest (carried out twice)

A towel cloth (20cmx30cm, different colour to cover) was handed to the child so that they could handle it and become familiar with it.

The cloth was placed on the tray and the toy placed on top of it, at the far end of the cloth from the infant. It was ensured that the child was watching while this was done.

The tray was pushed towards the child so that end of the cloth became within reach of the infant, but not the toy (forcing the infant to pull the edge of the cloth towards them bringing the toy with it; Figure 20, middle).

Thirty seconds were allowed for the child to solve the problem and pick up the toy by pulling the cloth close enough to reach the toy.

Part 3 – Barrier Pretest (carried out twice)

A foam barrier (Figure 20, right) was presented to the child to handle for a short while.

The barrier was then placed on the tray and the toy placed directly behind it. The tray was pushed towards the child so that the barrier was within easy reach.

Thirty seconds were allowed for the child to solve the problem and pick up the toy by picking up and removing the barrier in order to reach the toy.

Part 4 – Two Step Problem (repeated 5 times)

With the infant watching, the towel was placed on the tray with the toy on the cloth at the far end of the table, so that the infant could not reach the toy. The cover was then placed over the toy as shown in Figure 21.

The tray was pushed towards the infant so that the near edge of the cloth became within reach.

The child was allowed thirty seconds in which to solve the two step problem and retrieve the toy by pulling the cloth close enough to reach the cover (Figure 21) and then remove the cover to pick up the toy.

Figure 20: From left to right the cover pretest, clothpretest, and barrier pretest steps

Figure 21: Two-step problem test setup and challenge

Scoring

For the 2-step trials, each of three behaviours while solving the problem - 1) behaviour with the cloth/cover, 2) fixation on the toy, 3) and behaviour with the toy - was scored on a scale of 0 to 2. "0" was given for no evidence of intention, "1" for possible intention, and "2" for clear evidence of intention for retrieving the goal.

A brief description the behaviour scores are given in Box 2.

The two outcome measurements were as follows:

a) Trial behaviour scores were averaged to give a mean "total intention score".

b) Each trial in which there was a score of "1" or more for all three behaviours was considered to be an intentional solution. The number of trials in which the child showed some sign of intention became the "total intentional solution score".

However, the results from the pilot study indicated that the scores obtained on the pretest trials were useful as they contained a large amount of information which appeared to have good discriminatory power for distinguishing between infants of differing degrees of problem solving abilities. Thus the average that infants obtained across all pretest trials attempted was used as a third outcome called "pretest average".

Full scoring instructions and the scoring sheets are provided in Appendix 6.

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Box 2: Infant behaviour scores

Cover/Cloth behaviour:

Score=0:   Child failed to -

a) contact the cover/cloth,

b) bring the toy within reach/reveal the toy by lifting the cover, or

c) engage in playing or examining the cloth at any time.

Score=1:

a) Infant pulled the cloth/lifted the cover without any play or examination and brought the toy within reach/revealed the toy, but began an activity which may have been playing with or examining the cover/cloth, but did not carry it through to completion, and

b) did no let go of the cloth/cover for more than 1 sec before the toy was within reach.

Score=2:

The infant pulled the cloth/lifted the cover without any play or examination and brought the toy within reach/revealed the toy.

Fixation behaviour:

1. Score=0:   The infant looked away from the toy for more than 2 seconds.

2. Score=1:   The infant briefly looked away from the toy, but looked back within 2 seconds.

3. Score=2:   The infant was continuously looking at the toy.

Toy Behaviour:

1. Score=0: Infant failed to contact the toy or touched the toy but made no attempt at grasping it.

2. Score=1:   Infant attempted to grasp the toy but did not pick it up.

3. Score=2:   Infant successfully grasped the toy and picked it up.

3.7.2.3   Toddler Attention Assessment

A "V-Tech® Nursery Rhyme" toy, as in Figure 22 below, was presented to the infant for free play. The Nursery Rhyme is a complex electronic toy with various interesting features such as colourful buttons, songs and sounds, which stands in front of the infant on the table. After the toy was presented to the child they were encouraged to play with it. The tester would demonstrate by pressing some of the buttons. The infant was then allowed five minutes to explore and manipulate the toy freely. Precautions were taken to prevent any distractions during this time of play. At the end of five minutes the filming was discontinued and the test procedure complete.
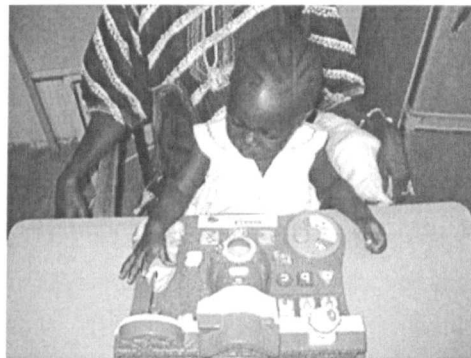
Figure 22: Infant playing with V-tech® toy during an attention assessment

Scoring

The video recording was played back using "Virtual Dub mpeg2" software which allowed frame-by frame playback viewing, so that each frame corresponded to a given time-interval. When the video playback was stopped at an event (start of trial, start of look, end of look) the frame number was noted and copied into a Microsoft Excel spreadsheet set up for the scoring purpose (see example in Appendix 7). (Looks/looks away shorter than 1 second were not noted). The duration of each look at the toy was then automatically calculated by Excel.

The two outcome measurements were:

a) Mean Length of Looks at the toy (or "mean look duration"): total looking time divided by the number of looks at the toy.

b) Inattention Rate: number of looks away from the toy per minute.

3.7.2.4   Rationale for using the mentioned tests

A variety of different cognitive tests has been used in infants at this age, some composite, others specific. Global measures of development and intelligence, (e.g. Griffith, Bayley and Gesell scales) are popular tests, which have been thoroughly validated and frequently used, and are so useful for making population comparisons. However, they generally give aggregate measures of different psychological processes, including motor and perceptual skills, consequently making it difficult to infer from the results which specific cognitive, psychological, or behavioural processes may have been affected by the exposures in question. Furthermore, the literature suggests that even during infancy cognitive functions develop independently (238), and particular kinds of interventions affect certain specific cognitive functions differentially.

Considering the above, and after receiving advice from an expert in early child development and nutrition (Prof Sally McGregor) it was decided that a non-global measure, allowing a more specific understanding of the physiological functions of n-3 LCPs in infant cognition, was appropriate. The Willatts Test was then chosen as most suitable because of the following advantages:

1. It has been used in several studies to assess cognitive behaviour in young infants.

2. It has proven sensitive to past nutritional interventions in infants (131, 195) and has been successfully used in developing countries (239), where it was able to detect differences between groups of infants aged 7 mo after a two-month intervention.

3. Unlike most of the composite measure assessments, it is relatively easy and quick to perform, therefore not requiring intensive training regimes for testers.

4. It may be scored from videotapes, facilitating in standardisation and quality control.

By conducting this test at 12 mo, it was possible to combine it with the infant attention assessment, which was a recommendation made by Peter Willatts. Given the simplicity of the test, and the suggested important role of DHA in attention in childhood, it was considered warranted to include the Toddler Attention Assessment to our outcome measures.

3.7.3 Acute-phase proteins

Plasma samples shipped to the MRC Human Nutrition Centre in Cambridge were also analysed for their inflammatory markers, once FA measurements were complete.

One of the consequences of increased gut permeability is the translocation of antigenic macromolecules, which stimulate and perpetuate local and systemic inflammatory responses (8). In order to test whether n-3 LCP supplementation is associated with reduced infant systemic inflammation (either via immune-modulation, or by reducing gut leakiness), the concentrations of the acute-phase proteins plasma CRP, AGP and albumin were assessed.

3.7.3.1 C-reactive protein

CRP levels rise dramatically (up to 50 000 fold in acute inflammation) during general, non-specific inflammatory responses to infectious and non-infectious insults, and is so classified as a positive acute-phase reactant. This increment, rising above normal within six hours and peaking at 48 hours, is due to an increase in the plasma concentration of interleukin-6, produced predominantly by macrophages (240). CRP levels are largely determined by the rate of production, which is determined by the severity of insult, and so are useful in detecting and monitoring inflammatory processes.

The protein is thought to play an important role in innate immunity, in assisting in complement binding to foreign and damaged cells, and in enhancing phagocytosis by macrophages (240).

CRP was measured using a commercial colorimetric immunoassay (Dimension® CardioPhase® high sensitivity C-reactive protein method, Siemens Healthcare Diagnostics Ltd, Camberley, UK).

Using this method, the sample was incubated with chromium dioxide particles and a β-galactosidase conjugate, which were each coated with a monoclonal antibody specific for CRP. CRP therefore became conjugated to both the chrome particle and conjugate during incubation. Unbound conjugate was removed by magnetic separation and washing, and then bound conjugate combined with a chromogenic substance (o-nitrophenyl-beta-D-galactopyranoside). β-galactosidase was then allowed to catalyze the hydrolysis of the chromogenic substance, resulting in a colour change read at 405/510nm. The

subsequent change in absorbance was finally taken to be directly proportional to the CRP concentration of the sample.

3.7.3.2   α-1-Acid glycoprotein

Similar to CRP, AGP is a positive acute-phase protein, rising in response to systemic tissue injury, inflammation or infection. Its biological function is not clear, but data support an anti-inflammatory and immunomodulating role. Glucocorticoids and a cytokine group of mainly interleukin-1 beta, tumour necrosis factor-alpha, and interleukin-6 and its related cytokines regulate AGP gene expression (241).

The determination of AGP is based on an immuno-turbidimetric specific reaction, using a commercial kit sold by Sentinel Diagnostics (Sentinel CH. SpA, Milan, Italy), which contains specific anti-ACP polyclonal antiserum. Antiserum and its corresponding antigen react in optimal pH conditions and in the presence of polyethylene glycolepolymer. The turbidity of the resulting immuno-complex is proportional to the concentration of AGP in the plasma sample.

3.7.3.3   Albumin

Serum albumin is produced in the liver and is the most abundant blood plasma protein. Its levels decrease in the blood during the acute-phase response. Hypoalbuminaemia may be caused by protein-losing enteropathy, malnutrition, and genetic variations.

Plasma concentration was measured using the Flex® reagent cartridge (Siemens Healthcare Diagnostics Ltd, Camberley, UK), employing an adaptation of the bromocresol purple dye-binding method (242, 243). In the presence of a solubilising agent, the die binds to albumin at pH 4.9, and the amount of albumin-dye complex (read at 600nm) is proportional to the albumin concentration.

3.7.4 Intestinal inflammation

The calcium-binding, anti-microbial protein, calprotectin, is found primarily in neutrophils, but lower concentrations are found in monocytes and macrophages. It competes for zinc to inhibit zinc dependent enzymes and so kill microorganisms. Bowel content is rich in bacteria and other microorganisms releasing substances which may be toxic, and so stimulate

leukocytes, in particular polymorphonuclear granulocytes, to migrate to the gut lumen where they release their contents, including substances like calprotectin.

Mothers were asked to collect a sample of their infants' stool into a stool pot provided the evening before they were due to come to Keneba for the clinic visit, and to continue the collection during the clinic visit if no stool was passed the previous evening. Calprotectin is extremely stable and stool samples can be stored for six days at room temperature.

Samples were homogenised by vigorous stirring for five minutes to remove any possible clusterings of protein pockets. Homogenised stool (roughly 5-7ml) was transferred to a pre-weighed 30ml tube. They were then weighed again, covered with parafilm with three holes punctured into the top, covered lightly with the lid, and frozen at -80°C. The regular weighing was done in order to calculate the moisture loss and so determine the water content of stools.

Frozen stools were freeze-dried overnight, or until all sample matter had dried through. Although not specified in the kit protocol, drying of stools was intended to correct for differing stool water concentrations so as to be left with similar dilutions of inflammatory products across otherwise variably dilute stools. A high inter- and intra-variation in moisture content of stools in this population is common, which becomes amplified by regular diarrhoea episodes. An example of two such different stools is shown in Figure 23. Once dried, the stools were each weighed again and transferred to a mortar and pestle where they were homogenised into a fine powder, transferred to 2ml tubes, and stored at -20°C for later analysis.

Figure 23: Example of two stools of different dilution moisture content

Calprotectin concentration was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Phical, CALPRO AS, Oslo, Norway). The kit is used primarily for determining disease activity and monitoring response to treatment in adult IBD patients, and those with colorectal cancers.

For this assay, extracted re-suspended stool samples were incubated in 96-well plates coated with polyclonal antibodies against calprotectin. After washing, the epitope bound calprotectin was allowed to react with immunoaffinity purified enzyme labelled anti-calprotectin. The amount of enzyme left bound after incubation and washing is roughly proportional to the amount of calprotectin in the sample or standard. A substrate for the enzyme was then added and allowed to react, resulting in a colour change proportional to the enzyme (and so calprotectin) concentration in wells. A concentration of greater than 50mg/kg wet stool is regarded as a positive test in adults (median is 25kg/mg). In active IBD conditions, the concentration can reach up to 200 – 20 000mg/kg (220, 244-246).

3.7.4.1 Laboratory work in Keneba

Before the start of the sample analysis, preparations were made to test methods, and adapt the protocol where needed. It was known that paediatric calprotectin levels are higher than those of adults, but the only information available on the normal calprotectin concentrations in the study population being the highly variable and questionable results from the pilot study - achieved using un-optimised techniques - these had to be measured in order to choose appropriate dilutions. Adaptations to the protocol to accommodate freeze-drying of stools, suitable homogenisation methods and incubation times etc were assessed, and also determinations of the average water content of stools. Adjustments to fit the available equipment (e.g. shaker/centrifuge/agitator pins) and relatively warm laboratory temperature were made and optimised to write a modified protocol.

Ten stool samples were collected on the same morning from 10 different study infants for assay workup purposes. A 5g aliquot of each sample was transferred to a clean stool pot and mixed thoroughly with a wooden spatula. The mixed stool was separated into 4 separate, pre-weighed 30ml tubes, weighed again, and frozen at -80°C overnight, before freeze-drying. Weighed, freeze-dried stools were then homogenised and the fine powder transferred into four separate 2ml tubes and used for all assay workup purposes.

Firstly, extraction conditions were tested. This was done by applying different extraction buffer volumes with or without added (improvised) agitator pins, and different vortexing and shaking times and conditions, to samples grouped into different batches. Variability was greatest amongst batches using lower buffer volumes, no agitator pins, and shorter vortexing and shaking speeds, suggesting an incomplete extraction. A shaking time of 45 minutes (as opposed to the 25 minutes recommended), a doubling of the recommended vortexing time, and a slightly higher extraction buffer-to-sample-ratio than protocol specifications gave the least variable results.

Secondly, dilution extent and incubation times were optimised. When matching degrees of dilution and incubation times to those stated in the protocol, adjusting for a roughly 80% average moisture loss during freeze-drying, calprotectin concentrations fell on the very high end of the standard curve, and the highest standard's reading exceeded an optical density of "2". In order to determine which dilution would bring the average down towards the middle of the curve, and ensure that the highest standard reading was brought down, batches of serial dilutions were prepared and resulting concentrations determined. From the readings, the optimal dilution was (accounting for extraction as well as sample dilution buffers) more than three times more than the recommended. The accompanying ideal final incubation time was found to be 30% shorter than the kit protocol instructions, which was most likely due to the warm laboratory temperature which accelerated the reaction.

Once conditions had been optimised, intra-assay variability was assessed: one of the mixed stool samples was divided into four, and one of these parts further divided into four samples, as illustrated in Figure 24. Samples were extracted, and for one sample, the extraction supernatant was separated into four and further processed. For samples "ii" and "iii" a serial dilution series was prepared to assess whether the readings correspond well to the different dilutions. The samples, controls, blanks and standards were plated out as illustrated below (in Figure 24).

The standard curve R^2 correlation coefficient value was 0.99, demonstrating a good fit, as shown in Figure 25. Optical densities, with corresponding concentrations read from the standard curve are shown in Figure 26.

Interpretation

"Control" concentrations (218ng/ml – 232ng/ml) were well within the range required, and the average of 223ng/ml was close to the target of 222ng/ml. Blanks read well below an optical density of 0.2, as was desired.

Percentage CVs between replicates were high, but inconsistently so, varying between less than 1% and greater than 30%. The higher CVs were contributed mostly by replicates repeated across eight wells, and by the most dilute standard. Because of the time it took to plate out across eight wells from left to right, CVs were likely influenced by resulting differing incubation times. To avoid this in future work, half-plates rather than full plates were run. In addition, intervals between pipetting of sample or standard into wells, and filling with conjugate or substrate solutions were timed carefully. Careful attention was paid to pipetting the most dilute standard for future analyses, but despite this, high CVs between replicate of this standard were hard to avoid some of the times. Because of the very small quantity of calprotectin it contained its susceptibility to even minor pipetting differences was high.

Unexpected was that samples which had been weighed out separately ("i"-"iv") displayed lower variation than those that had not ("a"-"d"). Samples that had been taken from the same extraction homogenant and centrifuged separately were exposed to some source of variation which was most likely due to sampling from different parts of the 14ml of homogenate. For future work, aliquots were always taken from the same part of the extraction homogenate solution. One of the samples ("i") failed to result in a reading as the calprotectin concentration lay below the detectable range. Because all four replicates were affected, but not samples "ii" – "iv", the problem most likely occurred during the final sample dilution. It could be that too little sample was unwittingly added to the sample diluent, or that the dilution step for "i" was completely missed.

The most concentrated dilutions of the two dilution series were too high for the standard curve. The most dilute sample (1:200) replicate average was roughly half (120) of the doubly concentrated sample (1:100; 260), as was hoped.

After studying the results and adjusting the protocol by means of further assay piloting, the following method was followed:

Adapted protocol

- To minimise costs, a combined stool sample for each infant at each time-point was measured, rather than the individual samples each at baseline and endpoint. The dried stool powders of each infant per time-point (a maximum of 3 at baseline and 3 at endpoint) were weighed in a 1:1:1 (w/w/w) ratio into a clean eppendorf tube and vortexed thoroughly to mix.

- 15mg of the mixed dried stools (as opposed to the usual 100mg wet stool) were weighed out from the eppendorfs into 14ml falcon tubes, two agitator pins added, and 4985µl of extraction buffer pipetted into each falcon tube, before vortexing to mix, and transferring to a shaker to agitate for 45 minutes at maximum speed.

- 1.4ml of extraction homogenate was centrifuged at 10 000g for 20 minutes, and 500µl of the resulting supernatant transferred to an eppendorf and kept for ELISA analysis.

- Controls, samples and standards were determined in duplicate, following the principles already described. Plates were read at a wavelength of 405nm using an Appliskan plate reader (Thermo Scientific, part of Thermo Fischer Scientific, Waltham, MA, USA).

- Optical density readings were given in duplicate, and read from the standard curve to give a calprotectin concentration expressed in ng/ml.

- The values of the diluted samples were adjusted for the dilutions and converted to mg/kg dry stool by multiplying by the total dilution factor (8300) and then multiplying by the 0.001 conversion factor, so that, for example, a reading of 100ng/ml became 830mg/kg.

- For group comparisons, this expression in dry weight was adequate, but to enable the comparison with reference values, stools were also expressed per kg of wet stool. This was done by calculating the moisture content of stool by subtracting their dry weights from their wet weights, and converting dry stool weights into their equivalent wet weights. For example, for a stool with 80% moisture, 830mg/kg dry weight = 830mg/5kg wet weight = 166mg/kg wet weight.
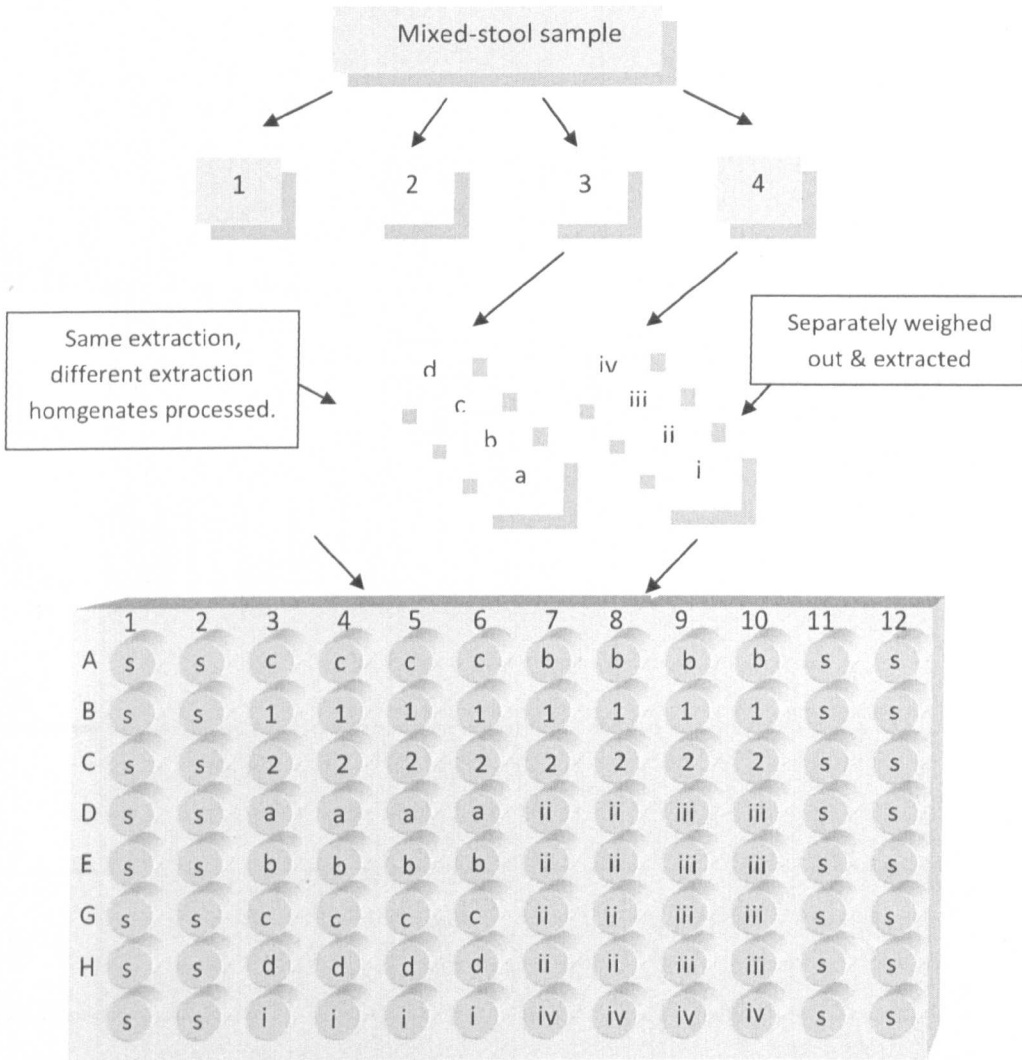
Figure 24: Illustration of intra-assay variability test procedure. "s"- standard, "c"-control, "b" – blank
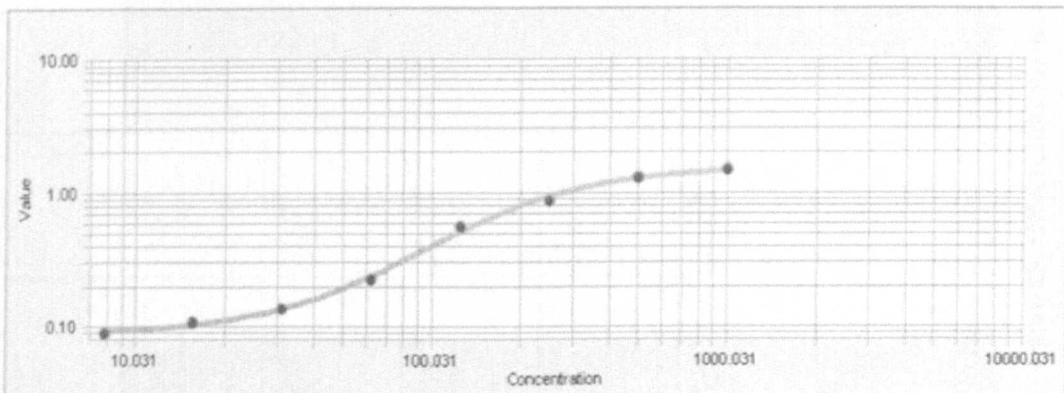
Figure 25: Standard Curve from assay work-up

Figure 26: ELISA results for assay workup. Each block represents a well with top row sample name, second row optical density, and third row concentration. "NaN" – reading out of standard curve range.

Neopterin

A breakdown product of cyclic guanosine monophosphate released from activated phagocytic cells, neoopterin is an early biochemical marker of cellular immunity (247). This marker's main determinant is stimulation of cells by interferon-γ (IFN- γ) and has been used in monitoring the course of inflammation associated with increased cellular immune activity. Neopterin was therefore chosen as a marker of intestinal cell-mediated inflammation.

A commercial ELISA kit (IBL, Hamburg, DE) designed for measuring neopterin concentrations in serum and urine was adapted for faecal measurements during the pilot study. The pilot study stool analyses took place in the UK. During the main study in Keneba, however, numerous difficulties were experienced in setting up the assay and in achieving satisfactory standard curves and assay CVs although the same protocol was followed as in the UK. The difficulties were probably due to the kit components being vulnerable to damage during transit between the UK and Keneba and so not responding optimally during analysis, or because of some other effect in the laboratory in Keneba influencing the assay.

When considering these problems in the light of the number of other outcomes already incorporated in the study, including an alternative marker of intestinal inflammation, and the costliness of the neopterin kits, it was decided to remove faecal neopterin as a marker of intestinal inflammation and rather focus on the other endpoint and laboratory assessments. Work on the assay was therefore discontinued before the cause of the problem could be found.

3.7.5 Morbidity assessments

A general health questionnaire (designed with input from the trial statistician and database developer; Appendix 8) was administered by trained fieldworkers every day after infants were supplemented so as to assess their general health. The mother was the usual respondent, but when she was not available the nursemaid or other member of the compound caring for the infant stood in. Mothers/caretakers were asked whether the infant had experienced any form of un-wellness during the past 24 hours, and about the occurrences of diarrhoea, vomiting, cough, fever, and abnormal bleeding. This monitoring was done both to assess any observed effect of treatment on morbidity patterns in infants, and to ensure a high

standard of safety monitoring. Morbidity data was sent to the trial statistician at two to three monthly intervals. A morbidity report, showing group differences, was then prepared and sent to the safety monitor for evaluation.

If a mother felt that her infant was in need of medical attention she was free to take her infant to the clinic in Keneba to be seen by a doctor, or request a visit by a nurse. If the infant was seen by a nurse or doctor, the diagnosis and treatment details were recorded on a "morbidity/adverse event form" (Appendix 9).

3.8 Other measurements and observations

3.8.1 Cognitive development in relation to umbilical cord and maternal serum DHA and AA concentrations

Previous data have found that in an Inuit population in Arctic Quebec, cord DHA concentration was related to later infant development whereas breast-milk DHA was not (248). This suggests that DHA exposure during pregnancy may be more strongly related to later developmental outcomes than exposure postnatal, and indeed a recent review on DHA supplementation in pregnancy and lactation also confirms evidence to suggest this (249).

Maternal and cord serum samples were collected at the delivery of infants born to mothers taking part in the previously mentioned peri-conceptual micronutrient supplementation trial. The PI of this study granted a request for using a number of these blood serum residuals in order to investigate their FA content. A sample of 104 maternal and cord serum residuals that overlapped with infants taking part in the n-3 LCP study were identified and sent to the MRC Human Nutrition Research Centre in Cambridge for FA analyses.

The maternal and cord samples were analyzed for their relative proportions of DHA and AA (also an important constituent of the brain) concentrations, and these values were correlated with the developmental scores that infants achieved on the Willatts and Attention Tests. Relative maternal and cord FA concentrations were further compared to assess how strongly maternal levels predicted infant ones.

It was hypothesised that because the cord serum would reflect maternal diet during late gestation, the difference between the cord and the 9 mo blood LCP levels would give a measure n-3 LCP exposure from breast-milk and diet post-natal. By using this measure, a similar analysis was performed, testing the relationship of post-natal n-3 LCP exposures on cognitive development.

3.8.2 Growth outcomes at 12 mo

During the 12 mo developmental outcomes testing clinic visit, infants were also measured to assess their growth. The same methods and equipment were used as described for anthropometric measurements at 3 and 9 mo of age. The measurements were taken only once, however, and by a trained fieldworker rather than the PI.

The availability of 12 mo anthropometric data meant that any effects of treatment which may have been detected between groups at 9 mo of age could be evaluated at 12 mo to investigate whether the differences where maintained, or indeed manifest only then.

3.8.3 Overall growth compared with WHO references at 3, 9 & 12 mo of age

Growth measurements were assessed against WHO reference curves in order to examine and contrast the patterns of growth at 3, 9 and 12 mo of age, evaluate them according to WHO recommendations, and compare the data with previous findings in West Kiang.

3.8.4 Weaning foods

A very basic questionnaire (Appendix 10) was administered to mothers in their own language every month, asking whether their infants had been fed anything other than breast-milk, and if so, what. From this a brief descriptive summary was compiled, indicating how many of the infants in the study had been weaned at each monthly interval, and what the majority had been weaned onto.

3.8.5 Relationship between calprotection levels and LMRs

Stool calprotectin concentration measures intestinal and colonic inflammation whereas urinary LMRs reflect the permeability and surface area of the small intestinal mucosa. The relationship between the integrity of the intestinal mucosa and intestinal inflammation was investigated by correlating urinary LMRs with stool calprotectin levels. This analysis also gave some suggestions as to whether calprotectin and LMR measures were interrelated or instead entirely independent indicators (whether due to the mechanisms which influence them, the differential timings of their physical expression, or the areas of the intestine they were reflecting), and whether one could be used to predict the other to any degree.

3.9   Statistical methods

The main analysis was intention-to-treat. Multiple, linear and log-linear regression, and negative binomial regression analyses, where appropriate, were used to test effect of treatment on all primary and secondary endpoints.

In order to look at the possibility of dose effects, a further analysis fitting the number of doses of treatment, controlled for compliance, in the regression models above were done for all primary and secondary endpoints. By adjusting for compliance and number of doses, the effect of treatment as it was received in each individual case was measured on the various outcomes.

Frequency distribution and normality of dependent variables were checked by plotting the data and by drawing histograms. Where the data were skewed, log transformations were used to both normalise the distribution, and stabilise the variance of the skewed variables.

3.9.1   Data cleaning

Growth, weaning foods and morbidity data were cleaned during the running of the trial, as the data were entered and accessible. The rest of the data were cleaned at the end of the study, or once laboratory analyses were complete. Owing to real-time checks facilitated by the double entry system, specific entry forms and automatic range checks, the number of errors that had to be dealt with during the later stages of data cleaning were limited.

Queries generated in Microsoft Access were used to test for any entries which were incompatible (e.g. "fever" and "child well over past 24 hours" on same record), erroneous (e.g. if a growth measurement at 9 mo was mistakenly entered at 3 mo, consequently showing 4 baseline and 2 endpoint entries), out of range (where normal data ranges were available), or mistakenly entered a second time. ID number discrepancies were also checked with the aid of check letters which were added on to the end of ID numbers at the start of the study. The ID numbers in each dataset (especially data arising from laboratory analyses) were checked against a set of correct ID numbers, and digit or letter discrepancies investigated and corrected. Data were then plotted in order to check for outliers and any noticeable errors.

Errors and outliers were cross-checked against hard copies of data records where available (i.e. not for laboratory results). Illegible hand-writing, incorrect dates, or misinterpretation of the coding system used on the forms were common sources of error. Mistakes which could not be rectified, and clear outliers, were dropped from the dataset.

3.9.2   Primary outcomes

Multiple regression analysis was conducted to test the effect of treatment on change in growth and anthropometric z-scores.

As urinary LMR, lactulose:lactose ratio, and lactose percentage recovery data were skewed, they were log transformed. Regression analysis was performed to test for treatment effects on intestinal integrity.

As explained in Section 3.6.2.1, free fructose and glucose urinary sugar concentrations were subtracted from the urinary lactulose and lactose concentrations. Because the urinary sugar concentrations are so small and their determinations therefore very susceptible to small technical errors, it occasionally happened that when free fructose/glucose values were subtracted from lactulose/lactose values, a negative end result was found. In order to compensate for such negatives, "+10" was added to the data before log-transformation, to solve the problem of negatives so that data was not lost in a possibly biased way.

Instead of using the calculated LMR for regression analysis, lactulose (logged) was regressed on mannitol (logged), so that mannitol concentration was controlled for. This was considered more efficient than calculating and comparing the ratio, as it did not assume a proportional relationship which may not be true, especially if there was error in the measurements.

For all analyses baseline values were fitted as covariates in order to correct for any modification of treatment effect they may have caused and to minimise variance introduced by different initial nutritional and intestinal statuses.

In a subsequent analysis, the following variables were added as extra covariates to the regression models above:

1. Breastmilk DHA and EPA

2. Sex

3. Season of birth

4. Age of commencement of treatment

These variables were identified as potential variance inflators or/and effect modifiers, and by adding them to the regression model the data could be corrected for such influences by these variables.

When a significant difference between groups was detected for any of the primary outcomes, the analysis was repeated, but fitting plasma DHA and EPA concentration (at 9 mo, controlling for 3 mo) as covariate. It was thus possible to show (if it disappeared) whether the observed treatment effect was indeed working via an increase in plasma DHA or EPA levels.

Seasonal effects were investigated on 3 mo growth measurements, using a Fourier series. Growth outcomes were plotted by month, and the seasonal effect tested using a Wald Test.

Breast-milk DHA and EPA, and season of birth, were examined for any interactions with treatment on outcomes by adding these terms to the regression models and inspecting for an interaction. For fitting season of birth a simple sine wave model (first two Fourier variables of the Fourier series) was used.

3.9.3 Secondary outcomes

Plasma fatty acids

n-3 LCP concentrations were skewed, the data consequently being log transformed.

As mentioned in section 3.7.1.1, absolute FA values were obtained, and from these the relative percentage FA values could be calculated by dividing the absolute FA values by the total FAs. It was not known to what extent absolute versus relative values would be related to treatment and other variables, and so the following analysis strategy was chosen: in order to optimise the balance between using absolute FA values and % FA values in the analyses when

plasma FA was treated as the outcome, "(log)absolute FA" was used as the dependent variable, controlling for "(log)total FA". For the main LCPs, however, percentage and absolute value differences were analysed and presented separately.

When plasma FA was fitted as a covariate, both percentage and absolute values were fitted. This choice was made based on the relatively independent relationship of these two variables.

Cognitive development

As summarised in Section 3.7.2.2 and 3.7.2.3, three outcome measures were obtained for the Willatts Test and two for the Attention Assessment. Group comparisons were made between these scores using regression analysis. The analyses were repeated fitting the mother's highest degree of education as covariate.

The relationships between the various outcome measures were examined by first plotting them against each other and then correlating, also comparing the relationship between achievements on the two different tests.

Systemic and intestinal inflammatory markers

Calprotectin, AGP and CRP were log transformed due to skewed distributions. Group means were compared using regression analysis, and the population above the normal was also compared by group. Baseline measures were controlled for.

Morbidities

Binomial regression was used to test group differences in the number of visits at which one or more symptom was recorded, and in the number of visits at which infants were reported to have specific complaints e.g. vomiting, fever, cough etc. Interaction of season of measurement with treatment was tested.

3.9.4 Overall growth compared with WHO reference standards at 3, 9 and 12 mo of age

Baseline, 9 mo, and 12 mo anthropometric z-scores were plotted against WHO growth standards to assess the study population's growth and nutritional status in comparison to reference data. Anthropometric indices at 12 mo were tested for treatment effects using multiple regression, controlling for baseline values.

3.9.5 Plasma and breast-milk fatty acids

3.9.5.1 Comparison of breast-milk fatty acid levels in this population with those of other populations

A summary of the average FA concentrations in this population was prepared and compared with data from other populations.

3.9.5.2 Correlations between pre- and post-treatment plasma fatty acid concentrations and between breast-milk and plasma fatty aicds

Indicative of the extent to which treatment altered FA profiles, over and above the influence of normal diet or biological determinants, the above-mentioned correlations were done with the aim of investigating how strongly pre-treatment and breast-milk DHA, EPA and AA fatty acid profiles predicted endpoint plasma ones.

3.9.5.3 How much variation in plasma fatty acid levels is explained by breast-milk versus treatment?

As a follow-up to the tests mentioned above, the partial R^2's in an analysis of variance test were compared with the purpose of comparing how much variation in plasma DHA and EPA fatty acid levels was explained by the breast-milk intake of these fats and how much was explained by treatment.

3.9.5.4 Do plasma or breast-milk fatty acids predict any of the other outcomes?

Different plasma and breast-milk PUFAs were tested to examine their relationship with the anthropometric z-scores, gut integrity, cognitive development, and markers of inflammation using simple linear regression on endpoint data, and a random effects regression on pooled 3 mo and endpoint data.

3.10 Ethical, safety, and other considerations

3.10.1 Ethical considerations

The trial complied with MRC GCP (good clinical practice) guidelines, the then current version of the Declaration of Helsinki and applicable local ethical and legal requirements. Ethical approval was obtained from both the LSHTM and the joint Gambian Government/MRC ethics boards (Appendix 11).

As part of community sensitisation, elders were visited in their respective villages before the start of the trial to explain the purpose of the study to them. They then gave their verbal approval for research to be conducted in their villages.

A standardised information sheet (Appendix 12) was read to the mother or caretaker, and father, if reachable, in their own language by a trained field-worker. After a clear explanation of the detail of the study, written parental consent (see Appendix 13 for consent form) for the child to take part in the study was sought from both parents. It was stressed that recruitment is voluntary and that a decision not to join would not jeopardise the provision of normal healthcare. Participants were made to understand that they were free to leave the study at any time, should they so wish.

Subject confidentiality and anonymity was protected as far as possible by following the above-mentioned standards. Samples taken were coded with an ID number. Personally identifiable information was never released to third parties.

Were there any cases of harm as a direct result of participating in the trial, medical care and treatment would be provided free.

3.10.2 Safety considerations

3.10.2.1 Potential adverse side effects

Safety concerns were considered as minimal as supplementation was with a naturally occurring food ingredient.

Combined DHA+EPA formulations have been safely used in infants, without adverse effects on growth, mental development, bleeding time, and lipid peroxidation, and several randomised controlled trials support their safety (57, 250). The supplement used in this trial was of high pharmaceutical grade. Its quality exceeded the stringent Norwegian Medicinal Standards and the European Pharmacopoeia Standards.

The dosage chosen was high in relation to recommended daily intakes; however, it was designed not to meet a requirement, but to attempt to account for potential fat losses and to induce a therapeutic effect. The dosage, nonetheless, fell within the high range of recommendations for daily omega-3 intakes of the US Institute of Medicine (251) of 500mg omega-3/day for infants aged 1 – 18 mo. Additionally, one of the word's most distinguished scientists in the area of FAs and infant nutrition (Prof Ricardo Uauy), was consulted, and the dose was found acceptable as long as safety was monitored, and any abnormal/nose bleeds or any other evidence of side effects were recorded.

Membranes enriched with PUFAs are more susceptible to oxidative damage and unless in the presence of appropriate antioxidants, high doses of n-3 FAs may possibly cause tissue oxidative damage. Another concern is that of increased erythrocyte haemolysis, unless accompanied by appropriate antioxidant supplementation. By including the antioxidant d-alpha-tocopherol in the oils, however, both these potential harmful effects were avoided.

There is some evidence that high doses of n-3 LCPs may increase bleeding time by most likely altering platelet-vascular interactions, but that the prolongation is modest and of no clinical significance (252). Nevertheless, including a bleeding time evaluation as an added safety measure was considered.

The only methods available for use, which would test platelet function related bleeding time, were a bleeding time evaluation or an ex vivo test using the PFA-100[®] system (Platelet Function Analyzer). Both these measurements, however, are invasive and present risks for

infection: The bleeding time evaluation requires that a measured incision be made in the skin on the forearm, and that the time to stop bleeding be measured. The PFA-100® system requires that a minimum of 800μl blood be drawn.

It was therefore decided, jointly with the trial safety monitor, that the only way to test bleeding time would be too invasive to warrant the risk involved for monitoring the unlikely case of increased bleeding time, and that instead the morbidity questionnaire will ask about nose bleeds, blood in the stools, or any other abnormal bleeding occurrences.

Infants in West Kiang do not routinely receive vitamin K at delivery. Being mostly fully breastfed, they are therefore, due to lack of vitamin K, at potential risk of haemorrhagic disease of the newborn (HDN) caused by abnormal clotting function. Such effects are likely to be extremely rare but potentially very serious for the individual concerned. Early HDN tends to present in the first week of life. Late HDN does occur but is rare, particularly after 8 weeks of age, although it has been described to occur up to 12 weeks of age mainly in breast-fed populations.

If, due to any bleeding prolongation caused by n-3 LCP supplementation, there were to be a potential for interaction, it was advised that the risk could easily be mitigated by offering oral vitamin K (1mg) supplementation to all infants at entry. This route was discussed with the safety and trial monitors, but was finally considered unjustified, given that late-onset HDN is so rare, particularly after 8 weeks. Because infants would be entering the n-3 LCP supplementation trial at 3 mo of age, the magnitude of risk was thought to be extremely small.

3.10.2.2 Other safety considerations

The risks of drawing blood from infants include temporary discomfort, potential bruising, bleeding, and, extremely rarely, infection. Venipuncture was conducted under hygienic standards by a qualified phlebotomist, and all attempts to minimise discomfort was made.

Participating children were visited daily by fieldworkers for health checks and in the event of illness were entitled to receive immediate medical attention. Mothers were given priority access to transport and the clinic, and clinical examinations took place on request.

3.11 Trial administration

The trial was registered with the ISRCTN[1] Register (ISRCTN66645725). It was conducted according to MRC GCP standards, as agreed and discussed with the MRC Clinical Trials Support Manager in The Gambia.

In the original trial registration with the ISRCTN Register a couple of errors were not noticed until later: gut integrity was listed as a primary hypothesis but a secondary outcome, and the stated dose was incorrect. When these errors were noticed later a letter was sent to amend these details and to update changes/additions made to the study (such as the cognitive development outcome add-on). The letter sent to the ISRCTN Register administrator in order to update the register is given in Appendix 14.

3.11.1 Quality assurance

A field supervisor carried out regular quality control visits to oversee the process of recruitment, the administration and handling of investigational product, morbidity and compliance monitoring, field anthropometry measurements, and equipment handling/calibration etc. The PI conducted similar visits on a monthly basis. At these visits the field supervisor filled out a quality control form (Appendix 15) for the fieldworker being reviewed, noting any concerns or areas needing improvement. These areas were then addressed with the fieldworkers.

Weekly team meetings were held where data forms were handed to the PI and looked over before submitting to the data office. Any error, queries or discrepancies were dealt with during the meetings. Queries generated by the data office because of incompatible information or measurements falling out of range were reviewed at the same meetings.

In instances where the PI was unable to conduct anthropometry measurements, due to travel or illness, infants were measured by a second observer. In order to measure the inter-observer variability, and to adjust for it later on if required, a 30% sub sample of infants was measured by both the PI and second observer.

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[1] International Standard Randomised Controlled Trial Number

A second observer, for quality control purposes, repeated a total of 305 compliance and morbidity questionnaires on a sample of 40% of the infants.

Oxidation values for the supplement oils were provided to the PI before the oils were sent to The Gambia. In order to comply with Norwegian Medicinal Standards and European Pharmacopoeia Standards, the peroxide value of fish oil has to be lower than 10 meq/kg. The peroxide value gives an indication of total current oxidation state of oils. The oils supplied by Nordic Naturals typically have a peroxide value of under 5meq/kg.

