# SAFETY AND EFFICACY OF HIGH DOSE DOCOSAHEXAENOIC ACID FOR THE PRETERM INFANT

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This thesis is submitted for the degree of Doctor of Philosophy

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SUMMARY

There has been substantial research demonstrating improvements in visual and cognitive performance of preterm infants after feeding formulas containing n-3 long chain polyunsaturated fatty acid (LCPUFA). The amount of docosahexaenoic acid (DHA) estimated to be accrued by the fetus in the last trimester of gestation is greater than that supplied in current preterm formulas and breast milk of average DHA content (~0.3% of total fat in Western women). Yet many trials have compared infants fed formula containing concentrations near 0.3% DHA with infants fed formula containing no LCPUFA. No research has addressed whether the average breast milk DHA milk results in optimal development of preterm infants. The focus of this thesis was to compare the efficacy and safety of supplementing preterm infants with milk containing docosahexaenoic acid (DHA) at concentrations that meet the estimated *in utero* accretion rate (~1%) compared with current clinical practices (~0.3%).

In a double-blind, randomised controlled trial (RCT), infants born <33 weeks gestation were assigned to receive milk containing one of two doses of DHA. Treatment group infants received milk containing high dose DHA (1%) and infants in the control group infants received milk containing standard levels DHA (0.2 - 0.35%). Lactating mothers consumed capsules containing either tuna oil (900mg DHA) or soy oil (no DHA) that resulted in breast milk with either a high or typical concentration of DHA. Standard preterm formula milk with a corresponding DHA composition was fed to infants if formula feeds were required. The intervention period was from five days of commencing enteral feeds through to the infants estimated due date (EDD). Primary efficacy assessment was sweep visual evoked potential (VEP) acuity at 4 months corrected age (CA). Secondary efficacy outcomes included VEP acuity at 2 months CA and VEP latency at 2 and 4 months CA. Infant anthropometry was assessed regularly throughout the trial and the primary safety outcome was weight at 4 months CA. Other clinical safety

data including incidence and severity of diseases commonly associated with prematurity were also assessed.

The success of the intervention was demonstrated with infants in the treatment group having a significantly higher level of erythrocyte membrane DHA at EDD compared with the control group (% total erythrocyte phospholipids (mean ± SD), treatment group 6.8 ±1.2, control group 5.2 ± 0.7, $p<0.0005$). The primary efficacy outcome of acuity at 4 months CA was significantly higher in the treatment compared with the control group infants (mean ± SD acuity (in cpd) treatment group 9.6 ± 3.7, control group 8.2 ± 1.8, $p = 0.025$). No significant differences were found in acuity at 2 months CA or latency at 2 or 4 months CA between infants in the control and treatment groups.

No significant differences in weight, length or head circumference were found between treatment compared with control infants at EDD or at 4 months CA. Nor were any differences found in other clinical outcomes commonly associated with prematurity including, tolerance, necrotising enterocolitis, sepsis, retinopathy of prematurity, bronchopulmonary dysplasia or intraventricular haemorrhage.

Increasing milk DHA to 1% of total fat suggests that the DHA requirement of preterm infants may be higher than the level available in preterm formula or breast milk of Australian women. Addressing both breast and formula milks demonstrates wide generalisability of these findings to common feeding practices in neonatal nurseries. Further studies are needed to determine whether this feeding strategy and dose of DHA is capable of improving other aspects of infant development.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying.

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'Fight through ignorance, want, and care
Through the griefs that crush the spirit;
Push your way to a fortune fair,
And the smiles of the world, you'll merit.

Long, as a boy, for the chance to learn
For the chance that Fate denies you;
Win degrees where the Life-lights burn,
And the scores will teach and advise you.'

Henry Lawson

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic acid |
| AC | alternating current |
| AFT | adaptive filter technique |
| AGA | appropriate for gestational age |
| ALA | Alpha linolenic acid |
| ANZNN | Australia and New Zealand Neonatal Network |
| AR | analytical reagent |
| BF | Breast fed |
| BPD | Bronchopulmonary dysplasia |
| BSID | Bayley Scales of Infant Development |
| CA | Corrected age |
| CDC | Centre for Disease Control |
| CDI | Communicative development inventory |
| CI | confidence interval |
| cm | centimetre |
| CNRC | Child Nutrition Research Centre |
| CPAP | continuous positive airway pressure |
| cpd | cycles per degree |
| CLD | Chronic lung disease |
| df | degrees of freedom |
| DHA | Docosahexaenoic acid |
| DPA | docosapentaenoic acid |
| EDD | estimated due date |
| EEG | electroencephalogram |
| EFA | essential fatty acid |
| EI | enteral intake |
| EPA | Eicosapentaenoic acid |
| ERG | Electroretinogram |
| Exc | Exclusive or exclusively |
| FAME | fatty acid methyl ester |
| FF | Formula fed |
| FFA | Free fatty acids |
| FT | full term |
| FTII | Fagan test of infant intelligence |
| GA | gestational age |
| GC | gas chromatograph |
| GI | gastrointestinal |
| HC | Head circumference |
| HDL | high density lipoprotein |
| HMD | Hyalin membrane disease |
| Hz | hertz |
| IQ | intelligence quotient |
| ITT | intention-to-treat |
| IV | intravenous |
| IVH | Intraventricular haemorrhage |
| kJ | kilojoule |
| kg | kilogram |

| | |
|---|---|
| L | litre |
| LA | Linoleic acid |
| LBW | low birth weight |
| LCPUFA | Long chain polyunsaturated fatty acid |
| LDL | low density lipoprotein |
| LED | Light emitting diode |
| LOS | length of stay |
| LPL | Lipoprotein lipase |
| MDI | motor development index |
| mL | millilitres |
| mo | months |
| MRI | Magnetic Resonance Imaging |
| msec | milliseconds |
| MUAC | mid upper arm circumference |
| ND | no difference |
| NEC | necrotising enterocolitis |
| NH&MRC | National Health and Medical Research Council |
| NICU | neonatal intensive care unit |
| NR | reported |
| NS | not significant |
| PC | Phosphatidyl choline |
| PCA | post conceptual age |
| PDA | patent ductus arteriosus |
| PDI | psychomotor development index |
| PE | Phosphatidyl ethanolamine |
| PET | Positron emission tomography |
| PI | Phosphatidyl inositol |
| PL | phospholipid |
| PMA | Post menstrual age |
| PS | Phosphatidyl serine |
| PUFA | polyunsaturated fatty acid |
| PVL | periventricular leukomalacia |
| RBC | red blood cell |
| RCT | Randomised Controlled Trial |
| RDS | Respiratory distress syndrome |
| REC | Research Ethics Committee |
| ROP | Retinopathy of prematurity |
| RR | Relative Risk |
| SD | Standard deviation |
| SES | socioeconomic status |
| SGA | Small for gestational age |
| SNR | signal to noise ratio |
| TLC | Thin layer chromatography |
| UV | ultraviolet |
| VEP | Visual evoked potential |
| VLBW | very low birth weight |
| yr | year |

# CHAPTER 1: LITERATURE REVIEW

## 1.1 Scope of the Review

During gestation, the fetus has a large requirement for long chain polyunsaturated fatty acids (LCPUFA) particularly in the last trimester of pregnancy. The placenta enriches fetal circulation with LCPUFA and demonstrates a preference for supplying docosahexaenoic acid (DHA) [1,2]. The infant born preterm has less time to accrue LCPUFA, has negligible reserves in adipose tissue and exhibits slow conversion of precursor fatty acids into DHA [3]. The concentration of DHA in preterm formula is similar to that present in breast milk and in this review I will show that both breast milk and formula supply less DHA than that transferred to the fetus during gestation [4]. The reduced opportunity to accrue DHA, the slow endogenous synthesis of DHA and their dependence primarily on milk to supply DHA renders preterm infants susceptible to inadequate dietary intakes of DHA.

There has been considerable research focused on the potential benefit of supplementing preterm infants with LCPUFA. In recent years, most trials have tested formulas that have included both n-3 and n-6 fatty acids, although in initial trials, efficacy was demonstrated after supplementing with n-3 LCPUFA alone [5-7]. The inclusion of n-6 LCPUFA, particularly arachidonic acid (AA) has resulted from observations of poorer growth when only n-3 LCPUFA have been included in formula [6,7]. The quality of evidence demonstrating the ability of AA to correct any growth deficit when milk is enriched with DHA will be the subject of further discussion in this review.

The majority of evidence presented in this review is based on human trials. Animal studies have only been included to provide additional data when trials in infants are not available. Similarly, LCPUFA supplementation trials in term infants are generally not considered in this review, unless they contribute information unavailable in preterm infants.

The review begins by identifying the location of DHA in human tissue and the demand for DHA during gestation. Estimates of placental transfer and supply of DHA for the preterm infant are discussed. Examining the responsiveness of infant's plasma and erythrocyte membrane phospholipids from dietary DHA follows. An evaluation of the effect of DHA on the visual development of preterm infants comprises a significant focus of this review and is followed with a brief discussion on LCPUFA and cognitive function. The safety of dietary DHA for preterm infant growth and other clinical outcomes are then examined.