Anasidine value, which measures the aldehyde production during oxidation of fats, is used as an indication of past oxidation, reflecting how the oil has been handled and stored. (However, once vitamin E has been added to oils the anasidine value becomes affected, so can only be measured in oils before vitamin E is added). An adjusted combination score for the anasidine and peroxide values is called the "total oxidation value" and describes the total oxidation to which oils have been exposed. According to the Council for Responsible Nutrition, oil has to have a combined oxidation value of 26 or lower. Nordic Naturals provided a value of 7 for their oils (tested before the addition of vitamin E).

After eight months of storage, supplement oils were sent to Cambridge, on ice, to be tested for their vitamin E and oxidation values. The Peroxide Value was tested using the Wheeler Method (253), and vitamin E concentration was tested using High Performance Liquid Chromatography.

3.11.2 Safety monitoring

An independent Trial Safety Monitor (TSM) was appointed at the start of the trial, according to the MRC GCP guidelines, in order to provide real-time safety oversight.

The TSM was responsible for reviewing SAEs immediately after they occurred. It was his task to follow these until the events resolved or stabilised, or until a non-study-related-cause was assigned, and to offer guidance on how to proceed. Any SAE found to be related to treatment would warrant suspension of the trial until a safety review was done.

The TSM also moderated the growth and morbidity results at regular intervals and was qualified to break blinding codes or stop the trial if considered necessary.

3.11.2.1 Interim safety monitoring reports

Safety monitoring reports were sent to the TSM at interims of 3 months. The report provided summary data on length, weight, knee-heel length, daily morbidities and nurse/clinic visits. The trial statistician, who was unblinded to which infants were in the same groups, but not to which group corresponded with active treatment, provided summary statistics by group (calling them group "A" and "B"), noting any significant differences. A typical example of such a report is given in Appendix 16.

3.11.2.2 Serious Adverse Events

The collection, recording, and reporting of serious adverse events (SAE) were under the direct responsibility of the PI.

In the case of an SAE the following steps were followed:

PI contacted by the fieldworker assigned to the infant as soon as the fieldworker became aware of the event. Treatment suspended.

$\downarrow$

SAE initial report form was filled out by the PI and sent immediately to the TSM, together with any available relevant documentation and information.

$\downarrow$

Once the patient had been treated, a follow-up SAE report was prepared by the PI and sent to the TSM, together with any relevant documentation e.g. doctors reports, test results etc.

$\downarrow$

The TSM assessed the SAE, requested further information if required, made an assessment of causality, and advised when treatment was allowed to resume. SAE filed in the subject's case record form.

The SAE initial and follow-up report forms are presented in Appendix 17. The definition of an SAE, as used for purposes of the trial, is described in Box 3, and the guidelines for assessment of causal relationship of SAE to administration of investigational product followed in Box 4.

Box 3: Definition of an SAE

(Abridged from Clinical Trial Protocol Template, MRC Laboratories, The Gambia)

Any untoward medical occurrence that at any dose:

- Results in death;
- Is life-threatening;
- Requires inpatient hospitalisation or prolongation of existing hospitalisation;
- Results in persistent or significant disability or incapacity;
- Is a congenital anomaly or birth defect.

Important medical events that may not result in death, be life threatening, or require hospitalisation may be considered an SAE when, based upon appropriate medical judgment, they may jeopardise the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed above.

An untoward medical occurrence that results in death from any cause at any time is considered as an SAE.

An event is considered as life-threatening if a subject was, in the view of the investigator, at immediate risk of death from the event that occurred.

Any requirement of hospitalisation is considered an SAE. However, hospitalisation for either elective procedure related to a pre-existing condition which did not increase in severity or frequency following initiation of the trial, or for routine clinical procedures are not considered as SAEs, but must be recorded in the case record form (CRF). The relationship to the investigational product will be checked "No".

The written report to the TSM included a follow-up SAE Form, photocopies of pertinent test results and medical notes, trial records, hospital report (if relevant and as soon as available), and a summary of the outcome of the event plus the PI's opinion of relationship of the investigational product to the event. The TSM would then review and make a final assessment of causality. Guidelines for such an assessment are given in Box 4.

Box 4: Guidelines for assessment of causal relationship of SAE to administration of investigational product

(Abridged from Clinical Trial Protocol Template, MRC Laboratories, The Gambia)

This interpretation will be based on the type of event, the relationship of the event to the time of trial intervention, and the natural history of the underlying diseases, concomitant therapy, etc, as follows:

Not related
No temporal relationship to investigational product; and
Event could be explained by alternate aetiology

Possibly Related
Reasonable temporal relationship to investigational product; but
Event could also be explained by alternate aetiology

Probably Related
Reasonable temporal relationship to investigational product; and
Event could not be explained by alternate aetiology

Definitely Related
Reasonable temporal relationship to investigational product; and
Event could not be explained by alternate aetiology; or
Event could be confirmed with a positive re-challenge test.

3.11.3 Data handling and record keeping

A central database with specific access entry forms and automatic range checks (for morbidity) was created for the trial. Trained data-entry staff at MRC Keneba double entered all data collected, and real-time checks were performed. The MRC Gambia database developer and the PI oversaw this work.

Where inconsistencies between double entered data were found, the original form was consulted and the incorrect entry amended. When data fell out of range, the PI advised whether the extreme measurement was real or due to error. As the PI was taking all anthropometric measurements it was not hard to distinguish between errors and measurement which were real.

3.11.4 Trial Monitoring

In accordance with MRC GCP requirements, and following advice given by the MRC Clinical Trials Support Manager in The Gambia, an independent Trial Monitor was appointed to monitor and supervise the progress of the trial, and ensure it abided by MRC GCP standards.

The Trial Monitor was involved with finalising and approving the clinical protocol before it was sent to the MRC prior to the start of the trial. Once the trial started, the Trial Monitor was required to monitor the progress of the trial, adherence to the protocol and patient safety, and to take consideration of new information relevant to the study. The PI sought consent from the Trial Monitor before making any updates and changes to the trial protocol. All protocol deviations were reported to the Trial Monitor.

A site visit was conducted half-way through the trial. The trial monitor was then given the opportunity to observe procedures on the ground. These involved outcome measurements, sample collection, processing and storage, and procedures in the field. It also involved an audit of documentation procedures, data handling and the paper trail. Trial progress was noted, and any problems and queries discussed at a meeting with the PI and her supervisor. Progress was also recorded on a monitoring report submitted to the MRC.

3.11.5 Fieldworker training

Field-workers had had some degree of field experience before joining the study. They had all previously worked for the MRC and as part of on-the-job training had completed fieldworker courses.

Details of additional training provided before the start of the trial are given below. With the exception of anthropometry and motorbike usage, the PI provided all training. Fieldworkers were also provided with a field manual specific to the study, containing reference material, answers to questions, and a trouble-shooting guide. The matters listed below were re-addressed, in turn, during the weekly team meetings for the duration of the trial.

Anthropometry

To teach fieldworkers how to most accurately collect anthropometric data in the field, for purposes of safety monitoring measurements, training was provided by an experienced Scientific Officer for measuring height, weight, and knee-heel length. A refresher course was provided mid-way through the trial.

Supplement administration and care

Fieldworkers were trained in administration technique and dosing the correct volume. They were also trained in safe and hygienic supplement storage practices, and instructed on supplement handling in the field and during transportation.

Morbidity assessments

Rigorous training was provided to ensure that fieldworkers understood how to correctly fill out the daily morbidity questionnaires. Great measures were also taken to make sure that proper and standardised wording were used while conducting these interviews.

Recruitment and consenting

Emphasis was given to making sure the information sheet was translated properly, and the fieldworkers were able to explain it clearly and accurately. They were also trained in answering

specific potential, foreseeable questions, and in acceptable standards for collecting informed consent.

Motorbike usage and transport

As stipulated by the MRC and station rules. Arrangements and procedures for the transportation of subjects and their mothers for study and clinic purposes were discussed.

Village assistants responsibilities

Fieldworkers were explained the tasks and responsibilities of village assistants.

3.11.6 Standard operating procedures

Standard operating procedures were accessible for the following trial-related activities:

- Recruitment
- Supplement administration
- Call days
- Blood processing
- Anthropometry measurements
- Stool collection and processing
- Calprotectin determination
- Lactulose-mannitol solution preparation
- Urine collection
- Breast-milk collection and processing
- Sample storage and shipping

3.11.7 Staff and scientists involved in trial

A graphic outline of staff and scientists who worked with or supervised the PI is given below in Box 5. Although not exclusively, the local supervisor as well as clinic, laboratory, data, field and driving teams were actively involved in the study during the running phase, while the advisory committee, trial statistician, trial monitors, and overall supervisor provided support during study design and trial setup phases.

3.12 Pilot studies

A pilot study (May – Sept 06) was carried out on ten 9 mo old infants and ten 3 mo old infants randomly recruited from Keneba and its surrounding villages in order to establish the discrimination ratios of the proposed outcome measures. Gut permeability and intestinal inflammation were measured three times in each infant, with an interval of one week between each. Plasma FA profiles and acute-phase markers were measured twice, three weeks apart.

The study also tested acceptability, ease of administration, and potential side effects of the fish oil supplement. Fish oil, or olive oil placebo, were given to infants every day for a week, after which a questionnaire was completed by mothers for the reporting of any side effects.

Box 5: Outline of staff and scientists involved in the trial

Head MRC International Nutrition Group (PhD Supervisor)
Overall supervisor

Head MRC Keneba field station
Local supervisor

Trial Statistician

Advisory Committee consisting of four LSHTM scientists

Database developer/manager

Trial/safety monitors

Various ground staff:
Data office, drivers, administrative support, laboratory, clinic etc.

1 Field Supervisor
6 Fieldworkers

16 Village assistants

Lastly, all mothers expressed a pooled sample of breast-milk (three times, one week apart) in order to evaluate its PUFA content.

The faecal and urinary assays showed very high variability within and between individuals. In order to use urinary LMRs and faecal calprotectin (and neopterin) as markers in our study the assays therefore required further work to investigate whether most of the variability was explained biologically or due to collection/processing/laboratory methods and whether their discrimination ratios could be improved.

The supplement was very well received by all infants. No side effects were reported.

The results of the sample assays follow in Table 6.

Table 6: Results of pilot study laboratory assays on urine, stool and plasma samples

	Mean/median	SD	Range	Normal healthy reference values	Inter-individual %CV	Intra-individual %CV	Inter-assay %CV	Intra-assay %CV
LMR	0.23	1.41	0– 8.2	<0.2	190	91	8	NM
CALPROTECTIN (mg/kg wet sample)	167	340	164- 1473	25	107	57	10	6.0
NEOPTERIN (mg/kg dry sample)	9.2	2.9	6 – 15	-	32	23	10	2.9
Plasma AGP (g/l)	1.5	0.3	0.8-2.4	<1.2	18	20	NM	NM
Plasma albumin (g/l)	39	2.7	36.8-44.3	34-54	7	6.5	NM	NM

NM: Not measured

Because of a hiatus caused by the resignation of the chief analyst at the Human Nutrition Research Centre at Cambridge, where the plasma and breast-milk samples were sent to be analysed, GC work was delayed until after the ethical approval of the study was granted. The samples were finally sent to King's College London but breast-milk and FA results were unfortunately only available after the start of the main study.

A later pilot study, led by the same graduate student who scored the cognitive tests and supervised by the PI, was conducted in September – December 2007. As both the Willatts Test and Infant Attention Assessment had not been used in The Gambia before, both were piloted in order to conduct the tests most effectively, particularly in terms of selecting the most appropriate age to test the children.

Seventy-five infants aged 10 – 12 mo (twenty-five children in each age-group) were tested with the following aims:

- Determine the most appropriate two-week age-interval (between 10, 11 and 12 mo of age) in which to test the children;
- Detect any problems with administering the test;
- Assess the suitability of chosen test equipment and toys to the Gambian context.

Results:

The infants responded better to Western than to Gambian home-made toys, provided these were presented by a Gambian fieldworker. They responded well to the complex V-Tech®toy, despite its novelty and loud sounds, as long as the tester made the child feel comfortable by encouraging them to play with and touch the toy.

In making a choice for deciding which age of testing was most appropriate, the following were considered:
- The frequency distribution scores at different age groups;
- The number of children unable to achieve any scores (attaining "0");
- The correlations between scores achieved on different components of the Willatts Test and the Attention Test at different age-groups.

Infants, on average, did not achieve even half of the maximum possible intention score. At 12 mo the best scores (43% of the maximum, on average) were achieved, compared to the lower 36% and 22% at the other ages. Similarly for the intentional solutions, average scores were low, but at 12 mo they were the highest at 30% of maximum score.

Similarly on the Attention Test, 12 mo old infants performed better than younger infants. The correlation between the two measures "look duration" and "inattention rate" was highest for tests performed at 12 mo ($r = -0.91$), and correlations between the Attention Test and Willatts Test, as well as the individual Willatts outcome measures, were also highest at 12 mo.

Because 1): the relationship between outcome measure were strongest at 12 mo; 2): the Willatts Test proved too difficult for many of the younger infants (with some achieving a total score of "0"); and 3): the higher response rate at 12 mo provided more information with which to discriminate between more and less intelligent infants, it was decided that, between the three ages assessed, 12 mo would be the most sensitive age-of-testing in this rural Gambian population for conducting the tests of choice.

CHAPTER 4: RESULTS AND SPECIFIC DISCUSSIONS

4.1 Trial profile

Assessed for eligibility (n=220)

Excluded (n=37)
Moved out of study area or travelling (n=21)
Not meeting inclusion criteria (n=7)
Died (n=6)
Refused to participate (n=3)

Randomised (n=183)

Allocated to receive Fish oil (n=92)

Received allocated treatment (n=90)
Did not receive allocated treatment (n=2)
Severely malnourished (n=1)
Not meeting inclusion criteria (n=1)

Lost to follow up (n=3)
Moved/travelled (n=1)
Died (n=1)
Did not come for endpoint
measurements (n=1)

Analysed (n=87)

Allocated to receive Placebo (n=91)

Received allocated treatment (n=90)
Did not receive allocated treatment (=1)
Did not come for baseline
measurements (n=1)

Lost to follow up (n=5)
Moved/travelled (n=3)
Died (n=1)
Mistakenly enrolled - did not meet
inclusion criteria (n=1)

Analysed (n=85)

Figure 27: CONSORT flow chart

Of the 220 assessed infants, 186 were eligible to take part in the trial. Three of these infants were not included because of parental refusal, either because the husband did not want his wife to spend time away from her house-hold duties, or because of distrust towards the MRC. This refusal rate of 3/186, or 1%, however, was very low. The high acceptance rate was probably a reflection of the good relationship between the MRC and local communities, the value the parents place on the medical treatment that they and their children receive from the MRC, well-trained fieldworkers with good community rapport, and the desire of individuals to contribute to the body of medical and nutritional research.

Figure 27 summarises the trial flow by means of a CONSORT[2] diagram. Of the 183 infants randomised, three did not receive any treatment. Reasons for this are given in the diagram. Of the remaining 180 subjects, data sets were available for 172 infants from which growth and other outcomes could be determined. Eight infants were lost to follow-up: three in the fish oil group and five in the control group. This drop-out rate was reasonably balanced between trial arms and was therefore unlikely to lead to bias in the final sample size.

One infant with congenital abnormalities was recruited by a fieldworker unsure of whether they were eligible to take part. When the infant was brought in to the clinic on his first due visit, before commencement of treatment, it was clear upon examination that the abnormalities were severe enough to influence growth and hence warrant the infant's exclusion from the study. Another infant was mistakenly recruited a month too early and went on to receive treatment. When the error was later discovered, the child was withdrawn from the study, as will be explained in Section 4.9. Given that he did not meet the inclusion criteria and because it was considered unethical to supplement an infant of 2 mo of age with a dose which was approved for infants of 3 mo of age, the trial and safety monitors agreed with his withdrawal from the study.

4.2   Dates of recruitment and follow-up

Age-eligible infants were recruited from May 2007 to January 2008. To allow for a 20% loss to follow-up, a final sample size of 180 was targeted. Eventually, 183 infants were recruited (when, towards the end of the study, the last list of potentially eligible infants was drawn up,

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[2] Consolidated Standards of Reporting Trials

and, from it, three more mothers than predicted were traced by different fieldworkers, and recruited).

To facilitate the most efficient use of transport, subjects were brought in for baseline and endpoint measurements in such a way that vehicle use between this and other studies, and between the various study areas, were co-ordinated. Thus, some infants were brought in up to ±7 days before or after turning 3 mo of age. All subjects were consequently supplemented for the duration of 24 to 25 weeks, starting one week after their first baseline measurement and receiving the last dose of treatment during their 9 mo endpoint clinic visit. The last supplement was administered in July 2008, and the final 12 mo cognitive and anthropometric measurements conducted three months later, in October.

4.3   Baseline characteristics of study participants

Table 7: Baseline characteristics of study participants

Characteristic	Fish oil group $\bar{x}$; SD	Placebo group $\bar{x}$; SD	Differences
Male	49	50	.
Female	38	35	.
Age (days)[1]	92.3; 4.25	93.2; 4.22	.
Anthropometric indices	(n=87)	(n=85)	
Weight (kg)	5.86; 0.84	5.75; 0.83	0.11
Length (cm)	60.1; 2.20	60.0; 25.6	0.10
HC (cm)	40.1; 1.24	40.0; 1.29	0.10
MUAC (cm)	13.2; 1.15	13.0; 1.12	0.22
Knee-heel length	16.1; 0.75	16.0; 0.90	0.10
Biceps skinfold	6.84; 1.24	6.72; 1.38	0.12
Triceps skinfold	8.84; 1.62	8.60; 1.71	0.24
Subscapular skinfold	8.24; 1.50	8.21; 1.79	0.03
Anthropometric z-scores[2]			
Weight-for-age	-0.55; 1.13	-0.75; 1.25	.
Weight-for-length	-0.29; 1.19	-0.43; 1.07	.
Length-for-age	-0.41; 0.95	-0.52; 1.26	.
HC-for-age	-0.11; 0.85	-0.26; 0.99	.
MUAC-for-age	-0.16; 1.07	-0.40; 1.11	.

Body-mass-index-for-age	-0.44; 1.19	-0.63;1.15	.
Triceps skinfold-for-age	-0.69; 1.09	-0.83; 1.11	.
Subscapular skinfold-for-age	0.27; 0.95	0.22; 1.15	.
Plasma FAs (ng/µl)	(n=83)	(n=85)	
DHA	70.2; 23.8	76.4; 23.2	6.2
EPA[3]	23.0 (14.5, 35.5)	24.3 (16.1, 40.4)	1.3
AA	125; 42.5	133; 39.9	8.0
(% total FAs)			
DHA	3.75; 0.89	4.00; 0.95	0.25
EPA[3]	1.15 (0.81, 1.66)	1.31 (0.82, 2.03)	0.16
AA	6.66; 1.41	6.90; 1.51	0.24
Acute phase proteins	(n=80)	(n=78)	
Plasma albumin (mg/L)	33.0; 3.01	33.0; 2.61	0.00
	Median	**Median**	
	(25, 75 pctile)	**(25, 75 pctile)**	
α-Acid glycoprotein(g/L)	0.95 (0.77, 0.11)	0.88 (0.69, 0.12)	0.77
C-reactive protein (mg/L)	3.02 (2.23, 5.31)	2.91 (2.22, 4.67)	0.11
Gut integrity	(n=85)	(n=82)	
Urinary LMR	0.15 (0.10, 0.22)	0.15 (0.12, 0.20)	0.00
Lactose:lactulose ratio	1.29 (0.88, 1.84)	1.35 (0.97, 1.87)	0.06
Lactulose % recovery	0.26 (0.19, 0.45)	0.24 (0.16, 0.34)	0.02
Mannitol % recovery[4]	7.02 (3.46)	6.21 (2.94)	0.81
Intestinal inflammation	(n=82)	(n=83)	
Calprotectin dry wt (mg/kg)	124 (75.5, 192)	128 (73.5, 198)	3.5
Calprotectin wet wt (mg/kg)	1029 (627, 1534)	1058 (610, 1639)	29

[1]Age on the first day of supplementation
[2]Measured against WHO reference standards
[3]Data were skewed so for this FA the median and 25th & 75th percentiles instead of the mean & SD are presented
[4]As these data were normal, the mean and SD are presented for mannitol recovery

Table 7 describes the age and sex characteristics of participants. Baseline measurements, separated by treatment group, are also given.

As expected, there were no important differences between treatment group means at baseline, demonstrating a successful randomisation procedure leading to good group comparability. Furthermore, as mentioned in the previous chapter, in most cases, baseline

values were fitted as covariates during analyses of endpoint variables, and so any modification of treatment effect or variance inflation they may have caused would have been corrected for.

The balance between male and female infants (99:73) in the overall sample size was uneven. This ratio (1.4:1) does not reflect the West Kiang demographic balance between male and female infants, nor of male-to-female refusal rates for inclusion into the study, and was most likely due to chance. The difference, however, lay only on the border of statistical significance, and, importantly, the male-to-female ratios were similar between treatment groups. The sex ratio, therefore, did not lead to bias when making group comparisons.

EPA, AGP, CRP, lactulose-mannitol, attention assessment and calprotectin data were skewed and so for these the median and 25% and 75% percentiles, rather than the mean and SDs, are given as an indicator of spread and of central distribution.

4.3.1 Discussion

Although significance tests are often used to identify differences between baseline characteristics, CONSORT and others (254-257) advise against this practice due to its inappropriateness. If randomisation has been performed correctly, the only explanation for group differences would be chance, in which case significance tests become redundant. Additionally, when presenting twenty or more baseline characteristics, as is the case below, a strong probability exists that at least one characteristic will be significantly imbalanced due to chance alone. Differences between group means were, therefore, presented and inspected for any clinically significant differences, but not compared by statistical testing.

On average, infants were three months old when commencing with supplementation. The youngest age was 2.8 mo (12 wks), and the oldest was 3.3 mo (14 wks).

All growth index z-score averages were negative, except subscapular skinfold. Thus even at 3 mo, some growth faltering could be detected in these infants. The growth faltering was, however, not severe and no z-score average was smaller than -0.8.

Plasma FA levels are given both in absolute values and as relative percentage FAs. It is difficult to make comparisons with other populations because of the limited data which exist on infant plasma lipid n-3 LCP levels.

A third and nearly half of infants had higher than normal CRP and AGP levels, respectively, and 15% had lower than normal albumin levels.[3] These acute phase protein levels were comparable to those previously reported in Gambian infants (9, 258, 259).

Lactulose percent recoveries were similarly comparable to those previously measured in Gambian infants of approximately this age, while LMRs were somewhat lower on average (9, 30, 258-260). The improved LMRs were due to higher mannitol % recoveries which were nearly twice the amount of those previously reported, suggesting an improved villous surface area in this population, but a similar degree of leakiness compared to previously studied Gambian populations. The ratio was still, however, significantly higher than the ratio of <0.07 reported in healthy European children (261, 262).

Finally, calprotectin levels could not be compared to previous Gambian data as none are available. However, the median of 1033mg/kg in the Gambian infants is significantly higher than the normal healthy reference of 263mg/kg (wet stool) in 3 mo old European infants (263), and only nine infants had calprotectin levels falling below this cut-off.

4.4   Number of participants

The primary analysis was intention-to-treat and involved all subjects who were recruited and for whom 9 mo outcome measurements were available. Of the 183 subjects originally recruited, three were withdrawn before they started receiving treatment. Of the remaining 180 infants, eight were withdrawn or lost to follow-up during the 6 mo intervention period. A total of 172 subjects completed the study and were evaluated for the planned study outcomes at 9 mo, where information was available. The study dropout rate was therefore 8/180, or 4.4%.

This dropout was far below the expected 20% dropout rate. The following four factors presumably contributed to the successful participant retention in the study:

1. Mothers were asked for a high degree of commitment at time of recruitment, and were encouraged to decline from participating in the study if they foresaw the need to travel for more than two or three weeks in the ensuing six months.

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[3] Using standard adult cut-off ranges from healthy individuals: CRP≤5mg/L; AGP≤1g/L; albumin≤30mg/L.

2. The daily cups of tea and bread provided for mothers when they brought their infants for supplementation, as well as the high standard of medical care offered served as incentives which led to greater cooperation in mothers' attendances of supplementations and a high proportion of mothers remaining in the study throughout their infant's treatment period.

3. Because resources allowed for a fieldworker to be stationed at the coast full-time towards the end of the study, the mothers who travelled there during the dry season could remain in the study even though they were away from the ordinary study area for more than two or three weeks. Had this not been the case, the infants of these mothers would have been lost to follow-up.

4. Field-workers were experienced and skilled in maintaining good relations with study participants. Although participants understood that they were free to withdraw from the study should they wish, when cases of discontent arose, every attempt was made to understand what was causing the dissatisfaction, and to find a solution, so that they could continue taking part without complaints. Also the PI met with participants who expressed valid concern or complaints, and these were then addressed. One such grievance, for example, was dissatisfaction with nurses who, the mothers felt, were not giving their infants timely or acceptable standards of care. As mothers were promised a high standard of medical care for their infants if they took part in the study, this complaint was taken very seriously, and the nurse concerned consulted together with the head nurse and mothers so that a solution could be found.

A total of 143 infants came for their outcome measurements at 12 mo of age, viz. cognitive development and anthropometry. Dropout at this 9 mo follow-up was, therefore, 20%, calculated from the original 180 participating infants, and was again reasonably balanced between treatment groups. Thirteen infants from the fish oil group and sixteen from the placebo group were lost to follow-up, primarily due to mothers travelling, or not turning up on the day that their infants' testing was due.

This higher drop-out rate could be expected because parents were not involved in the study in any way during the three months before cognitive testing, and so would more easily leave to travel or fulfil family or other commitments.

Anecdotal information from field-workers also indicated that some mothers did not understand the value of the cognitive test, and so undermined its significance. They felt that a few minutes' watching their infants playing with a toy could not be worth a great deal, and so did not feel compelled to bring their infants in when requested.

4.5 Compliance

Compliance was measured as the percentage of doses taken versus doses offered during each infant's supplementation period, as is summarised in Table 8. The distribution of compliance is presented in Figure 28, showing a high density of compliance between ~97% and 100%, and a long tail extending at lower compliances of ~80 – 95%.

Any infants who were supplemented less than 90% of the time were classified as non-compliant. Twenty-three infants fell into this category. Compliance and non-compliance were individually regressed on treatment group to test for differences. There were no significant differences in compliance and non-compliance rates between intervention groups (Table 8).

The majority of instances (74%) of non-compliance were due to infants temporarily travelling with their mothers out of the study area. Other missed doses were mainly due to serious illness, where the protocol was suspended on advice of the TSM, and infants not being traced in their villages when the fieldworker arrived for supplementation.

Table 8: Compliance with treatment, by treatment group

	Compliance by treatment group[1]		
	Fish oil group	**Placebo group**	**95% CI (p-value)**
Mean (SD, std error)	95.8 (5.00, 0.54)	96.1 (4.37, 0.48)	-1.76 - 1.08[2] (0.639)
Non-compliant	13/87	10/85	-0.07 - 0.13[3] (0.543)

[1]Measured as proportion of doses taken vs. doses offered
[2]CI for difference in compliance rate (% of doses taken) between treatment groups
[3]CI for difference in percentage of non-compliant infants between treatment groups
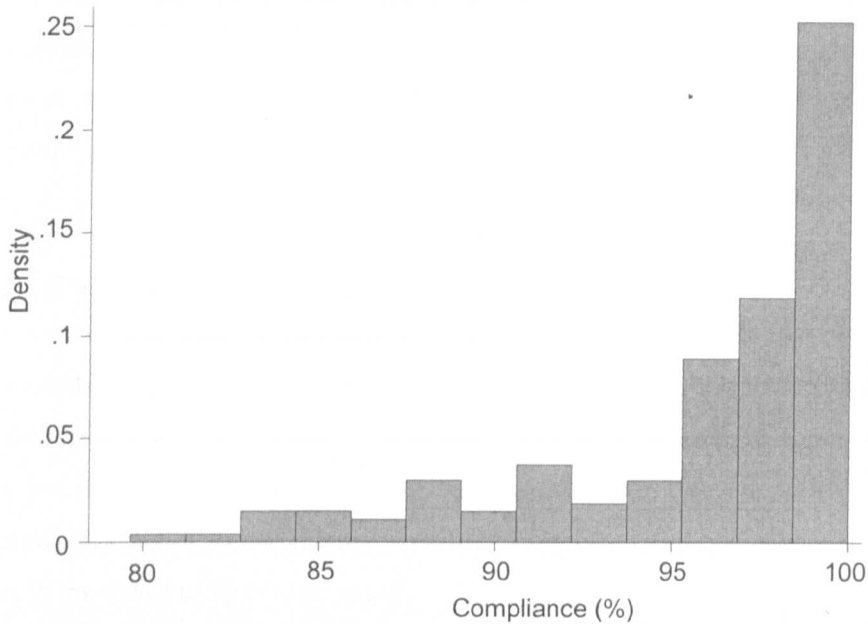
Figure 28: Distribution of compliance

As judged from compliance records, reports from fieldworkers, and observations by the PI, the oils were generally well accepted by the infants, and the supplementation regimens by the mothers. Thirteen cases of an infant vomiting the oil out after administration were reported in total, and in only one instance did a mother object to having her infant dosed. This objection arose because her infant had been experiencing diarrhoea and the mother was concerned that the supplement was causing it. After three days, however, the diarrhoea cleared up and the mother gave her approval for the supplementation to be continued.

Quality control of compliance estimates were conducted by a second observer who returned to compounds and asked mothers/caregivers whether their infants had been supplemented. This was done a few hours to a day after the relevant fieldworker had supplemented the infants assigned to him. A total of 305 compliance report sheets were repeated in this way. Four percent of infants were reported to have had no supplement given when recorded by the routine fieldworker. In contrast, 7% of infants were reported to have had no supplement given when recorded by the second observer. The calculated overall discordance between observers was 3%.

Compliance was correlated with the infant's village, sex, and date of recruitment by fitting the variables in a regression model, to test whether any of these covariates influenced the degree of doses taken. No associations between compliance and the named covariates were observed.

4.5.1 Discussion

A similar compliance rate between intervention groups shows that group comparison assessments were not affected by compliance bias. With an average compliance rate of over 95% and only one infant complying less than 80% of the time, poor compliance was not a cause for concern in this population. It also suggests that the supplements did not cause noticeable discomfort to the infants, as mothers (apart from the one passing occasion) did not object to the daily dosing of their infants.

The daily village visits made by fieldworkers to administer and record dosages to each participant, together with strict weekly monitoring of each infant's compliance, served to maximise the overall supplementation adherence. Any doses missed were noted and discussed at the weekly team meetings and special attention paid to ensure doses were not missed unnecessarily. If a mother travelled with her infant outside of the study area, she was contacted daily by telephone if she or her hosts owned one, in order to ask about the wellbeing of her child and to verify her return travel plans.

The first two factors which most likely contributed to the low study participant drop-out rate (the high degree of commitment asked from mothers, and daily cups of tea provided, listed under Section 4.4) also likely added to the successful compliance rate.

From quality control reports it appeared that compliance may have been over-reported. However, when the second observer returned to compounds for quality control visits, he occasionally did not find the mother at home, and someone who was perhaps not present when the infant was supplemented a day or a few hours earlier may have assumed that the child received no supplement simply because they were unaware of the routine fieldworker's earlier visit. Regardless of whether this was the case, even accounting for a 3% over-reporting by fieldworkers, compliance was still high.

4.6   Primary outcomes

4.6.1 Growth

There were no statistically significant differences in the growth indices of weight, length, HC, biceps- and triceps-skinfold thickness and knee-heel length using intention-to-treat analysis (Table 9). A significantly larger MUAC z-score (95% CI: 0.06,0.56; p=0.017) and a borderline significant increase in MUAC growth rate (95% CI: 0.00,0.51; p=0.049) was, however, detected in the intervention group at the usual 5% significance level. (The more pronounced treatment group difference in the MUAC z-score than absolute growth in MUAC could be explained by the removal of variation due to sex differences and age, and by a normalisation of the distribution by the z-score calculations).

A small significant difference for triceps skinfold-for-age was also detected, but the lower limit of the 95% CI was very close to zero (0.00 - 0.55), and the p-value was only borderline significant (0.048).

Principal components analysis was used to predict the combined fatness scores of the three skinfold measures: triceps, biceps and subscapular. When tested, there were no differences in this combined fatness score between treatment groups (95% CI: -0.05,0.79; p= 0.086).

The analysis was repeated, controlling for breast-milk DHA or EPA, sex, season of birth, and age of commencement of treatment. Because z-score growth indicators were already corrected for age and sex, the analysis was only repeated on absolute measures of growth. The results were similar with respect to the significance of treatment effects (Table 12). However, the (borderline) significant treatment effect seen on MUAC was changed to borderline non-significant (95% CI: -0.02,0.48; p=0.074) when controlling for the listed covariates and breast-milk DHA. The diminishing effect was smaller but similar when controlling for breast-milk EPA rather than DHA.

When testing them individually, breast-milk DHA and EPA were the covariates which had the greatest influence on diminishing treatment effect. When investigating the distribution of breast-milk DHA and EPA between treatment groups, a significantly different n-3 LCP concentration was, indeed, detected in the mothers of infants in the control group versus mothers of infants in the fish oil group (EPA: 95% CI 0.61-0.93, p=0.008; DHA: 95% CI 0.72-0.96,

p=0.013). Mothers with infants in the control group had higher breast-milk concentrations of both EPA and DHA than mothers with infants in the fish oil group.

The adjusted analysis results are summarised in Table 13, showing the effects of treatment controlled for compliance, compared to the unadjusted analyses. The table also shows the results of additionally fitting "doses of treatment taken" to the regression model, which gives an indication of the dose effect over and above what is explained by the treatment groups.

In contrast to the intention-to-treat analysis, which answered the question of whether the intervention had an impact (in this case on growth), the secondary analysis answered the question of whether receiving the intervention had an impact, and whether compliance modified this impact. Not surprisingly, owing to the high compliance rate and narrow distribution of compliance, the secondary analysis produced similar results to the primary analysis, and there were no interactions found between compliance and treatment on growth.

When controlling for plasma n-3 LCPs (Table 12), the treatment effect seen on MUAC z-scores was removed by plasma EPA (95 % CI: -0.09,0.49, p=0.168), indicating that the effect was working via this FA. It was to be expected that the effect would be working via n-3 LCPs, but interesting that most of this effect was explained by EPA rather than DHA or their combination.

Controlling for AA did not diminish the treatment effect.

Breast-milk DHA and EPA, and season of birth, were examined for any interactions with treatment on outcomes by adding these terms to the regression models and inspecting for an interaction. Season was weakly significant with length (p=0.0445) and knee-heel length (p=0.0494). This provides weak evidence that infants who received treatment had a different pattern of seasonal growth compared to infants who received placebo.

Because knee-heel length had not been used as a marker of growth in this population before, and is not a commonly used indicator in paediatrics but has been reported to be a sensitive and precise marker of short-term linear growth in infants (264), its relationship with length was investigated. The two measures indeed displayed a strong linear relationship

(r=0.95), more so than any of the other growth variables. A scatter plot of length versus knee-heel length is shown in Figure 29.
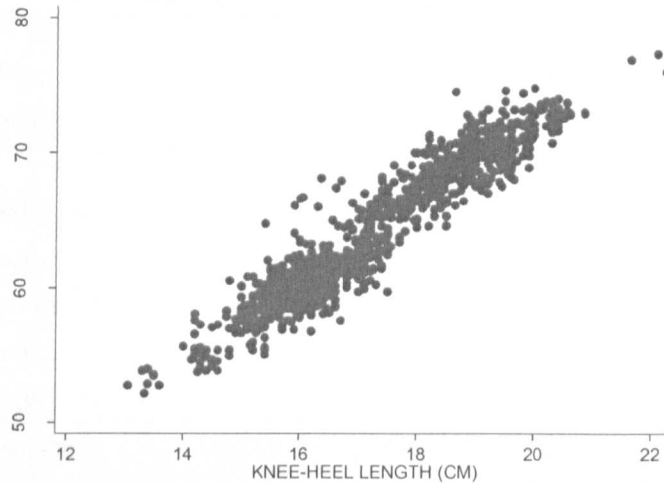
Figure 29: Graph showing length plotted against knee-heel length (baseline and endpoint measures combined)

Table 9: Effect of n-3 LCP supplementation on primary endpoints, by treatment group

Endpoint	Fish oil group	Placebo group		
	$\overline{\chi}$; SD (n=87)	$\overline{\chi}$; SD (n=85)	Effect size (95% CI)	p-value
Anthropometric indices				
Weight (kg)	7.79; 0.99	7.56; 1.08	-	-
Length (cm)	69.1; 2.45	68.6; 3.03	-	-
HC (cm)	43.8; 1.21	43.6; 1.48	-	-
MUAC (cm)	14.1; 1.02	13.7; 1.25	-	-
Knee-heel length	18.8; 0.88	18.7; 1.12	-	-
Biceps skinfold	6.71; 0.98	6.46; 1.12	-	-
Triceps skinfold	8.64; 1.42	8.22; 1.73	-	-
Subscapular skinfold	7.53; 1.77	7.39; 1.63	-	-

Anthropometric z-scores[1,2]				
Weight-for-age	-0.90; 1.09	-1.20; 1.28	0.15 (-0.08, 0.38)	0.208
Weight-for-length	-0.57; 0.99	-0.76; 1.10	0.12 (-0.14, 0.38)	0.377
Length-for-age	-0.79; 1.02	-1.07; 1.30	0.79 (-0.27, 0.90)	0.084
HC-for-age	-0.52; 0.84	-0.64; 1.06	-0.01 (-0.18, 0.17)	0.954
MUAC-for-age	-0.27; 0.92	-0.66; 1.18	0.31 (0.06, 0.56)	0.017*
Body-mass-index-for-age	-0.60; 0.99	-0.79; 1.12	0.08 (-0.16, 0.33)	0.503
Triceps skinfold-for-age	-0.01; 0.85	-0.30; 1.09	0.27 (0.00, 0.55)	0.048*
Subscapular skinf-for-age	0.41; 1.12	0.27; 1.29	0.16 (-0.16, 0.48)	0.326
Change in growth				
Weight (g)	1935; 669	1805; 635	135 (-62, 332)	0.179
Length (cm)	9.04; 1.67	8.56; 2.00	0.49 (-0.50, 1.04)	0.076
Head circ (cm)	3.68; 0.87	3.69; 0.68	0.00 (-0.23, 0.23)	0.991
MUAC (cm)	0.87; 0.90	0.68; 0.94	0.26 (0.00, 0.51)	0.049*
Knee-heel length (cm)	2.76; 0.61	2.63; 0.61	0.12 (-0.06, 0.31)	0.190
Biceps skinfold (mm)	-0.13; 1.10	-0.26; 1.33	0.20 (-0.08, 0.48)	0.152
Triceps skinfold (mm)	-0.20; 1.60	-0.38; 1.60	0.30 (-0.11, 0.71)	0.149
Subscapular skinf (mm)	-0.71; 1.65	-0.82; 1.51	0.12 (-0.31, 0.55)	0.580
Gut integrity	(n=86)	(n=81)		
Mannitol % recovery	4.22; 2.48	4.51; 2.04	-0.34 (-1.04; 0.35)	0.332
	Median	**Median**		
	(25, 75 pctile)	**(25, 75 pctile)**		
LMR[3,4,5]	0.22(0.14,0.37)	0.22 (0.15,0.29)	0.96 (0.87,1.07)	0.507
Lactose:lactulose ratio[3,5,6]	1.80 (0.99, 2.69)	1.49 (1.02, 2.35)	1.00 (0.87, 1.14)	0.946
Lactulose % recovery[3,5]	0.21 (0.11, 0.38)	0.23 (0.15, 0.32)	0.91 (0.72,1.15)	0.434

[1]General least squares regression analysis, entering the dependent variable and treatment. Median growth measurements of each individual were used, controlling for baseline.
[2]Using WHO reference curves
[3]Data were log transformed
[4]Regression analysis, fitting (log) mannitol on (log) lactulose, controlling for baseline values
[5]For skewed data, effect size and confidence intervals are expressed as the anti-log of calculated CIs and regression coefficients.
[6]Regression analysis, fitting (log) lactose on (log) lactulose, controlling for baseline values
*$p<0.05$

The effects of fish oil on growth will be further addressed in Section 4.8.2, where growth outcomes at 12 mo will also be discussed.

4.6.1.1   Measurement reliability

The variation between similar anthropometric measurements taken on the same individual on alternative days was calculated using data before it was corrected for outliers and other obvious errors. This variation, also termed "measurement error" (265), was quantified by calculating intra-class correlation coefficients and intra-subject SDs, as recommended by Bland and Altman (266), using a one-way analysis of variance.

The intra-class correlation coefficient, calculated as the ratio of the within-subject to total sample variance, estimates the combined variance due to measurement error (e.g. variability in measuring technique and equipment) and small short-term random fluctuations (e.g. level of tissue hydration, slight growth between days one and five of baseline and outcome measures), and is calculated from the equation:

Correlation coefficient = (m*SSB - SST) / ((m-1) * SST)

Where m = number of measurements per subject; SSB = sum of squares between subjects; SST = total sum of squares.

Measures with the highest correlations discriminate best between subjects i.e. carry the most information (267).

Seen in Table 10, all measurements apart from triceps- and biceps-skinfolds, had an intra-class correlation coefficient of at least 0.90, averaged across baseline and endpoint measures. Weight and head-circumference had the highest coefficients. The table also presents intra-class SDs, used to determine the size of the measurement error in straightforward clinical terms.

Although only the PI took most of the anthropometric measurements (>75%), a (trained) second observer was required to stand in in cases where the PI was ill or travelling. A 30% sub sample of measurements taken by both the PI and second observer were compared for correlation and bias using Pearson product-moment correlations and two-tailed t-tests, the results of which are presented in Table 11.

Table 10: Anthropometric intra-class correlations and SDs, calculated from three independent measurements, using a one-way ANOVA

	Baseline	Endpoint	Combined
	Intra-class correlation coefficient (95% CI)	Intra-class correlation coefficient (95% CI)	Intra-class SD
Weight (g)	0.96 (0.95-0.97)	0.98 (0.98-0.99)	147g
Length (cm)	0.87 (0.83-0.90)	0.93 (0.91-0.94)	0.86cm
MUAC (cm)	0.94 (0.92-0.95)	0.92 (0.91-0.95)	0.30cm
HC (cm)	0.95 (0.94-0.97)	0.93 (0.91-0.94)	0.33cm
TSF[1] (mm)	0.78 (0.73-0.86)	0.76 (0.70-0.81)	0.83mm
BSF[2] (mm)	0.85 (0.81-0.88)	0.84 (0.80-0.87)	0.48mm
SSF[3] (mm)	0.91 (0.88-0.92)	0.90 (0.87-0.92)	0.53mm
Knee-heel length (cm)	0.92 (0.90-0.94)	0.94 (0.92-0.95)	0.25cm

[1]Triceps skinfold thickness
[2]Biceps skinfold thickness
[3]Subscapular skinfold thickness

Table 11: Comparison of anthropometric measurements taken by the PI and second observer

	Correlation between observer 1 & 2	Difference between observers' measurements[2]		
	r[1]	$\bar{x}_1 - \bar{x}_2$	95% CI	p-value
Weight (g)	1.00	0.58	-3.64 - 4.80	0.785
Length (cm)	0.99	0.16	-0.03 - 0.35	0.106
MUAC (cm)	0.96	0.06	-0.03 - 0.15	0.200
HC (cm)	0.98	0.16	0.05 - 0.26	0.005**
TSF (mm)	0.79[¶]	-0.09	-0.37 - 0.19	0.513
BSF (mm)	0.88[¶]	0.02	-0.12 - 0.16	0.789
SSF (mm)	0.87[¶]	-0.24	-0.46 - 0.02	0.031*
Knee-heel length (cm)	0.98	0.25	0.17 - 0.32	0.000***

[1]Pearson correlation coefficient
[2]From paired t-test; comparison between observer measurements
[¶]$r < 0.95$
*$p < 0.05$; **$p < 0.01$ ***$p < 0.001$

4.6.1.2 Seasonality

Baseline growth measurements were plotted by month and fitted, using a Fourier series (Figure 30). A statistically significant seasonal effect on growth was detected on all growth indices.
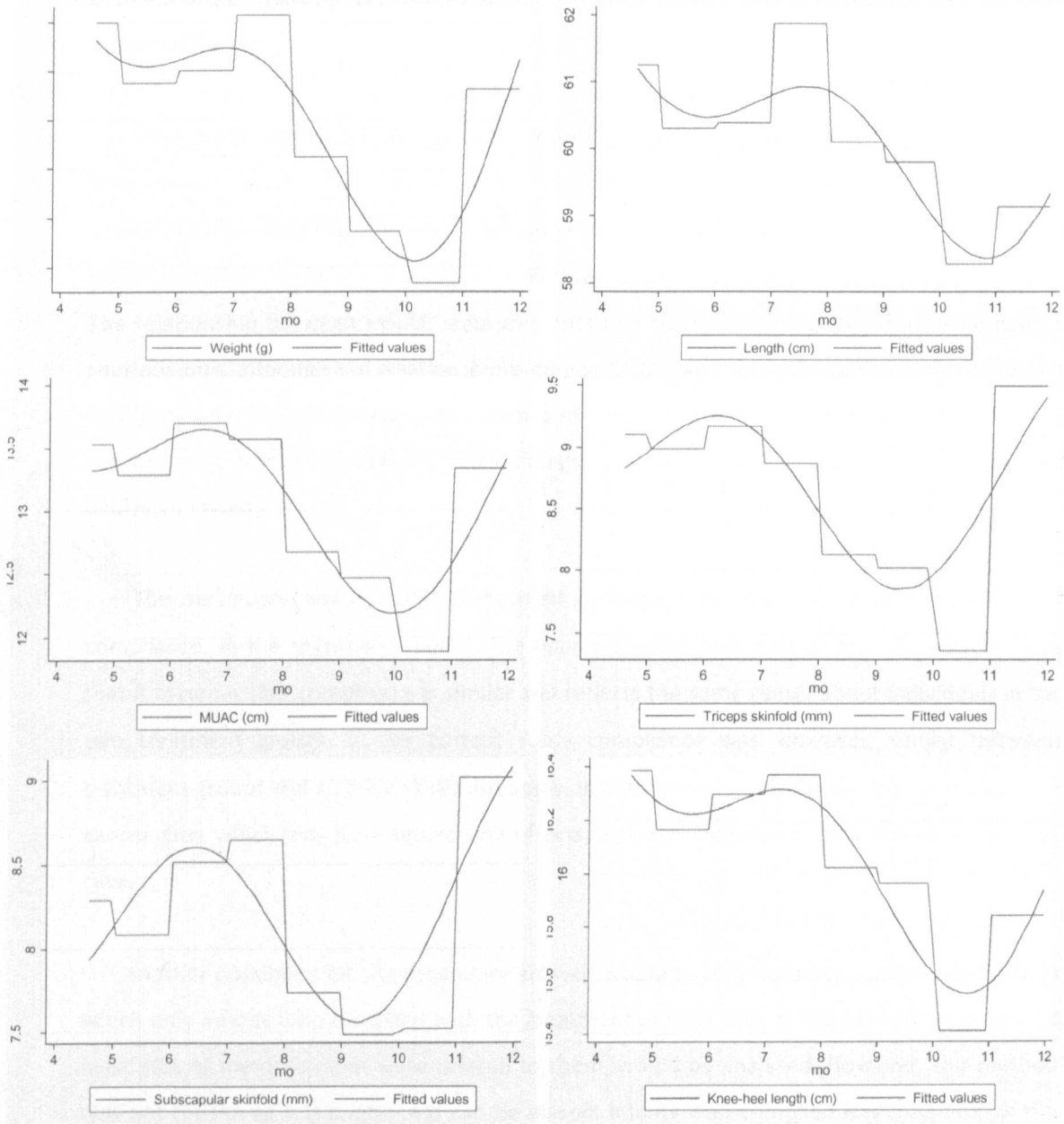
Figure 30: Clockwise from top-left: Weight, length, triceps-skinfold, knee-heel length, subscapular skinfold, and MUAC measurements, fitted by calendar month. Significance of seasonal effect on growth for all outcomes above was p<0.001, tested using the Wald Test. (Month "4" corresponds to May, "5" to June etc.)

4.6.1.3 Discussion

Although the fish oil group averages were consistently higher than the olive oil group averages, group differences were not significant and the lower limit of the 95% CIs stretched to below zero in most cases. At baseline the fish oil group average was higher for all anthropometric indices except length, which explains part of the reason why no growth benefits were found in the supplemented group although their anthropometric measures averaged higher.

Since breast-milk n-3 LCPs correlated with both the dependent variable (MUAC) and the independent variable (treatment group), without lying on the causal pathway, they were possibly confounding the relationship between treatment and MUAC. The observed borderline treatment effect might not have been attributable to the intervention, but to breast-milk FAs. The relationship between MUAC measurements and treatment could therefore have been a spurious one, although the relationship between MUAC and n-3 LCPs was not. However, the unadjusted model only shows weak evidence of a treatment effect, so only minimal inferences can be made from the relatively small change in treatment effect differences the adjusted analysis showed.

The secondary analysis was performed by fitting "number of doses received", and compliance, in the regression model. The main potential weakness of this analysis could be that it assumes that compliance is similar and reflects the same things about individuals in the two treatment groups. In the current study compliance was, however, similar between treatment groups and so this risk did not apply. In another scenario, e.g. testing the effects of a cancer drug which may have severe side effects, a similar compliance may not have been the case.

Another possibility for the secondary analysis would have been a per-protocol analysis, in which only infants who complied with the treatment at least 90% of the time, or who took at least 90% of the doses that were offered to them, would be analysed. However, this method was not chosen as it is cruder and can be biased: infants who complied less than 90% of the time may be different in some unknown way to infants who did not.

Adding dose-effects to the regression model as covariate was instead chosen as it was a more efficient way of testing the effect of treatment whilst controlling for compliance, and in

so doing not only testing whether treatment had an effect when administered per-protocol, but testing whether compliance did indeed modify the effect of the treatment offered. Essentially, by fitting the number of doses of treatment received to the model, the interaction between treatment group and compliance was assessed.

Measurement reliability

The importance of anthropometry in nutritional assessment as an indicator of health status has been well described in the literature (268, 269). Its advantages as a measurement of nutritional status include the relative speed, simplicity, and inexpensiveness with which it can be measured (270). Anthropometry is, however, prone to measurement error and inter-observer bias, which may limit its interpretability.

In the present study, although accuracy (the extent to which measurements reflect "true" values) was important for comparison of data with other populations, it was not as critical to the ability to detect group differences as measurement precision (measurement error variance (271)) due to intra- and inter-observer variability (270) was. The precision indicated by the intra-class correlation coefficients of three repeat measures was generally high, although for triceps- and biceps-skinfold measures it was only moderately high.

By using the median of three measurements the most flawed and outlying readings were in all likelihood avoided, deflating some of the variance introduced by measurement errors and hence increasing the precision.

Some of the lengths and the weight of the intra-subject SDs may appear quite large, but in comparison to the overall inter-individual variance in the sample they are not significant contributors to the overall variance.

It is interesting to note the comparison between knee-heel length and length. Knee-heel length measurements were biased between observers whereas length measurements were not, and the correlation between observers was no better for knee-heel length (r=0.98) than for length (r=0.99). Moreover, the intra-class SD expressed as percentage of the mean for knee-heel length was larger (9.77%) in comparison to that for length (9.26%). Intra-class

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correlation was the only area where knee-heel length out-performed length in measurement reliability estimates. These results therefore contradict suggestions that the precision of knee-heel length measurements may be superior to those of length.

The repeatability was, nevertheless, higher for knee-heel length than length (0.93 vs 0.90). Given its high repeatability, strong correlation with length measurements, and simple and cheap method of measurement, knee-heel length may serve as a useful alternative to heel-crown length as an indicator of linear growth in infants in settings when it is impractical or otherwise difficult to measure length.

As is to be expected, given their susceptibility to inter- (and intra) observer variation, skinfold measures had the weakest strength of association (0.79 – 0.88) when comparing measurements between observers. All other measurements had strong correlations of r>0.95 between observers. As for bias, head-circumference and knee-heel length measurements were taken consistently significantly higher by the first observer (p=0.005, p<0.001, respectively), while subscapular-skinfold measurements were taken consistently higher by the second observer (p=0.031). Because there was no statistically significant difference in the number of measurements taken by the observers between groups, this bias would have been only a very minor variance inflator and so there was little justification for fitting "observer" as covariate when testing for the effect of treatment on growth. However, for analyses where randomisation was not considered (observational analyses), adjustments for observer were made where appropriate.

Seasonality

In The Gambia, as in many other developing countries, growth rate has repeatedly been shown to differ between the rainy and dry seasons due to the differences in parasite load, infections, food availability, and child care standards which the seasons bring (272). The rainy season starts in June, and during this time crops are first planted. As the rainy season begins, food supplies start to run low as reserves from the previous season's harvest become limited. Simultaneously, the burden of infection, particularly of diarrhoea, peaks, resulting in weight loss and growth restriction (273).

Many families in West Kiang can now afford to buy enough food when supplies run low, due to, amongst others, remittances from family members living abroad or in the city.

Additionally, the MRC station in Keneba continues to provide medical care to an increasing number of families. Nevertheless, the influence of season is still prominent: Figure 30 shows clearly that those infants who were born between June and August (i.e. in height of the rainy season), had achieved the lowest gains in growth by 3 mo of age compared to those born before or after this time.

Table 12: Effect of n-3 LCPs on growth & gut integrity, adjusting for the covariates breast-milk n-3 LCPs, sex, season of birth, & age first supplementation

Endpoint	Unadjusted analysis		Controlling for breast-milk DHA, sex, season of birth, age of first supplement		Controlling for breast-milk EPA, sex, season of birth, age of first supplement		Controlling for plasma DHA		Controlling for plasma EPA	
	Effect size (95% CI)	P	Effect size (95% CI)	P	Effect size (95% CI)	P	Effect size (95% CI)	P	Effect size (95% CI)	P
Growth indices										
Weight (g)	135 (-62.5,332)	0.179	108 (-81.8, 299)	0.262	117 (-74.7, 308)	0.230	138 (-64.0, 340)	0.179	93.0 (-129, 315)	0.410
Length (cm)	0.49 (-0.05,1.04)	0.076	0.36 (-0.16,0.88)	0.177	0.39 (-0.13,0.92)	0.143	0.40 (-0.15,0.95)	0.152	0.17 (-0.43,0.77)	0.577
HC (cm)	0.00 (-0.23,0.23)	0.991	-0.03 (-0.27,0.19)	0.740	-0.04 (-0.27,0.19)	0.724	0.00 (-0.23,0.24)	0.986	-0.08 (-0.34, 0.18)	0.530
MUAC (cm)	0.26 (0.00, 0.51)	0.049	0.23 (-0.02,0.48)	0.074	0.24 (-0.01,0.59)	0.065	0.28 (0.02, 0.54)	0.036	0.20 (-0.09, 0.49)	0.168
Knee-heel length	0.12 (-0.06,0.31)	0.190	0.10 (-0.08,0.27)	0.291	0.10 (-0.08,0.28)	0.272	0.12 (-0.06,0.31)	0.194	0.09 (-0.12,0.30)	0.404
Biceps skinfold	0.20 (-0.08,0.48)	0.152	0.21 (-0.07,0.49)	0.144	0.21 (-0.07,0.51)	0.138	0.23 (-0.05,0.51)	0.103	0.21 (-0.10, 0.52)	0.183
Triceps skinfold	0.30 (-0.11,0.71)	0.149	0.34 (-0.07,0.75)	0.105	0.37 (-0.04,0.78)	0.075	0.30 (-0.12,0.72)	0.155	0.22 (-0.25,0.68)	0.357
Subscapular sf	0.12 (-0.31,0.55)	0.580	0.15 (-0.29,0.59)	0.506	0.18 (-0.26,0.62)	0.428	0.14 (-0.29,0.57)	0.523	0.06 (-0.42,0.54)	0.812
Gut integrity										
LMR	0.96 (0.87,1.07)	0.507	0.96 (0.86,1.07)	0.445	0.96 (0.86; 1.07)	0.418	1.02 (0.88, 1.09)	0.692	1.01 (0.87,1.12)	0.852
Lactulose % recovery	0.91 (0.72,1.15)	0.434	0.93 (0.94,1.05)	0.794	0.99 (0.94,1.05)	0.781	1.00 (0.94,1.06)	0.982	1.01 (0.94,1.06)	0.856
Mannitol % recovery	-0.34 (-1.04; 0.35)	0.332	-0.31 (-1.05,0.41)	0.394	-0.31 (-1.04,0.41)	0.394	0.31 (-1.01,0.40)	0.393	-0.55 (-1.33,0.22)	0.160

Table 13: Adjusted analysis: testing the effect of n-3 supplementation when adjusting for compliance and doses of treatment received

Endpoint	Unadjusted analysis		Fitting compliance only		Interaction between compliance & treatment (fitting compliance & doses of treatment received)	
	Effect size (95% CI)	p	Effect size (95% CI)	p	Coefficient (95% CI)	p
Anthropometric indices						
Weight (g)	135 (-62.5, 332)	0.179	133 (-65.5, 331)	0.188	-4.91 (-25.1, 15.3)	0.632
Length (cm)	0.49 (-0.05,1.04)	0.076	0.48 (-0.07, 1.03)	0.084	-0.00 (-0.07, 0.05)	0.728
HC (cm)	0.00 (-0.23,0.23)	0.991	-0.01 (-0.24, 0.23)	0.955	-0.01 (-0.03, 0.02)	0.541
MUAC (cm)	0.26 (0.00, 0.51)	0.049	0.26 (-0.00, 0.51)	0.051	-0.01 (-0.03, 0.02)	0.555
Knee-heel length (cm)	0.12 (-0.06,0.31)	0.190	0.12 (-0.07, 0.30)	0.203	-0.01 (-0.03, 0.01)	0.345
Biceps skinfold (mm)	0.20 (-0.08,0.48)	0.152	0.22 (-0.06, 0.50)	0.116	-0.00 (-0.03, 0.03)	0.834
Triceps skinfold (mm)	0.30 (-0.11,0.71)	0.149	0.29 (-0.12, 0.71)	0.163	-0.01 (-0.05, 0.03)	0.664
Subscapular sf (mm)	0.12 (-0.31,0.55)	0.580	0.15 (-0.28, 0.58)	0.488	-0.00 (-0.05, 0.04)	0.879
Gut integrity						
LMR	0.96 (0.87,1.07)	0.507	0.97 (0.87, 1.08)	0.932	1.01 (1.00, 1.02)	0.012*
Lactulose% recovery	0.91 (0.72,1.15)	0.434	1.00(0.94, 1.74)	0.971	1.00 (1.00, 1.00)	0.126
Mannitol % recovery	-0.34 (-1.04; 0.35)	0.332	-0.35 (-1.06, 0.35)	0.319	0.04 (-0.03, 0.11)	0.255

*P<0.05

4.6.2 Gut integrity

No group differences between LMRs (95% CI: 0.87,1.07; p=0.507) or percentage recoveries of lactulose (95% CI: 0.72,1.15; p=0.434) and mannitol (95% CI: -1.04,0.35; p=0.332) were evident. (Because data for the LMRs and lactulose percentage recoveries were log-transformed, CIs are given as the anti-log of calculated CIs, indicating the ratio of the geometric means of fish oil to placebo groups (274, 275). The effect size similarly shows the untransformed regression coefficient, indicating the same ratio). The lactose:lactulose ratio was also investigated and again, there was no evidence of an effect of treatment on gut integrity. These results are given in Table 9.

Fitting the same covariates as were fitted in the growth regression models, the results were similar (Table 12). There was no statistically significant interaction between season, breast-milk DHA, or breast-milk EPA and treatment on LMR or lactulose and mannitol recoveries (p>0.09 for all).

A significant interaction between compliance and treatment (p=0.012) was found for LMR. The CI, however, included "1" and the effect size was trivial (1.01, Table 13). There were no compliance-treatment interactions for lactulose or mannitol recovery.

When urinary lactulose and mannitol concentrations were compared, a significantly higher mannitol concentration was detected in the urine of the control group than in that of the intervention group (95% CI: 0.62,0.95; p=0.017; Table 14).

Table 14: Urinary lactulose and mannitol concentrations

Endpoint	Fish oil group (n=85)	Placebo (n=83)		
	Median (25, 75 pctile)	Median (25, 75 pctile)	Effect size (95% CI)	p-value
Urinary lactulose (mg/100ml)	14.9 (10,24)	18.2 (12,26)	0.88 (0.70, 1.07)	0.184
Urinary mannitol (mg/100ml)	68.2 (48,109)	90.8 (59,136)	0.79 (0.62, 0.95)	0.017

4.6.2.1 Measurement reliability

The average intra-assay % CV, calculated across all replicates run during sample analysis, was 3.01% (urine samples) and 3.97% (standards) for the lactulose assay, and 1.89% (samples) and 2.36% (standards) for the mannitol assay. These can be seen plotted in Figure 31 (lactulose) and Figure 32 (mannitol).

The calibration curve comprised seven individual standards. The concentration of the fourth standard[4] (which lay midway on the calibration curve), as measured, was compared across all plates and the overall % CV calculated. This standard was chosen as it fell in the middle of the standard curve. For the lactulose assay it was 6.26% (plates without B-galactosidase) and 10% (plates with B-galactosidase), and for the mannitol assay it was 4.46%.
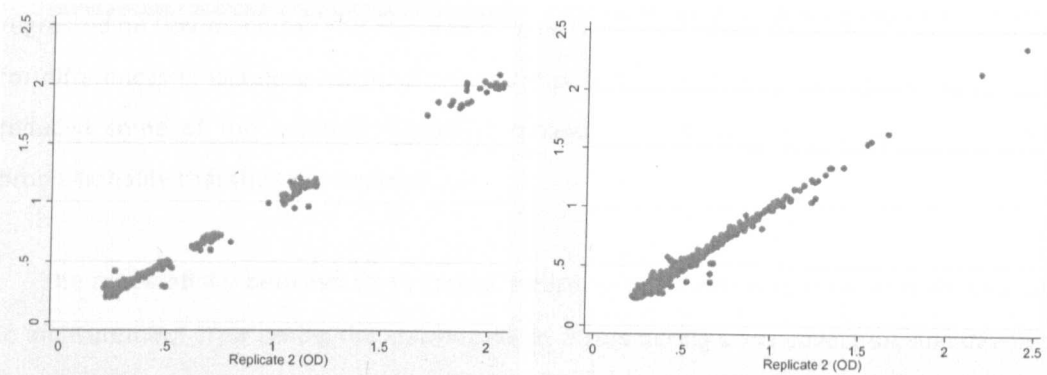
Figure 31: Correlation of ODs measured between two replicates. Left: lactulose assay standards; right: lactulose assay urine samples.
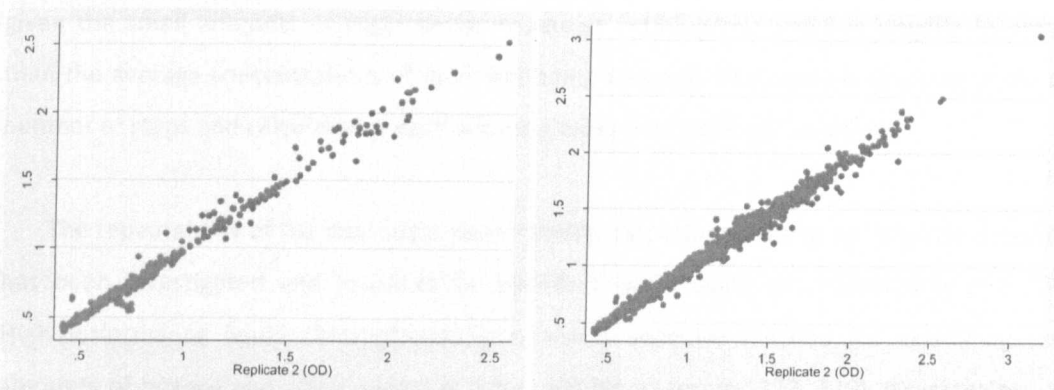
Figure 32: Correlation of ODs measured between two replicates: Left: mannitol assay standards; right: mannitol assay urine samples

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[4] Because standards kept well and did not need frequent replacing, only four batches of sets of standards were required to be made up in total, at sample analysis during the end of the study.

The estimation of the repeatability of LMR within individual subjects at each time-point was repeated on the complete set of samples, in the same manner as for the power calculation described in the Methods section, using a one-way analysis of variance. The intra-class correlation coefficient was 0.26 and the estimated reliability of an individual mean was 0.49. A high amount of error was therefore introduced during measurement.

Upon inspecting the intra-class correlation coefficients separately for lactulose and mannitol, it was evident that most of this error was brought in by the lactulose part of the assay.

4.6.3 Discussion

Instead of comparing (log)LMR by fitting LMR into the regression model, (log)lactulose was regressed on (log)mannitol. This regression model was used as an alternative way of examining for differences in lactulose-mannitol relationships between groups, but, unlike the ratio, it a) reduced some of the between-individual variance, and b) relaxed the tight restriction of proportionality that the ratio imposes.

The repeatability between three repeat measures on infants was not very high, due partly to measurement error during the assaying stage, errors during urine collection, and day-to-day biological variation.

The lactulose part of the assay introduced a high degree of error, which is not surprising, given the small amounts of sugar being measured (~150μg/ml, which is roughly 5x smaller than the average concentrations of mannitol being assayed). The assay is also comprised of a number of steps and calculations, each allowing entry for additional variation.

The repeatability of the dual-sugar permeability test, also in regards to lactulose detection, has been investigated and found to be excellent when using gas chromatography (276). High-performance liquid chromatography is also a sensitive method for examining small amounts of lactose and other sugars in urine and blood serum (277, 278). Nevertheless, the low cost and relative speed of using the enzymatic method for sugar determination makes it attractive in comparison to these other methods, particularly where, as in the present study, large numbers of samples are required to be analysed.

Certain difficulties with urine collection present challenges for obtaining valid data: urine can become contaminated with faeces; some infants do not pass urine, even for the duration of five hours; water can filter into the urine bags when mothers wash their children after they have passed stool. Most problematic, though, and certainly a factor introducing errors, is the spillage, or missed collections of entire passages of urine, due to urine bags which become detached, urine leaking out at the mouth of the bag - particularly in girls, or infants passing further urine immediately after a bag is removed and just before the next one is attached.

Due to differences in absorption and excretion rates of the two sugars (due to e.g. varying gastric emptying rates and intestinal transit times) (39), the final ratio can become distorted if part but not all of the entire volume of urine passed over five hours is collected. Indeed this was a common and seemingly unavoidable difficulty of the test, which would not be overcome with the most sensitive or precise of assays.

These problems with the test are not unknown, and the study design allowing for three urine collections at each time-point was constructed to minimise some of the errors and increase the test's reliability. Nonetheless, the measurement error, combined with other components of variance inflation, remained large. Inspection of the group LMR means and 95% CI, however, reveals that it is unlikely that a real difference remained undetected, i.e. that a type II error occurred. The limits of the 95% CI show the ratio between the geometric means of LMR in the fish oil to placebo groups to be 0.87 – 1.07, and even the lower limit precludes a real clinically significant treatment effect.

An interesting finding was the significant difference in urinary mannitol concentrations. Some allowance needs to be made for the possibility that the effect could be attributed to chance - an artefact of multiple testing, particularly when this comparison of mannitol concentrations assessed independent of the LMR was not stated as a primary outcome. Furthermore, if the effect was real, one would expect to see the same difference in urinary lactulose concentration.

However, it could be that such a difference in lactulose concentrations was not detected due to the very low concentrations of lactulose being assessed, and the high amount of error introduced during its assaying. At any rate, the difference would not be expected for LMRs,

because urine concentration would affect both lactulose and mannitol equally and hence would be cancelled out. (Or, alternatively, dividing lactulose concentration by mannitol concentration may introduce so much error that the effect is lost).

What is clear, though, is that this difference has more to do with urine concentration than an effect on mannitol uptake, and so has little to do with gut integrity. Even so, the possibility of an effect of fish oil supplementation on urine dilution might be an important finding and could raise interesting questions regarding its other, yet unknown, physiological effects.

4.7   Secondary outcomes

4.7.1   Plasma fatty acids

Fish oil supplementation resulted in a significant increase in the percentage of DHA (95% CI: 0.31,0.58; p<0.001) and EPA (95% CI: 1.5,1.9[5]; p<0.001) in the plasma total lipids, confirming that tissue uptake of n-3 LCPs in the fish oil supplemented infants had taken place.

Expressed in absolute amounts, the plasma EPA concentration was similarly statistically significantly elevated, while the DHA concentration difference lay only on the cusp of significance (95% CI: -0.03,11.2; p=0.051). Results are tabulated in Table 15.

Figure 33 shows the graphs of plasma concentrations of DHA and EPA at 9 mo against 3 mo, scattered by treatment group. The elevated n-3 LCPs at 9 mo in the fish oil compared to olive oil group can clearly be observed. There is also some indication that the control group DHA levels at 3 mo have an association with those at 9 mo (r=0.44). This is, unsurprisingly, less clear in the fish oil group (r=0.17). The relationship between DHA at 3 mo and 9 mo will be assessed later, in Section 4.8.6 (Plasma and breast-milk fatty acids: further considerations).

Blood AA levels remained unchanged by the intervention. Similarly, total n-6 FA levels did not differ between olive and fish oil groups. When expressed as a ratio, however, a marked difference in n-6 to n-3 LCPs (95% CI: -0.35,-0.18; p<0.001) and AA/(EPA+DHA) (95% CI: -0.32,-0.16; p<0.001) were detected, with lower ratios found in the fish oil group.

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[5] Ratio geometric mean intervention group:placebo group

Table 15: Fatty acid endpoints, by treatment groups

Endpoint	Fish oil (n=86)	Placebo (n=83)	Effect size (95% CI)	p-value
	$\overline{\chi}$; SD	$\overline{\chi}$; SD		
(% plasma total FA)				
DHA	4.87; 1.01	4.44; 0.81	0.53 (0.28, 0.79	<0.001**
EPA[1]	2.13	1.34	1.65^2 $(1.45, 1.88)^3$	<0.001**
	(1.66,2.95)	(0.94,1.72)		
AA	7.28; 1.52	7.25; 1.35	0.09 (-0.33, 0.51)	0.680
(ng/µl)				
DHA	70.0; 20.6	65.4; 16.3	5.54 (-0.03, 11.2)	0.051
EPA[1]	31.1	19.3	1.61 (1.38, 1.84)	<0.001**
	22.8, 43.7	14.3; 25.0		
AA	104; 29.0	107; 27.6	-2.38 (-10.8, 6.05)	0.578
Other FA indicators (ng/µl)				
Essential:nonessential PUFAs[4]	1.88; 0.39	1.75; 0.34	0.14 (0.02, 0.25)	0.018*
DHA:22_5n-6	28.4; 7.34	21.5; 7.31	6.85 (4.64, 9.05)	<0.001**
Total n3 FAs (ng/µl)	123; 41.2	104; 27.7	19.7 (9.08, 30.4)	<0.001**
Total n6 FAs (ng/µl)	440; 118	452; 119	-9.77(-46.0, 26.5)	0.595
AA/(EPA+DHA)	1.05; 0.26	1.28; 0.31	-0.24(-0.32, -0.16)	<0.001**
Total n-6 ≥ C20/ Total n-3 ≥C20	1.10; 0.26	1.34; 0.32	-0.25 (-0.35, -0.18)	<0.001**

[1]Due to a skewed distribution for this FA, the median (25 percentile, 75 percentile) is presented
[2]Effect size (untransformed regression coefficient) indicating the ratio "fish oil group geometric mean:control group geometric mean"
[3]CI limits (untransformed calculated 95% CIs) for the ratio "fish oil group geometric mean:control group geometric mean"
[4]Ratio of (n-6 + n-3) /(n-9) unsaturated PUFAs. n-7 PUFAs should ordinarily be included in the ratio as non-essential FAs but data for these were unavailable
*p<0.05; **p<0.001

The ratio DHA:C22_5n-6 is lower in conditions of DHA functional shortage. The increase in this ratio in the fish oil group (95% CI: 4.64,0.05, p<0.001) shows that DHA was not merely absorbed into the bloodstream, but also used efficiently by cells and tissues.

Overall PUFA status was measured by the ratio essential:nonessential PUFAs, which increases as essential FA status increases. There was a statistically significant higher essential FA status in the intervention group (95% CI: 0.02,0.25; p=0.018), although this difference may only have been as small as 0.02, the lower limit of the 95% CI.
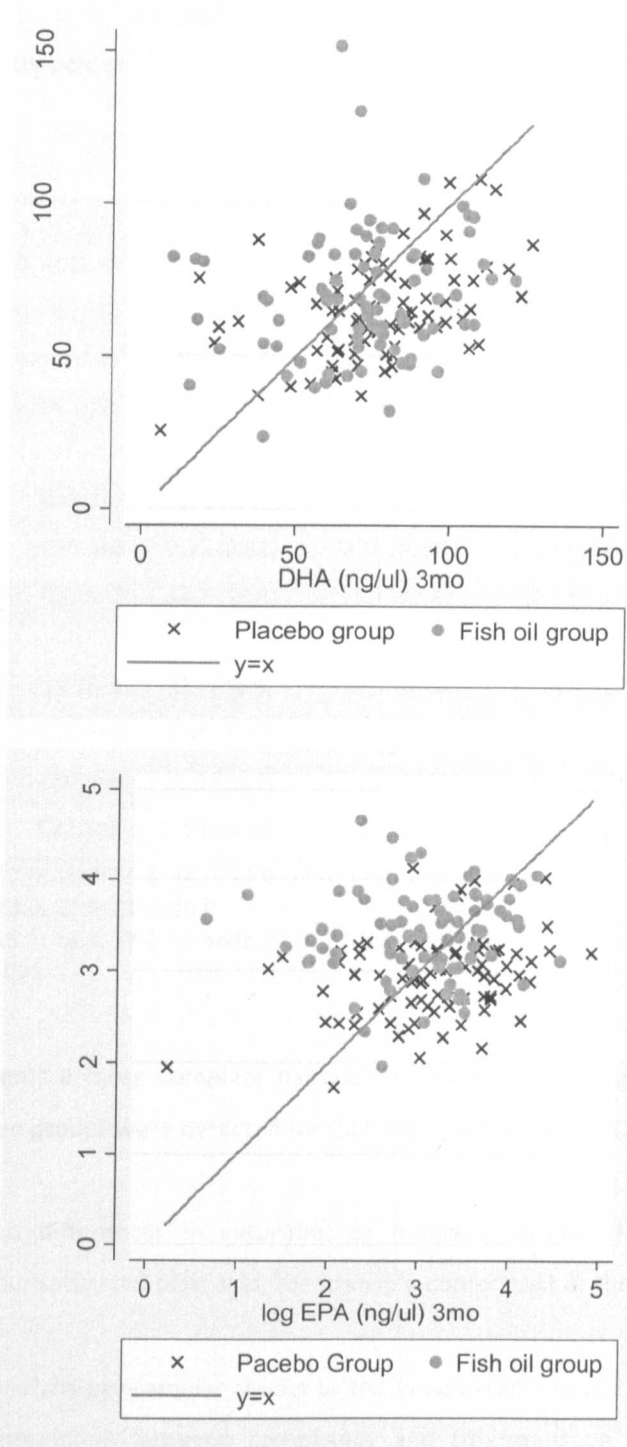
Figure 33: Plasma DHA (above) and EPA scattered at 9 mo vs. 3 mo, by treatment group

Table 16: Other fatty acid profiles

FA (ng/μl)	Fish oil (n=87)	Placebo (n=85)	Effect size (95% CI)	p-value
	$\bar{x}$; SD	$\bar{x}$; SD		
Saturated (C8:0 – C18:0)[1]	516 (150)	541 (137)	-27.0 (-70.8, 16.7)	0.224
Saturated (C20:0 – C24:0)[2]	11.6 (2.79)	11.8 (3.60)	-0.17 (-1.17, 0.83)	0.739
Monousaturated[3]	350 (99.7)	373 (106)	-23.4 (-54.4, 7.67)	0.139
Oleic acic (C18:1n9c)	307 (90.8)	325 (99.8)	-19.0 (-47.8, 9.81)	0.195
n-3 PUFAs				
C18:3n3	4.86 (2.87)	4.76 (2.16)	0.05 (-0.73, 0.84)	0.893
C20:3n3	0.73 (0.81)	0.74 (0.87)	0.02 (-0.24, 0.28)	0.878
C22:5n3	13.2 (4.64)	11.9 (3.82)	12.8 (8.42, 17.2)	<0.001**
n-6 PUFAs				
C18:2n6c	317 (93.8)	322 (91.9)	-4.40 (-33.0, 24.2)	0.761
C18:3n6	3.01 (2.40)	3.62 (4.77)	-0.76 (-1.89, 0.37)	0.184
C20:3n6	17.1 (5.80)	19.1 (6.49)	-1.93 (-3.67, -0.10)	0.039*
C22:5n6	2.59 (1.00)	3.32 (1.24)	-0.76 (-1.07, -0.44)	<0.001**

[1]Includes 18:0; 17:0; 16:0; 15:0; 14:0; 13:0; 12:0; 11:0; 10:0; 8:0
[2]Includes 24:0; 23:0; 22:0; 21:0; 20:0
[3]Includes 14:1; 15:1; 16:1; 17:1; 18:1n9c; 20:1; 22:1n9; 24:1
*p<0.05; **p<0.001

Table 16 presents a more complete overview of FA profiles, by group. The only other differences between groups were detected for C22:3n-3, C22:5n-6, and C20:3n-6.

There were no differences in saturated or monounsaturated FAs between groups, including the monounsaturated oleic acid, the principle component of the olive oil placebo.

The adjusted analysis gave similar results to the unadjusted analysis (Table 17), and some evidence for an interaction between compliance and treatment on DHA and EPA levels (p=0.029 and p=0.026, respectively).

Table 17: Fatty acids adjusted analysis: fitting compliance and doses of treatment received

Endpoint	(% total)	Unadjusted analysis Effect size (95% CI)	p	Fitting compliance only Effect size (95% CI)	p	Interaction between compliance & treatment (fitting compliance & doses of treatment received) Effect size (95% CI)	p
Plasma FAs							
	DHA	0.53 (0.31, 0.58)	<0.001	0.49 (0.22, 0.76)	<0.001	-0.03 (-0.06, -0.00)	0.029
	EPA	1.65 (1.45, 1.88)	<0.001	1.65 (1.45, 1.89)	<0.001	-0.01 (-0.03, -0.00)	0.026
	AA	0.09 (-0.33, 0.51)	0.680	0.01 (-0.43, 0.45)	0.959	-0.01 (-0.06, 0.03)	0.540
(ng/µl)							
	DHA	5.54 (-0.03, 11.2)	0.051	5.70 (0.16, 11.2)	0.044	0.02 (-0.54, 0.58)	0.940
	EPA	1.61 (1.38, 1.84)	<0.001	1.59 (1.38, 1.84)	<0.001	-1.01 (-0.98, 1.01)	0.204
	AA	-2.38 (-10.8, 6.05)	0.578	-2.52 (-11.0, 5.93)	0.556	0.27 (-0.57, 1.12)	0.525

4.7.1.1 Discussion

At least eight different biomarkers for measuring n-3 LCP status have been used in the literature (279). This makes comparisons of dose effects on plasma FA status, and of FA profiles of this sample of Gambian infants with those of other populations of infants difficult (as the distribution of FAs among different biological compartments can vary substantially (280)), especially since the literature on infant plasma FA profiles is limited to start with. In addition, as with any biomarker, measurement errors and variability introduced by sampling, transport, sample handling, the use of different laboratories, and total analyte present in the sample (93), present drawbacks to repeatability and comparability of results.

Plasma FA n-3 LCP status was a secondary endpoint, and no allowance was made for multiple testing. Where p<0.001, the likelihood of a chance significant finding is very small, however. But in making inferences about the difference in essential FA status (p=0.018), the above should be kept in mind.

That the DHA% elevation was highly significant, while the absolute concentration of DHA was not, suggests that DHA partly displaced other FAs in the blood tissues and hence formed a larger proportion of the overall lipids, without increasing significantly in absolute concentration. For example, the activity around the n-3 LCPs EPA, C22:5 and DHA metabolism is catalysed by the same enzyme as the n-6 LCP metabolism of AA, C22:4, and n-6 docosapentaenoic acid (C22:5).

Figure 34, which was already presented in Chapter 1, is repeated here for reference. It is known that in conditions where DHA is in limited supply, n-6 docosapentaenoic acid synthesis is increased. It follows that in ample DHA supply, docoasopentaenoic acid levels will decrease. Indeed, this n-6 LCP was significantly lowered in the fish oil group (Table 16), perhaps explaining a component of the relative increase in DHA.

Additionally, DHA is sometimes retro-converted back to EPA in humans. Studies have found increases in both DHA and EPA in blood and cell lipids, for example, when supplementing with DHA only (281). Considering that more EPA relative to DHA was administered daily, it would seem unlikely that this retro-conversion occurred to any

significant degree in these Gambian infants. Nevertheless, if it had, this could further explain the lack of substantial increase in relative DHA levels in the plasma.
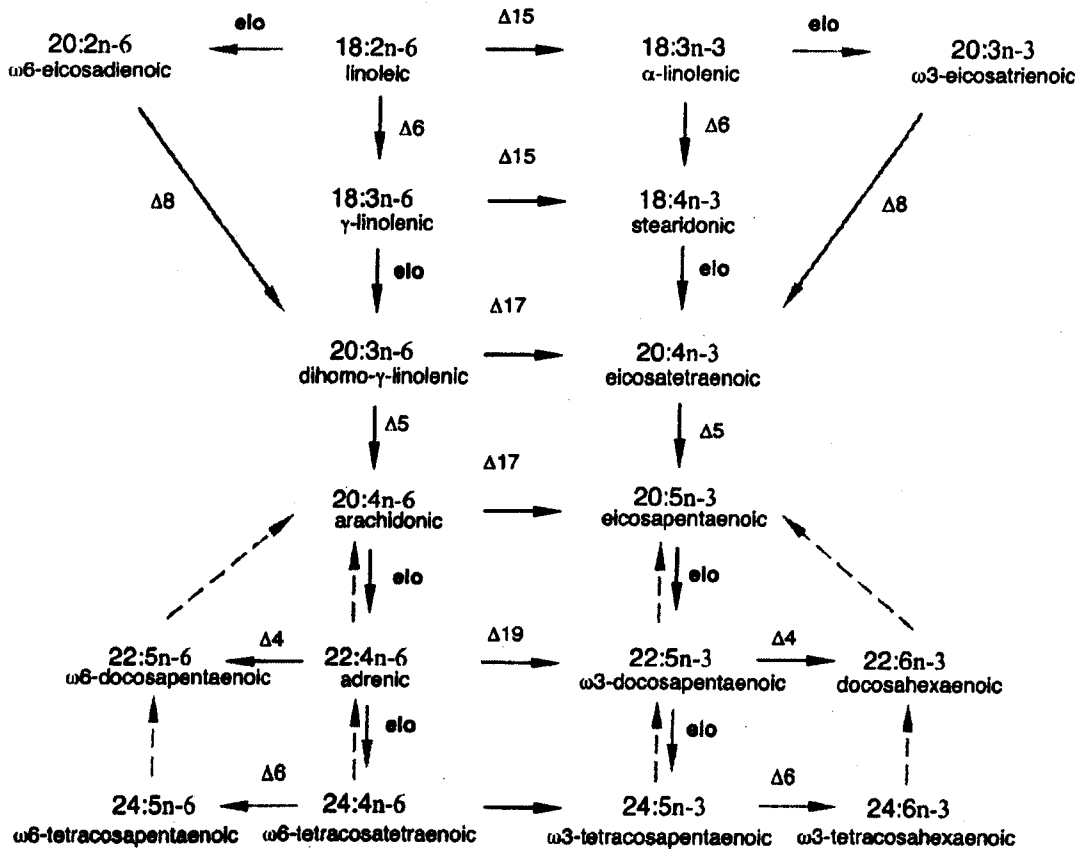
Reprinted from reference (58)

Figure 34: Metabolism of fatty acids to form LCPs

The strong relationship between DHA at 3 mo and 9 mo in the control group (r=0.44) implies some stability of DHA in the blood. Whether this was mostly because of steady breast-milk DHA concentrations rather than blood lipid profiles is uncertain, but will be further addressed in Section 4.8.6. It is remarkable that some infants had roughly five times the DHA concentration and nearly ten times the plasma EPA concentration in their plasma than others. Figure 33 demonstrates this very wide inter-individual range of LCP concentrations. It is surprising that even a 6 mo-long daily FA supplementation regimen evidently had no impact on reducing the extent of this inter-individual variation.

LCP incorporation into plasma phospholipids and other cell membranes is strongly dependent on the diet (75). Yet, even when the overall intakes of these fats in infants were matched, most likely fairly narrowly, to considerably high doses, some infants still had very low n-3 LCP plasma concentrations while others had considerably high ones.

Possible explanations for these large inter-individual differences could be differences in a child's ability to absorb, process and retain the administered LCPs. There is evidence for a reduced fat absorption in children with acute infections (282), diarrhoea (283, 284), intestinal infections and tropical enteropathy (92, 285), and helminth infections (284, 286). A large part of this malabsorption in situations of persisitent intestinal infection is explained by morphological alterations in the mucosal villous, which affect digestive-absorptive functions (92). Additionally, small-intestinal bacterial infections associated with environmental enteropathy can lead to a deconjugation of the bile salts required for the formation of micelles necessary for fat digestion. This deconjucation action in turn leads to maldigestion of fats (92). Differing severities of these conditions leading to varying degrees of reduced nutrient absorptions or steatorrhea in different children, most likely play a significant role in determining overall lipid status.

The high variability in plasma LCP concentrations may also be a reflection of the great variability known to exist in breast-milk LCP concentrations (287, 288). However, DHA is the most variable breast-milk FA and yet EPA was far more variable than DHA concentration in the plasma of these infants. Furthermore, one would expect to see a minimisation of this variability in the plasma of the treatment group if the variability was explained to such a large extent by the diet or breast-milk intakes.

Certainly genetic and metabolic factors would be considered potential determinants of PUFA status. Although the genetic effects of LCPs on molecular activities have been and continue to be examined extensively, the genetic determinants of LCP status remains largely under-explored. Recently, however, several polymorphisms and haplotypes of the genes encoding desaturases - delta-5 and delta-6 - involved in catalysing the conversion of C18 PUFAs to their longer chain derivatives have been identified. *FADS1* and *FADS2* encode delta-5 and delta-6, respectively (289). Evidence that polymorphisms of the *FADS1* and *FADS2* gene clusters are associated with serum phospholipid PUFA concentration has been found (290, 291). For example, high AA concentrations were associated with various *FADS* alleles.

However, although these polymorphisms can predict a large extent (roughly 28%) of the variation in AA plasma phospholipid levels in adults, they predict only about 2% of the DHA variation (291, 292), and so probably do not explain any major extent of the variation in Gambian infant DHA concentrations. Nevertheless, these findings show at least some genetic contribution to physiological plasma concentrations of FAs.