The importance of further research in the area of DHA supplementation in preterm infants is considered with emphasis given to the aims of the research presented in this thesis and its potential significance for the preterm infant. I specifically address the hypothesis that preterm infants given DHA at the level provided by the placenta during gestation will develop better visual acuity and visual evoked potential (VEP) latency than infants that receive lower levels of DHA present in breast milk and preterm formula without adverse growth or clinical outcomes.

## 1.2 DHA During Development: Location and Importance of DHA

### *Tissues Containing DHA*

DHA is accumulated in all cells as an integral component of the phospholipid bilayer of the membrane. Cell membranes that require efficiency of movement - cells that are highly reactive, have a high proportion of LCPUFA. With the exception of adipose tissue, the organ containing the most DHA is the brain, with much DHA localised in the synaptic membranes [8]. The tissue with the highest concentration of DHA is the retina. The outer segment of photoreceptor cells present on the retina is filled with lamellae which contain pigments that react structurally to light. This membranous structure is packed with DHA, which is necessary for reactivity to visual stimuli. The large quantity of DHA in the brain and

high concentration in retinal tissues led investigators to speculate that changes in dietary fatty acids might influence visual and cognitive function.

Whether a diet containing the essential fatty acid alpha linolenic acid (ALA) is capable of meeting tissue requirements for n-3 LCPUFA has been the subject of further research. Manipulation of the n-3 polyunsaturated fat content of animal diets has shown that diets supplying ALA result in lower retinal and neural tissue DHA compared with diets providing some DHA [9,10]. Changes in tissue function have been associated with diets devoid of n-3 LCPUFA. In rodents, a lower brain DHA was associated with reduced performance in a spatial task [11] and infant rhesus monkeys fed formula with AA and DHA demonstrate superior orientation and motor skills than those fed formula containing only the essential fatty acid precursors [12].

Although the effect of DHA on visual and neural development of preterm infants is presented further in Section 5, studies in animals demonstrate cognitive consequences of an inadequate supply of LCPUFA, in particular a source of preformed dietary DHA.

*Demand for DHA During Development*

The growth of the visual system and the brain is a unique occurrence that begins as a fetus and continues into early childhood. Both retinal and neural tissues begin accruing DHA during gestation [13]. Retinal accretion may be completed by full term gestation as no difference has been found in retinal function (measured using the electroretinogram) of term infants fed diets differing in the n-3 LCPUFA content [14], or in preterm infants after 36 wk post conceptual age (PCA) [15]. Further supportive evidence from post mortem tissue analysis showed no difference in the DHA composition of retinal tissue between term babies fed breast milk and those fed formula (with no n-3 LCPUFA) [16]. However, in preterm infants a postnatal shortage of n-3 fatty acids results in less retinal DHA [13]. By comparison,

deposition of DHA in neural tissue increases rapidly during the last half of gestation and continues more modestly in the postnatal period [13]. The tapering of DHA accrual from gestation to postnatal periods is supported with estimates of intrauterine and extrauterine accretion of DHA into infant brain [4,17]. Intrauterine accretion rates were estimated from post mortem analysis of 15 preterm infants born between 22 weeks gestation and term. These estimates were calculated by measuring the fatty acid content of cerebellum, frontal and occipital cortex tissue, then by multiplying these by the average weights of the brain lobes then summed to give a total brain estimate.

Whole body estimates of fatty acid accretion during gestation have been estimated in a similar manner[18]. Regression equations were developed from these data to predict perinatal requirements for fatty acids. It was estimated that 67 mg of n-3 fatty acids per day are needed to support neonatal growth of all organs and tissues of a preterm infant weighing 1300 g (or 52 mg of n-3 fatty acids/kg/day). However, this figure is likely to underestimate the actual n-3 fatty acid requirements, as it does not include a proportion of fatty acids oxidised for energy. Unfortunately, the accretion of DHA during development was not separated from total n-3 fatty acid accretion for whole-body estimates [18]. However, post mortem descriptions of brain tissue indicate that DHA is *the* primary n-3 fatty acid in brain tissue and is present in cortex tissue at far greater quantities than any other n-3 fatty acid [13,16]. During *in utero* development, DHA is also the primary n-3 fatty acid accrued by adipose tissue [18] and liver [19], far higher than any of the other n-3 fatty acids. Therefore, DHA is the major n-3 fatty acid required for fetal tissue development and a large proportion of the estimated accrual rate would be attributed to DHA.

### *Supply of DHA to the Fetus During Gestation*

The fetus relies on maternal circulation to supply n-3 fatty acids for growth and development *in utero* and to establish reserves for postnatal growth. Fetal blood levels of DHA are higher than those present in maternal blood [1] suggesting that the placenta actively concentrates n-3 fatty acids in favour of the

fetus. It appears as though the placenta itself does not contribute to fetal DHA through conversion of fatty acids to DHA [2,20,21], even though delta-5 desaturase and its messenger RNA is present in placental tissue [22]. Instead, other mechanisms are thought to direct the flow of DHA from maternal circulation to the developing fetus.

The first of these mechanisms is simple diffusion of free fatty acids (FFA). The fetus has increased levels of free albumin, which opportunistically capture passing FFA from the maternal circulation [23]. The second method of securing n-3 fatty acids is through lipoprotein lipases (LPLs) present on the microvilli of the placenta [24]. Lipoproteins in the maternal circulation carry fatty acids in the form of triglycerides. The lipoproteins bind to the microvilli of the placenta and once bound, LPLs are able to cleave the fatty acid from the triglyceride molecule. A third mechanism is through binding proteins located on the maternal surface of the placenta that also direct the transfer of LCPUFA [25]. A preference for binding fatty acids has been identified, with DHA having a higher affinity than other n-3 or n-6 PUFA [25].

Empirical evidence of *fetal* synthesis of LCPUFA is not available, but some data indicates both term and preterm neonates are capable of *in vivo* conversion of essential fatty acids into their long-chain derivatives [3,26]. Carnielli *et al* showed that one-month old preterm infants (gestational age ranged from 25 to 31 weeks at birth) were capable of desaturating and elongating a $^{13}$C-labelled ALA precursor into $^{13}$C-DHA [26]. Salem *et al* fed nine neonates a formula containing deuterium-labelled ALA and LA and then detected these fatty acids and their long-chain derivatives in plasma phospholipids [3]. Both term and preterm neonates involved in this study were between 1 and 6 days old at the time of testing and ranged in gestational age at birth between 32 and 41 weeks. Salem *et al* demonstrated that conversion of DHA from essential fatty acid was slower compared with AA. Studies from these neonates indicate that the fetus is probably capable of synthesising DHA, albeit in small quantities.

The numerous mechanisms present for movement of DHA in favour of fetal circulation and the observation of slow fetal synthesis of DHA suggests that it is likely the placenta directs the majority of DHA to the fetus. However, infants born prematurely do not have the placenta to channel the n-3 fatty acids. Since the demand for DHA remains high, other sources such as fat stores or diet need to be utilised to supply DHA to the preterm infant.

*Postnatal Supply of DHA for the Preterm Infant*

The fat stores of the fetus increase dramatically during the last trimester of pregnancy. A preterm neonate weighing 1000g has merely 1% body fat [27]. For comparison, the average fat stores of baby born at term have increased to 16% of body weight (to approximately 500 g). The adipose tissue of a preterm infant contains only 1.6% DHA[18], so there is very little DHA in reserve for future growth and therefore preterm infants rely heavily on nutritional sources to supply DHA.

After a preterm birth, the infant is stabilised before the introduction of enteral feeds. The feeds are cautiously increased to establish tolerance. Feeding guidelines suggest that an acceptable and achievable feeding plan for a preterm infant varies between 120 – 200 mL/kg/day [28,29]. Estimates of DHA intake have been made from these volumes and various DHA concentrations in milk (see Table 1.1).

Table 1.1: Estimated DHA Intake (in mg/kd/day) Based on Fluid Intake Recommendations for Preterm Infants According to Various Concentrations of DHA (% total fat) in Milk

| Reference | Fluid intake (mL / kg / day) | 1% DHA | 0.35% DHA | 0.2% DHA |
|---|---|---|---|---|
| 28 | 120 - 160 | 48 - 64 | 17 - 22 | 10 - 13 |
| 29 | 150-200 | 60 - 80 | 21 - 28 | 10 - 16 |
| 30 | 180 | 72 | 25 | 14 |

The average level of DHA in breast milk of Australian women is 0.2% (of total fat) [31] and the level in preterm infant formula is approximately 0.35%. Based on a milk fat content of 4%, a near-term infant weighing around 2.5 kg that consumes 150 mL/kg would receive approximately 12 mg DHA / kg if fully breast fed or 21 mg DHA / kg if fully formula fed. As previously discussed, the quantity of n-3 fatty acids accrued by the fetus during the last trimester of pregnancy has been estimated to be approximately 52 mg/kg/day [19]. If the DHA content was increased to 0.9 -1% of total fat, the milk could then provide 54 - 60 mg of DHA / kg of infant.