The effects which the n-6 and n-3 series PUFAs exert on each other during metabolism and synthesis provides an example of one of the metabolic influences PUFAs experience in determining their eventual cellular concentrations. Other metabolic determinants may, no doubt, exert similar suppression or enhancing effects on LCPs , their synthesis and effects.

Various studies have demonstrated that supplementation with n-3 PUFAs or n-3 PUFA containing foods resulted in increased n-3 concentrations of plasma lipids and phospholipids, and erythrocyte membrane FAs (293-295), and some have reported a simultaneous decrease in n-6 plasma fractions (173, 296-298). Figure 34 shows how the different n-3 and n-6 FAs undergo desaturation, elongation, and retroconversion. Delta-6 and delta-4 desaturases are competed for between the two omega-chains (297), explaining some of the decrease in n-6 FAs reported as a consequence of n-3 supplementation.

Although some studies of n-3 supplementation have resulted in a concomitant decrease in AA levels, this did not occur in the present study. A lowered n-6:n-3 ratio has indeed been associated with improved health outcomes, although AA remains a potent FA with biologically important functions in infant health and development. An adequate AA status is imperative if an infant is to thrive (83, 292, 299).

Both AA and DHA can be formed by endogenous conversion of their precursor essential FAs. However, AA is synthesised via a short enzyme-catalysed pathway of only three desaturation and elongation steps, whereas the synthesis of DHA is long, complex and indirect (84, 292), with the result that DHA endogenous synthesis is far less effective than that of AA. Indeed, as already mentioned, polymorphisms of the desaturase enzymes predict far more variation in AA plasma phospholipid levels than DHA levels.

The plasma of all infants, regardless of treatment, remained rich in linoleic acid (C18:2n-6; ~320ng/μl; Table 16), the precursor to AA. It is likely that a substantial degree of endogenous synthesis of AA took place, particularly if preformed AA from the breast-milk was in any way

insufficient. At any rate, there is no evidence to suggest that there was a physiological lack of AA, and even a large dose of n-3 LCPs given daily did not have the undesirable effect of reducing AA tissue levels.

4.7.2 Cognitive development

The secondary outcomes – cognitive development, acute phase proteins, and intestinal inflammation – are summarised by treatment group, showing significance test results, in Table 18.

On both the Willatts and Attention tests, infants who did not receive fish oil performed equally well as those who did (Table 18). There were no significant differences detected between intention scores (95% CI: -1.10,1.56; p=0.759), pretest scores (95% CI: -1.26,2.14; p=0.609), inattention rates (95% CI: -0.64,0.24; p=0.365), or the mean look times (95%CI: 0.86, 1.20; p=0.884).

In an attempt to retrieve the toy, infants pulled the cloth off the table an equal number of times in the placebo and fish oil groups (p=0.918), indicating that n-3 LCP supplementation had no impact on motor control (195).

The Attention Test data were skewed, and were thus log-transformed. The data for the Willatts Test were not distributed normally either. Unlike the Attention Test data, however, they appeared to be mixture distributions: uniform distributions with point density at zero. The truncation of the scoring range at zero, while the range theoretically reaches further below (for example, some infants succeeded in pulling the cloth or lifting the cover, but looked away for more than two seconds and so achieved a zero on the relevant score, whereas other infants made no attempt to pull the cloth whatsoever yet also achieve a zero) might have caused this. No simple transformation was available to efficiently normalise such data. Options were the inefficient approach of reducing to a binary variable, or the complex method of, e.g., likelihood approach modelling. However, as group means were being compared, with more or less the same number of infants in each group, the distribution of the mean should have followed the normal very closely and thus regression analysis was performed on the untransformed data.

Table 18: Secondary endpoints, by treatment group

Endpoint	Fish oil	Placebo		
	$\overline{x}$; SD	$\overline{x}$; SD	Effect size (95% CI)	p-value
Cognitive development				
Willatts test	**(n=73)**	**(n=65)**		
Average total Intention Score	4.63 (3.77)	4.45 (3.71)	0.20[1] (-1.10, 1.46)	0.759
Average intentional solutions	0.44 (0.52)	0.43 (0.54)	0.01[1] (-0.17, 0.19)	0.871
Pretests average score	8.61 (5.02)	8.16 (5.23)	0.44 (-1.26, 2.14)	0.609
Attention Test	**Median**	**Median**		
	(25, 75 pctile)	**(25, 75 pctile)**		
	(n=74)	**(n=69)**		
Inattention rate	2.38	2.37	0.98 (0.82, 1.81)	0.868
	(1.70, 2.91)	(1.70, 2.91)		
Mean look duration	23.8	23.2	1.01 (0.86, 1.20)	0.884
	(19.5, 33.9)	(17.2, 35,5)		
Acute phase proteins	**Mean; SD**	**Mean;SD**		
	(n=86)	**(n=83)**		
Plasma albumin (mg/L)	35.3;3.1	35.3;2.8	0.07 (-0.83, 0.96)	0.880
	Median	**Median**		
	(25, 75 pctile)	**(25, 75 pctile)**		
AGP (g/l)	1.01	1.03	0.97 (0.88, 1.08)	0.596
	(0.85, 1.34)	(0.82, 1.36)		
CRP (mg/l)	4.7(3.1, 8.2)	4.7(3.2,7.9)	0.98 (0.81, 1.19)	0.838
Intestinal inflammation	**(n=76)**	**(n=69)**		
Calprotectin dry weight (mg/kg)	78.0 (43,120)	66.5 (46, 138)	0.90 (0.86, 1.42)	0.427
Calprotectin wet weight	647 (357,996)	552(382,1145)	-	-
	Proportion abnormal	**Proportion abnormal**		
	(95% CI: % abnormal)	**(95% CI: % abnormal)**		
Plasma albumin (mg/L)[2]	5/86 (0.77, 10.9)	3/83 (-0.49, 7.71)		0.933[3]
AGP (g/L)[2]	44/86 (40.4, 61.9)	43/83 (40.8, 62.8)		0.501[3]
CRP (mg/L)[2]	39/86 (34.6, 56.1)	36/83 (32.5, 54.3)		0.796[3]
Calprotectin (mg/kg)[4]	70/79 (81.4, 95.8)	62/74 (75.2, 92.4)		0.386[3]

[1]Controlling for number of trials
[3]Using standard adult cut-off ranges from healthy individuals: CRP≤5mg/L; AGP≤1g/L; albumin≤30mg/L
[2] Group differences analysed by Pearson's chi squared test
[4] Using European healthy reference value (calprotectin≤263mg/kg)
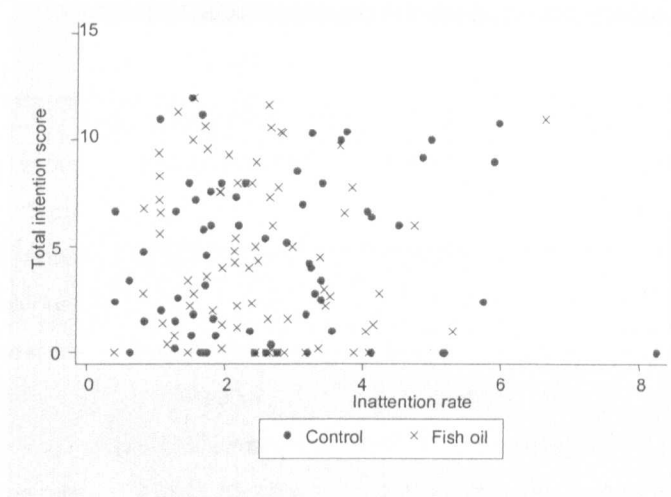
Figure 35: Intention score versus inattention rate, by treatment group

Judging from scatter plots and Pearson product-moment correlation coefficients, the Willatts and Attention tests had no relationship with each other. The correlations of inattention rates and mean look duration with total intention scores, intentional solutions, and pretest average scores, were r<±0.1 in all instances. The poor association, with inattention rate compared to total intention scores chosen as example, is graphically illustrated in Figure 35.

Sex differences between scores were tested using regression analysis. In the Willatts Test, the average total intentional solutions score was significantly higher in boys than in girls (p=0.0123), and for the intentional solutions and pretest scores the difference approached, but did not reach, statistical significance (p=0.0672, p=0.0835, respectively, Table 19). There were no sex differences in the Attention Test.

The mothers' highest level of education was determined by the highest grade or class she had completed in Arabic or English school, or both. Schooling is offered in either medium in West Kiang for a maximum of twelve years' Arabic tuition or/and nine years English tuition. (The final three years' English tuition may be taken at the coast or regions further inland, but none of the mothers had followed this route).

Table 19: Cognitive development scores, by sex

	Girls n=56	Boys n=82	Effect size (95% CI)	
Willatts test	$\bar{\chi}$ (SD; SE)	$\bar{\chi}$ (SD; SE)		P
Average total Intention Score	3.59 (3.74; 0.50)	5.20 (3.60; 0.40)	1.61 (0.35, 3.86)	0.012*
Average intentional solutions	0.33 (0.52; 0.07)	0.50 (0.53; 0.06)	0.17 (-0.01, 0.35)	0.067
Pretests average score	7.51 (5.45; 0.71)	9.02 (4.79; 0.53)	1.51 (-0.20, 3.21)	0.084
Attention Test	Median (25th;75^{th}percentile)	Median (25th;75^{th}percentile)		
Inattention rate	2.38 (1.70; 2.91)	2.37 (1.57; 3.39)	0.97 (0.80, 1.02)	0.739
Mean look duration	23.2 (17.2; 35.5)	23.8 (19.5; 33.9)	1.05 (0.88, 1.24)	0.595

*p<0.05

Data were available for 94% of mothers whose infants completed the cognitive testing. Twenty-five mothers received no form of formal schooling at all. The data are summarised in Table 20. There were no differences in education level between the two groups (p=0.782 and p=0.190 for Arabic and English school, respectively, using a Pearson's chi-squared test).

Table 20: Maternal years of education, by treatment group

Highest year completed	Fish oil (n=68)	Control (n=68)
Arabic School		
0-2	26	19
3-6	28	26
7-11	4	4
English School		
1-4	4	9
5-9	6	10

No association between the mothers' education levels and the performance of their infants on any of the tests was found.

The analysis was repeated, fitting mother's highest level of education (highest grade passed in Arabic school or English school) into the same regression models. The results were unchanged in regard to group significant differences (Table 21).

Table 21: Cognitive development regression analysis (repeated), controlling for mother's highest level of education

Endpoint	Adjusting for mother's education	
	Effect size (95% CI)	p-value
Total intentional solutions	0.53 (-0.75, 1.81)	0.414
Total solutions	0.06 (-0.12, 0.25)	0.482
Average pretest s core	0.57 (-1.19, 2.36)	0.522
Inattention rate	-1.77% (0.81, 1.19)	0.854
Mean look duration	1.64% (0.86, 1.21)	0.850

The adjusted analysis, summarised in Table 22, again showed similar results to the unadjusted analysis. This held true for all adjusted analysis results summarised in the table.

4.7.2.1   Inter-observer reliability

The scores of 39 infants marked by both the primary scorer and secondary observer were scattered, with the line Y=X superimposed (Figure 36). Correlations were examined by Pearson product-moment correlation coefficients. Ninety-five percent CIs for the correlation coefficients were estimated using the bootstrap approach. Bias between markers was tested with a paired t-test, to test whether, even though a strong linear relationship exists, one marker was consistently marking higher or lower than the other.

Inter-observer reliability was highest on the pretest (r=0.99) and total intention scores (r=0.97), Table 23. There was also no statistically significant evidence of bias between markers (p=0.121, p=0.2827, respectively). Total intentional solutions were correlated at r=0.92, with evidence of bias narrowly falling short of statistical significance (p=0.097).

Table 22: Secondary endpoints, adjusted analysis

Endpoint	Unadjusted analysis		Fitting compliance only		Interaction between compliance & treatment (fitting compliance & doses of treatment received)	
	Effect size (95% CI)	P	Effect size (95% CI)	p	Effect size (95% CI)	P
Cognitive development						
Willatts test	(n=73)		(n=65)			
Average total Intention Score	0.20 (-1.10, 1.46)	0.759	0.16 (-1.09, 1.43)	0.796	-0.02 (-0.15, 0.10)	0.703
Average intentional solutions	0.01 (-0.17, 0.19)	0.871	0.01 (-0.16, 0.18)	0.911	-0.01 (-0.94, 4.37)	0.587
Pretests average score	0.44 (-1.26, 2.14)	0.609	0.35 (-1.33, 2.04)	0.680	0.08 (-0.09, 0.25)	0.372
Attention Test	(n=74)		(n=69)			
Inattention rate	-1.55% (0.82, 2.00)	0.868	1.21% (0.82, 1.19)	0.897	-0.02 (-0.04, 0.00)	0.083
Mean look duration	1.23% (0.86, 1.20)	0.884	0.94% (0.85, 1.19)	0.911	0.01 (-0.00, 0.03)	0.076
Acute phase proteins						
Plasma albumin (mg/L)	0.07 (-0.83, 0.96)	0.880	-0.03 (-0.12, 0.06)	0.525	0.05 (-0.85, 0.95)	0.917
α-Acid glycoprotein(g/L)	-2.78% (0.88, 1.08)	0.596	2.9% (0.88, 1.08)	0.585	1.00 (0.99, 1.02)	0.320
C-reactive protein (mg/L)	-2.04% (0.81, 1.19)	0.838	-2.3% (80.2, 1.19)	0.819	0.99 (0.97, 1.01)	0.381
Intestinal inflammation						
Calprotectin dry wt (mg/kg)	10.0% (0.86, 1.42)	0.427	9.77% (0.86, 1.42)	0.445	1.00 (0.98, 1.03)	0.890
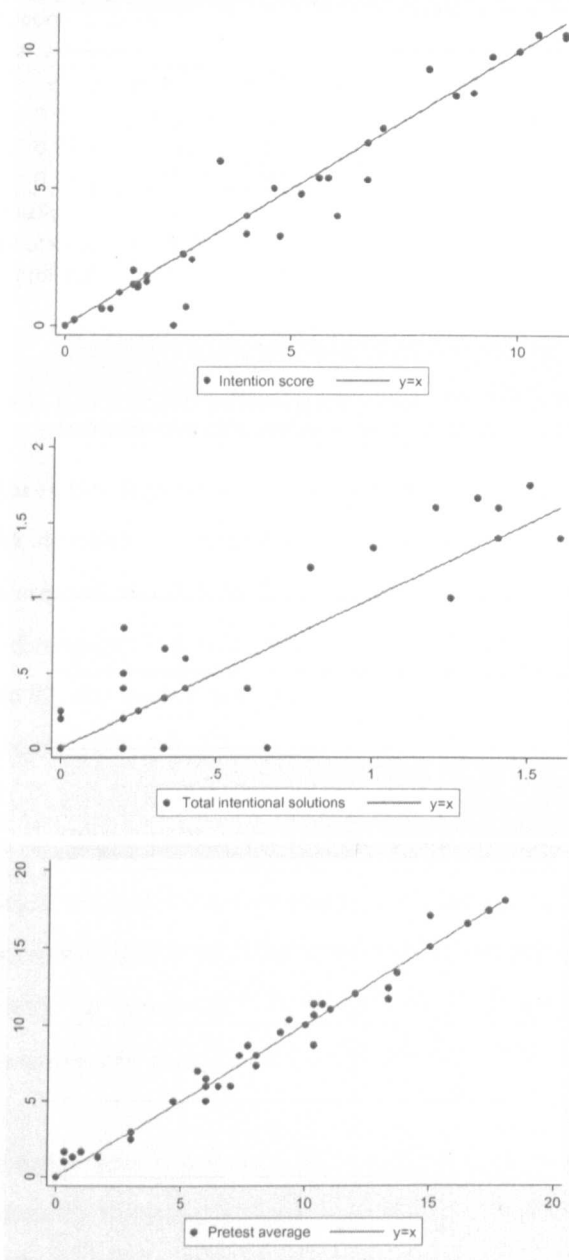
Figure 36: Scatter graphs showing scores of observer 1 plotted versus observer 2. Top to bottom: average total intention score; average total intentional solutions; average pretest scores.

Table 23: Inter-observer reliability, Willatts Test

| Score | Correlation scores between observer 1 and 2 | | Difference between observers' mean scores[3] | |
	r[1]	Bias-corrected CI[2]	$\bar{x}_1 - \bar{x}_2$ (95% CI)	P
r total intention score	0.97	0.936 - 0.989	0.21 (-0.06 − 0.49)	0.1210
r total intentional solutions	0.92	0.821 - 0.961	-0.06 (-0.14 - 0.01)	0.0973
r total pretests score	0.99	0.981 - 0.993	0.33 (0.33 − 1.00)	0.2827

[1]Pearson correlation coefficient
[2]Calculated using the bootstrap approach
[3]From paired t-test; comparison between means of scores

4.7.2.2 Discussion

Look duration decreases between roughly 2 mo and 9 mo of age (208). During this first year of life, shorter look duration (as measured during infant habituation studies when a stimulus is repeatedly presented) is believed to reflect more efficient information processing and a greater ability to disengage (131, 300). Accordingly, shorter look durations have been associated with childhood IQ, with infants who habituated faster (i.e. displayed shorter looking times) scoring higher IQ scores (208, 301, 302).

In contrast, beyond 9 mo of age and into the second year of life, looking and examining, as measured during free-play, is reflective of attention span or attention control, and an ability to stay focussed on a task, rather than processing speed (208). An increase in looking times measured during free-play is associated with more mature cognition, and positive developmental outcomes as this age and into early childhood (208, 303).

One would therefore expect longer look durations to reflect better attention to a task, and an enhanced cognitive maturity. However, not even a weak correlation was detected between scores attained on the Willatts and the Attention tests, as stated above. This could be because the Attention Test is not measuring what it aims to i.e. has a low degree of validity, particularly in the present population. Indeed, this particular single-object test has not been validated in further population groups, and its use has been reported very rarely since its first description in 2004.

Alternatively, there may be no real relationship between infant attention development and problem-solving behaviours in this population at this age. As described above, there is a

decline in looking time during the first year of life. This look time plateaus at around 9 mo of age, but then gradually starts to increase. By 12 and 18 mo, longer look times are believed to indicate enhanced attention. Given the poor performance the Gambian infants displayed on the problem-solving test in comparison to other infant groups studied (discussed below), it may be that they lag behind in their information processing and attention control domains so that the "look-time" plateau is reached at a later stage than typically seen in infants reported in the literature. Were the Gambian infants to reach this plateau at around 12 mo rather than 9 mo, some infants may still fall into the category of faster information processing linked to shorter look times. Some infants, on the other hand, may be at a point in their development which displays more mature attention behaviour, and where longer look times therefore indicate enhanced cognitive behaviour. Still others might be hovering between the two degrees, with their look times accordingly indicating very little.

In order to gain clarity on both the validity of the test and what it measures in this population, as well as the appropriate age that it should be employed, further research testing infants of different ages across the first years will be required.

The consistently higher average on all Willatts Test measures in boys compared to girls suggests that boys in the population were cognitively further developed than girls in the area of planning and problem solving behaviour. If this were true (and not merely due to chance) it could reflect a biological difference in rates of cognitive maturation in boys compared to girls, although this finding has not been made in the other studies testing problem solving behaviour in infants, and the consensus view is that there are no sex differences in intelligence (304). The finding may, on the other hand, be rather related to different standards of care, or different modes of interactions which caregivers display towards girls compared to boys. It is well known that parents tend to interact differently with their male children than their female children (305, 306). In rural Gambia, gender differences appear to be particularly pronounced and marked differences in gender roles are the norm. Certain behaviours might be encouraged and expected in boys but not in girls, or boys might receive preference treatment or attention from other family members compared to girls.

Whatever the case may be this gender difference in cognitive development first needs to be verified before inferences about its causes and consequences can be made.

Sixteen percent of infants were unable to demonstrate any form of intention for retrieving the toy whatsoever (obtaining an overall score of "0"), and only two percent were able to obtain the maximum intention score. In comparison to infants from other populations the scores compared poorly.

Infants who were investigated as part of a well-designed n-3 LCP supplementation study in Denmark (n=73) obtained a similar intention score as the Gambian infants of 4.5, and a higher number of intentional solutions - 37% - compared to the 29% achieved in the Gambian sample (307). However, these infants were 3 mo younger than infants in the present cohort, completing their assessments at 9 mo of age. A group of 29 American infants obtained an average score of 7.4 for intention, and made intentional solutions on 52% of trials (308). These infants, too, were 9 mo of age. In a similar study lead by the developer of the test in the UK, 9 mo old infants (n=40) achieved an average intention score of 7.4, and intentional solutions on 38% of trials (131).

In non-developed countries the literature is scarce. One study in Kingston, Jamaica, investigating 135 low birth weight and 87 normal birth weight infant, similar procedures as the "pretest" scoring procedure described were used, providing scores comparable to "pretests average score" above. The Gambian infants achieved a mean of 1.9 on the support step, and 2.4 on the cover. In the Jamaican sample the scores were 2.5 and 2.9, respectively, even though these infants were on average 7 mo old and mostly born at low birth weight (239). In a large population-based study in Bangladesh, nearly 2116 seven month old infants achieved a roughly 2.8 mean on the support step and 3.2 on the cover step (309, 310).

The comparatively poor performance of the Gambian infants is difficult to elucidate. Because all scorers used the same scoring protocol, and all tests were validated by a second observer, it would seem unlikely that markers varied greatly in their strictness. Infants in developed countries may be more accustomed to toys of the nature used, and environments such as the settings where the test was conducted, compared to the rural African infants, explaining some of the difference in results between these infants. However, even 7 mo old infants from Bangladesh and Jamaica performed better on these tests than did the Gambian infants. No doubt the reason, simple or complex as it may be, may be understood through further research on the topic, but the scope of this thesis does not permit it here.

4.7.3   Acute phase proteins

The results of plasma acute phase proteins (Table 18) provide evidence that there is no impact on a reduction in systemic inflammation by fish oil in the infants studied.

A recent study involving infants living in the same area (258) found mean AGP and CRP concentration, at 9 mo of age of 1.25mg/l and 7.78mg/l, respectively. This is not comparable to the median concentrations presented in Table 18, but compared to the calculated mean concentrations of 1.13g/l and 7.4mg/l for AGP and CRP, the findings are similar.   Using identical cut-offs, the same recent study surprisingly found somewhat different proportion of abnormally high levels of acute phase proteins: 68.5% for AGP and 33% for CRP, compared to the 52% and 45% in the present study.

4.7.4   Intestinal inflammation

There were no differences in average calprotectin levels between infants in the different treatment groups (95% CI: 0.86,1.42[6]; p=0.427; Table 18), and elevated calprotectin levels were equally distributed between groups (p=0.386)

Only 21 out of 153 infants had calprotectin levels lower than a European reference of 263mg/kg for infants at 3 mo of age (263), and none were lower than the 79mg/kg for infants of 6 mo of age, or 67mg/kg for infants of 12 mo. These calprotectin concentrations are indicative of the high degree of gastrointestinal tract inflammation characteristic of Gambian infants.

The median calprotectin concentration was 614mg/kg. This is significantly less than the median of 1033mg/kg found at baseline, but younger infants are known to have higher physiological levels of calprotectin. The variation between infants is immense, as the 25th and 75th percentiles in Table 18 show. The minimum and maximum calprotectin wet weight concentrations were 125 and 5200 mg/kg, respectively.

There were no sex differences for this inflammatory protein.

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[6] Ratio of geometric means (treatment group/placebo group)

4.7.4.1 Measurement reliability

A control used on eighteen individual plate runs was used to calculate an inter-assay % CV of 7.43%.

Additionally, a set of 21 samples was assayed twice, and from the concentrations calculated in Run 1 versus Run 2, an overall mean inter-assay % CV of 9.79% and Pearson correlation coefficient of >0.99 calculated. However, the CV distribution was skewed to the right, so the median was calculated and found to be 6.1%.

All OD data on replicates of stool samples run on each plate were used to calculate an overall mean intra-assay CV of 3.5%, and median CV of 2.6%, between replicates.

The concentrations of the 21 samples obtained in Run 1 versus Run 2 are plotted in Figure 37. Replicate ODs for all samples and standards are shown plotted in Figure 38.
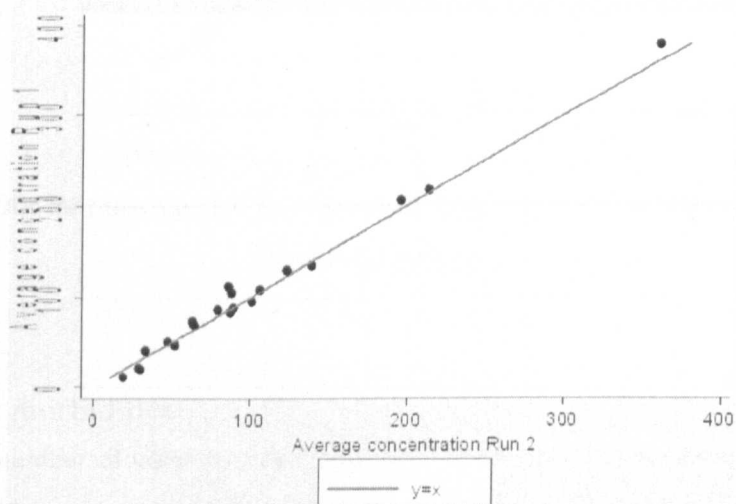
Figure 37: Calculated concentrations of 21 samples read on two independent plate runs, showing average concentrations from Run 1 on the Y-axis, and average concentrations from Run 2 on the X-axis
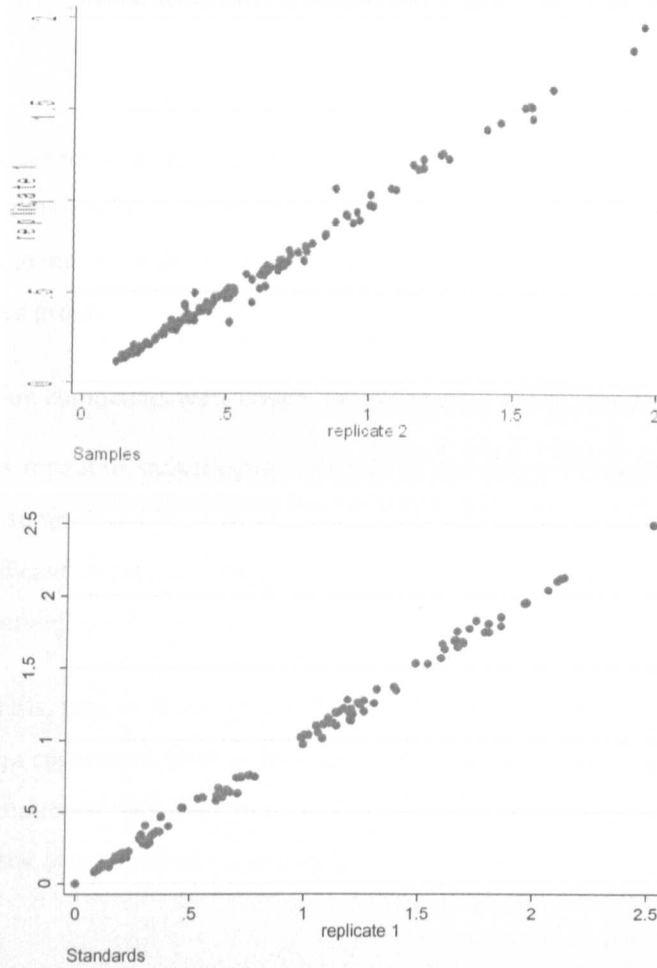
Figure 38: ODs for replicates 1 & 2 plotted against each other for samples (above) and standards (below)

4.7.5 Infant morbidities

Using the number of visits an infant was reported ill with any symptom as a negative binomial variable, group comparisons showed evidence of no treatment effect. Similar negative binomial regression analyses indicated no statistically significant impact of the intervention on rates of fever, respiratory complaints, vomiting, or nurse visits/doctor visits/referrals amongst infants. Although the difference was not statistically significant, diarrhoea rates were far higher (roughly 25%) in the control than the fish oil group. These results are presented below, in Table 24.

Because the number of times a particular complaint was reported increased with the number of times the infant was visited - but possibly not in a straightforward multiplicative

way, since the risk may change with age - log(number of visits) was controlled for in each of the analyses.

Nose/abnormal bleeding and miscellaneous complaints were not observed frequently enough to analyse meaningfully, so for this only the total number of observations in each treatment group is given in Table 24. There was no evidence of increased bleeding in the fish-oil supplemented group.

The most common complaints were cough and respiratory conditions, followed by fever.

The analysis was repeated, adjusting for compliance and fitting compliance and treatment doses taken, and is summarised in Table 25. The results when adjusting for compliance were similar, and no significant interactions between compliance and treatment group on morbidity outcomes were observed.

In a similar analysis, season of visit was controlled for by adding the first two pairs of the Fourier series as extra covariates. Some evidence, but just statistically significant (p=0.0489), of an interaction of treatment with season was seen for fever, and there was no interaction observed on any of the other morbidity outcomes.

Table 24: Morbidity endpoints, by treatment group

	Fish oil	Control		
Morbidity rates	Total recorded (n=87)	Total recorded (n=85)	**Effect size (95% CI)**	**p-value**
Unwell, any complaint[1]	3603	3088	0.13 (-0.08, 0.34)[2]	0.238
Diarrhoea[3]	474	605	-0.27 (-0.61, 0.06)	0.110
Fever[3]	1479	1297	0.11 (-0.12, 0.34)	0.338
Respiratory complaints[3]	1645	1503	0.06 (-0.2, 0.32)	0.670
Nurse/doctor visits[3]	186	161	0.13 (-0.10, 0.36)	0.268
Vomiting[3]	251	268	-0.12 (-0.56, 0.03)	0.570
Abnormal bleeds/other complaints[3,4]	12	17	-	-

[1]Number of visits at which one or more symptom was recorded
[2]The number of times a condition was recorded for each individual was analysed as a negative binomial variable
[3]Number of visits at which any infant was reported to have this complaint
[4]Too few observations to make meaningful statistical comparisons

Table 25: Morbidity outcomes, adjusted analysis

Morbidity rates	Adjusting for compliance		Interaction between compliance & treatment (fitting compliance & doses of treatment received)	
	Effect size (95% CI)	p-value	Effect size (95% CI)	p-value
Unwell, any complaint	0.12 (-0.09, 0.33)	0.275	0.02 (-0.01, 0.04)	0.223
Diarrhoea	-0.28 (-0.62, 0.05)	0.096	0.03 (-0.01, 0.07)	0.114
Fever	0.10 (-0.13, 0.33)	0.402	0.00 (-0.02, 0.03)	0.754
Respiratory complaints	0.05 (-0.22, 0.32)	0.725	0.02 (-0.01, 0.05)	0.144
Nurse/doctor visits	0.13 (-0.09, 0.37)	0.242	-0.00 (-0.03, 0.23)	0.772
Vomiting	-0.13 (-0.56, 0.30)	0.563	0.21 (-0.03, 0.08)	0.438

The markedly higher diarrhoea incidence in the control group compared to the fish oil group may have been an explanation for why this group of infants also had lower MUAC and skinfold thickness measurements than infants supplemented with fish oil. A reduction in diarrhoea incidence over the 6-month period might have had an influence on body fat accumulation, so that fat gain increased, or fat loss decreased, during this time. To investigate the hypothesised mechanism, an analysis was performed to investigate group differences in MUAC and skinfold measurements and z-scores, while controlling for diarrhoea incidence. These adjusted analyses are presented alongside the unadjusted analyses in Table 26. The results observed when adjusting for diarrhoea incidence did not differ to any large extent to those observed when adjustment was not made.

Table 26: MUAC and skinfold thicknesses at 9mo of age, adjusted for the incidence of diarrhoea between baseline and endpoint

Anthropometric endpoint	Unadjusted analysis[1]		Adjusted analysis, controlling for diarrhoea incidence	
	Effect size (95% CI)	p-value	Effect size (95% CI)	p-value
MUAC-for-age	0.31 (0.06, 0.56)	0.017*	0.28 (0.03, 0.53)	0.031
Triceps skinfold-for-age	0.27 (0.00, 0.55)	0.048*	0.26 (-0.02, 0.53)	0.065
Subscapular skinf-for-age	0.16 (-0.16, 0.48)	0.326	0.13 (-0.20, 0.45)	0.434
MUAC (cm)	0.26 (0.00, 0.51)	0.049*	0.22 (-0.03, 0.48)	0.085
Biceps skinfold (mm)	0.20 (-0.08, 0.48)	0.152	0.17 (-0.12, 0.45)	0.231
Triceps skinfold (mm)	0.30 (-0.11, 0.71)	0.149	0.28 (-0.13, 0.70)	0.177
Subscapular skinf (mm)	0.12 (-0.31, 0.55)	0.580	0.07 (-0.36, 0.50)	0.748

*P<0.05
[1]General least squares regression, entering the dependent variable and treatment

4.7.5.1   Measurement reliability

Morbidity questionnaires were repeated by a second observer on a sample of 40% of infants and discrepancies between observers examined. The second observer filled out a total of 305 questionnaires.

For each pair of visits, the reports of whether an infant was ill with a particular complaint (e.g. fever) were compared between observers. Either a "yes" or a "no" was recorded by each observer. When a "yes / no" or "no / yes" combination between the quality control and routine reports occurred, it was counted as a discordant result. Such discordances between observers were totalled, and compared for each complaint.

The results of discordances are given in Table 27. This table also gives the percentage of times a complaint was recorded as "no", by observer.

Table 27: Compliance and morbidity questionnaire quality control (QC) results

	%discordant	% reported "no" - QC	% reported "no" – routine
Diarrhoea	6	93	96
Vomiting	3	96	95
Cough/rapid breathing	20	73	84
Fever	17	75	83
Unwell, any complaint	25	61	74

4.7.5.2   Discussion

The fish oil supplement had little impact on the prevalence and patterns of common morbidities, proving it to be neither harmful nor beneficial in this aspect in the population of infants studied, also in regard to bleeds. There was, however, some possible effect of fish oil on reducing diarrhoea incidence, as seen by the large difference in average incidence between the study groups. This is an interesting observation, and difficult to explain, especially since diarrhoea is a known potential side-effect of fish oil supplementation in adults. In order to verify whether this was a chance finding, and to investigate the biological mechanism for the reduction if real, further research is needed. Nevertheless, regardless of whether a significant reduction in diarrhoea may be affected by n-3 LCPs, it has been shown that even a large daily dose of fish oil did not cause any diarrhoea hazard in the studied infants.

When an analysis was done in which MUAC and skinfold thickness measurements were adjusted for diarrhoea incidences, the borderline significant difference between groups observed for triceps z-score and MUAC were lost. For MUAC z-score, the p-value for the group difference remained significant but increased (0.017 to 0.031). The p-values for the other measurements all increased. These results suggest that diarrhoea incidence did explain some of the effect of treatment seen in MUAC and skinfold thicknesses. However, since the occurrence of diarrhoea was weakly correlated with treatment, and treatment had only a weak effect on these anthropometric measures in the first place, the resulting small change in significance might have been expected when diarrhoea was added to the regression model. The results therefore do not prove a causal relationship between diarrhoea and MUAC/skinfold thickness, since diarrhoea incidence could have acted as a crude surrogate measure for treatment. The results do, nevertheless, provide some evidence for the hypothesised mechanism to be true.

Discordances flagged up by comparing quality control and routine morbidity data (particularly for respiratory complaints, fever, and unspecific complaints) is a reminder of how difficult it is to make reliable estimates of morbidity events through the use of morbidity questionnaires. Extensive reviews on the subject have highlighted the methodological issues, inherent problems, and lack of standardisation and validation in relation to morbidity measurements (311). Kroeger (1983) (312) noted that morbidity interviews rely on "people's memory, their willingness to communicate with the interviewers and – at least for children – on proxy reporting." Furthermore, interviews are susceptible to differences in perception and reporting which are influenced by social and cultural factors (312, 313).

In the present study the daily interviews, apart from facilitating estimates of illness durations and more straightforward data analyses, also placed little demand on mothers' memory, unlike interviews which ask about events which have taken place far further back. A mother had simply to recall the previous 24 hours, and also knew to expect an interview each day. Recall bias would therefore not be an important cause of inaccuracies. When the quality control interview was conducted, it sometimes fell on the second day, though, and a mother may have become confused by the time, or vague as to what symptoms her child had displayed two days earlier.

A mother or caregiver's willingness to communicate with the interviewer may have differed between mothers, between fieldworkers, and between the beginning of her 6 mo participation and the end. An unwilling mother, or one in haste, may have said that there is nothing the matter with her infant because she felt the interviewer was disturbing her with what she experienced as annoying and time-consuming daily probing, and she may not have been as cooperative after a certain period of daily appearances by the fieldworker as in the beginning. On the other hand, when the second observer visited her, she may have been more willing to cooperate, seeing the visit as special and serving a more important function than the routine visit. When comparing the results in Table 27, it is clear that a greater number of complaints were reported during the quality control visit than the routine one.

The daily visits were therefore useful for minimising recall bias, but mothers may have found the interviewing burden high and so answered as briefly as possible, consequently leading to a greater number of false negative reports.

Over-willing mothers, on the other hand, may have felt that they are showing the field-worker some interest and answering more favourably if they list at least some complaint during visits. She may have felt that by repeatedly reporting no incidences whatsoever, the fieldworker might interpret this as lying, forgetfulness, or unwillingness to cooperate. Yet other mothers may have felt embarrassed to report a symptom of illness in their children because of fear that it might reflect badly on her capability as parent. A child that is repeatedly reported to be ill might, the mother could fear, lead to some or other negative consequence to herself or to her family. In contrast to this, it was found during the trial that some mothers report symptoms when none are present in order to be given some medicine. She may report nightly fevers, for example, and after some time request a nurse's visit in the hope that he will give her child medication. This kind of bias leads to false positive reports.

Bias and false reporting lead to a degree of clouding of the results of morbidity surveys and paint an inaccurate picture of a child or community's burden of morbidity, making especially observation data vulnerable to errors. When performing simple treatment group comparisons however, the implications of these errors become less serious because they are likely to occur with the same frequency in each group. It is unlikely that over and under-reporting will influence results to the extent that significant group differences are found in error, or that significant group differences that in truth exist are not detected.

4.8   Other observations

4.8.1   Cognitive development in relation to umbilical cord and maternal serum DHA and AA concentrations

Cord and maternal sample laboratory analyses gave 104 sets of results, providing relative percentages of serum FAs. Combining these prenatal data and the available cognitive development results, 83 complete sets of overlapping data were obtained.

Maternal and cord percent FAs are summarised in Table 28. DHA and AA, and the ratio AA:DHA, were consistently higher in cord than in maternal serum. All LCPs were correlated between mothers and infants, as expected. Plasma DHA and the ratio AA:DHA were most strongly correlated.

Table 28: Maternal and cord serum DHA and AA concentrations

% FA	Maternal Serum (n=104) Mean; SD	Cord Serum (n=104) Mean; SD	Correlation coefficient (p)
DHA	3.76; 0.91	5.93; 1.85	0.366 (0.0001)
AA	6.20; 1.15	11.0; 2.83	0.260 (0.0076)
AA/DHA	1.74; 0.53	1.97; 0.63	0.594 (<0.0001)

DHA and AA, which are important constituents of the brain's grey matter, and believed to be important for adequate cognitive development, was correlated against each of the cognitive test results. No clear pattern of association was detected for any of the combinations.

Via regression analyses, partial correlation coefficients were assessed for DHA, AA, and AA:DHA, fitting Willatts and Attention Test outcome measures as the dependent variable in separate models. Maternal education level was added to all models, and when testing total intention and total intentional solutions, the trial count was also fitted (Table 30).

As a measure of post-natal n-3 LCP exposure, cord serum FA concentrations were subtracted from 9 mo plasma FA concentrations, and this variable used in further analyses. They are summarised in Table 29, together with breast-milk LCP concentrations and the Pearson correlation coefficient for their linear relationship. Breast-milk LCP measurements,

due to the limited samples drawn from each mother and the typically large day-to-day variation characteristic of breast-milk, would most likely be subject to a high degree of variability. This blood based measure of post-natal LCP exposure, in contrast, provided the advantage of integrating the breast-milk changes to show how the combination of dietary intake of LCPs and their metabolism altered FA status since birth.

Table 29: Plasma-based indicator of post-natal LCP exposure and breast-milk LCP concentrations at 3 mo, and the correlation between them

	Plasma Mean; SD	Breast-milk Mean; SD	Correlation coefficient (p)
DHA	4.15; 2.09	0.77; 0.39	-0.03 (0.769)
AA	3.79; 3.03	0.49; 0.11	-0.10 (0.328)
AA/DHA	0.96; 1.07	0.77; 0.35	0.15 (0.127)
EPA	0.68; 1.25	0.36; 0.29	0.01 (0.957)
DHA/EPA	7.36; 58.2	2.69; 1.08	0.19 (0.059)

In a similar fashion to above, this change in FA status, both for DHA and AA, was plotted against the developmental outcome scores, and regression analysis performed.

Of the FAs tested, there were no significant associations with cognitive development, apart from an inverse relationship between cord DHA and total intention score (partial r= -0.2528, p=0.031). This relationship was plotted. There was only a very small degree of noticeable association, as seen in Figure 39. The figure also shows some of the distortion of the distribution caused by the truncation of the intention score data at zero (referred to earlier).

4.8.1.1 Discussion

The correlation between cord and maternal serum DHA and AA, also found in other populations, points to the dependence of the foetus on its mother for DHA, and, less so, AA supply. In a study involving 210 infant mother pairs from five different countries, for example, highly significant positive correlations (p<0.001) were found between the relative values of the n-6 (r=0.35) and n-3 (r=0.39) FAs (after correction of gestational age at birth and the effect of countries) (314). The higher DHA and AA concentrations in cord vs. maternal serum give indication of a preferential placental uptake of the LCPs from the mother (315).