The amount of DHA provided in milk in the post-natal period falls short of the estimated accrual rate during gestation. In the postnatal period, the concentration of DHA in plasma and erythrocyte phospholipids decrease [32,33] in both breast fed and formula fed preterm infants. This decreasing concentration of DHA is the combined effects of meagre DHA stores, slow conversion of ALA to DHA and a low dietary supply of DHA. The low supply of DHA is occurring at a critical period of brain and visual development that may have consequences on the functional capacity of these tissues. It is hypothesised that milk providing DHA at 1% of total fat would match the estimated n-3 fatty acid accrual that occurs during gestation and might protect the infant from unfavourable functional changes to developing tissues.

## 1.3 Improving Infants' DHA Status Through Nutrition

### *Achieving High Levels of DHA in Milk*

Breast milk DHA levels show remarkable malleability to dietary manipulation. The average breast milk DHA of Australian women is approximately 0.2% of total fat [31]. Higher levels of DHA (0.9%) are found in women consuming a high fish diet [34,35], however regular fish intake is necessary to maintain this level of DHA in breast milk. Supplementing lactating mothers with capsules containing fish or algal oil

provides an alternative for manipulating breast milk DHA. Makrides *et al* has demonstrated a dose response relationship between breast milk DHA and maternal supplementation with algal oil [36].

*Digestion and Absorption of DHA*

The absorption of DHA from breast milk and a modern formula preparation has been investigated in preterm infants through dietary intake and faecal recovery analysis [37]. Preterm infants were fed breast milk, formula containing no LCPUFA, or formula enriched with DHA and AA from either egg phospholipid (PL) or in triglyceride form from algal oil (TG). The TG formula contained 0.64% of total fat as DHA and 0.84% AA compared with 0.2% DHA and 0.35% AA in PL formula. Infants that received the TG formula had the lowest intake of milk, the highest intake of DHA and AA and significantly higher faecal losses of DHA and AA when compared to the PL and breast fed infants. Nevertheless, DHA absorption was high from all sources; approximately 81% absorption from TG, 78% from preterm breast milk and 88% from phospholipids, demonstrating that like other fats LCPUFA is well absorbed.

*Dietary DHA Incorporation into Brain and Retinal Tissue*

Although dietary DHA is readily taken up into the circulation, at this point in time there are no data to demonstrate the incorporation of DHA into brain or retinal tissue. Some work has confirmed the endogenous manufacture of DHA from dietary ALA through deuterium labelling techniques, but this does not demonstrate dietary DHA being laid down in brain and retinal tissue of preterm infants [38]. Positron emission tomography (PET) scanning is a non-invasive radiological technique that is able to demonstrate the rate and distribution of a radio-labelled tracer in brain tissue. In the future, this technology may be applied to describe deposition of DHA or other fatty acid into neural tissue [39].

Evidence from post mortem studies suggests that dietary sources of DHA may contribute to cortical DHA. Breast fed infants were reported to have higher brain cortex DHA than infants fed formula with no

LCPUFA [16]. In the study by Makrides *et al,* the fully formula fed infants did not receive any dietary LCPUFA after birth. Instead any LCPUFA present in their brain was from endogenous conversion of essential fatty acid (EFA) precursors present in infant formula. Although the breast fed infants would have also received EFA from breast milk, it is present in smaller quantities than levels in infant formula. This may indicate that formula fed infants are not able to synthesise enough DHA to meet tissue requirements, despite higher levels of dietary ALA. As supported with evidence from animal studies [40,41] the higher cortex DHA in breast fed infants could be due to utilisation of preformed DHA present in the infant diet. Interestingly, a similar relationship between dietary LCPUFA and cortex AA was not observed, which may indicate that factors other than breast or formula feeding might influence the accretion of AA into neural cortex tissue.

*Blood Phospholipids Reflect Dietary LCPUFA Intake in Breast Fed Infants*

Since it is not possible to test LCPUFA concentrations of brain or retina, infant plasma and erythrocyte phospholipid fatty acids are frequently used to demonstrate the success of a LCPUFA intervention. Breast milk DHA is highly correlated with the DHA content of maternal diet. The DHA content of breast milk naturally affects the dietary DHA intake of the infant, in turn changing the plasma and erythrocyte phospholipid profile of the infant. This sequence of events means that the infant DHA status can be manipulated by increasing maternal DHA intake.

Gibson *et al* supplemented mothers with different doses of DHA, resulting in changes to breast milk DHA from 0.2% (of total fat) after placebo supplementation to 1.7 % after a dose of 1.3 g DHA / day [42]. The supplement contained DHA in triglyceride form purified from algal oil, with almost no EPA. The infants showed subtle decreases in plasma and erythrocyte phospholipid EPA, as the increasing DHA concentration displaced EPA at high levels of supplementation (Table 1.2). Small decreases in

Table 1.2: Infant Erythrocyte LCPUFA Status after Feeding Milk Containing Approximately 1% (of total fat) as n-3 LCPUFA

| Reference | 42 | 42 | 43 | 44 | 32 | 45 |
|---|---|---|---|---|---|---|
| n-3 LCPUFA | 0.9% DHA | 1.1% DHA | 3% (1.3% DHA) | 1% (0.4% DHA) | 1.1% (0.5% DHA) | 0.7% (0.6% DHA) |
| Source of LCPUFA | Algal Oil | Algal Oil | Fish Oil | Fish Oil | Fish Oil | Fish Oil |
| Duration (wk) | 12 | 12 | 17 | 3 to 5 | 6 | 4 |
| Infant Details | Exc BF term | Exc BF term | BF Term | FF Preterm | FF &BF preterm | FF Preterm |
| Number of Infants | 10 | 8 | 39 | 12 | 13 | 13 |
| RBC DHA | 9 ± 2 | 10 ± 1 | 9 ± 2 | 4 ± 2‡ | 5 ± 2‡ | 4 ± 2 |
| RBC EPA | 0.6 ± 0.2 | 0.5 ± 0.2 | 1 ± 0.6 | 1 ± 0.4‡ | 0.3 ± 0.5‡ | 0.2 ± 0.2 |
| RBC AA | 15 ± 1 | 14 ± 1 | 15 ± 2 | 14 ± 3‡ | 13 ± 1‡ | 10 ± 2 |

Results reported as percentage (mean ± SD) of total erythrocyte membrane phospholipid fatty acids, or as red cell lipids‡

Abbreviations; Exc BF, exclusively breast fed; FF, formula fed; DHA, docosahexaenoic acid; LCPUFA, long chain polyunsaturated fatty acids; NR, not reported; RBC, red blood cell

phospholipid AA were also observed with increasing dose of DHA. Infant plasma and erythrocyte phospholipid DHA reached a plateau with maternal breast milk levels supplying 0.8 to 1.1% DHA.

Lauritzen *et al* has tested the highest level of n-3 LCPUFA supplementation (1.5 g/day) in lactating women reported to date [43]. In this trial, pregnant Danish women that had daily fish consumption at or below the 50$^{th}$ percentile of the national population (estimated n-3 LCPUFA intake of < 0.4 g/day) were eligible. If these women intended to breastfeed, they were randomly assigned after birth to consume an n-3 LCPUFA supplement or olive oil placebo for 4 months. The supplement was provided as muesli bars, cookies or as capsules containing microencapsulated fish oils. Women with a fish intake in the upper quartile of the population (estimated n-3 LCPUFA intake >0.8 g/d) were enrolled as a reference group. This intervention resulted in significantly higher breast milk DHA in the treatment compared with the control group (1.3% in treatment, 0.4% in placebo). Infant erythrocyte fatty acids measured at 4 months of age showed the supplemented group had higher DHA and EPA, and lower AA compared with the placebo group (Table 1.2). The intervention was also effective at raising erythrocyte membrane DHA and EPA in supplemented infants above that of the high fish diet reference group.

Despite large differences in the total n-3 LCPUFA supplements, the DHA content of supplements in the Gibson *et al* and Lauritzen *et al* trials were similar and resulted in comparable levels of erythrocyte phospholipid DHA (Table 1.2) [42,43]. However, Lauritzen *et al* used a fish oil supplement with a considerable quantity of EPA, and this resulted in a larger increase in erythrocyte phospholipid EPA compared with the Gibson *et al* trial. Surprisingly the erythrocyte AA content remained very similar despite the large differences in total n-3 LCPUFA of the interventions. It is clear from these trials that infants who receive 1% of total fat as DHA from breast milk achieve high levels of DHA in plasma and erythrocyte membranes.

*Blood Phospholipids and High Dietary DHA Intake in Formula Fed Preterm Infants*

As with trials manipulating breast milk LCPUFA, most formula trials use infant plasma and erythrocyte phospholipid content to demonstrate the success of their intervention. Trials that test low levels of DHA in formula are not included in this section as the purpose is to evaluate biochemical status of formula fed infants after high dose DHA supplementation. Along with data from breast fed infants, the erythrocyte phospholipid fatty acid status of formula fed preterm infants have been summarised in Table 1.2.