No group in the literature has related maternal and cord LCP concentrations with either infant planning tests or the Attention assessment at 12 mo. Associations between prenatal DHA concentrations and other infant cognitive measures have been measured, however. Positive relationships have been found in some studies but not in others. The study by Jacobson *et al.* (2008) (248), finding positive associations between cord plasma DHA and infant cognitive performance has already been discussed in the previous chapter. In the USA, it was found in 17 mother-infant pairs, that infants born to mothers with higher DHA status displayed a greater central nervous system maturity, as measured by sleep-state patterning (correlations between maternal DHA and wakefulness: r=0.42, not significant) (316), and that motor indices in 44 infants at 12 mo correlated positively with plasma phospholipid DHA content at 4 mo of age (p~=0.02) (317). In Canada, a prospective study of 83 infants exclusively breast-fed for at least 3 mo, found the measured infant DHA status to be significantly related to visual acuity at 12 mo of age (r=0.30, p=0.03), and although the extent to which the results could be attributed to other confounding factors is not certain, the data suggest that maternal DHA breast-milk levels may influence the development of visual acuity and neural pathways in breast-fed infants (174).
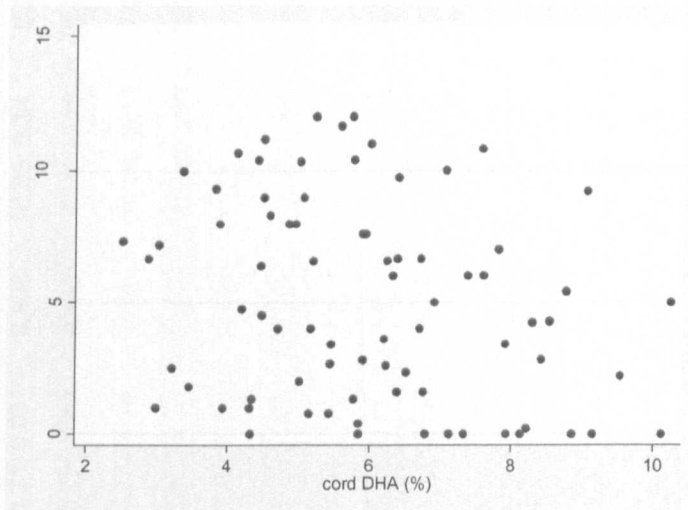
Figure 39: Scatter plot for average intention scores vs. cord DHA %

Table 30: Regression coefficients for the relation between maternal and prenatal LCP status, and postnatal LCP exposure, to cognitive performance

n=83	Total intention score[1]		Intentional solutions[1]		Pretest average[2]		Inattention rate[2,3]		Mean look duration[2,3]	
Serum %	β; parial r	P	β; partial r	P	β; partial r	p	β; partial r	P	β; partial r	p
Maternal DHA	1.24; 0.03	0.808	0.09; 0.14	0.230	-0.44; -0.07	0.526	1.05; 0.07	0.500	0.95; -0.08	0.483
Maternal AA	-0.29; -0.09	0.432	-0.02; -0.05	0.662	-0.80; -0.18	0.113	1.04; 0.09	0.423	0.95; -0.11	0.333
Maternal AA/DHA	-0.49; -0.06	0.61	-0.14; -0.11	0.327	-0.92; -0.001	0.942	0.99; -0.01	0.917	1.00; 0.00	0.979
Cord DHA	-0.54; -0.25	0.031*	-0.05; -0.16	0.190	-0.58; -0.20	0.084	1.03; 0.10	0.379	0.97; -0.11	0.294
Cord AA	-0.10; -0.08	0.505	-0.00; -0.02	0.896	-0.01; -0.00	0.972	1.01; 0.03	0.786	0.99; -0.04	0.723
Cord AA/DHA	0.93; 0.14	0.246	0.08; 0.08	0.485	1.87; 0.21	0.062	0.95; -0.05	0.641	1.05; 0.06	0.570
Postnatal DHA	-0.41; -0.21	0.066	-0.04; -0.13	0.272	-0.29; -0.11	0.324	1.04; 0.15	0.201	0.96; -0.12	0.269
Postnatal AA	-0.01; -0.01	0.937	0.01; 0.04	0.764	0.13; 0.08	0.503	1.06; 0.04	0.759	0.99; -0.01	0.875
Potsnatal AA/DHA	0.24; 0.06	0.604	0.05; 0.08	0.495	0.28; 0.05	0.642	1.03; 0.03	0.794	0.99; -0.08	0.461

β: regression coefficient

[1] Controlling for trial count and mother's highest education level

[2] Controlling for mother's highest education level

[3] Variable was log-transformed

*p<0.05

However, Ghys, Bakker and colleagues in the Netherlands, found no association between umbilical cord LCP levels and later cognitive performance at 4 and 7 years of age (318, 319). In 128 infants, the association of cognitive development at 4 years with DHA at birth measured close to zero (r<0.05) in bivariate and multiple regression analyses, controlling for relevant confounders. Similarly at 7 years, no associations with DHA were found for various cognitive scores, after correcting for confounders (p>0.2 for all).

The current study has found evidence of a negative correlation between cord DHA and later cognitive development. This result is not strongly significant (p=0.031), and is not supported by additional associations between either cord, maternal, or postnatal DHA statuses on the same outcome. Furthermore, none of the other outcomes were influenced by infant or maternal DHA levels. In the current section, more than 40 statistical tests were done to investigate the relationship between LCPs and cognitive development. One would expect to find, therefore, at least one or two significant relationships due to chance only.

This given, in addition to a lack of similar evidence from the literature, it is very unlikely that a true association between cord DHA and infant cognitive development exist as measured by the tests chosen. Furthermore, when removing the 22 observations for which the total intention score was "0", in an attempt at normalising the data, the association between DHA and this score was lost (p=0.882).

4.8.2 Growth outcomes at 12 months

The results of the anthropometric measurements taken during the cognitive testing visits are presented in Table 31.

Treatment effects seen on MUAC z-scores at 9 mo were maintained at 12 mo (increasing from 95% C:I -0.06-0.56, p=0.017). Those effects seen on triceps skinfold z-scores at 9 mo were amplified at 12 mo (increasing from 0.00-0.55, p=0.048), and a new group difference was observed for subscapular skinfold thickness z-score at 12 mo, not previously seen at 9 mo.

These effects were echoed when investigated as length in centimetres, with significant group effects detected not only for MUAC, triceps and subscapular skinfolds, but also for biceps skinfold thicknesses.

Principal components analysis was performed, as before, combining triceps, subscapular, and biceps-skinfold thicknesses. The result was regressed and a significant treatment effect observed (95% CI: 0.09,0.98; p=0.019).

Table 31: Anthropometric indices at 12 mo of age, by treatment group, controlled for baseline measures

Endpoint	Fish oil group (n=79) $\bar{x}$; SD	Placebo group (n=76) $\bar{x}$; SD	Effect size (95% CI)	p-value
Anthropometric indices				
Weight (g)	8323; 1120	8041; 1093	109 (-112, 329)	0.332
Length (cm)	71.8; 7.54	71.9; 3.21	-0.50 (-2.22, 1.21)	0.564
HC (cm)	44.8; 1.30	44.6; 1.49	-0.04 (-0.31, 0.22)	0.751
MUAC (cm)	14.3; 1.08	13.8; 1.06	0.29 (0.04, 0.55)	0.023*
Knee-heel length (cm)	20.0; 1.09	19.8; 1.15	0.12 (-0.12, 0.36)	0.307
Biceps skinfold (mm)	6.70; 1.19	6.24; 1.10	0.41 (0.09, 0.74)	0.014*
Triceps skinfold (mm)	8.48; 1.35	7.90; 1.29	0.45 (0.07, 0.84)	0.022*
Subscapular skinfold (mm)	6.93; 1.39	6.47; 1.17	0.44 (0.08, 0.80)	0.018*
Anthropometric z-scores				
Weight-for-age	-1.09; 1.13	-1.41; 1.21	0.11 (-0.13, 0.35)	0.357
Weight-for-length	-0.79; 1.10	-1.03; 0.97	0.17(-0.09, 0.43)	0.207
Length-for-age	-.1.00; 1.11	-1.30; 1.32	0.08 (-0.15, 0.32)	0.489
HC-for-age	-0.61; 0.87	-0.72; 1.02	-0.04 (-0.24, 0.16)	0.713
MUAC-for-age	-0.20; 0.94	-0.64; 0.98	0.33 (0.09, 0.57)	0.008*
Body-mass-index-for-age	-0.69; 1.12	-0.90; 0.94	0.09 (-0.15, 0.34)	0.459
Triceps skinfold-for-age	0.19; 0.79	-0.15; 0.82	0.31 (0.06, 0.55)	0.014*
Subscapular sf-for-age	0.23; 1.05	-0.14; 1.02	0.36 (0.06, 0.67)	0.019*

*p<0.05

4.8.2.1 Discussion

That the treatment effects at 12 mo were maintained - and amplified - provides further evidence for an effect of fish oil supplementation on increasing MUAC and skinfold thicknesses.

Whilst an increase in muscle-mass would be reflective of an improved nutritional status, increased subcutaneous fat accumulation would not necessarily be so.

n-3 LCP supplementation studies have found either no change or a decrease in skinfold measurements in infants, as summarised in a review by Lapillonne (320). Only one other study, in 72 Danish infants (321), found an increase in body-mass-index (BMI) and subscapular skinfold in infants whose mothers were supplemented with 4.5g of fish oil during 4 mo of lactation. This difference was detected two years after completion of the intervention, but not during the intervention or at the 9 mo follow-up. The authors suggest that the late onset of the effect could indicate some kind of programming effect, and others have also suggested that n-3 LCPs regulate growth by influencing gene transcription activity (320). In the light of a potential late-onset (inflated) treatment effect, interesting results may be found if the infants in the present study were to be re-visited and assessed at a follow-up two or three years from the date that intervention was ended.

Although the skinfolds were increased, BMI scores were not significantly elevated by treatment, which one would perhaps suspect. However, when investigating the number of infants in the treatment group with BMI z-score>0.5, there were three times more in the fish oil group than the control group (12 and 4, respectively), and a chi-squared test gave some evidence for a relationship between BMI z-score>0.5 and treatment (p=0.4).

The fish oil group MUAC average was moved towards the WHO reference mean, improving this growth indicator with regard to the reference norms. Even so, MUAC in the treatment group was still relatively low on average, at -0.20 z-scores. The skinfolds, in contrast, were pushed from a negative average in the control group to a positive average in the intervention group. It is difficult to know whether these observed increases would potentially have harmful or beneficial effects.

According to the WHO reference curves, skinfold thicknesses and BMI are declining at 12 mo of age. An alternative explanation to an increased fatness is that the fish oil was delaying this decline, and could potentially delay the later adiposity rebound, lowering the risk of adult obesity (322, 323). This possibility has been mentioned by the Danish research group mentioned above. Alternatively, the increased fatness could be maintained into later life, with associated disease risks.

4.8.3   Overall growth compared with WHO references at 3, 9 & 12 months of age

Growth indicators of weight, length and HC compared well with WHO references at 3 mo of age (Figure 40). By 9 mo, however, growth faltering had become noticeable, with z-score distributions shifting towards the negative side of the x-axis (Figure 41). This shift moved further left at 12 mo of age (Figure 42).

Using WHO recommendations for stunting (length-for-age z-score <-2) and wasting (weight-for-length z-score <-2), the stunting rates (13% at 9 mo, 21% at 12 mo) were more pronounced than wasting (9% at 9 mo, 17% at 12 mo). HC measurements dropped severely from baseline to 12 mo, with a mean z-score at baseline of -0.2 falling to -0.7 at 12 mo of age. By 12 mo 9% of infants had a HC z-score of <-2.

MUAC scores were slightly higher than weight, length and HC scores. The triceps skinfold z-score mean improved from being close to -1 at baseline (-0.8 z-scores) to close to zero (-0.2 z-scores) at the other time points. Average subscapular skinfold measurements were slightly higher than WHO means at baseline (0.2 z-scores) and 9 mo (0.3 z-scores), but moved towards the norm at 12 mo (0.04 z-scores).
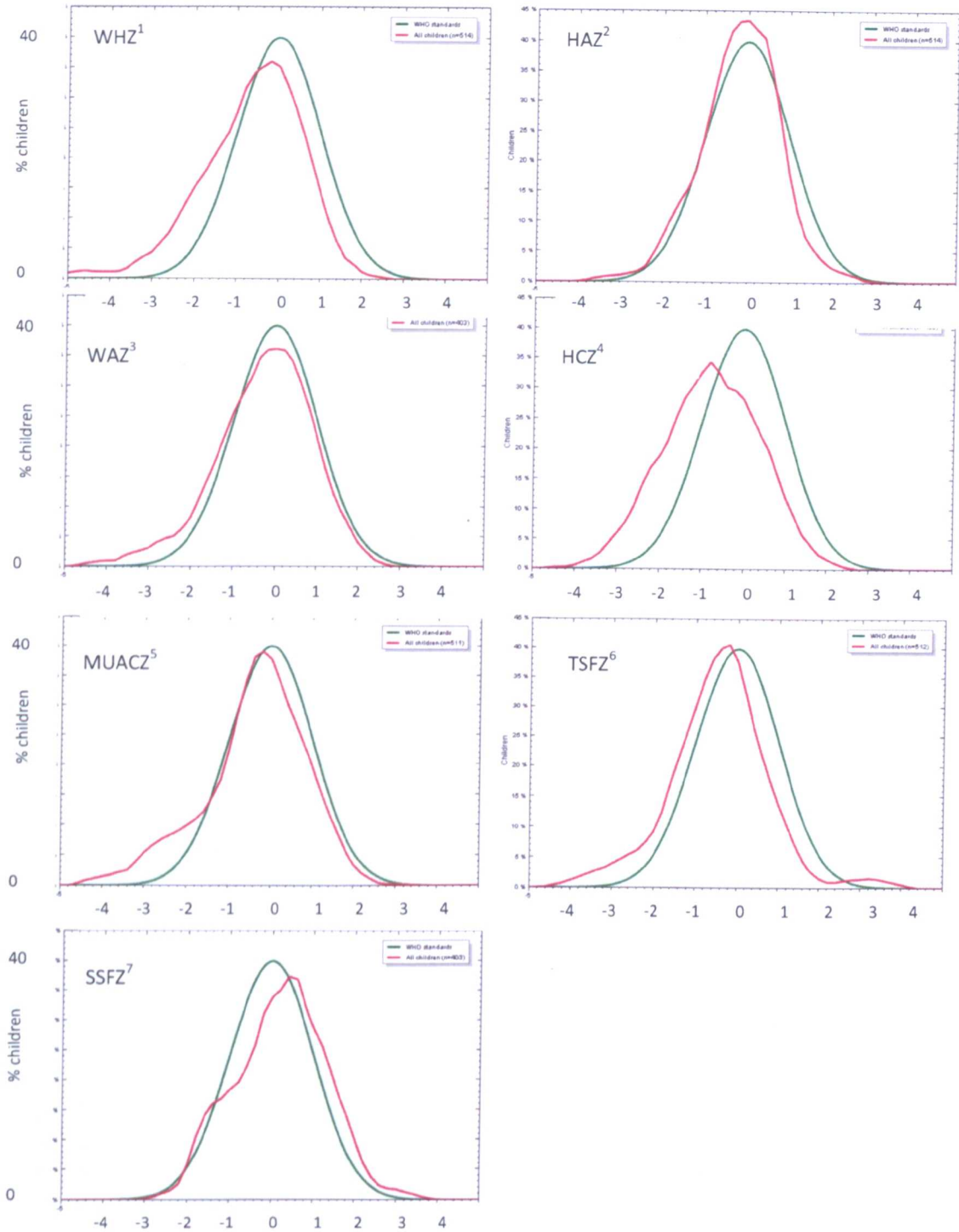
Figure 40: Baseline growth measurements plotted (red) against WHO standards (green). Z-scores are given along the x-axis.

[1]weight-for-length, [2]length-for-age, [3]weight-for-age, [4]HC-for-age, [5]MUAC-for-age, [6]triceps skinfold-for-age, [7]subscapular skinfold-for-age
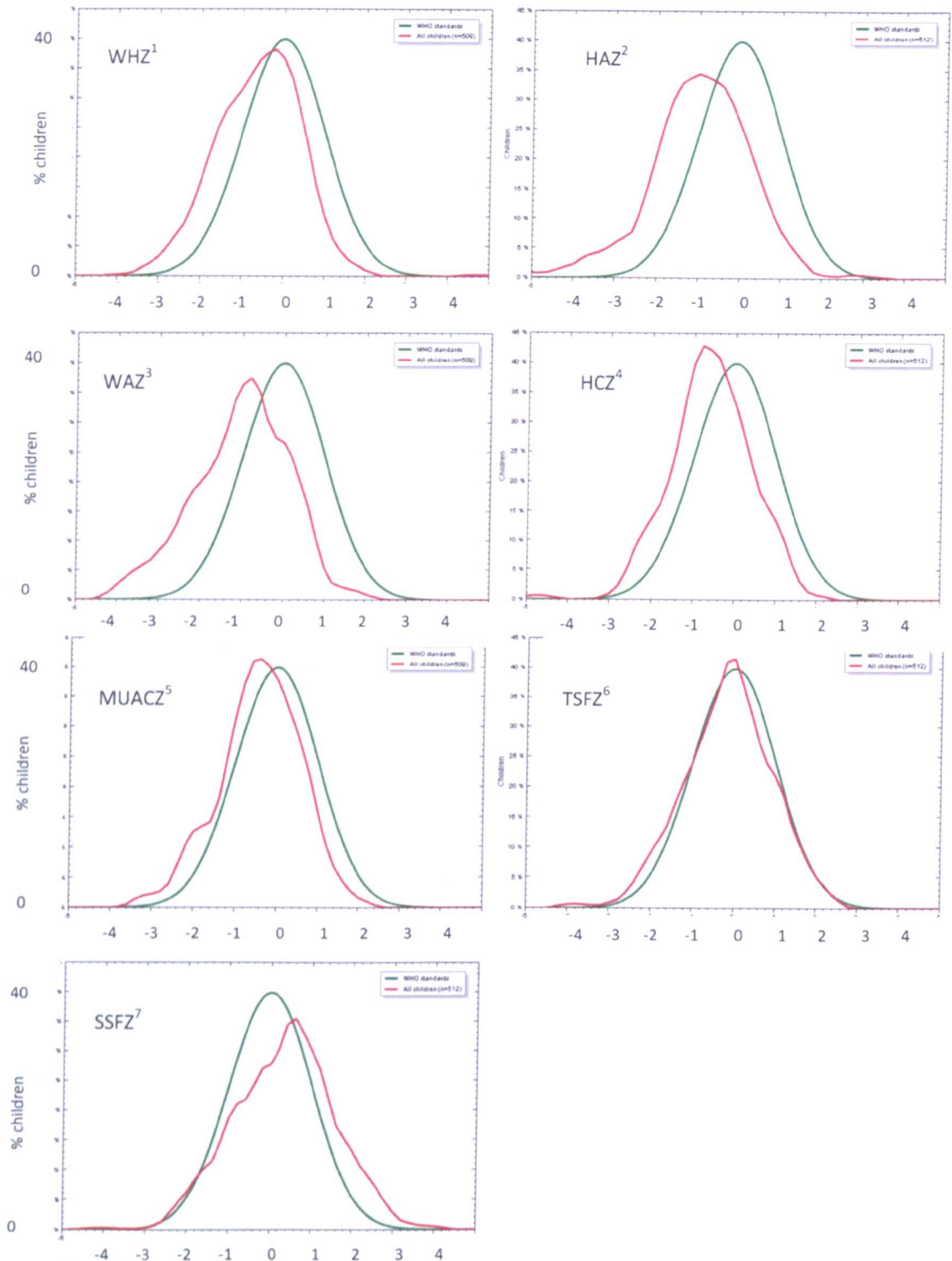
Figure 41: Growth measurements at 9 mo of age, plotted (red) against WHO standards (green). Z-scores are given along the x-axis.

[1]weight-for-length, [2]length-for-age, [3]weight-for-age, [4]HC-for-age, [5]MUAC-for-age, [6]triceps skinfold-for-age, [7]subscapular skinfold-for-age

180
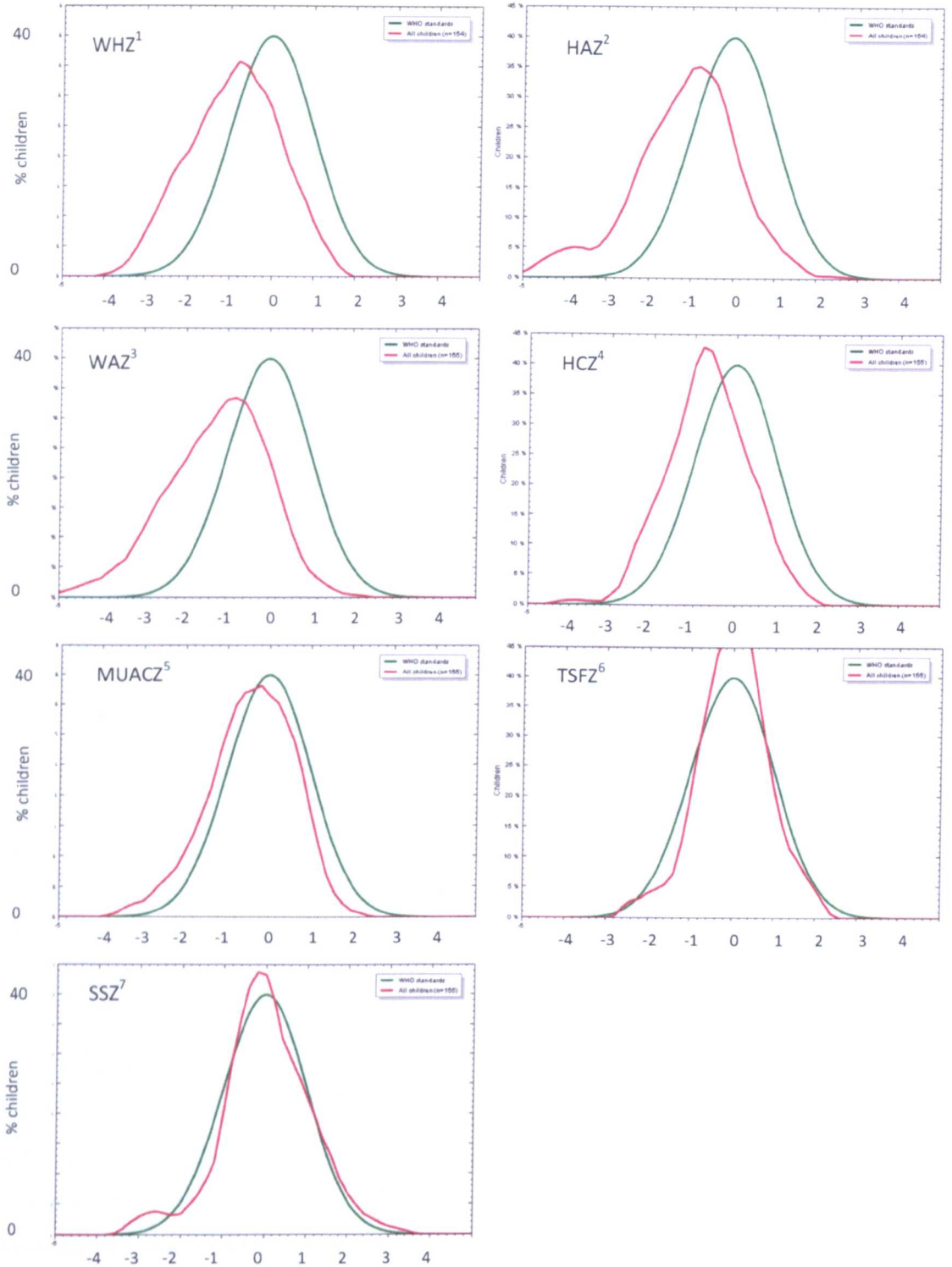
Figure 42: Growth measurements at 12 mo of age, plotted (red) against WHO standards (green). Z-scores are given along the x-axis.

[1]weight-for-length, [2]length-for-age, [3]weight-for-age, [4]HC-for-age, [5]MUAC-for-age, [6]triceps skinfold-for-age, [7]subscapular skinfold-for-age

4.8.3.1   Discussion

It is not surprising to see this pattern of growth faltering, with relatively good growth present at 3 mo and dropping to average z-scores of lower than -1 for weight and length indices at 12 mo. As mentioned in the introduction, this pattern has typically been observed in the West Kiang population, with growth faltering setting in at time of weaning, at around 3 or 4 mo of age.

Interestingly, the smallest z-score observed was for triceps skinfold thickness-for-age (-0.76 z-scores), whereas the subscapular skinfold distribution was the largest. These two measures, seemingly indicating conflicting information on the subcutaneous fat accumulation of these infants, could be due to a systematic error in measurement of one (or both) of the skinfolds. However, when compared to a small sample of previous data in this population, the subscapular:triceps ratios were not dissimilar.

Assuming the measurements were not systematically erroneous, the trunk and extremity fat deposition in this population differs at 3 mo from the norm in the WHO standards. Ethnic differences in skin folds have been documented by various groups (324-328) who found that racial differences in subscapular:triceps ratios exist.   Certain African and Caribbean populations, for example, have a higher subscapular:triceps skinfold ratio than American and British children and adolescents. Although a global database is used in the new WHO growth standards, the high subscapular:triceps skinfold ratio in the West Kiang population at 3 mo is still prominent when compared with the other, essentially well-nourished populations, and probably an adaptive attribute of young Gambian infants.

Evidence to support this phenomenon as an adaptive attribute is given by Yajnik *et al.* (2003) (328). In a study comparing the body size measurements of 631 term babies born in rural India with those of 338 term babies born in Southampton, UK, Indian babies had markedly reduced visceral sizes and MUAC measurements, but similar subscapular skinfold thicknesses compared with British-born infants. Thus, although these babies were much smaller or, apparently, "thinner" than the Southampton babies, they were simultaneously relatively adipose, as judged by their central fat adiposity. The evolutionary benefit of this described "thin-fat" baby syndrome was explained by the advantage which fat stores provide for neonatal survival, providing energy reserves, insulation, and a depository of precursors for

brain development. The authors went on to propose that this phenotype results from persistence of central fat laid down in utero.

4.8.4 Weaning foods

Exclusive breastfeeding was reported by 52.7% of mothers when their infants were 3 mo of age (intervention group: 54.2%; placebo group: 49.4%). Of the infants who were not being exclusively breastfed at 3 mo, 28.6% had been consuming water as the only weaning substance.

Cessation of full breast-feeding occurred by the time their infants had reached 4 mo of age in 71.9% of mothers, and 5 mo of age in 83.9% of mothers. Only 2.68% reported exclusively breastfeeding their infants by the time they had reached 6 mo. No infants were still being exclusively breast-fed at 7 mo. Of the infants who had been weaned at 4 mo and 5 mo, 24.7% and 17.6%, respectively, had received water only.

Solid foods were reportedly introduced in 31.5% of 3 mo olds, 31.5% of 4 mo olds, 66.9% of 5 mo olds, 81.2% of 6 mo olds, and 91.5% of 7 mo olds. By 8 mo, 97.9% of infants were eating solid foods, and all infants were eating solid foods by 9 mo of age.

Fourteen percent of infants had had some form of egg in their diet by 6 mo. Roughly the same number had shared the family bowl, including dishes that contain small amounts of dried fish. By 9 mo roughly 80% of infants had had some egg yolk or some form of fish.

The most common solid weaning food fed, by far, was a rice-based porridge. Bread was the next most common weaning food. Other frequently-fed foods were margarine, mayonnaise, bananas, and cooking oil. The dishes commonly eaten in the household were the next most common weaning food.

Formula milk was not popularly fed, particularly to younger infants. One infant had been fed formula milk by 3 mo of age, five by 5 mo of age, and nineteen by 7 mo of age. By 9 mo, however, nearly 20% of infants had been given formula milk.

Milk powder (made from cow's milk) can easily be bought in the villages (albeit at a high price) and is often added the infant's porridge or tea. Eight and fourteen infants had been fed

powder milk by 3 and 5 mo, respectively, and 39 by 7 mo. Twenty-nine percent of infants had been fed milk powder by age 9 mo.

4.8.4.1 Discussion

Even the most thoroughly-trained fieldworkers cannot overcome the well-known difficulties of poor validity and bias associated with food-frequency and recall questionnaires. A mother may report that she has given her child nothing but breast-milk when the infant is indeed being fed small quantities of other foods, because she believes the intakes of weaning foods are negligible in comparison to breast-milk, or because she wants to give the "correct" answer. It is also known to be very difficult for a mother to remember specifically what her child has eaten in the past month.

The questionnaire administered was not intended for collecting detailed or highly accurate data, and the results should be interpreted as only a rough indication of infant weaning practices in this population. Nevertheless, these estimates, even if imprecise, indicate that a considerable proportion of women were not adhering to the WHO recommendations of exclusive breast-feeding for 6 mo (329, 330). Despite high initiation rates and a long total duration of breast-feeding amongst Gambian women (331), only 53% of mothers were still exclusively breast-feeding their infants at 3 mo. By 6 mo of age, only 3% of infants were reportedly exclusively being breast-fed.

The Baby Friendly Community Initiative project, rooted in an adaptation of the United Nations Children's Fund (UNICEF)/WHO Baby-friendly Hospital Initiative, was first launched by the Gambian government and the Gambian National Nutrition Agency in the Lower River Division more than 10 years ago. This community based health programme has now expanded to 219 villages countrywide, and, amongst other activities, strongly promotes exclusive breast-feeding up to 6 mo. It may be surprising, therefore, that so many mothers – despite being educated on the benefits of exclusive breast-feeding and warned of the potential dangers of early weaning – wean their infants by 3 mo of age regardless.

However, it is well known that knowledge does not necessarily equate to changed attitudes or practices. Traditional and cultural beliefs and customs remain strong influences for determining a mother's decisions regarding raising her child. Social practices may, e.g., require a mother to feed her child water and complementary foods before the infant reaches 6 mo, if

she is to be regarded as an adequate mother by many of her peers and elderly relatives. Such customs limit the decision making process of women (332). Additionally, demands placed on women living in areas where subsistence farming is imperative to survival often force decisions on breast-feeding (331). A mother who does not have the assistance of a daughter to follow her to the rice field and act as nurse-maid while the mother cultivates the rice (whether because she does not have a one of the appropriate age or because the daughter is attending school), may have no choice but to leave her infant at home under the care of an elderly relative while she goes off to work in an often far away rice field. The infant will consequently necessarily survive off paps and other complementary foods during the day, while awaiting the return of their mother at sunset.

Clearly, further energy into appropriate and persistent strategies are required to be invested to break long-standing infant feeding practices and result in community and, ultimately, nation-level change.

4.8.5 Relationship between calprotection levels and LMRs

The relationship between intestinal integrity as measured by LMRs, and intestinal inflammation as measured by stool calprotectin concentrations was investigated graphically and by statistical correlations.

No relationships were detected in the scatter plots drawn between calprotectin concentrations and LMRs, or lactulose and mannitol recoveries, as seen in Figure 43. Correlation coefficients between pooled 3 mo and 9 mo data are also shown in Figure 43, all of which were small ($r^2 \leq 0.06$ for all) and statistically insignificant.

When restricting the analysis to relationships at outcome, a weak but borderline significant relationship was found between LMR and calprotectin ($r^2 \leq 0.16$; p=0.048).
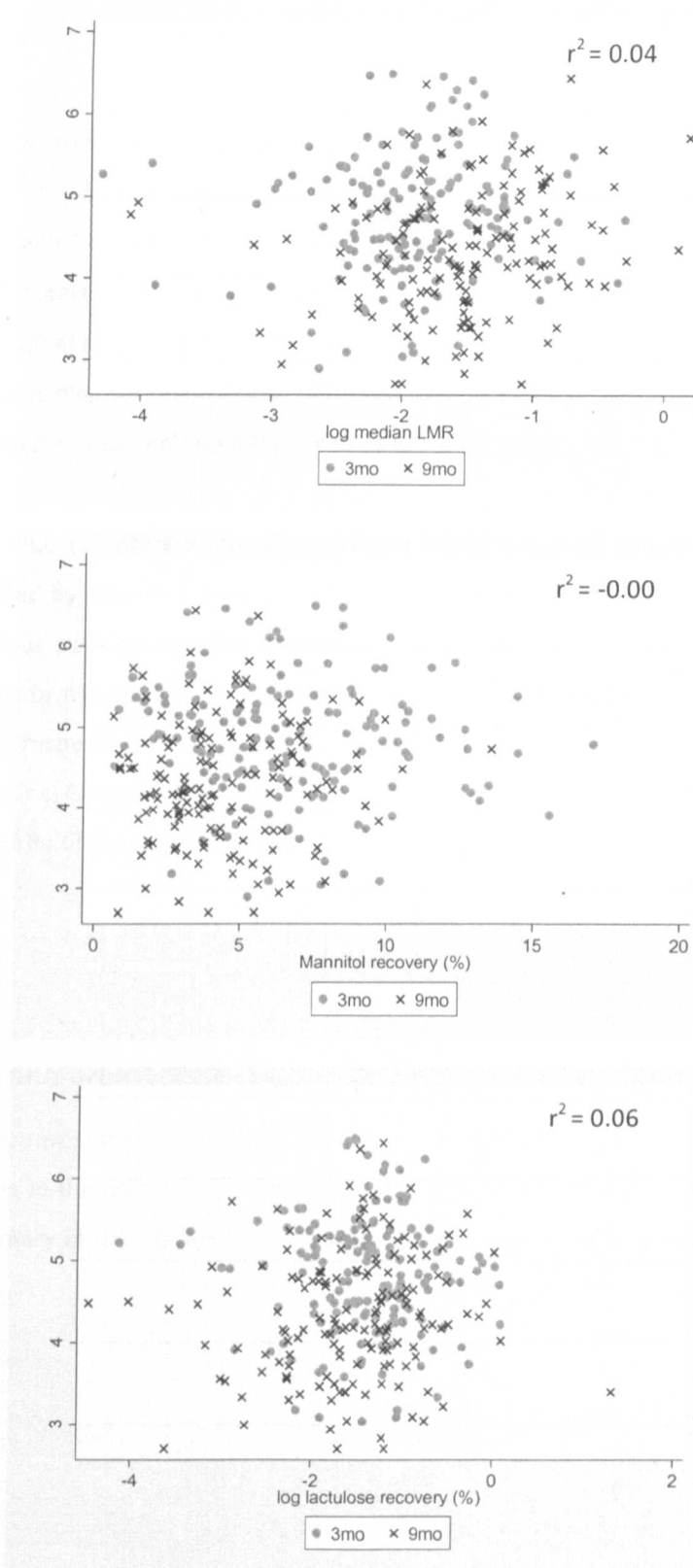
Figure 43: Scatter plots of stool calprotectin concentrations versus LMRs (top), mannitol recovery (middle) and lactulose recovery (bottom), by time-point

4.8.5.1 Discussion

Even when investigating the relationship between pooled (>300 observations) data, no associations were detected between these two markers of enteropathy. At outcome, a weak association was seen between LMR and calprotectin, but the overall results of this analysis suggest that calprotectin and LMR measures are unrelated and instead are independent indicators of intestinal state or that one or both of the measures is so variable as to obscure any underlying physiological relationship. Stool calprotecin measurements, therefore, cannot be used as a predictor of current gut integrity or as proxy for LMRs.

This may be due to different reasons: a) These markers can be influenced by different factors or increased by different mechanisms (e.g. the intestinal mucosa may be damaged by infection by various pathogens, while calprotectin levels may increase due to inflammation which is allergenic or not necessarily pathogen-induced); b) Calprotectin elevation occurs as a relatively quicker response to an insult than gut damage, which is a more gradual result of repeated insults; or c) Calprotectin largely reflects inflammation of the large intestine, whereas LMRs reflect integrity of the small intestine.

4.8.6 Plasma and breast-milk fatty acids: further considerations

4.8.6.1 Breast-milk fatty acid profiles

The main LCPs in the breast-milk of mothers at baseline and endpoint are summarised in Table 32. A summary of the other FAs was prepared, by time-point, and is presented in Table 33.

Table 32: LCPs in breast-milk at baseline and endpoint, relative percentages

Endpoint	3 mo (n=86)	9 mo (n=83)	
Plasma FA (% total lipid)	$\bar{\chi}$; SD	$\bar{\chi}$; SD	p-value[1]
DHA	0.77; 0.39	0.80; 0.38	0.496
EPA[2]	0.27 (0.16,0.44)	0.26 (0.18, 0.39)	0.489
AA	0.49; 0.11	0.50; 0.11	0.520
Other FA indicators (% total lipid)			
Essential:nonessential PUFAs[2]	0.44; 0.12	0.52; 0.63	0.155
DHA:22:5n-6	13.7; 5.85	13.5; 5.56	0.704
Total n3 FAs (ng/μl)	1.93; 0.84	1.91; 0.78	0.873
Total n6 FAs (ng/μl)	10.6; 2.45	11.2; 2.32	0.031
AA/(EPA+DHA)	0.56; 0.29	0.54; 0.24	0.489
Total n-6 ≥ C20/ Total n-3 ≥C20	0.77; 0.39	0.72; 0.31	0.180

[1]Differences between 3 mo and 9 mo FA concentrations, tested by ANOVA
[2] Geometric mean (25th, 75th percentiles)

Table 33: Other breast-milk fatty acids

Fatty acid (% total lipid)	3 mo (n=87)	9 mo (n=85)	
	$\bar{\chi}$; SD	$\bar{\chi}$; SD	p-value
Saturated (C8:0 – C18:0)[1]	0.34; 0.15	0.40; 0.20	0.003*
Saturated (C20:0 – C24:0)[2]	54.5; 6.12	53.9; 6.50	0.450
Monousaturated[3]	32.1; 5.10	32.1; 6.00	0.928
Oleic acic (C18:1n9c)	29.2; 5.20	29.1; 5.20	0.836
n-3 PUFAs			
C18:3n3	0.40; 0.18	0.42; 0.20	0.459
C20:3n3	0.03; 0.01	0.03; 0.02	0.055
C22:5n3	0.36; 0.18	0.33; 0.14	0.581
n-6 PUFAs			
C18:2n6c	0.02; 0.02	0.02; 0.02	0.819
C18:3n6	0.12; 0.04	0.11; 0.04	0.010*
C20:3n6	0.38; 0.10	0.34; 0.10	<0.001*
C22:5n6	0.06; 0.03	0.06; 0.03	0.439

[1]Includes 18:0; 17:0; 16:0; 15:0; 14:0; 13:0; 12:0; 11:0; 10:0; 8:0
[2]Includes 24:0; 23:0; 22:0; 21:0; 20:0
[3]Includes 14:1; 15:1; 16:1; 17:1; 18:1n9c; 20:1; 22:1n9; 24:1
*p<0.05

Breast-milk samples taken at baseline and endpoint differed significantly in relative percentages by time-point for combined short and medium chain saturated fats, C18:3n6, C20:3n6, and total n-6 FAs. There were no significant differences in relative percentages of DHA and EPA measured at 3 mo and 9 mo.

4.8.6.2 Breast-milk LCP levels in rural Gambian mothers compared to other populations

Brenna *et al.* (2007) (288) studied the breast-milk DHA and AA content (%w/w of total fatty acid) from 2472 women in more than thirty different countries around the world, including Europe, Asia, Africa, America, Australia, and the Canadian Arctic. The summary of this study's finding is presented in Table 34, together with summary statistics from the Gambian sample for comparison. Because the majority of breast-milk samples in the meta-analysis were taken from mothers with infants closer to 3 mo than 9 mo of age, baseline breast-milk values are given below.

A graphical illustration by Brenna *et al.* of the same breast-milk DHA and AA levels is given in Figure 44. An indication of where the Gambian means fall in relation is shown on the graph.

Table 34: Breast-milk DHA and AA concentrations worldwide and in rural Gambia

Data from Brenna *et al.* (2007) (288)

	AA (% total FA)		DHA (% total FA)	
	Wordwide	Rural Gambia	Worldwide	Rural Gambia
Mean; SD	0.47; 0.13	0.49; 0.11	0.32; 0.22	0.77; 0.39
Median	0.46	0.48	0.26	0.67
Range of population means	0.24-1.0		0.06-1.40	
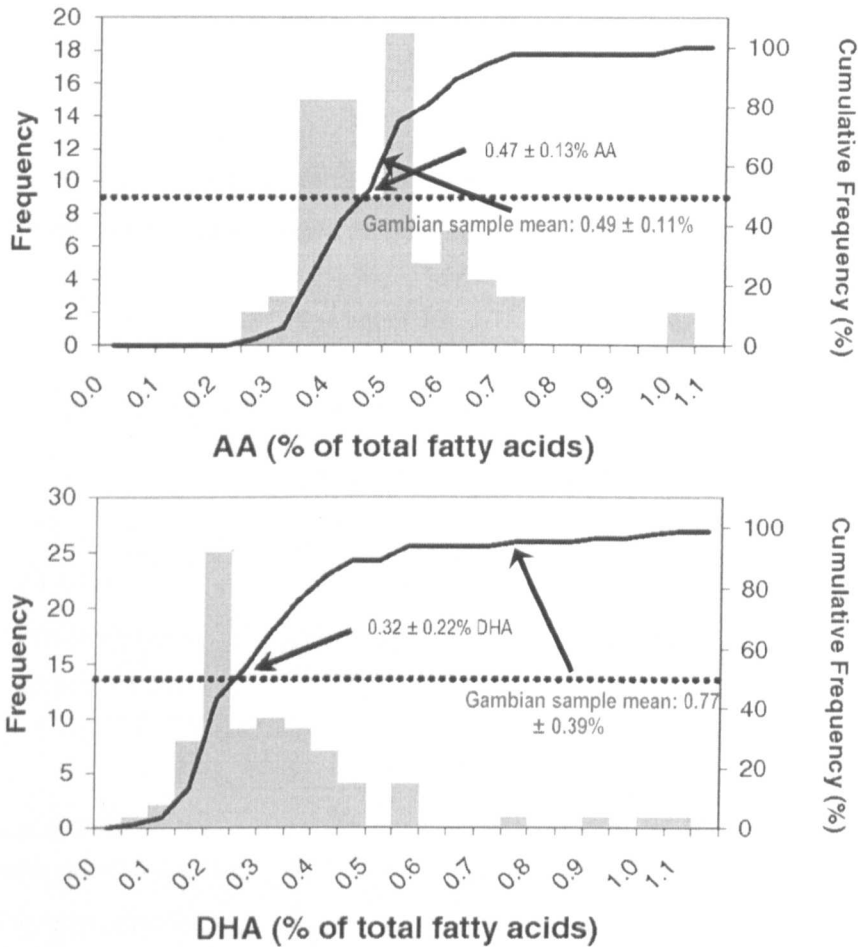
Adapted from Brenna (2007) (288)

Figure 44: Distribution of AA (above) and DHA (below) in breast-milk, presented as a histogram. The arrows show the average fatty acid concentrations of the entire sample (from 2472 women), and the Gambian sample means

A study by Yuhas *et al.* (2006) (287) compared FA profiles in the breast-milk of women from nine different countries in America and Canada, Latin and South America, Asia and Europe, and found the average of the nine population means for EPA to be 0.10±0.001% (w/w Total FA). The Gambian population average was, in contrast, 0.36±0.29%. This EPA comparison and comparisons between three different FA ratios (including α-linolenic to linoleic acid) is summarised in Table 35.

Table 35: Mean breast-milk EPA and fatty acid ratios in women in nine different countries (Yuhas *et al.,* 2006), and in rural Gambia

	Nine countries	Rural Gambia
	% total FAs	
	Mean; SD	Mean; SD
EPA	0.10; 0.001	0.36; 0.29
AA:DHA	1.54; 0.43	0.77; 0.35
EPA:DHA	0.35; 0.02	0.43; 0.16
18:3n3/18:2n6	13.2; 0.55	27.5; 10.3

4.8.6.2.1   Discussion

The Gambian mother's breast-milk contained a very high concentration of n-3 LCPs relative to data from women globally.

The highest mean breast-milk DHA levels (seen to the right side of the x-axis of the histogram in Figure 44) were reported in women from Japan (0.53, 0.99 & 1.10), Philippines (0.74), Canadian Arctic (1.40), and Dominican Republic (0.91). Other countries where levels where comparable to those in The Gambia, but not as high, were Congo (0.55), Sweden (0.53), and St. Lucia (0.53). All of these mentioned countries are bordered entirely or partly by ocean, and have populations with a high fish intake.

It has been established that breast-milk DHA levels are strongly associated with dietary intake of the FA, and breast-milk content increases linearly in a dose-dependent manner with maternal DHA intake (200, 333). The existent data on fish consumption in rural Gambia are not current, and have led to conclusions that have perhaps resulted in an underestimation of the breast-milk n-3 LCP content of Gambian women, more recently at least (88, 334, 335). Based on the premise that the breast-milk DHA content is a reflection of dietary DHA (i.e. marine) intake, the breast-milk results from the current study suggest that the Gambian women have very high intakes of DHA, comparable with intakes of women from countries with the highest

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fish consumption in the world, such as Japan. The average DHA level of 0.77% is nearly two-and-a-half times higher than the global average of 0.32%, and the contribution that this level makes to infant n-3 LCP status is undoubtedly significant (as breast-milk DHA has been shown to elevate infant DHA status (333)).

There is a smaller literature on breast-milk EPA than DHA, but Gambian mothers' breast-milk EPA content was more than three times higher than that of the populations studied. The comparative ratios of AA:DHA confirm the high DHA intake in Gambian mothers, and the higher (>2×) α-linolenic to linoleic acid ratio shows that the intake of precursors to DHA and EPA in relation to precursors to AA, or n-3 essential FAs versus n-6 essential FAs, is significantly higher in Gambian women than in the average woman in the populations studied.

These results are surprising, particularly when taking into account the large amount of palm oil consumed by Gambian mothers, which is high in the n-6 linoleic acid, but devoid of the n-3 α-linolenic FA. Additionally, the amounts of fish eaten with meals is typically very small, and average portions far smaller than portion sizes in the developed world.

There is evidence that habitual intake of fish is also an important factor in determining breast-milk n-3 LCP contents (336, 337), and perhaps the consistency of the fish intake throughout life contributes greatly to the breast-milk n-3 LCP content. The high intakes of leafy green vegetable in The Gambia may also be a significant contributor to the high breast-milk DHA content, as these vegetables contain α-linolenic acid which may be converted to their longer chain derivatives. There is also the possibility that laboratory or calculation methods could cause artificially elevated FA results. The ratios investigated, would, however, be expected to flag up these kinds of errors, but in the present study the ratios have consistently pointed to high breast-milk n-3 PUFA levels.

The substantial amount of n-3 LCPs delivered to the infants in West Kiang leads one to conclude that there are very few deficiencies existent in the infant population, and that the LCP requirement is adequately met for the vast majority of infants. It could be the case that infant tissues are largely saturated with these FAs at baseline, and that further supplementation therefore adds little clinical benefit.

Nevertheless, it is not certain how efficiently the infants absorb and utilise breast-milk LCPs. It may be that a lot of fat is excreted in the stool, malabsorbed due to a poor villous

surface area or some other reason (although treatment administered as oil successfully increased infant n-3 LCP status, so it appears unlikely that breast-milk LCPs would not). To investigate this question, the variation in plasma LCP levels explained by breast-milk compared to that explained by treatment will be examined shortly.

4.8.6.3 Correlations between pre- and post-treatment plasma fatty acid concentrations, and breast-milk and plasma fatty acids

Indicative of the extent to which treatment altered FA profiles, over and above the influence of normal diet or biological determinants, product-moment Pearson correlations were calculated to investigate how strongly pre-treatment LCP levels predicted endpoint ones, and to test the relationship between breast-milk and plasma FA levels at 9 mo.

DHA and EPA concentrations were not correlated in the treatment group, while in the control group DHA and EPA levels remained fairly stable (Table 36). AA remained as stable in the control as the fish oil group, and was unaffected by n-3 LCP administration. Similarly, the ratio AA:n-3LCP remained somewhat stable even in the fish oil group. The ratio DHA:C22_5n6 was significantly altered in the fish oil group but not in the control group, an indication that n-3 LCP cell usage was improved by treatment alone.

However, in contrast, the relative percentages of DHA and EPA were correlated in the baseline and endpoint plasma of infants in the fish oil group.

Treatment was therefore strongly predictive of the absolute concentrations, but not relative percentages, of endpoint FA profiles in individual subjects. Attention will now be drawn to how strongly breast-milk FAs predicted plasma FA levels.

Table 36: Correlations between pre and post-treatment plasma LCPs, by treatment group

	Control				Fish oil			
	ng/µl FA		Relative % FA		ng/µl FA		Relative % FA	
	Correlation coeff. (p-value)		Correlation coeff. (p-value)		Correlation coeff. (p-value)		Correlation coeff. (p-value)	
DHA	0.44	(<0.001*)	0.56	(<0.001*)	0.17	(0.129)	0.39	(<0.001*)
EPA	0.33	(0.002*)	0.30	(0.006*)	0.07	(0.541)	0.23	(0.041*)
AA	0.27	(0.013*)	0.30	(0.005*)	0.27	(0.013*)	0.31	(0.004*)
AA/(DHA+EPA)	0.36	(<0.001*)	0.36	(<0.001*)	0.33	(0.003*)	0.33	(0.003*)
DHA/C22_5n6	0.40	(<0.001*)	0.38	(<0.001*)	0.20	(0.065)	0.21	(0.059)

*p<0.05

Table 37: Correlations between breast-milk and plasma LCPs at 9 mo

	Control				Fish oil			
	ng/µl FA		Relative % FA		ng/µl FA		Relative % FA	
	Correlation coeff. (p-value)		Correlation coeff. (p-value)		Correlation coeff. (p-value)		Correlation coeff. (p-value)	
DHA	-0.14	(0.203)	-0.09	(0.419)	0.19	(0.081)	0.11	(0.297)
EPA	-0.13	(0.235)	0.01	(0.928)	0.03	(0.777)	0.04	(0.697)
AA	0.16	(0.155)	0.01	(0.947)	0.06	(0.590)	-0.06	(0.606)
AA/(DHA+EPA)	0.10	(0.357)	0.10	(0.358)	0.07	(0.496)	0.07	(0.505)
DHA/C22_5n6	0.34	(0.001*)	0.34	(0.001*)	0.08	(0.439)	0.08	(0.485)

*p<0.05

Breast-milk LCP concentrations at 9 mo did not predict any of the assessed LCP concentrations in plasma, even in the control group. DHA:C22_5n6 was the only FA indicator which was predicted by breast-milk levels (in the control group).

Thus, higher concentrations of particular LCPs in a mother's breast-milk did not predict higher concentrations of the same FA in the plasma of her infant.

Although breast-milk is very variable in its FA levels one would expect to see at least some predictive power, certainly in the control group, given the strong relationship of n-3 LCP status to diet and the great contribution of breast-milk to the infant diet.

Of course correlations are not the ideal way of testing the relationship, and are limited to one FA at one time-point. As a follow-up therefore, the partial R^2 in an analysis of variance, including more terms, were compared in the following section.

4.8.6.4 How much variation in plasma fatty acid levels is explained by breast-milk versus treatment?

With the aim of comparing how much variation in plasma LCP fatty acid levels was explained by the breast-milk intake of these fats and how much by treatment, a model was built: each 9 mo plasma LCP was controlled for its baseline concentration and the total plasma fat concentration. Treatment, the relevant breast-milk LCP relative percent concentration at 9 mo and the total breast-milk lipids were then added. Various other PUFAs were tested for their effects but these were all marginal and thus dropped from the model.

Treatment and the relevant breast-milk FAs were held constant. The partial R^2 was calculated separately for variation (in the dependent variable) due to treatment, and due to the respective breast-milk FAs.

The bootstrap method was then used to calculate 95% confidence intervals for the partial R^2 estimates. These are presented in Table 38.

For plasma EPA, a much larger portion of variation in plasma concentrations could be explained by treatment than by breast-milk EPA. Only minor variation was explained by either treatment or breast-milk for DHA and AA.

4.8.6.5   Do plasma or breast-milk fatty acids predict any of the other outcomes?

A cross-sectional evaluation of relative percentages of PUFAs[7] in plasma and breast-milk was tested to examine the relationship with anthropometric z-scores, gut integrity, cognitive development, and markers of inflammation.

The following FAs were tested in separate analyses:

a)   Sum of n-3 FAs and sum of n-6 FAs ("DHA + c22_5n3 + EPA + c20_3n3 + c18_3n3" and "c18_2n6c + c20_3n6 + AA + c22_5n6")

b)   EPA (log) and DHA

c)   Ratio n-6 LCP:n-3 LCP ("c22_5n6 + AA + c20_3n6" : "DHA + c22_5n3 + c20_3n3 + EPA")

A linear regression model was used to test the relationship between each outcome measure at endpoint with the various FAs at 9 mo.

The statistically significant results are presented in Table 39.

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[7] Relative percentages were used rather than absolute values fatty acids because they were slightly better correlated with anthropometric measures.

Table 38: Partial R^2 attributed to breast-milk fatty acids versus treatment, explaining the variation in plasma LCPs. Calculated from ANOVA, controlling for 3 mo plasma LCP concentrations and total plasma lipids

n=165	Breast-milk FAs			Treatment	
Total degrees of freedom fitted: 5	Partial R^2 (Bias-corrected CI)	Regression coeff. (p-value)		Partial R^2 (Bias-corrected CI)	Regression coeff. (p-value)
Plasma FA		Fitted FA	Total breast-milk lipid		
DHA	-0.02 (-0.04, -0.02)	-2.55 (0.467)	4.88 (0.361)	0.01 (-0.01, 0.05)	9.26% (0.002)**
EPA	-0.00 (-0.02, 0.05)	-2.37% (0.637)	-25.5% (0.034)*	0.20 (0.12, 0.30)	51% (<0.001)**
AA	-030 (-0.04, -0.03)	1.39 (0.844)	2.72 (0.624)	-0.01 (-0.02, -0.01)	-1.33% (0.659)

*p<0.05
**p<0.001

Anthropometry

Total n-3 plasma FAs were significantly positively related to MUAC z-scores (95% CI: 0.02,0.163; p=0.018), while plasma EPA was significantly positively related to MUAC z-score (95% CI: 0.12,0.92; p=0.012) and marginally positively significantly related to height-for-age z-score (95% CI: 0.01,0.98; p=0.046). Breast-milk PUFAs were only significantly related to height-for-age z-score, but these relationships were stronger than those plasma PUFA relationships mentioned above: breast-milk DHA was negatively associated (95% CI: -2.10,-0.34; p=0.007), and EPA positively associated with height-for-age z-score (95% CI: 0.22,1.21; p=0.005). Neither the plasma nor breast-milk n-6 LCP:n-3 LCP ratios predicted any of the anthropometric z-score outcomes.

Gut intergrity

None of the tested PUFAs were significantly related to gut integrity as measured by LMR, and lactulose and mannitol recoveries vs. plasma and breast-milk PUFAs.

Cognitive development and calprotectin

The five main cognitive outcomes measures: the Willatts test's "total solutions", "total intentional solutions" and "pretest average", and the "average look duration" and "inattention rate" of the Attention Assessment, were assessed against breast-milk and plasma PUFAs (by regression analysis and scatter plots) to examine their relationships. No relationships (neither positive nor negative) or patterns were detected.

Stool calprotectin concentrations in relation to PUFA levels were similarly assessed. No relationship between plasma or breast-milk PUFAs and calprotectin concentration was observed.

Acute phase proteins

Although not strongly so, plasma n-6 LCP:n-3 LCP ratio was a significant (positive) predictor of plasma albumin (95% CI: 0.24,3.00, p=0.021). The same ratio was negatively correlated to (log) CRP (untransformed 95% CI: 0.48,0.94; p=0.021). These were the only acute phase protein relationships with plasma PUFAs. Breast-milk total n-6 FAs were (weakly) positively related to (log) AGP (untransformed 95% CI: 1,1.04; p=0.038).

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To investigate whether these and other associations existed before the introduction of treatment, and to increase the power of the analysis, a random effects regression model (allowing for repeated observations on the same individual) was used to assess cross-sectional relationships on pooled data. The relationship of 3 mo and 9 mo FAs with the corresponding baseline and endpoint outcome measures were examined. Any significant relationships were further investigated by testing for an interaction between the relevant FAs and time-point, and by drawing scatter plots. The statistically significant relationships found are presented in Table 40. Scatter plots for relationships of outcome variables with plasma PUFAs are shown in Figure 45 - Figure 49 under Section 4.8.6.5.1, and those with breast-milk PUFAs in Figure 50 - Figure 53, under Section 4.8.6.5.2.

Table 39: Statistically significant regression analysis results for PUFA and outcome measure relationships, using endpoint data

n=169	Regression coeff. (95% CI)	P-value	Partial r
Plasma FAs (9 mo)			
Total % n-3 FAs[1]			
MUAC z-score	0.09 (0.02, 0.163)	0.018	0.18
% EPA[2]			
Height-for-age z-score	0.50 (0.01, 0.98)	0.046	0.15
MUAC z-score	0.52 (0.12, 0.92)	0.012	0.19
Ratio n-6 LCP:n-3 LCP[3]			
Albumin (g/L)	1.60 (0.24, 3.00)	0.021	-
(log) CRP (mg/L)	0.68 (0.48, 0.94)	0.021	-
Breast-milk FAs (9 mo)			
Total % n-6 FAs[1]			
(log) AGP (mg/L)	1.02 (1, 1.04)	0.038	0.16
% EPA[2]			
Height-for-age z-score	0.72 (0.22, 1.21)	0.005	0.21
% DHA[2]			
Height-for-age z-score	-1.21 (-2.10, -0.34)	0.007	-0.21

[1]Regression analysis adding "anthropometric z-score" or "acute phase protein" at 9 mo as dependent variable, and "total % n-6 acids (c18_2n6c + c20_3n6 + AA + c22_5n6)" and "n-3 FAs (DHA + c22_5n3 + EPA + c20_3n3 + c18_3n3)" at 9 mo as independent variables
[2]Regression analysis adding dependent variable as in "1" above, and "% (log) EPA and % DHA (9 mo)" as independent variables
[3]Regression analysis adding dependent variable as in "1" above, adding "ratio n-6 LCP (c22_5n6 + AA + c20_3n6):n-3 LCP (DHA + c22_5n3 + c20_3n3 + EPA)" at 9 mo as independent variable

Table 40: Statistically significant PUFA & outcome measure relationships, using pooled data

	Regression coeff. (95% CI)	P-value	Interaction[1]
Plasma FAs (pooled 9 mo & 3 mo)[2]			
Total % n-3 FAs[3]			
Weight-for-age z-score	0.06 (0.02, 0.11)	0.006	0.679
Height-for-age z-score	0.06 (0.02, 0.10)	0.005	0.970
HC z-score	0.04 (0.01, 0.07)	0.009	0.562
MUAC z-score	0.08 (0.03, 0.12)	0.001	0.940
(log) CRP	1.07 (1.03, 1.11)	<0.001	0.514
Total % n-6 FAs[3]			
Albumin	0.10 (0.00, 0.21)	0.045	0.325
% EPA[4]			
MUAC z-score	0.03 (0.02, 0.46)	0.030	0.705
Albumin	-1.01 (-1.78, -0.245)	0.010	0.838
% DHA[4]			
Albumin	0.96 (0.46, 1.46)	<0.001	0.547
Ratio n-6 LCP:n-3 LCP[5]			
Height-for-age z-score	-0.24 (-0.44, -0.04)	0.018	0.262
HC z-score	-0.16 (-0.31, -0.01)	0.041	0.789
MUAC z-score	-0.25 (-0.46, -0.04)	0.022	0.396
(log) CRP	0.75 (0.62, 0.90)	0.002	0.587
Breast-milk FA (9 mo & 3 mo)[2]			
Total % n-3 FAs[3]			
Height-for-age z-score	0.11 (0.01, 0.21)	0.026	0.199
(log) CRP	1.08 (1.03, 1.12)	<0.001	0.318
% EPA[4]			
Height-for-age z-score	0.26 (0.06, 0.46)	0.012	0.477
(log) CRP	1.37 (1.12, 1.69)	0.003	0.254
Ratio n-6 LCP:n-3 LCP[5]			
Height-for-age z-score	-0.40 (-0.63, -0.17)	0.001	0.431
(log) CRP	0.66 (0.52, 0.83)	<0.001	0.361

[1]P-value for interaction between time-point and FA

[2]Random-effects regression using pooled 3 mo and 9 mo data, treating "outcome measure" as dependent variable, "relative FA level" and "time-point" (3 mo or 9 mo, a fixed effect associated with age) as main effects, and "individual infant" as the random effect

[3]Adding "total n-3 FAs (DHA + c22_5n3 + EPA + c20_3n3 + c18_3n3) and total n-6 FAs (c18_2n6c + c20_3n6 + AA + c22_5n6)

[4]Adding "log EPA (%) and DHA (%)"

[5]Adding "ratio n-6 LCP (c22_5n6 + AA + c20_3n6) : n-3 LCP (DHA + c22_5n3 + c20_3n3 + EPA)

4.8.6.5.1   Scatter plots of statistically significant plasma PUFA relationships with outcomes
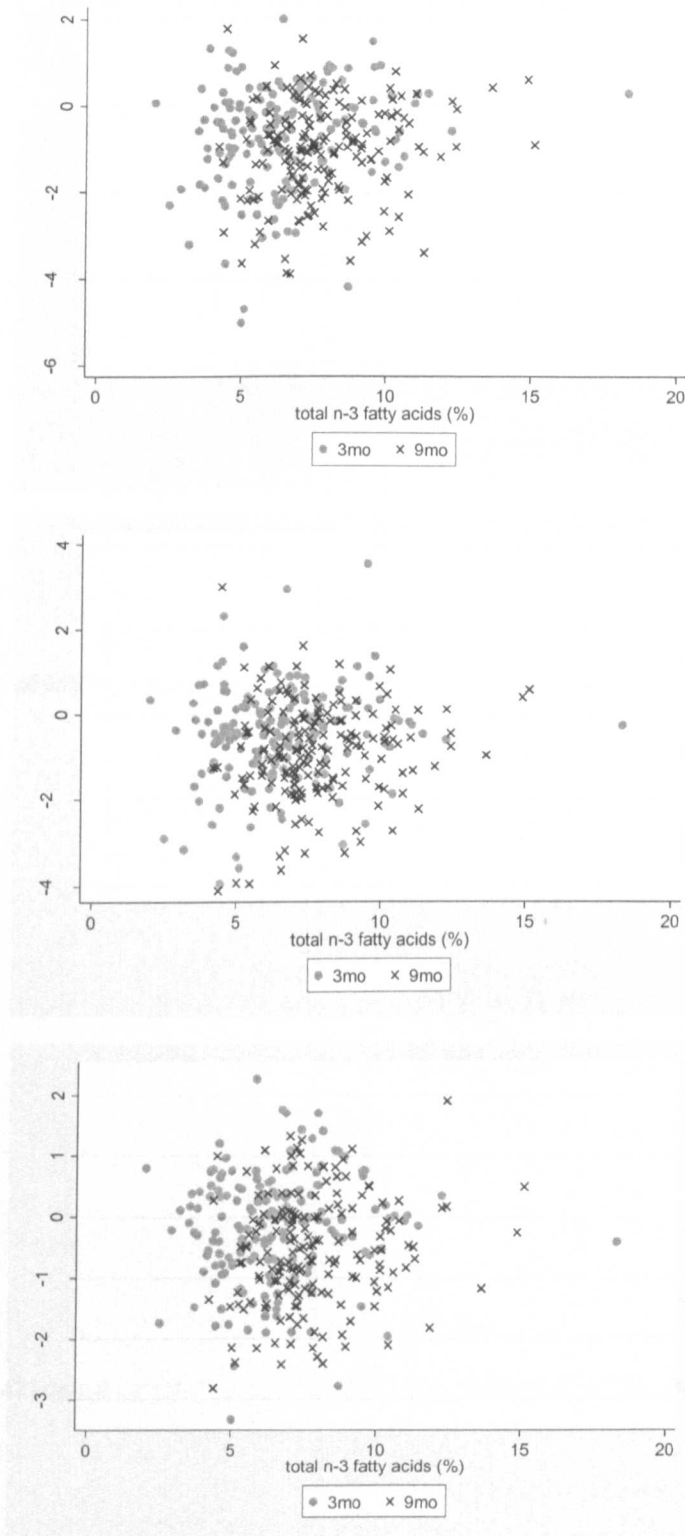
Figure 45: Graphs of weight-for-age z-score (top), height-for-age z-score (middle), and HC z-score (bottom) vs. relative total plasma n-3 fatty acid, by time-point
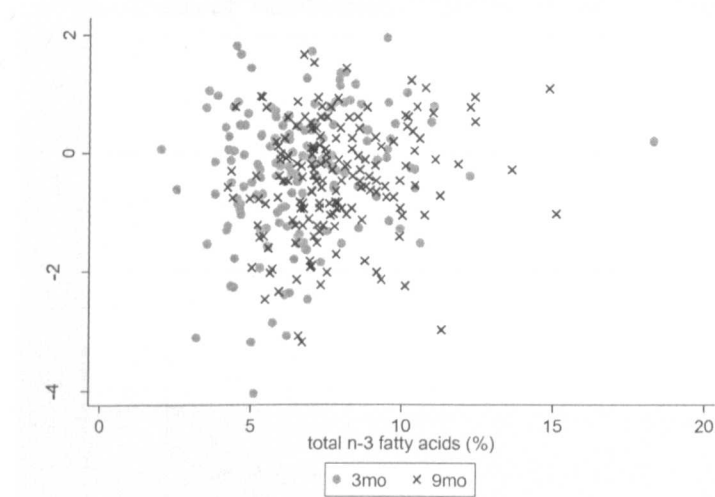
Figure 46: Graph of MUAC z-scores vs. relative total plasma n-3 fatty acids, by time-point
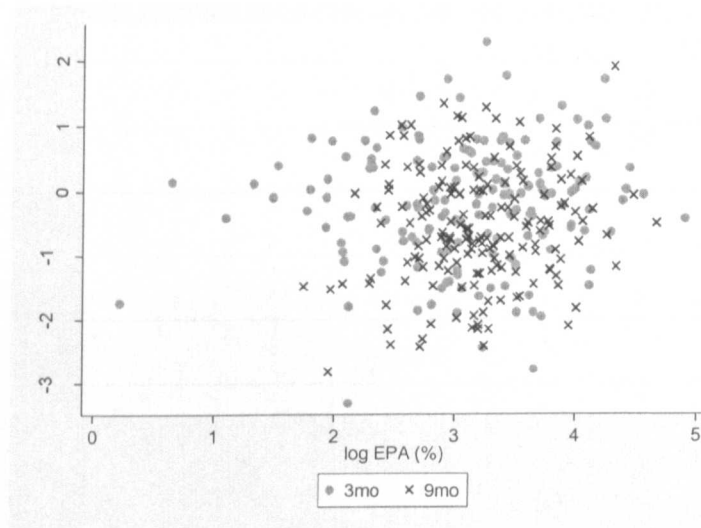
Figure 47: Graph of HC z-score vs. relative plasma EPA (log), by time-point
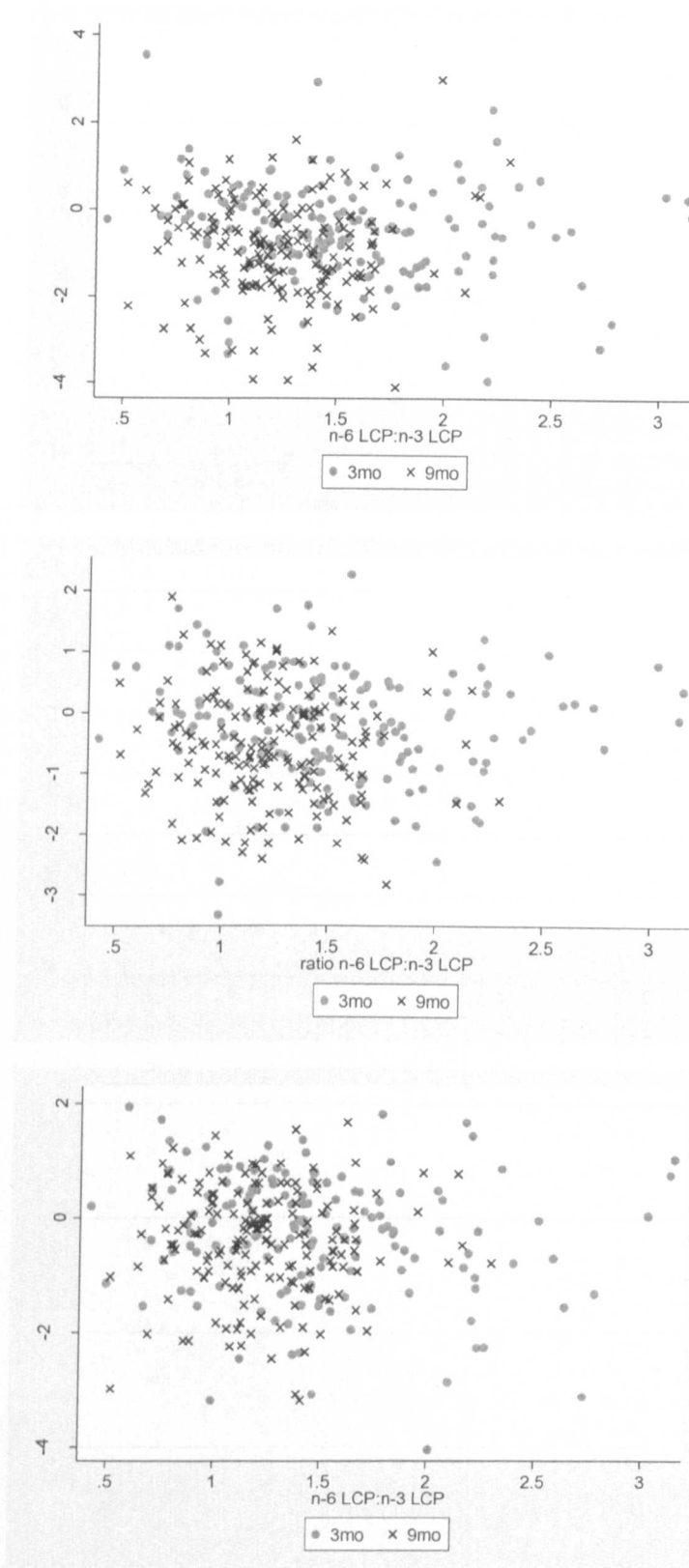
Figure 48: Graphs of height-for-age z-score (top), HC z-score (middle) and MUAC z-score (bottom) vs. plasma n-6 LCP:n-3 LCP ratio, by time-point
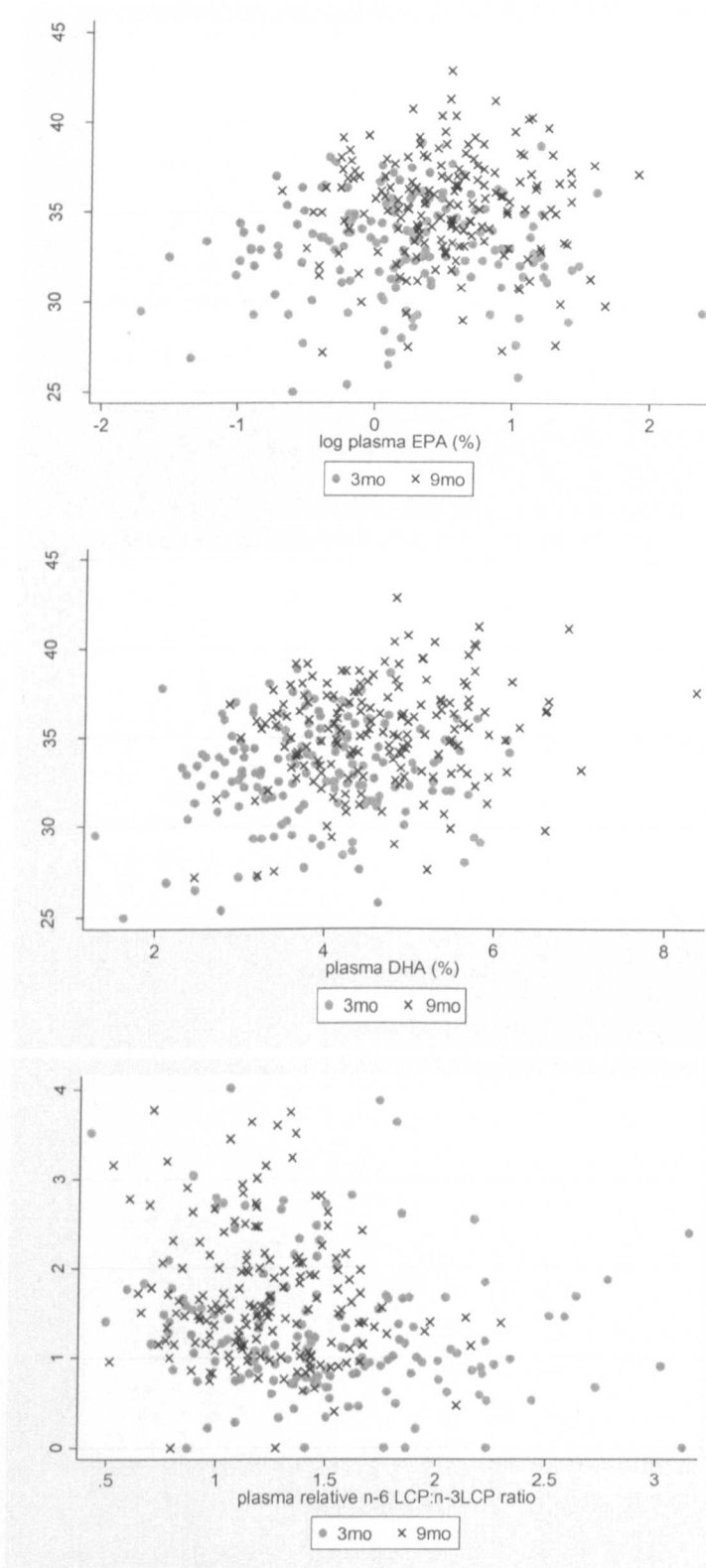
Figure 49: Graphs of plasma albumin vs. EPA (top) and DHA (middle), and plasma CRP vs. n-6 LCP:n-3 LCP ratio, by time-point

4.8.6.5.2   Scatter plots of statistically significant breast-milk PUFA relationships with outcomes
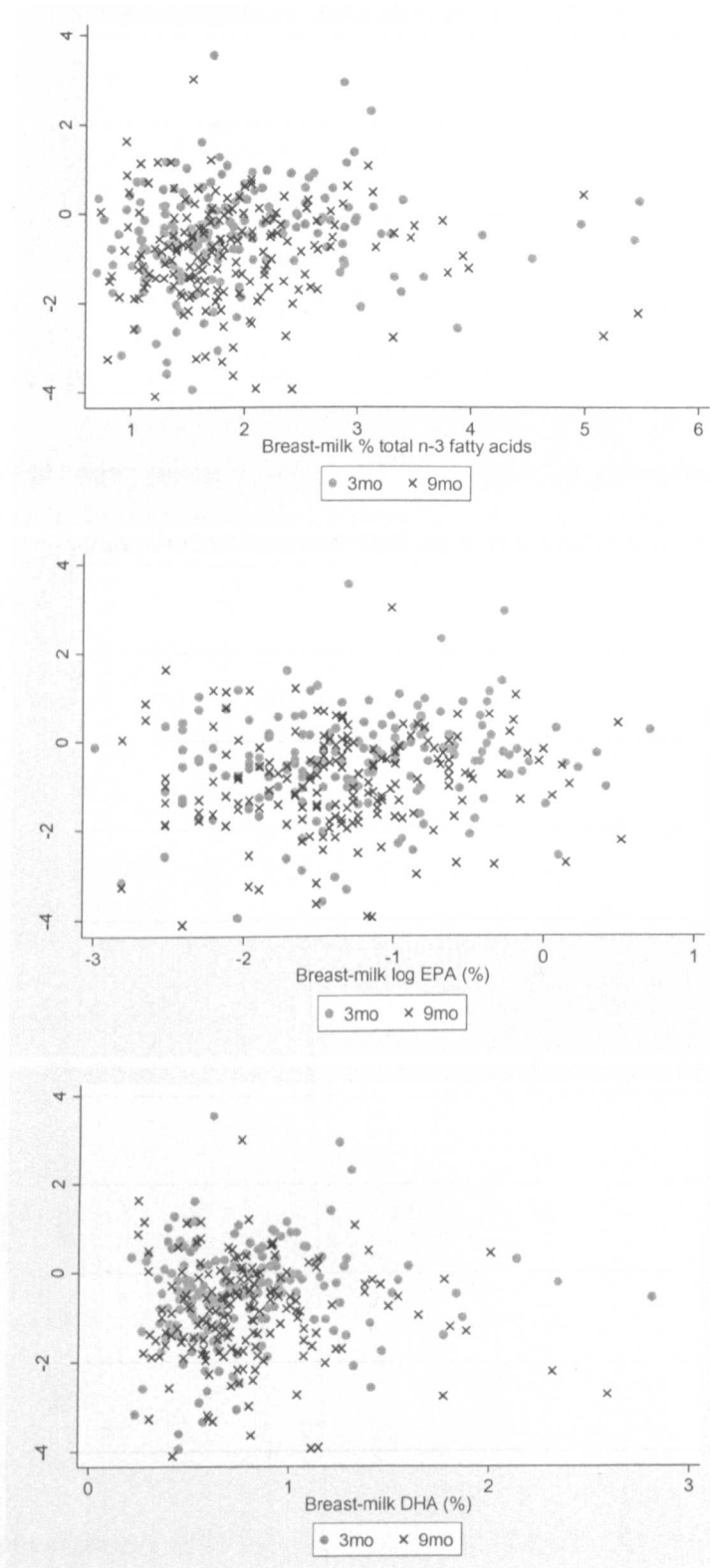
Figure 50: Graphs of height-for-age z-score vs. breast-milk total n-3 fatty acids (top), % EPA (middle), and % DHA (bottom), by time-point
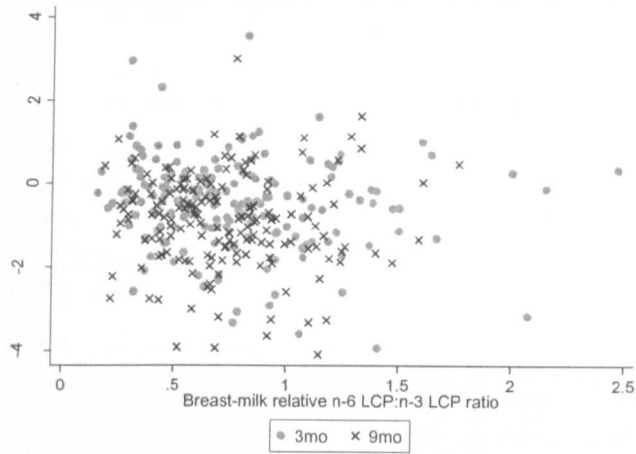
Figure 51: Graph of height-for-age z-score vs. breast-milk n-6 LCP:n-3 LCP ratio, by time-point
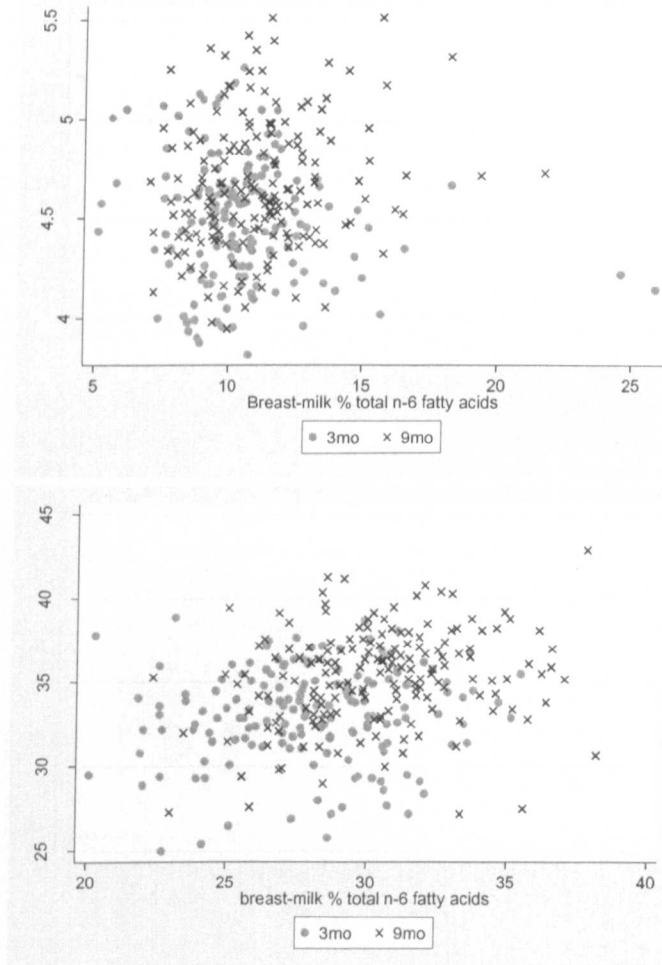
Figure 52: Graphs of plasma AGP (top) and albumin (bottom) vs. breast-milk total n-6 fatty acids, by time-point
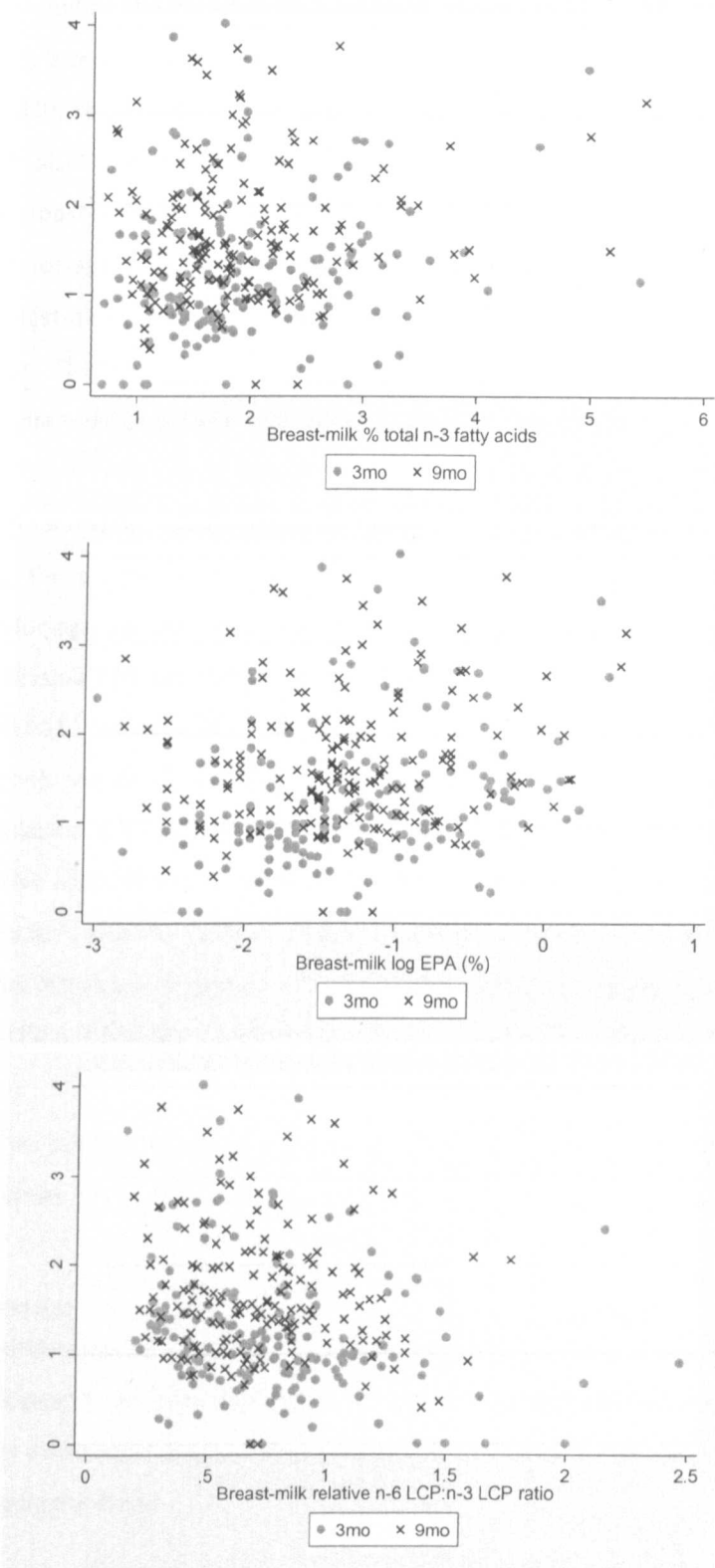
Figure 53: Graphs of plasma CRP vs. breast-milk total n-3 fatty acids (top), breast-milk EPA (middle) and breast-milk n-6 LCP:n-3 LCP ratio (bottom), by time-point

Also when including the data before treatment, plasma total n-3 FAs and EPA significantly predicted MUAC z-scores in a positive direction (95% CI: 0.03,0.12; p=0.001 and 95% CI: 0.02,0.46; p=0.030, respectively). The negative association between plasma n-6 LCP:n-3 LCP ratios and CRP also remained (95% CI: 0.62,0.90; p=0.002). The only relationship which remained with breast-milk FAs was the statistically significant correlation between breast-milk EPA and height-for-age z-score. The relationships between plasma EPA and height-for-age z-score, and breast-milk total n-6 FAs and plasma AGP – which were only marginal before - were, thus, lost. The negative correlations between breast-milk DHA and height-for-age z-score and plasma n-6 LCP:n-3 LCP with plasma albumin were also lost.

Significant relationships which were not observed at 9 mo (after treatment), but indeed when analysing the pooled data, were found between plasma n-3 FAs and weight-for-age z-score, height-for-age z-score, HC-z-score, and CRP (all positive). Further new relationships were those of plasma EPA and DHA with albumin (95% CI: -1.78,-0.245, p=0.010 and 95%CI: 0.46, 1.46, p<0.001, respectively), and plasma n-6 LCP:n-3 LCP ratio with height-for-age (negative), HC (only marginal), and MUAC (negative) z-scores. A weak positive correlation was seen between plasma n-6 FAs and plasma albumin. Concerning breast-milk, new statistically significant positive correlations between breast-milk n-3 FAs and height-for-age z-score (95% CI:0.01,0.21; p=0.026) and CRP (95% CI: 1.03,1.12; p<0.001), and breast-milk EPA and CRP (95% CI: 1.12,1.69; p=0.003) were observed once data were pooled. A negative association between breast-milk n-6 LCP:n-3 LCP and height-for-age z-score and CRP was also observed.

There were no positive interactions between FAs and time-point on any of the significantly correlated outcomes.

4.8.6.5.3 Discussion

No relationships were observed between plasma or breast-milk n-3 FAs and gut integrity, cognitive development or calprotectin concentrations. Breast-milk and plasma FAs did, however, predict anthropometric z-scores (except for skinfold thicknesses) and plasma acute phase protein concentrations.

Apart from the association between plasma EPA and MUAC z-score, there was little evidence that these relationships were due to treatment: associations did not appear to be stronger after treatment, and the interaction between treatment and time-point was never significant.

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Associations with anthropometric z-scores were not strong, though, as demonstrated by low regression correlation coefficients, lack of marked associations in the scatter graphs, and low partial correlation coefficients (where calculated). The limits of 95% CI's also often approached "0" or "1". Given the size of these associations, even those which were strongly significant and even if the relationship were causal, n-3 FAs would thus not have been responsible for large differences in absolute terms.

Acute phase proteins were more strongly predicted by PUFAs in regards to certain relationships. Apart from CRP, which displayed a consistently negative relationship with the n-6 LCP:n-3 LCP ratio both in plasma (in the pooled sample) and breast-milk (in both samples), no clear patterns were discernable. For example, plasma albumin was negatively correlated with plasma EPA but positively with plasma DHA in the pooled sample, and not with plasma EPA or DHA in the 9 mo sample, and, again, due to multiple tests (eighteen outcomes each tested for relationships with six different FA determinants) some of these significant results may have been due to chance.

Although the relationship between treatment and plasma FA levels at 9 mo was generally strong, as shown below in Table 41 and discussed in Section 4.7.1 and Section 4.8.6.3, associations between treatment and most anthropometric outcomes and acute phase proteins were absent. This could be said to be an indication that the relationship between plasma FA levels and these outcomes was likely confounded, and is therefore not causative. The same factors which influenced growth and/or acute phase protein levels either positively or negatively (e.g. poor overall nourishment, physiological stresses), may have influenced plasma FA levels. However, it needs to be taken into account that, given the size of the effects of plasma FAs on anthropometry and acute phase proteins overall, the influence of treatment on anthropometry would not be expected to be very big. The effect of supplementation may have been unable to modify plasma FA levels sufficiently to have a significant effect on these outcomes.