Three trials have reported erythrocyte fatty acid status of preterm infants after feeding formula enriched with approximately 1% n-3 LCPUFA [32,44,45]. All trials used fish oil supplements, with only one using a low EPA supplement [45]. Uauy *et al* reported increased erythrocyte EPA and DHA after supplementation. Although infants received the trial diet for only 20 – 35 days before blood sampling, there were significant differences in the phospholipid profiles between the control and supplemented groups. All trials tested formulas containing 0.4 – 0.6% DHA. The three formula trials reported comparable erythrocyte DHA concentrations, however the concentration of DHA was lower than the trials intervening with 1% DHA [42,43] (Table 1.2). This is likely to be a result of the low level of DHA in the intervention, but could also be related to the short duration of the intervention compared with the trials in breast fed infants by Gibson *et al* and Lauritzen *et al*. For two of the trials, it may also be a result of the DHA estimated as a percentage of total erythrocyte lipids [32,44] and not erythrocyte membrane phospholipids as reported in other trials.

Information for comparing different doses of DHA in formula and changes to lipoproteins and erythrocyte phospholipids of preterm infants has also been published by Clandinin *et al* [46,47]. This data has not been included in Table 1.2 as erythrocyte phospholipids were reported in separate categories of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol

(PI) and not as total phospholipids as reported in other trials. Clandinin *et al* tested four formulas ranging in concentrations from no DHA or AA, to 0.76% and 1.1% (of total fat) respectively. Both DHA and AA were sourced from single cell oils. Infants in this trial were supplemented with the test formulas for 6 weeks. This duration of feeding was shown to be effective for incorporating the fatty acids into erythrocyte membranes. After the 6-week intervention, infants that received no LCPUFA in formula had lower DHA content in all phospholipid fractions tested. Infants given the formula with the highest concentrations of DHA and AA (0.76% and 1.1% respectively) had significantly higher levels of these lipids in plasma and total phospholipids compared with a reference group of breast fed infants. The outcome of these different diets on the fatty acid composition of lipoprotein classes has been reported and a dose-response effect of DHA upon the phospholipid fractions of the HDL and LDL lipoproteins was found [46]. Clandinin *et al* considered the mid-range DHA and AA formulas as optimal because they exhibited fatty acid status most similar to breast fed infants.

Other reports of plasma and erythrocyte phospholipids after feeding formulas containing LCPUFA have been published, however most have not investigated high dose DHA supplementation. As previously discussed, milk containing 1% DHA would meet the estimated gestational accrual of n-3 LCPUFA for preterm infants[•]. Breast milk DHA reaching 1% of total fat is feasible with DHA capsule supplementation [36] and fat content of infant formula is readily enriched to 1% DHA. It is well accepted that dietary DHA, regardless of the source, is readily absorbed. And that infant plasma and erythrocyte DHA concentrations are responsive to dietary manipulation of DHA from either breast milk or formula.

[•] Calculation based on a 2.5 kg infant consuming 150 mL / kg / day and milk fat of 4%

## 1.4 Visual Development and Influence of LCPUFA in Preterm Infants

For some time there has been controversy amongst visual experts regarding the development of vision in preterm infants. It has been suggested that preterm infants have increased visual experience and therefore have better acuity at young ages than their full-term counterparts [48]. Others have found no difference in the visual performance of full-term and preterm infants [49]. However, an observational study conducted in 9-year-old children showed that acuity was poorer in low birth weight (LBW) infants than those born at term [50]. Irrespective of this debate, there is now little argument that the visual performance of preterm infants can be improved with DHA supplementation. A number of RCTs involving preterm infants have tested acuity after DHA supplementation. This following section reviews trials that measure acuity; other visual measurements such as latencies and electroretinograms (ERGs) are reviewed later.

### *1.4.1 Visual Acuity and Influence of n-3 LCPUFA in Preterm Infants*

Before discussing the DHA intervention trials in preterm infants it is necessary to describe the two principal techniques used for measuring acuity in infants. These techniques are founded on either physiological or behavioural responses of the infant [51]. The physiological techniques elicit a change in electrical potential at the primary visual cortex in response to a visual stimulus, hence are called visual evoked potentials (VEP). The response is detected by attaching electrodes to the infants scalp over the visual cortex. The stimulus is often a grating pattern, although checkerboard patterns have also been used [52]. An adaptation of the technique is to span a range of different sized gratings over a short testing period, this is called sweep VEP [53]. It provides the advantage of collecting acuity data quickly, which is important in a test where infants are required to be attentive. Behavioural techniques require less equipment and expense to set up than physiological methods. They rely on an innate preference to view a patterned stimulus. One of the most commonly used behavioural techniques is the rapid Teller acuity card procedure [54]. In this technique two cards, one plain grey and the other with the test grating,

are placed near each other in the infants' field of view. An observer watches the infant through a small peephole between the cards and judges which card the infant prefers. To estimate acuity, a series of different sized gratings are presented with the grey card and the infant's responses are collected. If the infant looks at the test grating only 50% of the time, then there is no preference for the grating and the infant is unlikely to be able to see the pattern. A probability of choosing the finest test grating 75% of the time is often used to estimate the limit of acuity. The gratings are usually presented a number of times, especially near the acuity limit, to gain confidence in the acuity estimate. There are large differences in acuity measurements generated between the physiological and behavioural techniques, with physiological acuity maturing at a faster rate than behavioural acuity [55-58]. It is not known exactly why there are differences between the techniques but it is thought that it may be a result of the different stimulus conditions, that one techniques taps into different underlying visuo-neural abilities or the additional time required for an infant to master control over looking at a preferred stimulus [58,59].

Trials in formula fed preterm infants that measure acuity after n-3 LCPUFA supplementation have varied widely in their dose of LCPUFA, the duration of supplementation, the methods used to measure acuity and overall trial quality (Table 1.3). One of the first published trials supplemented preterm infants with a formula containing 1% of fat as n-3 LCPUFA (0.4% as DHA, 0.6% as EPA) [5]. The visual development of supplemented infants was compared with two other groups of formula fed preterm infants, one group received formula with fat predominantly from corn oil (providing linoleic acid (LA) and almost no ALA) and the other received formula with fat predominantly from soy oil (providing both LA and ALA). Details of the randomisation procedure and allocation to treatment groups are not clear. At the beginning of the trial, 73 preterm infants were enrolled and randomised to receive one of the three formulas. The number of infants allocated to each formula and the success of the follow up was not reported. An increase in the infant's erythrocyte membrane n-3 LCPUFA demonstrated the effectiveness of the feeding regime. Interestingly, no differences were reported in n-6 LCPUFA content between any of the

randomised formula groups, implying the 1% LCPUFA supplementation regime did not displace the n-6 LCPUFA even after 21 weeks of receiving formula. Acuity assessments were performed masked to the treatment group at 36 and 57 weeks PMA using both electrophysiological and behavioural techniques. After just 4-5 weeks of supplementation, the preterm infants that received the n-3 LCPUFA formula exhibited a small but significant difference in pattern reversal VEP acuity compared with the infants that received the corn oil formula. This result was confirmed at 57 weeks PMA after 21 weeks of supplementation, when infants in the n-3 LCPUFA supplemented group demonstrated acuity over 4 cpd higher than the other formula groups that did not receive any LCPUFA. Less pronounced effects were observed on behavioural acuity at 57 wk PMA where infants that received the n-3 LCPUFA had better acuity than infants that received the unsupplemented formulas, although this did not reach statistical significance. This trial tested one of the highest concentrations of n-3 LCPUFA used in preterm infant formula to date and a sizeable effect on VEP acuity was found. However, specific and important details relating to trial quality and power are sketchy due to absence of information regarding concealment, allocation to the randomisation groups and flow of trial participants.

Improved acuity was subsequently reported by Carlson *et al,* after supplementing preterm infants with 0.5% n-3 LCPUFA (0.3% EPA, 0.2% DHA) until 9 months CA (Table 1.3) [7]. Seventy-nine preterm infants were enrolled but not all infants continued in the trial. After enrolment, 10 infants were excluded for medical reasons and lost to follow up by 4 months CA. These excluded infants were replaced to the group from which they were lost. Important trial information such as the randomisation schedule, allocation and how blinding were protected are not reported. Other pertinent trial information is that the second Teller Acuity Card tester may not have been blinded to the group allocation. The number of tests this investigator performed was not specified. Compared with the control group of infants that did not receive LCPUFA in formula, the treatment group infants demonstrated significantly higher acuity at 2 and 4 months CA but not when tested at 6.5, 9 and 12 months CA. The length of time on oxygen

therapy was related to poorer acuity in all infants at term age and in the control group at 2 and 4 months CA.

The complex relationships between acuity, oxygen therapy and n-3 LCPUFA supplementation were investigated further by Carlson *et al* in a trial in which a more modest dose of n-3 LCPUFA was tested [6]. Marine oil was used to enrich the trial formula, but this time it contained less EPA than DHA (0.06 % and 0.2% of total fat respectively) and the trial formula was fed to infants only until 2 months CA. To represent the general preterm population, study infants that developed bronchopulmonary dysplasia (BPD) were retained, but because BPD was associated with poorer acuity in previous trial, these infants were considered in separate subgroup analyses. There was a high dropout rate this study, with 35 of the 94 infants (37%) lost before 2 months CA. Nineteen infants dropped out for medical reasons (7 died) and a further 14 were not followed up for family reasons, withdrawn or became ineligible during the trial. The high dropout rate became clear to the investigators who attempted to improve follow up at 12 months CA by covering transport costs in attending trial appointments. Sample sizes ranged from n = 3 treatment group infants with BPD at term, to n = 18 control groups infants with no BPD at 2 months CA. The largest samples sizes were at 2 months CA in all groups, resulting in a total follow up of 56 infants. It was reported that the supplementation regime was successful in elevating the DHA component of erythrocyte PE and PC at 2 months CA. No effect of diet was observed on AA content of erythrocyte PE or PC either during the intervention or through follow up to 12 months CA. Comparisons of Teller acuity of the healthy infants showed that the supplemented infants had better acuity than the control infants at 2 months CA. Generally, poorer acuity was found in the subgroup of infants with BPD and acuity was lower in the BPD infants that received the supplemented compared with the control formula. However, the small sample sizes might have reduced the opportunity to detect an effect on acuity at assessments after 2 months CA.

A similar dose of DHA was tested in another trial in which preterm infants receiving no LCPUFA in formula were compared with infants that received formulas containing 0.26% DHA and 0.4% AA until term followed by 0.16% DHA and 0.4% AA until 12 months CA [60]. The trial protocol had two treatment groups; one had their LCPUFA sourced from fish and fungal oils (FF group) and the other from egg triglycerides and fish oils (Egg group). A computer generated the randomisation schedule and enrolments were stratified for site, gender and birth weight. Sample size calculations were based on recruiting a large number of infants to assess developmental outcome and acuity was measured in a subgroup of infants. This trial involved a substantial number of study subjects (n=470) compared with previous RCT in this area. Erythrocyte membrane AA and DHA in the control group were significantly lower than the infants that received formula with LCPUFA. Visual acuity was assessed at 2, 4, and 6 months CA using Teller Cards in 373 infants and at 4 and 6 months CA using sweep VEP acuity in 126 infants. The difference in Teller acuity between treatment and control groups was not statistically significant, however the VEP technique showed higher acuity in supplemented infants at 6 months CA. The dose of DHA given was similar to levels found in breast milk (0.16 - 0.26%) and although O'Connor *et al* argued the importance of supplying preterm infants with preformed n-3 LCPUFA in infant formula, the rationale for testing this low level of DHA was not described.

A further two RCT have tested acuity of preterm infants after feeding formulas containing 0.34% of fat as DHA [61,62]. Acuity was a primary outcome of the trial by Innis *et al* and sample size estimates were based on detecting a difference in Teller Card acuity at 2 and 4 months CA. This trial enrolled many more infants (n = 194) than the van Wezel-Meijler *et al* trial (n = 55) for which structural brain maturation at 3 and 12 months CA was the primary outcome and Teller Card acuity measured at 3, 6, 12 and 24 months CA was a secondary outcome. Both trials were conducted blind and both had follow up rates around 70 to 80% of infants enrolled. Although the duration of feeding the trial formula was very different (28 days [62] compared with 6 months CA [61]), neither trial detected a difference in acuity at any

time. The duration of feeding may not have been sufficient to detect an effect of treatment in the trial by Innis *et al* and the small sample numbers would have reduced the power to detect a significant effect of the treatment in the van Wezel-Meijler *et al* trial. When the more powerful repeated measures test was used to compare acuity at 3, 6, 12 and 24 months between control and treatment groups, the acuity approached statistical significance in favour of LCPUFA supplementation ($p = 0.07$) [61].

Of all the RCT described, two have assessed acuity using VEP *and* behavioural techniques [5,60]. Although there are large differences in design, both of these trials found significantly better acuity in supplemented infants using the VEP technique and no difference in acuity using the Teller card technique. It may appear that the VEP technique is more sensitive in detecting subtle changes in acuity. However, normative Teller acuity data suggests an alternative explanation. Infants at the age of 2 months have a standard deviation (SD) between 0.7 - 0.9 octaves [54]. To detect a difference in acuity of 0.4 cycles per degree, 159 infants would be required to achieve confidence to reject the null hypothesis with 80% power and 95% confidence. At 4 months of age (SD = 0.5 – 0.7 octaves), a sample size of 62 infants would be required to detect a difference in means of 0.5 cycles per degree. With this information, it seems that many studies using the Teller acuity technique have been underpowered. This view is supported with other opinions and evidence in literature [63,64].

Other factors relating to trial quality, power and confounding factors may have influenced the findings from n-3 LCPUFA trials. The conduct of the trial is paramount to the validity of the data generated. It has been recommended that reporting of specific information relating to enrolment procedures, protection of randomisation, allocation and concealment are necessary to demonstrate the validity of findings from a RCT [65]. Incomplete reporting of all this information has been noted in the LCPUFA acuity trials in preterm infants (Table 1.3). Recent trials have shown small or no improvements in acuity, which could

Table 1.3: Acuity Measures from RCT in Preterm Infants Fed Formula Containing LCPUFA

| Reference | 5 | 7 | 6 | 60 | 62 | 61 |
|---|---|---|---|---|---|---|
| LCPUFA - n-3 (DHA) | 1% (0.4% DHA) | 0.5% (0.2% DHA) | 0.26% (0.2% DHA) | 0.35% (0.26% DHA) | 0.34% (0.34% DHA) | 0.34% (0.34% DHA) |
| - AA | 0% | 0% | 0% | 0.42% | ± 0.6% | 0.7% |
| - Fat Source | Fish oil | Fish oil | Fish oil | EggTG /Fish /Fungal | Algal / Fungal | Algal / Fungal |
| Duration (wk) | 4 mo CA | 9 mo CA | 2 mo CA | 12 mo CA | ≥28 days | 6 mo CA |
| Infants - enrolled | n = 73* | n = 89* | $n_C$ =94* | $n_C$ =142 $n_T$ =278 | $n_C$ =62 $n_T$ =132 | $n_C$ =26 $n_T$ =29 |
| - assessed | n = 73* | $n_C$=34 $n_T$ =35 | $n_C$ =33 $n_T$ =26 | $n_C$ =39 $n_T$ =87 | $n_C$ =51 $n_T$ =102 | $n_C$ =20 $n_T$ =22 |
| Acuity Method | VEP & Teller | Teller | Teller | VEP & Teller | Teller | Teller |
| Acuity improvement in | 4mo | † 2mo; ≈ 0.6, p<0.014 | 2mo; 0.7, p<0.02 only | 4mo; Teller VEP NS | 2 & 4 mo; NS | 3, 6, 12 and 24 mo CA; |
| treatment compared | † VEP ≈ 4.5, p<0.002 | † 4mo; ≈ 0.5, p<0.002 | in healthy infants | 6 mo; VEP 3 to 4 cpd | | NS |
| with control (cpd) | † Teller ≈ 1.0, p<0.061 | 6.5, 9, 12 mo; NS | 4, 6, 9, 12 mo; NS | p<0.01. Teller NS | | |
| Trial Details | | | | | | |
| Allocation | NR | Randomly assigned | Randomly assigned | Computer, stratified | Computer, stratified | Random list |
| Concealment | Unclear | Inadequate | Unclear | Adequate | Adequate | Adequate |
| Blinding | Yes | Yes | Yes | Yes | Yes | Yes |
| Sample Size Estimate | No | 20 per group | 15 per group | Not for acuity | 32 per group | No |
| Drop outs | Unclear | 10 replacements | 35 (37%) | Unclear for acuity | 46 (24%) | 13 (24%) |
| ITT | Yes | No | No | Yes | Yes | No |

ctl, control; BM, breast milk; cpd, cycles per degree; ITT, intention to treat; NR, not reported; NS, acuity difference not significant; TG, triglyceride

$n_C$ = control group, $n_T$ = treatment group

*number of infants in control and LCPUFA groups not reported separately, † approximate difference estimated from published graph

be a result of low dose or short duration of the intervention. However, the suitability of the method used for detecting differences in acuity must be carefully considered, along with the sample sizes and the most sensitive age to detect a difference in acuity. Furthermore, other issues such as DHA status at study onset, gender effects and perhaps even the effect of breastfeeding may have contributed to the outcome of these trials. These variables are important to consider as differences in acuity of boys and girls [66] have been demonstrated in infants born at term. The gender balance of trial groups should be stratified at enrolment or may need to be controlled in analyses [67].

To address differences in trials design, acuity data collected from the first 3 acuity trials were combined in a meta-analysis [63]. These trials each reported improved VEP acuity [5] or behavioural acuity [6,7]. Not surprisingly, when combined in the meta-analysis LCPUFA supplemented infants showed improved acuity with both behavioural and electrophysiological techniques. Comparisons were made on the differences in acuity between treatment and control groups in each trial, with separate analyses conducted for VEP and behavioural data. This helped to overcome the larger differences in absolute acuity found when comparing acuity techniques and differences between sites. The updated Cochrane review published in 2004 included five new trials but reported that there was not sufficient evidence to endorse the addition of LCPUFA in formula for preterm infants [68]. For the acuity component of the review, data from the Birch *et al* trial was not available and the two trials reported by Carlson *et al* were not included in the review tables as they were presented as graphs and therefore could not be incorporated into the analysis [5-7]. The reviewer's comments on the effect of LCPUFA on acuity were based upon the 3 RCT that supplemented infants with merely 0.3% n-3 LCPUFA [60-62].