Table 41: Correlation coefficients for plasma fatty acid levels at 9 mo with treatment

Plasma %	r^2 (p-value)	Plasma %	r^2 (p-value)
DHA	0.23 (<0.002)	n-3 fatty acids	0.38 (<0.001)
EPA	0.50 (<0.001)	n-6 LCP:n-3 LCP	-0.40 (<0.001)

4.9   Trial monitoring

There were no concerns expressed by the trial monitors about the progress of the trial, adherence to protocol and MRC GCP requirements, or patient safety through the duration of the trial.

A trial monitoring report serving to inform the MRC of progress of the trial, any significant complaints arising, and any new information that had a bearing on safety or ethical acceptability of the trial, was prepared by the trial monitor, after a site-visit midway, and submitted to the MRC. It can be seen in Appendix 18.

All protocol deviations were reported to and discussed with trial and safety monitors. Two deviations occurred as follows:

Deviation 1

Initially, all infants were recruited from the database of the Peri-conceptual Micronutrient Supplementation Trial, as stated in the trial protocol.

However, for reasons previously explained in Chapter 3, potential subjects were later drawn from the West Kiang Demographic Surveillance System rather than the originally stated cohort.

Deviation 2

A recording error listed a child as being born a month earlier than in reality. This infant was subsequently recruited into the trial a month early, and baseline measurements taken at 2 mo rather than 3 mo of age. The error was later noticed, and reported to the trial and safety

monitors who advised the withdrawal of the infant due to not meeting the age criteria. Treatment was stopped after 3 weeks.

4.10 Safety and ethical concerns

4.10.1 Safety of the supplement oils

There were no concerns about the safety of the supplement arising from trial safety monitoring data. Growth, morbidity, and nurse-visit data did not differ significantly between groups. Furthermore, analyses of outcome data showed no harmful effects of receiving fish oil.

However, giving 2ml of oil to young infants is not without a risk of aspiration or choking. The cases of respiratory complaints was high (roughly 3000 days' worth of reports of this complaint in total), and there was a concern that the oil supplementation may be related to these respiratory complaints. Therefore the incidences of respiratory reports were investigated in relation to the reports of "choking during supplementation."

A total of 40 reports of choking were recorded, distributed amongst 28 infants. These 40 reports were listed, and the first 26 cases - a 65% sub-sample, distributed amongst sixteen infants - examined for coughs or respiratory complaints reported within seven days after the report of choking.

In five of the sixteen infants a respiratory complaint was reported within seven days. These are summarised in Table 42, together with a comparison of average respiratory complaint rates in individuals in the entire population who were ever reported to choke versus those who were not.

The rate of respiratory complaints in infants who had ever choked was not significantly higher than in those who had not (95% CI: -0.24,0.68, p=0.348).[8] In eleven of the sixteen infants analysed, no respiratory complaints were reported in the seven days after the choking incident.

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[8] Using negative binomial regression

Given these figures, it is unlikely that choking on the supplement increased the risk of respiratory complaints.

Table 42: Respiratory complaint reports in individuals who choked versus those who did not

Sub-sample examined: 26 reports of choking distributed across 16 individuals		
Cases of a respiratory complaint being reported within 7 days of a choking report		
	Duration of the respiratory condition (days)	Total cough/respiratory complaint ever reported (days)
Individual 1	2	14
Individual 2	1	13
Individual 3	2	12
Individual 4	1	11
Individual 5	8	62
Average cough/respiratory complaint report rates in days/individual		
Individuals in study population who ever choked ($\overline{\chi}$: SD)		Entire study population ($\overline{\chi}$: SD)
19.6: 15.6		18.3: 15.3

As discussed under "Safety Considerations" in the Methods section, membranes enriched with PUFAs are more susceptible to oxidative damage, and the inclusion of d-alpha-tocopherol in the oils was intended to avoid this potential harm.

When blood was drawn from infants, some degree of erythrocyte haemolysis occasionally occurred. The samples which were haemolysed (observed by a red-tinged plasma) were recorded and compared by time-point and treatment group.

At baseline there were 5 occurrences of haemolysis, compared to 3 cases at endpoint.

At endpoint, there were 2 cases of haemolysis in the control group, and 1 case in the fish oil group.

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These numbers are very small, and no important conclusions should be drawn from them, but nevertheless, these figures indicate it to be unlikely that fish oil supplementation, in the presence of added vitamin E, increased erythrocyte oxidative damage.

4.10.2 Serious Adverse Events

A total of nine SAEs occurred, all of which were reported to the TSM. Four of these were in the treatment group, and six in the control group. These numbers and the diagnoses and relationship with intervention product are given in Table 43.

Table 43: SAEs by treatment group

Fish oil group (n=4)		Placebo group (n=6)	
Diagnosis	Causal relationship with intervention product[1]	Diagnosis	Causal relationship with intervention product[1]
Pneumonia	Unlikely	Pneumonia	Unlikely
Pneumonia	Unlikely	Skull injury	Not related
Pneumonia	Unlikely	Pneumonia	Unlikely
		Severe pneumonia	Unlikely
		Bronchiolitis	Unlikely
SAEs resulting in death			
Nephrotic syndrome	Not related	Sudden collapse & severe respiratory difficulty	Unlikely

[1] As judged by TSM

Two SAEs resulted in death. The first was due to nephrotic syndrome, complicated by diarrhoea, vomiting and bacterial sepsis. The original diagnosis of glomerulonephritis was made shortly after starting supplementation, and not long after a urinary tract infection was diagnosed, prior to supplementation. After careful inspection of the information surrounding the SAE, the (blinded) TSM concluded that it was most likely not related causally to the investigational product.

In the second fatal SAE, the cause of death was not sure, but on the basis of probability was diagnosed as sepsis, the origin of which was unclear (although the chest was the likely source). The case, together with test reports, clinic notes, and medical history, were

thoroughly reviewed by the TSM with assistance from the MRC Keneba medical team. No evidence was found to suggest that the death was related to the investigational product.

Briefly, the child was brought in to the MRC Keneba clinic on 20th and 30th October with fever, couth and vomiting. On 8th November the mother took her child to the resident MRC nurse in her village during mid-morning. The field-worker, aware of the child's critical condition, contacted the PI. The infant was picked up and reached Keneba by noon, suffering from severe respiratory difficulty and sudden collapse. After tests and attempts at resuscitation the child was transferred to a hospital in the country's capital in the afternoon but died en route. It appeared that the sepsis had consumed her lungs and suffocation resulted.

4.10.3 Use of a placebo control group

Infants were not receiving any routine LCP supplementation in The Gambia, and by withholding supplement from placebo groups children were not placed in any danger. Additionally, LCP supplementation is not a proven therapy for infant health and development, so benefit of receiving the intervention could not be guaranteed.

4.10.4 Evaluation of Benefits and Risks[9]

The sample population was a highly vulnerable group of children without recourse to sophisticated medical investigation and treatment. The wellbeing of these subjects was considered the first concern of the investigating team. The trial proposal and protocol passed through scientific and ethical reviews in both The Gambia and the UK, which judged it safe on basis of information provided to them. The key external trial monitoring structures and relevant documentations, agreed in principle with the MRC Senior Scientific Officer for Clinical Trials and TSM and in keeping with the principles of the MRC GCP Guidelines, were in place. These measures all served to minimise any risks associated with trial participation.

The use of low dose supplementation of infant formulae in term and in preterm infants has been the subject of two Cochrane reviews, which concluded that supplementation was safe in the populations of babies on which it was assessed but that its benefits were unproven.

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[9] The author received significant input from the head of clinical services and resident paediatrician in Keneba, Dr Stephen Owens, for this section

Although the supplement was not a pharmaceutical, the study hypothesis stated that it would have beneficial physiological effects on gut immune function and on growth. With such an impact, the possibility that it might also have significant detrimental effects was in turn considered.

These potential harmful effects were discussed in Chapter 3. In short, they included the risk of increased bleeding time (potentially leading to catastrophic effects in the event of Haemorrhagic Disease of the Newborn), oxidative damage, and increased erythrocyte haemolysis. Finally, taking part in the study required the drawing of blood, introducing an additional slight risk of bruising or infection.

However, the magnitude of the increased bleeding risk was thought to be extremely small, particularly at 3 months of age (as discussed in Chapter 3). The addition of the antioxidant d-alpha-tocopherol to the oils offset the risk of oxidative damage and increased erythrocyte haemolysis, and venipuncture was conducted under hygienic standards by a qualified phlebotomist to minimise the (already small) risk of infection.

There was a concern that weaning diets in rural Gambian infants contain sub-optimal levels of n-3 LCPs, and experimental arguments to suggest that supplementing such infants would be beneficial to their gut function and growth. Expected benefits derived from the intervention were improved infant growth rates and gut integrity. Additionally, decreased intestinal and systemic inflammation, improved cognitive development, and lower rates of morbidity were possible advantages expected. Although these benefits were not strongly supported by the literature, if they did occur, they would potentially be highly advantageous to the individual trial subjects. Moreover, it is a generally accepted fact that infants taking part in trials normally enjoy better health than those who are not because of the greater clinical attention they receive.

The rationale for beginning with treatment at 3 mo was to prime and protect the gut before any damage set in, or at least to delay the deterioration of gut integrity in the subjects. However, because exclusive breast-feeding for 6 mo is advised by the National Nutrition Agency of The Gambia to mothers in the community, there were concerns that supplementation before this time would send out conflicting messages, and the suggestion for beginning at 6 mo instead was put forward. By means of negotiations with the Nutrition

Agency, however, it was agreed that the supplement - which would be viewed as a treatment or medication rather than a weaning food, and thus still fall within WHO guidelines – would be administered starting at 3 mo of age, but that a) the MRC fieldworkers administering it would heavily reinforce the message of 6 mo exclusive breast-feeding to mothers, and b) the MRC would pay for the repainting of Baby Friendly Community Initiative billboards/signs in Baby Friendly Community villages.

In light of all of the above arguments, it was considered that the potential benefits in generating knowledge and improving infant health and growth in the communities concerned outweighed the minimal risks involved for the subjects.

4.10.5 Quality assurance

Most of the results of quality assurance carried out have already been discussed above. Only the supplement oils' oxidation values and vitamin E contents measured after 8 mo of storage will therefore be addressed here.

Oxidation values for the supplement oils provided to the PI before the oils were sent to The Gambia were 5meq/kg. In order to comply with Norwegian Medicinal Standards and European Pharmacopoeia Standards, the peroxide value of fish oil has to be lower than 10meq/kg.

After eight months' storage, the fish oil peroxide value was still lower than 5meq/kg. The olive oil was higher, at 10.75 and 9.55meq/kg (Table 44).

Table 44: Average vitamin E and Peroxide values of stored opened and unopened supplement oils

	Average vitamin E % recovery	Average peroxide value (meq/Kg)
Fish oil (opened)	68.5	2.45
Fish oil (unopened)	96	3.1
Olive oil (opened)	66	10.75
Olive oil (unopened)	97	9.55

Fish oil was therefore still fresh at time of administration. Olive oil was on the border of falling above the cut off established for fish oil, but fell well within the range which most sources cite as being acceptable for extra virgin olive oil.

As oils become rancid, antioxidants such as d-alpha-tocopherol become consumed. At least 30% of the vitamin E in the opened oils had been consumed by the time of oil testing. Still, sufficient vitamin E was still left unconsumed for limiting further oxidation and for intake by the infants. How much vitamin E would have been consumed at the end of the storage period, or when opened bottles were nearly empty is not known; but judging from a 2% consumption in closed bottles over a period of 8 mo, and 30% consumption in bottles which had been stored for 8 mo and been in use for a couple of weeks, it seems likely that vitamin E levels at the end of the storage period would still have been adequate to protect both oils and infant tissues.

CHAPTER 5:   OVERALL DISCUSSION AND CONCLUSIONS

5.1 Discussion

Various study aspects, results and considerations have already been discussed in the previous chapter, also in relation to previous published work. Here, therefore, the discussion will be limited to considerations from a broader perspective.

5.1.1 Brief synopsis of key findings

To the knowledge of the author, this is the first study to investigate the effects of n-3 LCP supplementation during infancy on gut integrity, growth, and cognitive development in infants from a developing country. Although the effects of fish oil supplementation on DHA status in Pakistani infants have been investigated (338), previous studies investigating the effects of n-3 LCPs during infancy on the secondary outcomes of intestinal inflammation, systemic inflammation and morbidity in infants from developing countries have not yet been reported.

In the current study, at 9 mo follow-up, fish oil supplementation successfully increased infant n-3 plasma FA status as reflected in significantly higher relative concentrations of plasma EPA and DHA and significantly increased total n-3 FA plasma concentrations in the treatment group. At 9 mo, fish oil supplementation also resulted in a 0.3cm increase in MUAC. At 12 mo follow-up, significant increases in MUAC and subscapular, biceps and triceps skinfold thicknesses were detected in the intervention group. No further effects on growth were detected and the results of the trial provided insufficient evidence to support the primary hypotheses that dietary n-3 LCP supplementation improves rural African infants' growth performance or/and protects mucosal epithelial integrity, even at a very large dose.

Dietary n-3 LCPs did not lead to enhanced cognitive development, reduced degrees of intestinal and systemic inflammation, or reduced rates of morbidities in rural African infants, as measured by the chosen indicators.

Examination of associations between umbilical cord and maternal serum DHA and AA levels with later infant cognitive development provided no evidence of a relationship between these LCPs levels at birth and later development. Similarly, no evidence of associations

between plasma n-3 PUFAs at 3 mo or 9 mo and problem-solving or attention behaviour at 12 mo was found.

Investigating cross-sectional associations between outcome variables and PUFA status, no relationship between plasma or breast-milk PUFA at 3 or 9 mo and urinary LMR, lactulose or mannitol percent recoveries, or faecal calprotectin concentrations were found. Some evidence was found to support the existence of relationships between plasma and breast-milk n-3 FAs and certain anthropometric z-scores, but these relationships were weak. Plasma and breast-milk PUFAs and n-6 LCP:n-3 LCP ratios correlated more strongly with plasma acute phase proteins, but no clear patterns were observed and the associations were likely to be confounded.

Fish oil supplementation, at the chosen dose, proved safe, as indicated by safety monitoring analyses, SAE reports, and outcome results.

5.1.2 Considerations and possible explanations

The reasoning behind the described trial was, briefly, that because of the low n-3 LCP contents of weaning diets coupled with fat losses and malabsorption incurred by high rates of chronic environmental enteropathy and diarrhoea, the n-3 LCP status of rural Gambian infants may be low. A low n-3 LCP status, in turn, could contribute to increased intestinal inflammation, leading to exacerbated enteropathy, and, consequently, a negative influence on growth performance. It was hypothesised that supplementary n-3 LCPs would modulate immune system functioning and so reduce the persistent inflammation associated with environmental enteropathy. Simultaneously, gut damage was hypothesised to be minimised and growth performance improved. It was further hypothesised that an improved DHA status would enhance cognitive development and infant attention control.

However, breast-milk analyses revealed that the average mother's milk was considerably high in EPA and DHA compared to breast-milk of women in most other populations. The majority of infants aged 3 to 9 mo were, therefore, ensured diets supplying adequate amounts of n-3 FAs. Because almost all of the literature on infant fatty acid status in other countries supplies data on infant erythrocyte or plasma phospholipid fatty acid levels (and not total plasma fatty acid levels) and for reasons explained in Chapter 4 under Section 4.7.1.1, it is difficult to make comparisons of the Gambian infants' FA status with other populations. One group in Havana, Cuba, measured the plasma fatty acid status of thirty-one 2 mo old infants

(339) and found the following relative FA concentrations (mean ± SD): EPA 0.41 ± 0.17; DHA 2.82 ± 0.84; and AA 6.35 ± 1.81. Gambian infants, at 3 mo, had the following concentrations: EPA 1.52 ± 1.18; DHA 3.88 ± 0.93; and AA 6.78 ± 1.46. At 3 mo, then, at least compared to a sample of Cuban infants, Gambian infants indeed had far higher plasma n-3 LCP levels. At 9 mo, Gambian infant plasma n-3 LCP means may not be as high compared to, for example Cuban infant means, but it is likely that even at 9 mo of age, when weaning foods contribute a large part of the infant diet, breast-milk supplied most infants with substantial amounts of preformed LCPs even when it was consumed in smaller volumes.

It may be argued, therefore, that the intervention was targeted at infants who were already replete with n-3 LCPs. Had the intervention been targeted at infants who were instead older and fully weaned, or who were at least less dependent on breast-milk for their energy needs, it might indeed have had some of the hypothesised effects in this population. However, the size of the dose was large enough to still further increase in plasma n-3 LCP levels and warrant possible associated benefits. Secondly, delaying the intervention until later would have the disadvantage of necessarily attempting to redress a fully established enteropathy which is slow to repair (16). Once injury and inflammation is initiated in the gut, the vicious cycle of bacterial overgrowth, absorption of foreign proteins and toxins, malabsorption of nutrients, and perpetuation or aggravation of mucosal damage, sets in. Treatment at this stage will thus be required to reverse an extensive degree of mucosal injury which has proven to resist even intensive inpatient treatment with nutritional support, antibiotics, and antihelminthic therapy (16). Nonetheless, the effects of supplementary n-3 LCPs given at this later stage, may, by virtue of raising a low n-3 LPC status and supplementing a low n-3 LCP diet, prove more beneficial than when given at 3 mo, also for other endpoint measures.

Despite the daily health monitoring of infants, good primary health care provision, effective treatment of acute illness, and 2ml olive or fish oil daily, growth remained poor when compared to WHO standards. Given that growth faltering cannot, to any significant degree, be improved by energy or micronutrient supplementation (as shown in numerous studies (13-17)), and that even a high standard of medical care provision - which has previously been shown to have successfully reduced the prevalence of severe illness and mortality in this population (340), is unable to significantly dilute the impact of factors adversely affecting growth, it may be thought that this poor growth is normal for these infants, and that it is not pathological. It may be argued that slower, poorer increases in length and weight may be a

beneficial adaptation allowing the use of limited resources to the best advantage of the population and its individuals.

However, as argued by Poskitt (1999) (26), this is unlikely, given that growth is good during the first 3 or 4 mo of life. Similarly, growth is good during the dry season at later ages. Moreover, some children in the population do indeed grow normally, and the shape of the population growth curve is not equivalent to any known normal infant growth pattern. Furthermore, evidence shows that even mildly malnourished children are at increased risk of death from infection (341) and neurodevelopmental impairment (342), and suffer a greater propensity to conditions such as hypertention (343) and diabetes (344) in later life. For these reasons Poskitt asserts that the average growth patterns of Keneba children is abnormal and pathological.

Tanner (1989) (5), referring the work of Professor John Waterlow (345, 346), suggests that in infants who are malnourished (even to a mild degree), the restrictions on exploration, play and social interaction that this energy limitation causes may be a "more potent cause of delay in intellectual and emotional development than any nutritional effect on the nervous system." If this suggestion were true of the infants in West Kiang, their poor performance on the cognitive development tests would therefore to a greater degree be explained by these limitations on development than by a direct deficiency of nutrients, such as DHA and AA, which are important for central nervous system development. This might explain why some groups of infants in developed countries, who did not suffer the same restrictions to development caused by repeated infections and low-energy weaning foods, benefitted from LCP supplementation (108, 192, 195, 201) while the Gambian infants did not. During the perinatal period, n-3 FAs are required by the membranes of synapses for synaptogenesis. An infant who is thriving should experience a greater speed of synaptogenesis, and therefore has an increased LCP demand, compared to an infant developing less optimally. Alternatively, or in addition, infants in these study groups may have featured baseline tissue LCP levels far below the concentration in breast-fed Gambian infants, resulting in a greater benefit from LCP supplementation in the treatment group.

Another factor which may have diluted the observable effects of treatment was the use of olive oil as placebo. Olive oil, composed chiefly of monounsaturated FAs, is classically used as placebo in fish oil studies because monounsaturated FAs are regarded as being neutral FAs (347-349). Few other placebos offer the advantage of being quite as neutral, while

simultaneously being comparable in energy value to fish oil and suitable for keeping investigators blinded. Nevertheless, despite these advantages, the suitability of olive oil as placebo cannot be guaranteed. The neutrality of the oil may be debated as it has been suggested to indeed feature beneficial properties, including potential immunosuppressive actions. Although these require further investigation, effects of olive oil on immune function in rheumatoid arthritis have been suggested in human studies (350, 351). Oosthuizen *et al.* (1994) (352) found fibrinogen-lowering effects due to fish oil as well as olive oil supplementation, and a letter published in The Lancet, written by a research group who had studied the effects of fish oil in preventing the reoccurrence of blood vessel narrowing after angioplasty (353) suggested that future oil supplementation studies should not consider olive oil as a placebo until more research around the role of monounsaturated FAs in retarding atherosclerosis has been conducted (354). A study investigating the effects of olive oil on immune function (349) showed that, in middle-aged men, monounsaturated FAs alter the FA composition of plasma phospholipids, leading the authors to reinforce the recommendation against the use of olive oil for placebo in clinical studies. The same study found evidence to suggest that monounsaturated FAs affect immune function by decreased expression of some adhesion molecules.

Thus, although olive oil is regarded as a neutral food product, the possibility that it resulted in some beneficial effects in the infants in the control group, and therefore weakened some potential observable effects of fish oil, albeit small, cannot be ruled out.

It was essential for vitamin E (d-alpha-tocopherol) to be given in doses of 2IU/day to infants in both groups for reasons already discussed. Although it is unlikely, the small possibility that vitamin E also had beneficial effects in both groups which could have weakened observable effects of n-3 LCPs warrants consideration. The physiological role and importance of vitamin E remains unclear. A recent review by Traber and Atkinson (2007) concluded that "virtually all of the variation and scope of vitamin E's biological activity can be seen and understood in the light of protection of PUFAs and the membrane qualities (fluidity, phase separation, and lipid domains) that PUFAs bring about" (355). In contrast, Azzi (2007) argues that "α-tocopherol is not physiologically acting as an antioxidant" and that the natural function of α-tocopherol is that of cell signalling (356). The overall results of clinical trials to date do not support the use of vitamin E in cardiac health (357, 358), pregnancy (359), gastroinstestinal cancers (360) or for primary and secondary prevention of various diseases (361). The

reasonable conclusion to be made, therefore, is that it is unlikely that vitamin E improved any of the primary outcomes in infants studied. Due to controversy and uncertainties around its physiological roles, however, there still remains room for speculation around the possibility for unspecified effects to have occurred, whether beneficial or harmful, although the evidence to support this is weak.

5.1.3 Comparison of main findings with relevant findings from other published studies

A large number of early fish oil supplementation studies in relation to infant growth have previously been conducted, as described in Chapter 1. These studies differed from the present one in that infants were all from developed countries, and formula-fed. In these conditions, n-3 LCP supplementation of infant formula did not improve the growth of term infants in all studies considered. In preterm infants, mixed results were found but these infants are not considered comparable with the 3 mo – 9 mo old Gambian infants studied presently. In a study investigating breast-fed infants whose mothers were supplemented with fish oil during lactation (130), an increased subscapular skinfold was detected in infants of supplemented women, but only at follow-up two years after intervention was ceased. This result is similar to that found in the present study, where no difference in HC, growth in length or weight was found, but indeed an increased fatness, which, while not observed at endpoint, was observed in the skinfold thicknesses 3 mo after intervention was ceased.

Fish oil supplementation in relation to chronic environmental enteropathy has not previously been investigated and so no studies are available for comparison with the present study results in relation to n-3 LCPs. Other interventions which have attempted to reduce gut mucosal damage in infants or children from developing counries have, however, been undertaken using some of the following treatments: oral glutamine, probiotics, vitamin A with or without beta-carotene, and intensive inpatient treatment with nutritional support and antibiotics (and antihelminthic therapy where required).

Similar to results from the present study, glutamine administration was unsuccessful in improving gut integrity in Gambian infants (259). Probiotic administration for 30 days had no effect on intestinal integrity of 3 – 5 year old Malawian children (362). In India, vitamin A was given in two separate trials to two groups of infants: hospitalised infants (one 200,000 IU dose)

and infants attending a community health centre (16 700 IU weekly). All treated groups had more rapid improvement in gut integrity than the placebo groups, although no group had urinary LMR that reached those of UK standards (363). In a randomised controlled trial involving women infected with HIV, maternal vitamin A and beta-carotene administration (1.5 mg retinyl palmitate and 30 mg beta-carotene daily, plus 60 mg retinyl palmitate at delivery) was associated with significantly lower lactulose excretion in a sub-group of HIV-infected infants at one and fourteen weeks of age, but treatment did not decrease mucosal damage in uninfected infants (364). High dose vitamin A administration as recommended by the International vitamin A Consultative Group did not increase gut integrity in Gambian infants when compared to the lower WHO-recommended vitamin A dose (258). Intensive in-patient nutritional support with antibiotic medication (16) did not lead to significant improvements in LMRs after a month of rehabilitation. Finally, fish oil may now be added to this list as a further attempted intervention.

In Pakistan, breast-fed malnourished infants from the north of the country were found to have very low DHA status (365). This was linked to the low breast-milk DHA concentrations of their mothers (337). In an attempt to improve DHA status, ten malnourished infants (weight-for-age<-2 SD compared to US National Centre for Health Statistics references) were given 500mg fish oil providing 112mg DHA daily for nine weeks. A 50% increase in red blood cell DHA and total n-3 LCP was found in the treated group, while red blood cell n-6 FA concentrations were not changed. In the present study, significant increases in plasma DHA % were found in infants supplemented with 200mg DHA daily compared to the control subjects. Although the dose was nearly double that given to the Pakistani infants, plasma DHA levels increased by only 11% in supplemented Gambian infants. It is not easy to make valid direct comparisons between plasma DHA and red blood cell DHA levels, yet these results are not surprising considering the low baseline DHA status of Pakistani infants versus that of Gambian infants. The impact of a lower dose in deficient infants can reasonably thought to be greater than that of a higher dose in infants with a higher n-3 LCP status consuming breast-milk rich in DHA.

n-3 LCPs in relation to cognitive development in infants of developed countries have, similarly, shown mostly null effects, although a randomised controlled trial in 44 British term infants randomised to receive formula with or without LCP supplementation showed improved performance on the Willatts Test at 10 mo age (195). British infants were exposed to different

conditions than Gambian infants, however: British infants fed no preformed LCPs were compared with infants supplemented with LCPs. In the Gambian sample, not only were infants tested using a measure developed on infants from the developed world, but all infants in the sample were receiving some degree of preformed LCPs through the breast-milk. It is therefore not surprising that results from these two studies differed. No other fish oil trials in relation to cognitive development in infants in developing countries have been identified by the author for comparison purposes. However, observational studies assessing the relationship between prenatal DHA exposure and later cognitive development have been identified but these were already discussed in Section 4.8.1.

5.1.4 Strengths and limitations of the present study

The present trial was properly randomised and sufficiently powered, treatment allocation was well concealed, and outcome assessments performed blinded to treatment allocations. Randomised controlled trials, by virtue of their randomisation procedure, provide a direct test of the causal hypothesis that a certain exposure affects the outcome. Random allocations assure that the characteristics of participants are likely to be similar across groups at baseline, and so reduce the risk of serious imbalances in factors known or not, which could bias the results.

Random allocation, however, does not protect controlled trials against multiple types of bias which may occur at various stages of its setup, conduct and analysis. Poor compliance, loss to follow-up and exclusions may, for example, bias the results (although randomisation will neutralise at least the last of these). Such biases reduce the validity of inferences which may be made from studies. In the following section the overall strengths and limitations of the present trial will be discussed in the context of its validity, with reference to four different classes of validity.

5.1.4.1 Validity of the trial

Internal and external validity, as described by Campbell and Stanley (1966) (366) and summarised in Box 6, are important elements contributing to the overall quality of controlled clinical trials (367). Conclusion and construct validity were also later described by Cook and Campbell (1979) (368) and will be discussed below.

Box 6: Components of internal and external validity of controlled trials

Reproduced from Juni *et al.* (2001) (367)

Internal validity - extent to which systematic error is minimised in clinical trials:

1. Selection bias: biased allocation to comparison groups
2. Performance bias: unequal provision of care apart from treatment under evaluation
3. Detection bias: biased assessment of outcome
4. Attrition bias: biased occurrence and handling of deviations from protocol and loss to follow-up

External validity - extent to which results of trials provide a correct basis for generalisation to other circumstances

1. Patients: age, sex, severity of disease and risk factors, comorbidity
2. Treatment regimens: dosage, timing and route of administration, type of treatment within a class of treatments, concomitant treatments
3. Settings: level of care and experience and specialisation of care provider
4. Modalities of outcomes: type or definition of outcomes and duration of follow-up

Internal validity

Internal validity refers to the degree of confidence with which one can conclude whether there is any causal relationship between the dependent and independent variables (368). The present trial was little-affected by threats to its internal validity for the following reasons:

1. Subjects were allocated to comparison groups in a non-biased way (computerised block randomisation concealed from investigators recruiting subjects), facilitating the forming of groups that were comparable for potential confounding factors.
2. The PI, fieldworkers, village assistants and other investigators on the ground were blinded to treatment allocation, resulting in an identical division of medical care and other provisions between groups.

3. Outcomes were assessed in a non-biased way, approached identically across groups.

4. Similar compliance rates between groups enhanced comparability.

5. Finally, losses to-follow up were balanced between groups, limiting attrition bias.

These factors strengthened the internal validity so that systematic errors were minimised, and, apart from random errors and chance, any detected group differences could be explained by treatment alone.

External validity

In contrast, the external validity (the extent to which a research finding can be generalised across different circumstances (368)) suffered more threats. The extent to which present results may be extrapolated to apply to other age groups, populations, dosages etc. is not clear. The trial assessed and generated evidence for the effects of a daily dose of 500mg n-3 LCPs in a very specific group of infants living in one division of a small African country. The following questions about the relevance of this study in other settings might be raised:

1. Were the dose altered in its magnitude, DHA:EPA ratio, duration, regularity or mode of administration (e.g. administered via breast-milk), would results have differed, possibly profoundly?

2. Were fish oil to be introduced to children aged 18 mo rather than 3 mo, how would results have differed?

3. How applicable are the results of this trial to infants living in other rural areas of Africa, or indeed the world, where fish and breast-milk intakes, desaturase enzyme activity or other n-3 LCP status influences differ?

4. Would infants with greater or lesser degrees of growth faltering or gut damage, or who do not receive the same care as infants taking part in a study, respond differently to fish oil supplementation?

The generalisability of the results were, thus, limited. Unless the trial is replicated in other settings, or altered to match these determined circumstances of different dosages or durations, the answers to these questions cannot be given.

Statistical conclusion validity

Statistical conclusion validity is the extent to which conclusions about the existence and strength of co-variation between presumed dependent and independent variables can correctly be drawn from the data. Low statistical power, the use of inappropriate statistical tests and low reliability of independent and dependent variables are some of its threats. These and some further threats are summarised in Box 7 and shall be discussed in relation to the present study below.

Statistical power

Insufficient statistical power due to an inadequate sample size may lead to an inability to detect true differences, leading to Type II errors. The present study was sufficiently powered to detect, at 90% power and 5% significance levels, differences, determined before the start of the trial, to be functionally significant. A sample size of 150 would allow the detection of differences of 18 and 13% for change in weight and length, respectively, a 0.60 SD difference in LMRs, and a difference of 2.1 and 6.1 points in the Willatts and Attention tests, respectively, between groups.

Box 7: Threats to statistical conclusion and construct validity

Summarised from Cook & Campbell (1979) (368)

Threats to statistical conclusion validity

1. Inadequate study sample size and power
2. Use of inappropriate statistical tests
3. Multiple statistical testing
4. Poor measurement reliability
5. Poor reliability of administration of treatments
6. Heterogeneous sample

Threats to construct validity

1. Inadequate pre-operational explanation of concept
2. Mono-operational and method biases
3. Hypothesis guessing and evaluation apprehension
4. Experimenter expectancies

The use of appropriate statistical tests

Subjects were randomised at individual level into two treatment groups. The methods used for statistical analysis are described in Chapter 3. Briefly, using intention-to-treat analysis, effect of treatment on individual outcomes was examined using, chiefly, multiple linear or log-linear regression analysis. The outcome measure was added as dependent variable, and relevant covariates and treatment as the independent variables. All covariates were only added if identified as potential variance inflators or/and effect modifiers. CI limits were provided for all effect size estimates. The validity of the following regression assumptions were considered:

a) Sample representativeness: the sample comprised the majority of infants living in the population who met the inclusion criteria, and was thus representative of the population under examination.

b) Variables normally distributed: all variable distributions were examined. Apart from the Willatts Test results, all data were normally distributed or log-transformed, where appropriate, when entered into the regression model. The distribution of the Willatts Test results, and justification for adding the data as a mixed distribution to the model has already been discussed (Section 4.7.2).

c) Linear relationship between dependent and independent variables: all data were plotted before being analysed to examine, amongst others, the relationship between dependent and independent variables. STATA's "qnorm" command was used to draw scatterplots of the standardised residuals, and plots of standardised residuals as a function of standardised predicted values were used to test that relationships were linear (vs. curvilinear).

d) No error in the independent variables: the independent variable of "treatment" and covariates of sex, season of birth, and age of first supplementation were considered error-free.

Baseline values added as covariates were affected by measurement error and were thus not added to the models "error free". The error in the variable "growth" was, however, shown in the previous chapter to be small. FA levels, acute phase proteins, and stool calprotectin concentration measurement errors were difficult to estimate

because no repeat assessments were made. However, the laboratory methods used for their measurement were considered to be reliable, and the measurement errors small relative to the overall sample variation in these outcomes. The size of the errors introduced with the measurement of LMRs was discussed in Chapter 3. These errors increased the potential for both Type II (for LMR) and Type I (for other variables in the equation) errors. Where the concentrations of lactulose, and, to a lesser degree, mannitol, were added to regression models as independent variables, the correlation between the independent and dependent variables was reduced, and the variance introduced by error in the variable concerned possibly apportioned incorrectly. However, when baseline variables were added to regression models, little interest was placed in their effects, as they were only added to reduce the variance. Thus, measurement error in baseline values did little to bias the results.

e) Homoscedasticity: for relevant independent variables, the variance across all observations was assessed by plotting the standardised residuals against the regression standardised predicted value. No clear heteroscedasticity was observed.

Multiple testing

Because multiple testing of various outcomes causes an increased likelihood of falsely concluding that a statistical relationship exists when it does not, and no allowance was made for multiple testing, inferences about secondary outcome significant results had to be made with caution. Clear statements about these limitations were made when discussing results which were not primary outcome measures. There were, however, only a small number of significant results, not averaging more than what would be expected from the number of tests performed.

Reliability of measures and treatment administration

Unreliable measurements may inflate the standard errors and increase the variability of the estimates, increasing the potential for Type II errors. It increases unexplained variation within groups and reduces the power of the analysis. Error is generally inherent to measurement, resulting in often unavoidable imperfections in the reliability of estimates, as demonstrated in the present study where a degree of error did indeed enter into the variables examined. The reliability of plasma and breast-milk FA estimates, e.g., suffered constraints caused by numerous factors influencing measured FA levels, and high degrees of biological variability, particularly in breast-milk. Lipid levels vary due to differences in their absorption and

metabolism, and discrepancies in sample handling, storage, and laboratory methods (93). Efforts were, however, taken to minimise these errors, as described in the Methods Section. These included the collection of and adjustment for baseline information, the use of standardised measurement methods and refined instruments or procedures, and the use of a limited number of experienced and/or well-trained observers. Further efforts included the use of triplicate endpoint measures for all primary outcomes. Where possible, inter- and intra-observer reliabilities were assessed and quantified.

To minimise errors introduced by intervention implementation, treatment administration across all infants was stringently managed by ensuring doses were given only by fieldworkers, daily, using standardised, clearly defined procedures. Compliance was strictly monitored, and when adjusting for compliance in outcome analyses, the findings were not changed.

Sample heterogeneity

Sample heterogeneity also leads to increased unwanted variability. Homogeneity was improved by recruiting subjects within a narrow band of ages (3 mo ±7 days), all living in rural Gambia in communities sharing similar socioeconomic statuses, diets, breast-feeding rates and weaning practices. Sample heterogeneity was further minimised by ensuring adherence to clearly defined inclusion and exclusion criteria and by testing outcomes at similar ages. Variability was distributed evenly across treatment groups by implementing adequate randomisation procedures.

Construct validity

Construct validity of putative causes and effects addresses the conceptual validity of the research findings and so the degree to which interpretations and inferences drawn from the study findings can legitimately be made. It has been described by Barret (1984) (369) as the degree to which the conceptualisations of dependent and independent variables match the operationalisation of the measurements ("and therefore the extent to which the theoretical inferences we draw from the findings are empirically justified"). Some of its main threats, summarised in Box 7, will be discussed below.

Inadequate pre-operational explanation of concept

Proper analysis of the theoretical concept of the study and adequate conceptualisation of the dependent and independent variables are required for adequate pre-operational explanation of how the independent variable influences the dependent one.

The theoretical basis of the present study is described in Chapter 1. It was reviewed and approved by three panels on the basis of its scientific credibility (candidate's advisory committee and upgrading panel; MRC Scientific Coordination Committee), and two committees further endorsed the ethical justification for conducting the trial (London School of Hygiene and Tropical Medicine and Gambian Government ethics committees).

Many reasons exist why fish oil may be beneficial to the health and development of infants, and the physiological mechanisms of dietary LCPs have been well-described. The independent and dependent variables were relatively simple constructs, limiting the complexity required to conceptualise the influences of treatment on outcome variables.

Mono-operational and mono-method biases

When only one aspect of a dependent variable is measured, mono-operational biases can result. When only one form of assessment is considered to study the dependent variable while investigating treatment causal effects, mono-method biases can result (369).

Various dimensions of growth were measured, using validated techniques. Overall gut integrity was measured as a function of villous atrophy and intestinal leakiness. The dual-sugar permeability test assesses both gut integrity and absorptive capacity, and has been used in several studies characterising the aetiology of growth failure in The Gambia (30) and elsewhere (37, 38). Three different plasma acute phase proteins were measured for the secondary outcome assessing systemic inflammatory and acute phase markers. For morbidities, complaints of overall unwellness were assessed, and four common complaints as further components of this outcome. These variables, whether by their specific nature or by being assessed by multiple measures, therefore likely suffered only trivial degrees of mono-operational or mono-method biases.

Plasma FAs were measured to indicate infant FA status, and to assess corresponding changes due to treatment. Plasma levels of individual FAs reflect short term intakes over the

last few days (93) and thus are not an ideal indicator of overall FA status. (Adipose tissue is the preferred medium for the measurement of FAs as a reflection of long-term dietary intakes, but because of its invasive nature, its use is limited in studies involving infants). As the distribution of FAs differ among different biological compartments (280), the measurement of plasma FAs as the only indicator of FA status could lead to mono-method and operational biases if the outcome assessed was "(overall) fatty acid status". However, by confining the outcome definition to "plasma FA status" this kind of bias was reduced.

Cognitive development was assessed by two aspects of development: attention and problem-solving behaviour, representing a limited scope of cognitive functions and abilities. The narrow range of cognitive aspects measured were chosen for reasons already described, but the limitations of this decision were that construct validity of the study may have suffered because e.g. differences may indeed have occurred in other, unmeasured domains of cognitive functions, or in the functions tested if tests of a different nature were used. The same holds true of calprotectin as a measure of intestinal inflammation. It is only one aspect of a complex outcome, measured using one method only, and thus the extent to which the inferences about fish oil supplementation in relation to intestinal inflammation (as opposed to "stool calprotectin levels") may be drawn from the findings in this study are restricted.

Hypothesis guessing and evaluation apprehension

This threat is introduced when subjects guess the hypothesis being tested, and adjust their behaviour accordingly. The infants in this study were too young to guess the hypothesis and so influence outcomes assessed. Although evaluation apprehension may have affected morbidity assessments, it is unlikely that the effect was different between the two treatment groups.

Experimenter expectancies

Because investigators may expect a particular group of subjects to perform more favourably in outcome assessments, their observations may become biased. However, these expectancies were not able to influence the results of the present study as all investigators were blind to the treatment status of the infants.

5.1.4.2 Other strengths and limitations

Apart from the topics covered by the section above, other trial strengths and limitations include the following:

The cognitive tests used on these Gambian infants were developed on infants living in the UK and USA, where brain development and maturity possibly follow different trajectories to that in Gambian infants, and so this outcome may not be measured as sensitively by these tests in a rural African population. Inferences and comparisons with other studies are thereby limited.

The placebo used as control, although suitable for blinding purposes, may have contained compounds that benefited infants in unknown ways. The antioxidants supplied by both oils (0.5% rosemary oil and 1IU vitamin E/ml) may, similarly, have provided unidentified benefits (or harm) to both groups of infants, which could have diluted treatment effects.

As briefly discussed above, plasma FAs reflect short term FA intakes and are thus not an ideal indicator of overall FA status. Animal studies provide evidence that FA uptake kinetics differ across tissues, and that plasma does not reflect whole-body equilibrium (370). Although plasma n-3 FA levels were increased by supplementation, therefore, the true n-3 LCP status of infants could not precisely be known. The assessments of the effect of treatment on true overall FA status, and subsequent cross-sectional associations with outcome variables, could thus have suffered a compromised validity, i.e. they may not have been measuring what they were intended to measure.

The age-group assessed was probably inappropriate, given that breast-milk was high in n-3 LCPs and intakes at this age were still important. This assumed weakness could have been avoided had the initial pilot study breast-milk data been available at a sooner point. Analyses would have indicated that Gambian mothers' breast-milk was rich in LCPs and this would likely have led to a decision to increase age of recruitment into the study, and so the age of treatment initiation, to when breast-milk intakes were trivial. Because of time constraints placed on the PI for a timely completion of her PhD, it was imperative to continue with ethical approval seeking and trial set-up procedures even though this data were outstanding. However, the consequences of having proceeded regardless demonstrates the potential dangers of pursuing a trial without first having access to important relevant information, and the author has learned a valuable lesson through it.

5.1.5 Implications of the study

Despite supplementing young infants with a very large dose of fish oil, remarkably little effect was observed on the tested outcomes. The 95% CI calculated for most endpoints were, overall, fairly narrow, providing reasonable certainty that the differences observed were minimal. Previously there have been theoretical reasons for supposing that n-3 LCP supplementation might have observable impacts, but data have now been produced to disprove this.

Because so many interventions have failed to improve the growth and gut integrity of Gambian infants, it was hoped that n-3 LCP supplementation would indeed provide some beneficial impact. However, although n-3 LCP-rich fish oil was not successful at having the hypothesised effects at the end of the follow-up period, the study will contribute to the scientific body of literature by being the first trial addressing this specific subject, and providing definitive answers on it. Furthermore, the study will contribute to scientific knowledge by raising further questions; these questions, in turn, may lead to subsequent investigations which will build on the topic of fish oil in relation to infant health and development, and the subject of growth faltering and environmental enteropathy.

Certain analytic and measurement techniques (e.g. stool calprotectin assay, knee-heel length measures, problem-solving and attention behaviour assessment) have been tested or refined by this study, and data to support their validity or question it generated. For instance, triplicate measurements at baseline and endpoint produced good data on the reproducibility of the dual-sugar-permeability test, indicating its susceptibility to errors and thus its clear limitations to research studies when applying the methods described. Finally, this study has yielded data on African infants that were not previously available.