In recent years, we have seen the introduction of AA to formula. There is little evidence to support an efficacious role for AA in the development of acuity, as three trials that supplemented infants with only n-3 LCPUFA reported improvements in VEP and Teller card acuity [5-7] and small or no benefits to acuity

have been reported since the inclusion of AA [60-62]. Although AA was added to 'balance' the intake of n-3 and n-6 LCPUFA in formula, two of the acuity trials clearly showed that no changes in erythrocyte AA was observed after supplementation with n-3 LCPUFA in formula [5,6].

Over the last 15 years of LCPUFA research, a gradual decline in the dose of n-3 LCPUFA tested in RCT has been observed. The reduction in dose has been driven by designing interventions on the average breast milk DHA content (0.2 to 0.3% of total fat). Greater justification should be given to the dose of DHA tested. Of the all the LCPUFA trials in preterm infants that have detected a significant change in acuity, the greatest improvement remains with the trial that tested the highest dose [5]. Interestingly, this trial tested 1% n-3 LCPUFA in formula, a dose that would meet the estimated accrual rate during the last trimester of pregnancy.

*Breast Milk, DHA and Visual Acuity*

Formula intervention trials often include breast fed infants as a reference group and frequently test levels of DHA that comparable with average concentration in breast milk of Western women. However, observational studies have demonstrated a relationship between breast milk DHA and visual acuity in full-term infants [69,70]. Jorgensen *et al* found a significant ($p = 0.02$) but weak correlation ($R^2 = 0.09$) between breast milk DHA and sweep VEP acuity in 4 months old infants. This association was found in Danish women who typically consume a diet higher in fish than other Western women and had breast milk DHA levels ranging from 0.1 to 1.2% (of total fat). A strikingly similar association between erythrocyte DHA and Teller acuity was also found in Canadian children ($R^2 = 0.09$, $p<0.05$) fed breast milk ranging from 0.1 to 0.9% DHA [69].

The ability to change the concentration of DHA in breast milk offers a unique opportunity to investigate various doses for optimal acuity development. A number of trials in full term infants have measured

acuity after supplementing lactating mothers with n-3 LCPUFA [42,43,71]. Gibson *et al* used a transient VEP technique to measure acuity of infants at 3 and 4 months, Lauritzen *et al* measured sweep VEP acuity at 2 and 4 months and Jensen *et al* tested sweep acuity at 4 months and Teller acuity at 4 and 8 months of age. In these trials the supplementation period was long enough to detect changes in erythrocyte phospholipids, but no significant improvement in acuity was detected in the supplemented infants. A low level of DHA supplementation or low spatial frequency of the stimulus [71], problems with the VEP test [42], and perhaps a high baseline fish diet of mothers [43] could have resulted in an absence of a treatment effect in these studies. However, it is also possible that breast fed term infants are sufficiently well nourished to support optimal postnatal visual development, hence preventing acuity enhancement with increased DHA intake.

The relationship between DHA intake and acuity development in preterm infants has not been thoroughly investigated. One of the problems encountered when investigating breastfeeding in preterm infants is that the duration of breastfeeding is frequently shorter than for term infants and many preterm infants receive a mixed breast milk and formula diet [72-76]. One observational study of preterm infants has investigated the effect of breast milk DHA on visual acuity at 3 months of age using the Teller acuity card technique [77]. In this small study, most infants (n=14, 78%) received a mixed breast milk and formula diet. Average breast milk DHA content was 0.27% of total fat and supplementary formula feeds contained no DHA. The infants were dichotomised (into low or high breast milk consumption) for analysis and at 3 months CA, 7 of the 9 infants in the high breast milk group and 4 of the 7 infants in the low breast milk group were continuing to receive breast milk. A relationship between dietary breast milk consumption and plasma and red cell membrane DHA was reported, but higher DHA did not confer better visual acuity. This is not surprising given acuity testing used the Teller technique, the low number of study infants and most infants receiving mixed breast milk and formula diets. This combination of

study features has resulted in an inability to determine if breast milk DHA has an effect on visual acuity in preterm infants.

RCT investigating different doses of DHA have not been performed in breast fed preterm infants. Most intervention trials have been conducted in formula fed infants and low levels of DHA that are similar to average breast milk DHA in women consuming Western style diets are often tested. To date, there is no powerful evidence to suggest that the average DHA content of Australian breast milk is optimal for preterm infant development. The inadequacy of breast milk to meet nutritional demands of the preterm infants is not unexpected as it has been established that preterm infants require breast milk fortified with protein, minerals and fat soluble vitamins [28]. It seems logical that the level of DHA supplied during the last trimester of pregnancy would be an ideal model for predicting the requirements of a preterm infant. No trials have designed the DHA intake of the preterm infant to meet the expected levels of accretion *in utero* and the concentration of DHA in breast milk and its effect on visual acuity in preterm infants has not been well investigated.

### *1.4.2 VEP Latency and Influence of DHA and other n-3 LCPUFA*

As with studies of visual acuity in preterm infants, there is evidence to support and also to dispute that early visual experience hastens latency responses of preterm infants [48,49,78]. VEP latencies are reliable measurements that determine the speed of neural processing from the visual stimulus to the peak neuronal depolarisation in the primary visual cortex and are indicative of the integrity of the visual pathway. One of the advantages of latency measurements (over acuity measurements) is that the temporal component of a VEP is much less variable than the amplitude function on which acuity is dependent.

Latencies to VEP measurements have been described in LCPUFA trials in preterm infants. Latency is usually reported as the time to the first negative (N1 or N100) or positive peak of the response (P1 or P100). Flash latency, measured in response to diffuse bursts of light from a stroboscope placed in front of the infants eyes has been reported in two LCPUFA intervention trials [61,79] and using light emitting diode (LED) goggles in another trial [45].

In the first RCT of flash VEP latency, healthy preterm infants fed a formula containing 0.3% n-3 LCPUFA (0.2% DHA and 0.1% EPA) and 0.35% AA until 3 months CA were reported to have faster flash VEP latencies than infants fed a control formula with no LCPUFA [79]. Although the VEP tester performed the assessments without knowledge of the dietary group, randomisation and intervention were not masked. Of the 49 infants assigned to trial formulas, 46 were followed until 3 months CA and VEP data were collected from only 34 (74%) of these infants. The VEP observations were based on N2, P2, N3, P3, P4 and N4 components of the latency waveform. However, all components of the VEP response were not visible in all infants and consequently the significant difference in latency was based upon comparisons of the N4 and P4 latencies between 10 and 12 control infants and 14 and 17 treatment infants respectively. Compared with the control group, infants that received LCPUFA in formula had morphologically mature responses, more typical of a breast fed reference group that had been tested in parallel. However small sample sizes and the poor success rate of testing limits the interpretation and observations of this study.

A second RCT compared flash latency responses of infants fed no LCPUFA in formula with infants fed 0.34% DHA and 0.7% AA until 6 months CA [61]. Infants that developed any neural abnormalities or complications such as sepsis, necrotising enterocolitis (NEC), retinopathy of prematurity (ROP) or BPD were excluded, resulting in a large proportion (29%) of infants enrolled not followed up. The amplitude and latencies of the first positive wave, the subsequent negative wave were reported for 19 control and

20 supplemented infants at 3 months CA. No effect of the treatment was evident at this time, nor when followed up at 12 months CA.

No difference in latency responses were detected between control and DHA supplemented infants that received 0.7% n-3 LCPUFA (0.6%DHA and 0.1% EPA) and 0.1% AA [45]. Healthy preterm infants born <34 weeks PMA were given the trial formula for a minimum of 30 days. VEP latency responses were recorded from infants at 36 weeks PMA. The prematurity of the infants and their early assessment meant that for some infants the first peak (P1) component was not detectable, consequently only latency of N1 was reported. Testing infants before maturation of the required feature of the visual system reflects poorly on the design and feasibility of the study. It is also not clear how many of the 9 control and 13 supplemented infants remaining in the trial at 30 days were successfully tested, or how many infants were included in the statistical comparisons. Furthermore, the DHA content of plasma phospholipids and red cell membranes at study onset and after the intervention were not significantly different between groups. The authors suggest that infants receiving formula with no LCPUFA may be able to meet LCPUFA requirements through endogenous synthesis from EFA precursors. However, other evidence indicates the endogenous synthesis of DHA is slow [3]. Furthermore, an increase in plasma phospholipid and erythrocyte DHA occurs when feeding a DHA-enriched formula [47] and changes to plasma phospholipid DHA have been found over similarly short intervention periods [44]. Although it is possible that the short duration of the intervention in this study limited the opportunity for incorporation of DHA into red cell membranes, the lack of an increase in plasma phospholipid DHA is curious and may reflect some other problem in the trial such as issues with compliance or perhaps difficulties with fatty acid analysis.

All three trials investigating the effect of LCPUFA on visual latency have small numbers and large standard deviations, which would clearly minimise the likelihood of detecting differences between

treatment and control infants. Neither the Bouglé *et al* nor Faldella *et al* trials have determined the effect on the P1 component of latency. Furthermore, most trials have not included infants representing the usual range of morbidity of the preterm population, such that infants with common complications of prematurity including BPD, ROP, gastrointestinal complications and neurological abnormalities were excluded. The effect of DHA enriched formula on VEP latency has not been well investigated.

### *1.4.3 Effects of n-3 LCPUFA on Retinal Function in Preterm Infants*

An electroretinogram (ERG) is the total electrical potential released from the retina in response to flashes of light. In an ERG measurement the electrical response of the *fovea* is overshadowed by the full retinal response. ERG testing of infants according to the standard protocol described by the International Society for Clinical Electrophysiology can glean specific information relating to rod and cone function. In this protocol, the retina is stimulated with a standard sequence of light that measure dark-adapted, maximal rod, oscillatory single-flash cone and light adapted flicker responses. The onset of each response varies with age and at full term age usually the rod response is used for infants. Birch *et al* have described rod and cone ERG in a small number of preterm infants as guide for normal responses in this population [80]. The ERG response of preterm infants has been examined in two LCPUFA intervention trials.

Preterm infants that received n-3 LCPUFA for only a few weeks demonstrated better ERG responses than infants given formula with no LCPUFA [15,44,81]. At just 36 weeks CA, control infants had lower amplitudes and higher thresholds (requiring greater stimulus) of the rod response when compared with infants receiving the n-3 LCPUFA supplemented formula. The supplemented infants exhibited rod responses more like preterm breast fed infants. By 4 months CA, there were no significant differences in retinal function between the groups. No differences were found in cone function between the groups at either age tested. The importance of this outcome is unclear and further research is necessary to

explain this finding, however it is thought that differential effects on rods and not cones may be due to different rates of maturation [80]. Infants in the control group eventually reached similar retinal function as infants fed the enriched formula, indicating that rod maturation was delayed in infants receiving no n-3 LCPUFA [15].

The effect of DHA on ERG responses of preterm infants has also been investigated by Faldella *et al* [79]. Infants born <33 weeks gestation were randomly assigned to receive a formula containing 0.3% n-3 LCPUFA (0.2% DHA, 0.1% EPA) and 0.4% AA until 3 months CA or a formula containing no LCPUFA. Infants receiving the enriched formula had ERG wave morphology that was not different from the control formula group or a reference group of breast fed infants. However, the testing technique was not optimal as the infant's pupils were not dilated or dark adapted before testing and the positioning of the active electrode on the skin would have almost certainly reduced the sensitivity of the test [82]. This departure from the internationally accepted ERG protocol limits the validity and applicability of these data to the wider preterm community, comparisons with other research in this area and may explain the absence of difference between infants fed breast milk and those fed formula with no LCPUFA.

A relationship between rod function and DHA status has been demonstrated in breast fed preterm infants [77]. In an observational study, ERG responses of infants born 25 to 32 weeks GA (n = 18) were measured at term age. Nearly all infants received a mixed diet and the proportion of breast milk in the infants' diet was correlated with a higher DHA content in plasma and red cell phospholipids. This was probably an effect of breast milk, as the formula used to compliment feeding was devoid of LCPUFA. The DHA concentration of plasma ($r = 0.733$, $p = 0.001$) and erythrocyte membranes ($r=0.502$, $p = 0.04$) was also correlated with rod reaction time (scotopic implicit time).

With the relatively limited data available, dietary DHA appears to influence the retinal responses of preterm infants. In a small LCPUFA intervention trial and an observational study of breast fed infants, higher dietary n-3 LCPUFA intake was related to improved rod function. DHA has not been associated with improved cone function in intervention trials.

## 1.5 Global Development and Influence of DHA and other n-3 LCPUFA

### *Deficits of Development in Preterm Infants*

Epidemiological evidence and observational studies have shown that preterm infants have altered performance in many tasks when compared with term infants. Recently, physiological differences in volume of neural tissue have been found between preterm and full term infants observed using MRI analysis [83]. Other differences between preterm infants and their term counterparts in performance-based outcomes have also been identified. These include problems with cognition [84], language processing [85], visuo-motor [84], executive functioning [86], behaviour [87] and other indicators of general development and intelligence [88-90].

### *Breast Milk and Development of Preterm Infants*

Diet may have some influence on development as preterm infants fed breast milk have improved global development at 18 months CA compared with formula fed infants [91]. Development in infancy is a modest predictor of performance in childhood [92]. Follow up of formerly preterm infants into childhood has shown enhanced performance of breast fed preterm infants in intelligence tests (measured by the Wechsler Intelligence Scale for Children) compared with formula fed infants [93]. The improvement was related to intake of breast milk in the infants' diet in the first 28 days of life. Speculation began regarding the possibility that the LCPUFA component of breast milk was affecting later intelligence, and investigations into the effect of LCPUFA supplementation on cognitive functions were kindled.

*RCT of LCPUFA Supplementation and Development of Preterm Infants*

A number of different techniques have been employed to assess infant development in response to LCPUFA supplementation. Traditional tools used to measure infant development (such as the Bayley Scale of Infant Development, BSID) usually compare an individual's performance to a population norm in order to identify infants requiring therapy or interventions. Many of these types of tests were not designed to be predictive of later intelligence. However some of these techniques are well validated for measuring development and consequently are often used in LCPUFA trials to compare development between supplemented and control infants.

The Fagan Test of Infant Intelligence (FTII) is a test of cognitive function [94] that has been used in n-3 LCPUFA intervention trials. This test records an infants' preference for a new stimulus after a period of familiarisation to a picture, usually of a face. The duration and number of looks the infant makes to the new stimulus are measured. Infants usually prefer a new novel stimulus compared with a familiar one. When conducted in infancy the FTII has been shown to have a modest predictive validity to performance in childhood [92].

In two separate trials, preterm infants fed n-3 LCPUFA-enriched diets showed no difference in the proportion of time spent looking at a novel stimulus, but the way they looked at the new stimulus was different [95,96]. When presented with a new stimulus, preterm infants fed n-3 LCPUFA-enriched diets had a greater number of looks, with each glance of shorter duration. These observations were present at 2 and 9 months CA and are supported with similar evidence in primate infants [97]. A subsequent trial investigating the effect of supplementing infants with 0.3% DHA and 0.4% AA until reaching 12 months CA reported a significant difference in the novelty preference at 6 and but not 9 months CA [60]. The number of discrete looks at the novel or familiar stimulus was not reported, so these results could not be compared with previous work. Explanations put forth for the effect of LCPUFA on number of looks

include faster visual processing, altered attention or differences in the ability to disengage from a visual stimulus [98]. The difficulties in interpretation of FTII have contributed to a recent move away from recognition memory testing in favour of newer techniques that measure performance of specific neural domains such as attention, engagement and disengagement from a visual stimulus. These emerging tools may be better predictors of long-term development [99].

Five trials have used BSID scores to investigate preterm development after LCPUFA supplementation [60,61,100-102]. Three of these trials have reported no significant differences in mental development index (MDI) or psychomotor development index (PDI) of infants that receive no LCPUFA in formula with those supplemented with 0.2 to 0.3% DHA and 0.3 to 0.7% AA for up to 12 months CA [60,61,100]. One of these trials was not performed in an intention-to-treat manner and had a small sample size (42 infants) [61] and consequently was underpowered for detecting differences in infant development. Fewtrell *et al* reported no significant differences after feeding formula containing 0.2% DHA and 0.4% AA for only 1 month [100]. The low level of supplementation and the short duration of the intervention may have limited the opportunity to detect a significant improvement in neurodevelopment in this trial. Recently, Clandinin *et al* reported improved MDI and PDI in infants fed formula containing 0.3% DHA and 0.7% AA to 12 months CA compared with infants fed no LCPUFA in formula [102]. Unfortunately this trial was not conducted on an intention-to-treat basis, as only infants that were exclusively formula fed at EDD and had received 80% or more of their diet as trial formula at EDD were eligible for BSID outcome. In the trial by O'Connor *et al,* although no differences in intention-to-treat comparisons were found, subgroup analysis involving the smallest infants (those born at <1250g) showed a 9-point improvement in PDI (p=0.007) in infants fed formula supplemented with fish & fungal oils compared with infants fed formula with no LCPUFA [60].

Of the five LCPUFA trials assessing BSID, four have compared development of infants receiving n-3 and n-6 LCPUFA, but only one has supplemented infants with only n-3 LCPUFA [101]. The treatment formula in this trial contained the shorter-chain arachidonic acid precursor, gamma-linolenic acid (0.9%), as a strategy to increase endogenous synthesis of AA along with 0.6% n-3 LCPUFA (0.5% DHA, 0.1% EPA). No significant differences in intention-to-treat comparisons of BSID motor development index (MDI) or in psychomotor development index (PDI) were found at 18 months CA. However, subgroup comparisons showed a significantly higher MDI in boys that received the supplemented formula compared with boys that received the control formula.

The MacArthur Communicative Development Inventory (CDI) has been used to assess language development of preterm infants in LCPUFA intervention trials [60]. A subgroup of English-speaking singleton preterm infants that received a LCPUFA enriched formula had improved vocabulary comprehension in the MacArthur CDI than infants that received no LCPUFA [60]. Although increased vocabulary size was not seen in intention-to-treat group comparisons, the English format of the test may preclude fair assessment of infants exposed to languages other than English in the home environment.