5.1.5.1 Research implications and recommendations

Consideration of single nutrients in isolation does not take account of the influence of other nutrients in the diet, or combinations of nutrients. Jackson (2001) writes that deficiency in one nutrient may act as a marker of other dietary deficiencies, and that focusing on single nutrients limits the understanding of nutrient-nutrient interactions. Such an interaction may, for example, allow the effect of a limitation of one nutrient to be buffered by the ready

availability of another (371). The significance of nutrient-nutrient interactions in investigations of n-3 LCPs in relation to infant health and development, which may be highly intricate, is not known in any detail. This minimises the degree of inference that can be made from a trial supplementing with n-3 LCPs in isolation, without considering levels of other nutrients at the same time. An infant may have high plasma n-3 LCP concentrations, for example, but because of deficiencies existing in other nutrients or extra nutritional constituents (called "bioactive compounds" (372)) which may be necessary for proper usage of these fats or to act as co-factors in reactions in which LCPs are involved - facilitating their conversions or incorporations into cellular components - the benefits of supplementary LCPs may be restricted. Further knowledge of nutrient-nutrient interactions in this area may, in the future, facilitate trials which investigate n-3 LCPs and infant health in a broader context, and it is thus recommended that it be studied in greater depth.

The present study has suggested that fish oil administration may have a delayed effect on growth, as demonstrated by increases in skinfold thicknesses and MUAC seen at 12 mo but not at 9 mo of age. The n-3 LCP-supplemented infants had significantly more dilute urine than the olive oil-supplemented infants, as indicated by urinary mannitol concentrations. The implications of these finding are not known but could potentially be important, leading to investigations which further elucidate the still unknown impacts of fish oil.

Compared with populations already investigated, the breast-milk n-3 LCP levels of Gambian women were found to be of the highest in the world. The reasons for this are not certain. The Gambian diet is not considered to supply large quantities of n-3 LCPs, although precursor n-3 FAs are regularly consumed via green leafy vegetables. Conversion efficiency may be particularly high in these women, possibly due to polymorphisms of the *FADS1* and *FADS2* gene clusters mentioned above. Answers to these speculations remain to be given.

Cognitive maturity of the studied infants, as measured by the Willatts Test was found to lag far behind other population groups. The accuracy of this observation will require confirmation, but, if found to be true, reasons for this delay might be investigated and consequently lead to effective interventions for promoting optimum infant cognitive development in rural Gambian communities. The foundation for such future studies in rural Africa has now been laid by ground-work carried out during the pilot and main studies. It is

recommended that the predictive power of these tests for later cognitive performance in African infants be investigated.

The impact of early n-3 LCP supplementation may change over time, suggesting a physiological effect at the gene level. Not only might growth effects be delayed, but also effects on other outcomes such as cognitive development. Helland *et al.* (2001), for example, supplemented pregnant women with fish oil until 3 mo after delivery. At 6 mo of age no cognitive benefit was detected in infants of the supplemented group (172). However, at 4 years of age, children born to fish oil supplemented mothers showed significantly higher intelligence quotient scores than children born to mothers given placebo (173). In contrast, Agostoni and colleagues, using the Brunet-Lezine developmental quotient, reported a positive effect of supplementation at 4 mo (192) but not at follow up at 1 and 2 years (191). It is therefore recommended that these Gambian children be followed longitudinally to examine whether benefits which were not seen in the first year of life may manifest at a later age. Such a follow-up would also provide insights into the long-term effects of n-3 LCPs on skinfold thickness and MUAC measures (i.e., whether they are maintained, fade away, or become more pronounced).

A better understanding of the cause and pathogenesis of environmental enteropathy is recommended for assisting in the development of effective interventions. Etiological assumptions should be confirmed or refuted, and the mucosal immune response studied in further depth. The identification of specific gut pathogens leading to gut damage and consequent immunoprophylactic approaches targeting these and established pathogens are recommended. The increased power of analytical methods for studying the human microbiome may facilitate such exploration. The importance of attempts to address the worldwide problems of poor hygiene and sanitation - which lead to enteric infections in the first instance - should not be undermined, and their priority should be considered high.

5.2 Conclusions

A significant increase in relative concentrations of plasma n-3 LCPs were observed when providing a daily supplement of fish oil providing 500mg combined DHA+EPA to breast-fed rural African infants. No clear associated improvements in gut integrity and growth at 9 mo of age, and cognitive development at 12 mo of age, were observed when compared to endpoint measures in the olive oil-placebo control group. Although daily fish oil supplementation proved safe, no benefits were demonstrated in further outcome measurements of intestinal inflammation, acute phase plasma levels, and morbidity patterns, and the results of the trial do not support the use of supplementary n-3 LCPs in young, breast-fed, rural Gambian infants for the improvement of health and development. n-3 LCPs remain critical to human health and development, but breast-fed infants in rural Gambia are ensured an adequate supply of pre-formed LCPs by mother's milk which in the present sample proved considerably rich in these FAs.

Growth faltering and environmental enteropathy is the result of a complex set of mechanisms involving varied physiologic and environmental interactions, and much investigation is still needed before a full understanding can be reached. Although environmental enteropthy appears to be asymptomatic, its impact on growth remains big, and its full impact on the child cannot be known until an effective intervention to ameliorate or prevent the condition is found. The author believes that the problem should thus continue to be explored for the benefit of future generations in The Gambia and elsewhere.

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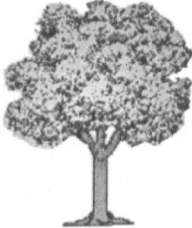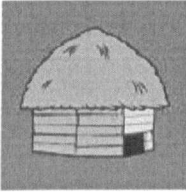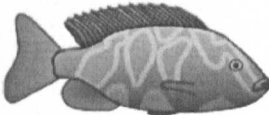
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APPENDICES

Appendix 1.   Treatment code pictures

Here in black and white but ordinarily in colour, representing treatment group allocations.

The labels below also appeared on the supplement bottles.

Infant Omega 3 Supplementation Study	Infant Omega 3 Supplementation Study
Tree	**Hut**
Infant Omega 3 Supplementation Study	Infant Omega 3 Supplementation Study
Fish	**Football**

Appendix 2.   Example of the treatment cards given out to the mothers

The image (ordinarily in colour) was folded onto the back of the card so that the PI was shown only the front of the card displaying demographic details, when needed.

Appendix 3.   Label which appears on the commercial Nordic Naturals "Omega-3 liquid"

Pure and Great Tasting Omega Oils | Pharmaceutical Grade* | Distilled for Purity

Nordic Naturals Omega-3 formula is a great tasting, fresh and pure source of Omega-3s and vitamin E. Omega-3s support proper cellular flexibility and cardiovascular health.*

*All Nordic Naturals products surpass all national and international pharmaceutical standards for freshness and purity, and are free from heavy metals, dioxins, and PCBs. Every batch is third party tested to guarantee exceptional freshness and purity levels.

Suggested Use: One-half to one teaspoon daily, with food, or as directed by your health care professional or pharmacist.

Do not take if tamper evident seal is broken or missing

Refrigerate after opening

Best if used within 3 months after opening

Warning: Consult with your physician before using this product if you are pregnant, diabetic, allergic to iodine, using blood thinners, or anticipate surgery.

Keep out of the reach of children

* These statement have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.

Distributed For the U.S. by:
NORDIC NATURALS INC.
Watsonville, CA 95076
Customer Service
800.662.2544
www.nordicnaturals.com

7 68990 02763 5

Natural Triglyceride Form

NORDIC NATURALS ®
OMEGA-3
LIQUID
FROM PURIFIED FISH OIL
With Enhanced Stabilizers

great lemon taste!

Dietary Supplement

Meets our daily needs for
Healthy Omega-3 levels

8 Fluid Ounces • 237 Milliliters

Supplement Facts

Serving Size: 1 Teaspoon (5 ml)   Servings per Bottle: 48

Amount Per Serving		% Daily Value*
Calories	45	
Calories from fat	45	
Total Fat	5.0 g	8%
Saturated Fat	1.5 g	8%
Trans Fat	0 g	†
Vitamin E (d-alpha tocopherol)	30 I.U.	100%

Omega-3s	Weight‡	Volume %
EPA (Eicosapentaenoic Acid)	825 mg	18%
DHA (Docosahexaenoic Acid)	550 mg	12%
Other Omega-3s	350 mg	8%
Total Omega-3s	1725 mg	38%
Oleic Acid (Omega-9)	290 mg	6%

* Percent Daily Values are based on a 2,000 calorie diet.
† Daily Value not established.   ‡ Natural Triglycerides.
Less than 5 mg of Cholesterol per serving.

Ingredients: purified deep sea fish oil (from anchovies and sardines), natural lemon oil, d-alpha tocopherol, rosemary extract.

No gluten, yeast, milk derivatives, artificial colors or flavors.
Contains vitamin E derived from refined soybean oil.

Please ♻ this container

ITEM #35

Appendix 4.   Information sheet & consent forms: cognitive development tests

SUBJECT INFORMATION SHEET
Developmental outcomes testing
(to be read to mothers in their own language)

You/your child are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to listen to this carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?
Some special kinds of oils contain benefits for the development of a child's brain. In order to test whether the oil your child was supplemented with for 6 months has had an effect on the development of his/her brain, we would like to observe them to measure their attention and problem-solving behaviour. You/your infant have been chosen to participate in this study as a follow-up from the omega-3 study you were involved in before.

The study involves the following if you take part:
We will provide transport and ask you to bring your infant to MRC Keneba on one occasion for a morning.

One of the Keneba nurses will examine your child to ensure he/she is healthy. It your child is found to be ill, they will not be tested although we may ask you to bring him/her in once they have recovered. If your child is ill when they visit Keneba they will be treated.

You will be seated at a table with your child while he/she plays with a toy and his attention to the toy is observed as he plays.

Three simple tests will be given to your child in which the toy is removed and he/she has to try and get it back.

Your child will be filmed as he/she plays, so that we can watch the test again on another day. This film will only be viewed by the project co-ordinators and will assist us in evaluating the children's behaviour.

All information which is collected about your child during the course of the research will be kept strictly confidential. You/your child will be identified by an ID number.

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Your decision will not affect the medical care we provide you in any way.

Thank you very much.

Principal Investigator: Liandré van der Merwe ; MRC Keneba ; tel 9878099

CONSENT FORM
Developmental outcomes testing

The information sheet has been read to me and I understand it. I understand what participation in the study means for me and the infant in my care. I understand that the information that is collected in the course of this study will remain confidential.

I understand that if I or the infant in my care gets sick during the study period, we can go to the clinic where study staff are providing care, and be examined and treated for free.

I understand that we are free to take part in the study or refuse, and that we can withdraw from the study at any time, and without giving any reason. Deciding not to take part or to withdraw from the study will not affect the care that we are normally entitled to.

I have had a chance to ask questions and have them answered.

Signature or thumb print of volunteer: _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

_____

This form has been read by / I have read the above to

_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _
(write name of volunteer)

in a language that he/she understands. The volunteer has understood what I have explained and he/she has freely agreed to take part in the study.

Name of infant: _

Signature of PI: _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

Name of field worker: _

Date: |__|__| / |__|__| / |__|__|__|__|

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Appendix 5. Mother's education questionnaire

Omega-3 Study

Mother name:
Baby name:
Village:
Study number:

What is the highest level of education you attained?
(Tick as appropriate)

Grade	Arabic	English	Any comments
0			
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			

List any other education (type and duration):

Appendix 6.   Detailed scoring instructions for the Willatts test

(Compiled by Dr Peter Willatts)

CLOTH (SUPPORT) STEP

CLOTH BEHAVIOUR – continue scoring until cover/base is contacted or trial ends.

Score 0
- No contact with cloth.
- Cover/base not within reach.
- Infant plays with or examines the cloth.

Score 1
Infant pulls the cloth without any play or examination, and brings the cover/base within reach, but:
- Infant begins an activity which might be play or examining, but does not carry it through to completion.
- Infant lets go of the cloth for more than 1 sec before the cover/base comes within reach.

Score 2
- Infant pulls the cloth without any play or examination, and brings the cover/base within reach. Short breaks in contact of less than 1 sec are allowed, provided the infant immediately regains contact. Pauses of any duration between movements of the cloth are permitted.

FIXATION – continue scoring until cover/base is contacted or trial ends.

Score 0
- Infant looks away from the cover/base for more than 2 sec.

Score 1
- Infant briefly looks away from the cover/base, but looks back within 2 sec.

Score 2
- Infant fixates the cover/base continuously.

COVER/BASE BEHAVIOUR – continue scoring until cover/base is picked up or trial ends.

Score 0
- Infant fails to contact the cover/base, or only touches the cover/base and makes no attempt at grasping.
- The cloth is pulled too far and the cover and toy are dragged off the edge of table.

Score 1
- Infant attempts to grasp the cover/base, but does not pick it up.

Score 2
- Infant grasps the cover/base and picks it up.

COVER (SEARCH) STEP

COVER/BASE BEHAVIOUR – continue scoring until toy is contacted or trial ends.

Score 0
- No contact with cover/base.
- Infant touches the cover/base, but fails to reveal the toy.
- Infant plays with or examines the cover/base.

Score 1
Infant removes the cover or tips the base to reveal the toy without any play or examining, but:
- Infant begins an activity which might be play or examining, but does not carry it through to completion.
- Infant knocks the cover away.
- Infant grasps the cover/base, but lets go for more than 1 sec before the toy is revealed.

Score 2
- Infant removes the cover or tips the base to reveal the toy without any play or examining. Short breaks in contact of less than 1 sec are allowed provided the infant immediately regains contact.

FIXATION – continue scoring until the toy is contacted or trial ends.

Score 0
- Infant never looks at the toy (either because it is not uncovered, or it is uncovered and ignored).
- From the moment the toy is uncovered or tipped out, the infant looks away from it for more than 2 sec.

Score 1
- From the moment the toy is uncovered or tipped out, the infant briefly looks away from it, but looks back within 2 sec.

Score 2
- From the moment the toy is uncovered or tipped out, the infant maintains continuous fixation on it until it is contacted or the trial ends.

TOY BEHAVIOUR - continue scoring until toy is picked up or trial ends.

Score 0
- Infant fails to contact the toy, or only touches it and makes no attempt at grasping.

Score 1
- Infant attempts to grasp the toy, but does not pick it up.

Score 2
- Infant grasps the toy and picks it up.

4

ONE-STEP PROBLEMS (PRETESTS)

BARRIER

BARRIER BEHAVIOUR – continue scoring until toy is contacted or trial ends.

Score 0
- No contact with barrier.
- Barrier not moved sufficiently to permit access to toy.
- Infant plays with or examines the barrier.

Score 1
Infant moves the barrier sufficiently to permit access to toy, and does not engage in any play or examination, but:
- Infant begins an activity which might be play or examining, but does not carry it through to completion.
- Infant lets go of the barrier for more than 1 sec before moving it away.

Score 2
- Infant moves the barrier sufficiently to permit access to toy, and does not engage in any play or examination. Short breaks in contact of less than 1 sec are allowed, provided the infant immediately regains contact.

FIXATION – begin scoring when the barrier is first contacted (or when barrier comes within reach if no contact) and continue until toy is contacted or trial ends.

Score 0
- Infant looks away from the toy for more than 2 sec.

Score 1
- Infant briefly looks away from the toy, but looks back within 2 sec.

Score 2
- Infant fixates the toy continuously.

TOY BEHAVIOUR – continue scoring until toy is picked up or trial ends.

Score 0
- Infant fails to contact the toy, or only touches the toy and makes no attempt at grasping.

Score 1
- Infant attempts to grasp the toy, but does not pick it up.

Score 2
- Infant grasps the toy and picks it up.

NOTES

1. Cover/base behaviour may receive different scores on each of the cloth and cover steps. For example, on the cloth step the infant may pick up the cover and play with it. A score of 2 would be given for cover/base behaviour (infant retrieved the cover/base at the conclusion of the cloth step), but a score of 0 would be given for cover/base behaviour on the cover step (infant played with the cover/base at the start of the cover step).

2. Play or examining behaviour includes:

- taking the object to the mouth
- striking the object
- scratching the surface of the object
- shaking or waving the object
- manipulating, crumpling, or fingering the object.

3. Also record:

(a) whether the cover was brought within reach (✓ x).

(b) whether the cloth was pulled too far so that the cover and toy were dragged off the table (✓ x).

(c) whether the cloth base was tipped to reveal the toy (✓ x).

4. If the infant pulls the cloth too far and the cover and toy fall off the table, give 0 scores for all behaviours on the cover step, and a 0 score for cover/base behaviour on the cloth step.

5. The coding scheme for the cover step allows for two methods of retrieving the hidden toy. Infants may reveal the toy by either removing the cover, or grasping the base of the cloth and tipping it so that the toy falls out. Either method is acceptable. Tipping is relatively infrequent and occurs on about 10% of trials.

6. The trial starts when the infant first makes any contact with the barrier. If the infant fails to contact the barrier, the start of the trial is the moment when the barrier first comes within reach. Trials last for a maximum of 30 sec. If the trial continues beyond 30 sec, scoring ends when the maximum time has elapsed.

7. Toy retrieval is defined as either an attempt at grasping or success at picking up the toy (i.e., toy behaviour score of 1 or 2).

MEASURES

We use two measures of means-end behaviour: total intention score and number of intentional solutions.

Total intention score on each step of the problem is defined as the sum of the 3 intention scores relevant to the step, averaged across trials (range 0 – 6). Intention scores are calculated for each step of the problem, and the total intention score for the entire problem is defined as the sum of the average intention scores on the barrier, cloth and cover steps (range 0 – 18).

Intentional solutions on each step are defined as the number of trials on which there is evidence of intention (scores of 1 or 2) on all 3 behaviours relevant to the step. Intentional solutions on the entire problem are defined as the number of trials on which an intentional solution occurred on each of the barrier, cloth and cover steps. This method of combining intention scores into a single measure offers a better way of capturing qualitative differences in behaviour than can be achieved by summing intention scores (total intention score). Total intention score might reflect the greater contribution of one behaviour over another, and would not necessarily be related to overall level of behaviour because the same total score could occur at different levels. An infant might retrieve the toy on every trial, but the number of intentional solutions indicates how often this was accomplished by intentional means-end behaviour. rather than by transitional behaviour.

CODER RELIABILITY

We normally have one observer score all the trials (this is usually the tester), and as a check on the reliability of scoring, a second trained observer scores a random sample of one third of all sets of trials. The second observer is blind to the aims of the study. We use a chance-adjusted statistic (Kappa) to estimate reliability on the percentage of agreement on intention scores for each of the 9 behaviours and identification of intentional solutions.

ONE-STEP PRETESTS

Infants' performance on the one-step pretests may be also be obtained using the scoring procedures given on pages 4 - 6.

Scoring sheet for the two-step problem

Cloth (Support) Step

	Trial						
	1	2	3	4	5		
Cloth							
Fixation							
Cover/base							
Within reach?							
Pull off table?							

Cover (Search) Step

	Trial						
	1	2	3	4	5		
Cover/base							
Fixation							
Toy							
Tip?							

0: no intention; 1: possible intention; 2: clear intention

Appendix 7.   Attention Assessment Scoring Sheet Example

Baby Number	Name	Age in days	DOB	Mother's ID	Date of Test		
					30/07/2008		
Trial Duration	Total Look Time	Total Looks	Mean Look Time	Looks Away	Inattention Rate	Max Look	First Look
300.0	252.88	16	15.81	15	3.56	67.40	28.68

Trial	Frame
Trial start	51403
Trial end	58903
Max end	58903

Looks	START		END		Look	Away
1	51403		52120		28.68	1.48
2	52157		52328		6.84	1.56
3	52367		52410		1.72	3.72
4	52503		52535		1.28	9.28
5	52767		53366		23.96	1.28
6	53398		53738		13.60	7.00
7	53913		55598		67.40	1.40
8	55633		56146		20.52	1.80
9	56191		56237		1.84	3.04
10	56313		56566		10.12	2.60
11	56631		57142		20.44	5.68
12	57284		57479		7.80	1.24
13	57510		57584		2.96	3.56
14	57673		57835		6.48	1.68
15	57877		58069		7.68	1.80
16	58114		58903		31.56	0.00
17					0.00	0.00
18					0.00	0.00
19					0.00	0.00
20					0.00	0.00

Appendix 8.   Morbidity questionnaire administered to mothers daily

Notes:

1. Dates and child details were pre-printed on the form.

2. Almost all the columns require either a yes or no answer. The exceptions are:

Supplementation problem – choose one from the list supplied. If the reason is not given there then supply the reason under items 5 or 6 and give the appropriate number in the supplementation problem column.

Other illness – here in the column you enter a letter corresponding to the condition you write in the list below. E.g. if on Monday the child is suffering from conjunctivitis then "conjunctivitis" is written against the letter "a" in the list and the letter "a" is written in the column. If on Tuesday the child still has the condition then you simply write the letter "a" in the column.

3. If the child is ill enough to require a doctor's attention, then a separate form should be filled in in order to request this. This would be sent back to Keneba immediately.

4. The supplementation and morbidity record forms are returned to Keneba at the end of each week for data entry.

IN3SS 2007 : Compliance and Morbidity Questionnaire

Study ID NO [LMS015Q]   Week beginning: [   ]   List of Monday's date [   ]   Name [   ]   Sex [F]   DOB [   ]

Mother [27-038-011N]   Mother Name [   ]   Father [   ]   Village [Janneh-Ku]   [01/03/2007]

Day of week	Date	Supplement Received	Supplement Problem	Unwell last 24 hour	Diarrhoea	Vomiting	Cough and or rapid breathing	Fever	Nose-bleeds or abnormal bleeding	Others Specify Below	Interviewer
Monday	11/08/2007										
Tuesday	12/08/2007										
Wednesday	13/08/2007										
Thursday	14/08/2007										
Friday	15/08/2007										
Saturday	16/08/2007										
Sunday	17/08/2007										

Supplementation problem codes:

0 = no problem
1 = child not found
2 = child travelling
3 = vomited oil out
4 = oil spillage
5 = other a: ....
6 = other b: ....

Specify other illnesses:

a = ...
b = ...
c = ...
d = ...

Appendix 9.   Morbidity/adverse event form

MORBIDITY/ADVERSE EVENT FORM
IN3SS – SCC 1061

Study No		Date of Birth	$\frac{\quad / \quad / \quad}{\text{(dd/mm/yy)}}$
Mother's name		Date of Report	$\frac{\quad / \quad / \quad}{\text{(dd/mm/yy)}}$
Child's name			
Interviewer's name			

DIAGNOSES:	KENEBA DIAGNOSTIC CODE:	SUMMARY/DESCRIPTION (please include details of history, treatment/referral etc.):

INVESTIGATOR/SAFETY MONITOR'S COMMENT:

Appendix 10.   Weaning foods questionnaire

IN3SS Introduction of weaning foods questionnaire

Infant name:

Study number:

Today's Date:

Child's age
3mo |
4mo ⌐
5mo |
6mo ⌐
7mo |
8mo ⌐
9mo |

Have you given your child any food/drink other than breast-milk (e.g. water or mono?).

YES/NO_____

If Yes, what?

Water	☐
Juice	
Tea	☐
Cow's milk (includes powder milk)	☐
Formula milk	☐
Mono/porridge	
Bread	☐
Mango	☐
Other fruit	☐
Egg yolk	☐
Rice dishes:	
Kino	☐
Tulo	☐
Tulsay	☐
Durango	☐
Jambo	☐
Nyankatang	☐
Fuuto	☐
Anything else?	

Interviewer name: _____

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Appendix 11. Ethical Approval obtained

LONDON SCHOOL OF HYGIENE
& TROPICAL MEDICINE

ETHICS COMMITTEE

APPROVAL FORM
Application number: 5072

Name of Principal Investigator   Liandré van der Merwe

Department   Epidemiology and Population Health

Head of Department   Pat Doyle

Title: n-3 long-chain polyunsaturated fatty acids (LC-PUFAs) in relation to
gut integrity and growth failure on rural African children.

Approval of this study is granted by the Committee.

Chair   T. W Meade
Professor Tom Meade

Date   9 January 2007

Approval is dependent on local ethical approval having been received.

Any subsequent changes to the consent form must be re-submitted to the
Committee.

The Gambia Government / MRC Laboratories Joint

ETHICS COMMITTEE

c/o MRC Laboratories Fajara
P. O. Box 273, Banjul
The Gambia, West Africa

Fax +220 – 4494498 or 4496 513
Tel +220 – 4494 073-9 ext 409

13th February 2007

Ms Liandre van der Merwe
PhD Student
LSHTM & Keneba

Dear Ms van der Merwe

Re SCC 1061 n-3 long chain polyunsaturated fatty acids in relation to gut integrity and growth failure in rural African children

Thank you for your letter dated 9 February 2007 in reply to our queries of 21st November 2006 on the above proposal. I also acknowledge receipt of the accompanying email sent by the Acting Director of NaNA. I note, with approval, your consultations with NaNA and their approval and support of the proposed study. I also note that you no longer plan to do rectal cather procedures.

I am therefore happy to record our committee's full approval for this study to proceed. You will be required to submit a summary report on the conduct and main findings at the end of the calendar year.

With best wishes

Yours sincerely

Mr. Malcolm Clarke
Chairman, Gambia Government/MRC Joint Ethics Committee

cc Prof Andrew Prentice
   Dr Sophie Moore

The Gambia Government / MRC Laboratories Joint Ethics Committee:

Mr Malcolm Clarke, Chairman	Professor Tumani Corrah
Mrs Kathy Hill, Secretary	Professor Hilton Whittle
Mrs Harriah Ait, 2nd Secretary	Dr Stephen Howie
Professor Ousman Nyan, Scientific Adviser	Dr Mariatou Jallow
Mr Dawda Jagne	Dr Lamin Sidibeh
Mrs Bertha Mbuge	Mr Malamin Sonko

The Gambia Government / MRC Laboratories Joint

ETHICS COMMITTEE

c/o MRC Laboratories |
P. O. Box 273, |
The Gambia, West

Fax: +220 – 4494498 or 449
Tel: +220 – 4494 073-9 ex

29ᵃ March 2007

Dr Liandre van der Merwe
C/O Dr. Stephen Owens
MRC Nutrition Programme
Keneba

Dear Dr van der Merwe

RE L2007.12 : 9ᵗʰ February 2007. Re SCC 1061 n-3 long chain polyunsaturated fatty acids in relation to gut integrity and growth failure in rural African children: Permission to amend protocol

Thanks you for submitting the above letter.

Your proposed study protocol amendments, including the increase in sample size (including the addition of three extra villages) and study procedures (constitution and dose standardisation of supplement, further anthropometric measurements, simplification of urine and stool collection procedures, daily home visits for morbidity assessments) were reviewed and approved by the Joint Gambia Government/MRC Ethics Committee at its meeting held on 23 March 2007.

Best wishes.

Yours sincerely

Mr. Malcolm Clarke
Chairman, Gambia Government/MRC Joint Ethics Committee

The Gambia Government / MRC Laboratories Joint

ETHICS COMMITTEE

c/o MRC Laboratones Fajara
P. O. Box 273, Banjul
The Gambia, West Africa

Fax: +220 – 4494498 or 4496 513
Tel: +220 – 4494 073-9 ext. 409

16th January 2008

Ms Liandre van der Merwe
c/o Nutrition Programme
MRC. Fajara

Dear Ms van der Merwe,

Re: L2007.100. 20th November 2007. Permission to add a developmental outcome to SCC 1061: n-3 long chain polyunsaturated fatty acids in relation to gut integrity and growth failure in rural African children.

Thank you for submitting the above project which was considered and approved at the recent GG/MRC Ethics Committee meeting held on January 11, 2008.

Best wishes

Yours sincerely

Mr. Malcolm Clarke
Chairman, Gambia Government/MRC Joint Ethics Committee

The Gambia Government / MRC Laboratories Joint Ethics Committee:

Mr Malcolm Clarke, Chairman
Mrs Karly Hill, Secretary
Mrs Norweh Ali, 2nd Secretary
Professor Ousman Nyan, Scientific Advisor
Mr Dawda Jagne
Mrs Bertha Mbogo
Malamin Senko
Mr. Modou Phall

Professor Tumani Corrah
Professor Hilton Whittle
Dr Stephen Howie
Dr Mariatou Jallow
Dr. Lamin Sidibeh
Mr.

LONDON SCHOOL OF HYGIENE
& TROPICAL MEDICINE

ETHICS COMMITTEE

APPROVAL FORM
Application number: 5072

Name of Principal Investigator   **Liandré van der Merwe**

Department   **Epidemiology and Population Health**

Head of Department   **Professor Laura Rodrigues**

Title: **n-3 Long-chain polyunsaturated fatty acids (LC-PUFAs) in relation to gut integrity and growth failure of rural African infants.**

The Committee has approved amendments to this study.

Chair   *T. W. Meade*
Professor Tom Meade

Date ....15 April 2008...

Approval is dependent on local ethical approval having been received.

Any subsequent changes to the application must be re-submitted to the Committee.

Appendix 12. Subject information sheet

n-3 Polyunsaturated fatty acids in relation to infant gut integrity & growth in rural Africa.

(to be read to mothers in their own language)

You/your child are being invited to take part in a research study. Before deciding it is important for you to understand why the research is being done and what it will involve. Please take time to listen to this carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

Certain types of fat, that occur especially in fish, are very important to children's diets. We are running a project to investigate whether feeding babies additional amounts of these special fats may strengthen their intestines and hence help them grow better.

Your infant has been chosen randomly to participate in this study which involves 150 infants. Half the babies will receive normal oil and half will receive the type of oil we wish to test to see if it is better. We will not know which oil your baby has until after the trial has been completed. Both types are believed to be safe and healthy.

The following will be required of you and your baby if you decide to take part:

1. We will provide transport and ask you to bring your infant to MRC Keneba six times: 3 times at the beginning, and 3 times at the end of six months. Here your infant will be given a small amount of sugar water to drink. We will then collect urine and stool samples for 5 hours. At the same time we will measure your child's growth. We will also collect a small amount of your breast-milk in order to investigate its fat content. On the first and last occasion we will collect 2-3ml (half a teaspoon) of blood from your baby to investigate how much of these special fats he/she has.

2. You will also be required to bring your child to the fieldworker in your village everyday for 6 months. The fieldworker will give a small amount of oil for your baby to take everyday, and ask you to answer 8 short questions about your child's health. If your child is ill we will provide treatment or transport to Keneba.

3. Lastly, the fieldworker in your village will measure your child's growth on 2 occasions (at 5 mo and 7 mo of age).

All information which is collected about you or your child during the course of the research will be kept strictly confidential. He/she will be identified by an ID number. If you have any questions, please ask.

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Your decision will not affect the medical care we provide you in any way. We hope that you will agree to participate.

Thank you very much.   **Principal investigator:** Liandré van der Merwe ; MRC Keneba ; tel 9878099

Appendix 13. Consent form

Infant Omega-3 Supplementation Study

Principal investigator:

Liandré van der Merwe; MRC Keneba

Mother's WK ID Number : |__|__|__|__|__|__|__|__|__|__|__|

Child's DOB: |__|__|__|__|__|__|

Child's Name:..

Mother's Name:..

Father's Name:..

Village Name: ....

I have read the information sheet concerning this study and I understand what will be required of me and my child if I take part in the study. You have answered all my questions and doubts. I understand that I can withdraw from the study any time I wish without giving a reason and this will not affect my normal health care I receive from your clinic.

I agree to take part in this study.

Mother's signature / thumb print:........................   **Date:** ....

Father's signature /thumb print:........................   **Date:**........................

Investigator's Signature: ....

Name of fieldworker: ....

Appendix 14. Letter submitted to ISRCTN administrator

The changes to be made, if possible and acceptable, are as follows:

Study hypothesis:

The primary/secondary hypotheses do not correspond to primary/secondary outcomes. We would like to leave our primary outcomes as is, but would like to line the hypotheses up with them, so that the hypotheses and outcomes are matched, and "Study hypothesis" reads as follows:

Primary hypotheses:
1. Dietary n-3 long-chain polyunsaturated fatty acid (LCP) supplementation will improve rural African infants' growth performances.
2. Dietary n-3 LCP supplementation will protect infant mucosal epithelial integrity.

Secondary hypotheses:
1. Dietary n-3 LCP supplementation improves infant plasma n-3 fatty acid status.
2. Dietary n-3 LCP supplementation will enhance the cognitive development of rural African infants.
3. Dietary n-3 LCP supplementation will reduce the degree of intestinal inflammation of rural African infants.
4. Dietary n-3 LCP supplementation will reduce infant systemic inflammation.
5. Dietary n-3 LCP supplementation reduces incidence and severity of morbidities in rural African infants.

Exclusion criteria:

The following addition was made:
1. Infants from multiple births.

Interventions:

The intervention is listed as 500mg EPH and 500mg DHA. This should be 500mg combined EPH + DHA, or 300mg EPH and 200mg DHA. Perhaps best if read as "300mg EPH and 200mg DHA".

Outcomes:

No change to 'primary outcomes'. We have added 'cognitive development' as a secondary outcome. We have removed the level 'tertiary outcomes' so that all non-primary outcomes fall under 'secondary outcomes'. Note that 'faecal neopterin' was not measured (due to assay problems) and is no longer listed under 'intestinal inflammation'.

Secondary outcome measures should now, therefore (corresponding to secondary hypotheses), read:

1. Plasma fatty acid status (Gas Chromatography [GC])
2. Infant cognitive development (infant planning test and attention assessment).
3. Systemic inflammatory markers (α-Acid GlycoProtein [AGP], C-Reactive Protein [CRP] and plasma albumin).
4. Intestinal inflammation (faecal calprotectin).
5. Infant morbidities (daily morbidity assessments, clinic/nurse visits).

Measures 1, 3 and 4 will be measured at 3 and 9 months of age (i.e. at baseline and 6-month follow-up).

Measure 2 will be measured at 12 months of age.

Appendix 15. Field Supervisor Quality Control Sheet

Date:...........................**Fieldworker reviewed:**......................................

Village:.............................

1. Supplementation

Accuracy of dosage:	*good/needs attention*
Technique for supplementing child:	*good/needs attention*
Mother breast-feeds child imm afterward:	*good/needs attention*
Infant acceptance:	*good/needs attention*
Random checks – child supplemented daily:	*good/needs attention*

2. Supplement handling

Labels visible or re-marked:	*good/needs attention*
Condition in field:	*good/needs attention*
Condition in fridge:	*good/needs attention*

3. Fridge

Temperature record sheet:	*good/needs attention*
Contents:	*good/needs attention*
Cleanliness:	*good/needs attention*
Freezer compartment:	*good/needs attention*
Handling:	*good/needs attention*

4. Motorbike

Condition of:	*good/needs attention*
Riding technique:	*good/needs attention*
Care of:	*good/needs attention*

5. Anthropometry

Overall technique:	*good/needs attention*
Repeat anthropometry forms attached:	*yes/no*

6. Anthropometry Equipment

Calibration:	*good/needs attention*
Condition/cleanliness:	*good/needs attention*
Care in field:	*good/needs attention*

7. Morbidity questionnaires:

	good/needs attention
Repeats attached	*yes/no*

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8. Recruitment information and consenting procedure   *good/needs attention*

Comment:..

9. Fieldworker-subject/community relationship

Comment:...

good/needs attention

10. Treatment group accuracy – treatment card/supplement:

Comment:...

good/needs attention

11. Bread and Tea supplying: distribution/subject satisfaction/supplies handling:

good/needs attention

Appendix 16.   Example safety monitoring report

The body of the statistical output has been omitted for the sake of brevity.

<u>Pufa supplemention study – Safety Monitoring summary, April 2008</u>

"Again there seem to be few safety concerns.

Below is a summary of the numbers of individuals experiencing the main types of adverse event seen – none (not even conjunctivitis) differ significantly between the groups. Most are similar to before but the incidence of diarrhoea seems to have increased a fair bit.

	A	B
Unclassified cough	5	6
Impetigo	9	4
Unspecified conjunctivitis	3	9
Diarrhoea (unknown cause)	28	27
Cold/coryza	29	38
Pneumonia/pleurisy	27	35
possibly due to intervention	22	20

Anthropometry output

```
-------------------------------------------------------------------------------
    -------------------
      log: C:\Documents and Settings\fulfordt.MRC-HNR\Desktop\Liandre - Pufa study\Liandre
    safety monitoring\Apr 2008\safety anthrops, Apr 2008.log
  log type:  text
 opened on:  29 Apr 2008, 21:13:43


. . .

end of do-file

. exit, clear
```

Morbidity output

```
-------------------------------------------------------------------------------
    -------------------
      log: C:\Documents and Settings\fulfordt.MRC-HNR\Desktop\Liandre - Pufa study\Liandre
    safety monitoring\Apr 2008\safety mobidity, Apr 2008.log
  log type:  text
 opened on:  29 Apr 2008, 10:38:33

.
. insheet using "safety morbidity.txt"
(10 vars, 173 obs)

. . .

. exit

end of do-file
```

SERIOUS ADVERSE EVENT – INITIAL REPORTING FORM
IN3SS – SCC 1061

Please send this information immediately by fax.
In addition please complete the adverse events section in the case report form.

Fax to:	From Investigator:	Country:
	Address:	
Date of receipt:	Telephone-No: Fax No. Email address:	

For the purposes of this form, a serious adverse event is defined as any untoward medical occurrence that at any dose results in death, is life-threatening, requires inpatient hospitalization or prolongation of existing hospitalization, results in persistent or significant disability or incapacity. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse event when, based upon appropriated medical judgment, they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above.

INSTRUCTIONS:

Infant omega-3 supplementation study	
PRINCIPAL INVESTIGATOR: *Liandré van der Merwe*	MRC Keneba Phone: +220 9878099 E-mail: Liandre.vanderMerwe@LSHTM.ac.uk.
SUBJECT'S INITIALS AND STUDY NUMBER:	
DATE & TIME OF SAE ONSET:	___/___/_____ __:____
LOCATION AT ONSET OF SAE:	
BRIEF DESCRIPTION OF SUBJECT:	SEX: M/F DOB OR AGE: _____ Diagnosis:
HAS THE BLINDING CODE BEEN BROKEN FOR THE SUBJECT?	No ☒ Yes ☐ Results:
First dose of study medication: Last dose given: Dose administered: No. of doses given so far: Batch number: Expiry Date:	 ....(date) ....(date) ____500_____ (mg) |__| _____ Lot# 1806_____ 04/09
NAME OF REPORTER	
BRIEF DESCRIPTION OF THE NATURE OF THE SERIOUS ADVERSE EVENT:	

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MEDICAL MANAGEMENT:	
CATEGORY (outcome) OF THE SERIOUS ADVERSE EVENT: [ ] death [ ] disability/incapacity [ ] life-threatening [ ] hospitalisation-initial or prolonged [ ] required intervention to prevent permanent impairment [ ] Medically important event [ ] Other	**PRELIMINARY EVALUATION OF CAUSAL RELATIONSHIP WITH INVESTIGATIONAL PRODUCT** [ ] 1 = unrelated (clearly not related to the research) [ ] 2 = unlikely (doubtfully related to the research) [ ] 3 = possible (may be related to the research) [ ] 4 = probable (likely related to the research) [ ] 5 = definite (clearly related to the research)
ANY CONCOMITANT MEDICATION/TREATMENT?	[ ] YES [ ] NO Description:
What steps do you plan to take as a result of the adverse event reported above?	[ ] no action required [ ] terminate or suspend protocol [ ] other, describe:
OUTCOME:	[ ] 1 = Recovered [ ] 2 = Not yet recovered [ ] 3 = Alive with sequelae [ ] 4 = Death [ ] 5 = Unknown
SUBMITTED TO:	[ ] Safety monitor [ ] Trial monitor [ ] SCC/ethics committee
SIGNATURE:	**DATE:**

SERIOUS ADVERSE EVENT – FOLLOW-UP FORM
IN3SS – SCC 1061

Please send this information immediately by fax.
In addition please complete the adverse events section in the case report form.

Fax to:	From	Country:
	Investigator:	
Date of receipt:	Address:	
	Telephone-No:	
	Fax No.	
	Email address:	

Infant omega-3 supplementation study		
PRINCIPAL INVESTIGATOR: *Liandré van der Merwe*	MRC Keneba Phone: +220 9878099 E-mail: Liandre.vanderMerwe@LSHTM.ac.uk.	
SUBJECT'S INITIALS AND STUDY NUMBER:		
DATE & TIME OF SAE ONSET:	___ / ___ / _____ ___ : ___	
LOCATION AT ONSET OF SAE:		
BRIEF DESCRIPTION OF SUBJECT(S)	*SEX:* M/F *DOB OR AGE:* _____ Diagnosis:	

Additional information on SAE and on actions taken *(including any new medication given as part of treatment for SAE:*

[ ] death
[ ] disability/incapacity
[ ] life-threatening
[ ] hospitalization-initial or prolonged
[ ] required intervention to prevent permanent   impairment
[ ] Medically important event
[ ] Other

CATEGORY (outcome) OF THE SERIOUS ADVERSE EVENT:	*EVALUATION OF CAUSAL RELATIONSHIP WITH INVESTIGATIONAL PRODUCT*
[ ] death	[ ] 1 = unrelated (clearly not related to the research)
[ ] disability/incapacity	[ ] 2 = unlikely (doubtfully related to the research)
[ ] life-threatening	[ ] 3 = possible (may be related to the research)
[ ] hospitalization-initial or prolonged	[ ] 4 = probable (likely related to the research)
[ ] required intervention to prevent permanent impairment	[ ] 5 = definite (clearly related to the research)
[ ] Medically important event	[ ] 6 = not assessable
[ ] Other	

OUTCOME:	[ ] 1 = Recovered [ ] 2 = Not yet recovered [ ] 3 = Alive with sequelae [ ] 4 = Death [ ] 5 = Unknown	
NAME AND SIGNITURE OF OF REPORTER		*DATE:*
INVESTIGATOR'S SIGNATURE:		*DATE:*

Please attach any relevant reports.

Appendix 18.   Trial monitoring report

Monitoring report on "Long chain n-3 polyunsaturated fatty acids in relation to gut integrity and growth failure in rural African infants", MRC Keneba

Name of trial: Long chain n-3 polyunsaturated fatty acids in relation to gut integrity and growth failure in rural African infants

ID numbers: Protocol number SCC 1061; ISRCTN 66645725

Date of monitoring visit: 4-7/12/07

Sample size sought: 150

Date recruitment started: May 2007

Proposed date for recruitment end: By end of 2007

Actual recruitment rate vs. target: The actual recruitment rate matches the target

Acceptance rate: 83%

Forecast of recruitment for the remainder of the trial: 148 infants have been recruited; recruitment is anticipated to be completed within a few weeks of the monitoring visit (i.e. by the end of 2007).

Losses to follow-up: 4

Number for which follow-up has been completed successfully: 26 infants have had their 9 month follow-up

Completeness of data collected: Anthropometric data is complete for those infants who have been studied at 3 months (n = 148) and 9 months (n = 26) of age. No laboratory data has been generated yet, but this will be initiated once all samples are collected. No developmental outcomes have been assessed yet; these will be assessed at 12 months of age

Any available results: None yet

Any organisational problems: None that I could see. I was impressed at the degree of organisation of the processes and researchers involved.

Specific issues: There was a problem with the initial shipment of fish oil capsules which did not arrive. The setting of the study (recruiting is spread across a number of villages) and its nature (i.e. supplementation in infants) mean that it is logistically difficult to conduct. However the available infrastructure coupled with the motivation, organisation and level of training of personnel involved appear to have overcome the difficulties imposed and the trial is progressing very satisfactorily. In

trial experiments there have been difficulties with measurement of intestinal (faecal) inflammatory markers; however the personnel involved are working towards resolving these difficulties. I am fully satisfied with the conduct of the trial, with procedures involved in recruiting and tracking subjects, with the field supplementation procedure, and with primary data recording and its transfer to electronic form.

P.C. Calder
University of Southampton
10/12/07