In summing the evidence from trials investigating the effect of LCPUFA on development of preterm infants there appears to be a trend towards improved development. Trials that have included both n-3 and n-6 LCPUFA have reported no improvements [61,98], or improvements to subgroups of infants [60]. Trials including n-3 LCPUFA trend towards improvements to development with n-3 LCPUFA supplementation [95,96,101]. Therefore it is not clear if n-6 LCPUFA have any efficacious role in preterm development. Clearly there is a lack of conclusive evidence, which has prevented endorsement for addition of LCPUFA to infant formula [68]. This is mainly due to factors relating to trial design, such as high quality intention-to-treat blinded RCT with adequate sample sizes. However, it is possible issues relating to DHA dose, duration of supplementation and the limitations of the assessment tools may have

subverted the opportunity to demonstrate efficacy. Many gaps exist in the current knowledge base, for example, it is not known if any improvements measured in infancy extend into childhood or even adulthood. And, it is not known if interventions should be aimed directly at the smallest and most vulnerable infants, although indications from the trials discussed here suggest that these infants may be more responsive to LCPUFA supplementation. Furthermore, the role of n-6 LCPUFA in preterm development is not clear and separating out the cognitive effects of n-3 from n-6 LCPUFA will need to be the subject of further work.

All of the trials that assess the relationship between DHA and preterm development have tested formula concentrations of DHA that are comparable to the DHA content of breast milk. Like the trials assessing infant acuity, none have considered reconciling the formula DHA content with *in utero* accrual rates. The greatest reluctance investigators may have in providing preterm infants larger doses of DHA is the potential to affect infant growth.

## 1.6 Growth of Preterm Infants Fed Formulas Enriched in LCPUFA

In this section of the review, I describe the effect of LCPUFA intake on growth of preterm infants. The discussion is limited to trials in formula fed infants as no similar reports exist in breast fed infants. Growth performance is often used as an indicator of safety in formula trials. Effects on preterm infant growth are extremely important and improvements are sought after, as these infants are often smaller through childhood than infants born full term [103].

### *1.6.1 Growth of Preterm Infants Fed Formula Enriched with n-3 LCPUFA (No n-6 LCPUFA)*

In the early 1990s, a trial investigating the effect of n-3 LCPUFA on visual performance of preterm infants also described poorer growth performance [104] (Table 1.4). It was reported that infants fed formula containing 0.5% n-3 LCPUFA compared with those fed no LCPUFA had lower weight, length

and head circumference from 2 months CA until completion of follow up at 12 months CA. The increased proportion of girls in the treatment group could have contributed to the finding. However, comparisons of z-scores which adjust for gender, also demonstrated poorer z-score weight, length and head circumference from 40 weeks PMA through to 12 months CA in the treatment group compared with control group infants. Since this study controversy regarding the growth of infants receiving formula containing n-3 LCPUFA has emerged.

The marine oil supplement used in the trial reporting poorer growth after n-3 LCPUFA supplementation contained a higher EPA content than DHA (0.2% DHA, 0.3% EPA). Carlson *et al* showed that the intervention significantly altered the infants' fatty acid composition of erythrocyte membranes to reflect the higher n-3 LCPUFA content of the formulas [105]. This occurred at the expense of the n-6 LCPUFA content of the membrane; as more n-3 LCPUFA were incorporated into the cell membrane, less n-6 LCPUFA were included. It was hypothesised that the prolonged feeding of the trial formula may have provided too much EPA consequently affecting growth. This theory was supported with evidence of reduced cell membrane AA content associated with decreased infant growth [106]. Carlson *et al* hypothesised that a balanced source of n-3 to n-6 LCPUFA was necessary for healthy growth in preterm infants [106].

A number of subsequent trials have reported growth of preterm infants after receiving n-3 LCPUFA enriched formula (Table 1.4). Two have reported poorer growth in preterm infants after supplementation with n-3 LCPUFA from fish oil compared with infants fed no LCPUFA [6,107]. In both of these trials the treatment formula contained 0.2% DHA, had comparatively low levels of EPA (0.04% and 0.06%, respectively) and no n-6 LCPUFA. The investigators designed the feeding regime to minimise deficits in growth seen in the previous trial by delivering a lower concentration of EPA and reducing the duration of supplementation [6]. In the trial by Carlson *et al,* the supplemented infants

exhibited lighter weight at 6 and 9 months CA, smaller head circumference at 9 months CA and lower weight-for-length at numerous times [6]. Ryan *et al* reported no differences in weight, length or head circumference of girls receiving the LCPUFA formula [107]. However, the treatment group boys had lower energy intake from formula ($p<0.05$), poorer weight ($p<0.05$), length ($p<0.01$), head circumference ($p<0.05$) and slower gains in weight, length and head circumference from compared with the control group. Infant fatty acid status was investigated in both trials. Erythrocyte phospholipids were changed in line with the increased dietary n-3 LCPUFA and despite the modified feeding regime, the ratio of AA:DHA was affected in supplemented infants [6]. Ryan *et al* reported no relationship between plasma AA and growth when all infants were considered, but described that increased plasma DHA was associated with lower weight and length gain in boys and paradoxically with increased head circumference in girls. These findings were based on relatively small sample sizes (between n=25 and n=31) and both trials observed high drop out rates (37% and 34%). Furthermore, Ryan *et al* did not estimate sample sizes based on gender, consequently any subgroup comparisons demonstrate substantially reduced power.

In contrast, three trials have reported no effect of n-3 LCPUFA in formula on preterm infant growth[108,32,62]. Uauy *et al* fed preterm infants formula containing 1% n-3 LCPUFA (0.4% DHA and 0.6% EPA) and found no differences in weight, length or head circumference between supplemented and control infants at 4 months CA [108]. A high dropout rate was observed with growth assessments performed on 43 of the 60 infants (70%) randomised, which did not meet sample size estimates. In another small study, Jacobs *et al* reported no differences in weight, length or head circumference in preterm infants fed 1.1% n-3 LCPUFA (0.5% DHA and 0.5% EPA) for 6 weeks during hospitalisation [32]. The frequency of recording growth measurements is unclear, as growth was reported as gain in weight and length during the intervention period and no follow up data was reported at completion of the intervention. Furthermore, the trial was not performed in an intention-to-treat manner as growth analysis

Table 1.4: Growth Performance of Preterm Infants Enrolled in RCT of n-3 LCPUFA Formula Supplementation

| Reference | 108 | 104 | 6 | 107 | 62 | 32 | 101 |
|---|---|---|---|---|---|---|---|
| DHA | 0.4% DHA | 0.2% DHA | 0.2% DHA | 0.2% DHA | 0.34% DHA | 0.5% DHA | 0.5% DHA |
| EPA | 0.6% EPA | 0.3% EPA | 0.06% EPA | 0.04% EPA | | 0.5% EPA | 0.1% EPA |
| Source | Fish Oil | Fish Oil | Fish Oil | Fish Oil | Algal Oil | Fish Oil | Fish Oil |
| Eligibility | 1000–1500 g & d10 EI = 70-120 kcal/kg | 748 – 1390 g & EI >110kcal/kg/d | <5d and EI =100 kcal/kg/d by 6wk | 750 – 2250 g AGA | VLBW & AGA, EI = 50 kcal/kg/d | 30 – 35wk GA | <35 wk GA, <2000 g and received formula |
| Intervention Period | <10 d to 4moCA | ≈32 to 9mo CA | <5d to 2mo CA | 35 to 19wk CA | ≥28 days | † 6 wk | 33 to 9 mo CA |
| Infants - enrolled | $n_C$ = 18 $n_T$ = 22 | n = 79* | n = 94* | $n_C$ = 45 $n_T$ = 45 | $n_C$ = 62 $n_T$ = 66 | n = 52* | $n_C$ = 116 $n_T$ =122 |
| - followed up | $n_C$ = 13 $n_T$ = 14 | $n_C$ = 34 $n_T$ = 31 | $n_C$ = 26 $n_T$ = 25 | $n_C$ = 32 $n_T$ = 31 | $n_C$ = 47 $n_T$ = 49 | $n_C$ = 15 $n_T$ = 13 | $n_C$ = 93 $n_T$ = 105 |
| Assessment age (PMA) | 40, 48, 57 | 40, 48, 57, 35, 79,92 | 40, 48, 57, 35, 79,92 | 37, 39, 43, 47, 51,59 | 40, 48, 57 | Frequency unclear | 79, 118 |
| Trial Details; | | | | | | | |
| Allocation | Randomly assigned | Randomly assigned | Randomly assigned | Computer, stratified | Computer, stratified | Random | Randomly assigned |
| Concealment | Unclear | Inadequate | Unclear | Adequate | Adequate | Unclear | Adequate |
| Blinding | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Sample size estimate | 15 per group | 20 per group | 25 per group | 32 per group | 50 per group | Not for growth | Not for growth |
| Not followed up | 13 (33%) | 10 replacements | 43 (46%) | 27 (30%) | 32 (25%) | 24 (46%) | 40 (17%) |
| ITT | Yes | No | No | No | Yes | No | Yes |

| Reference | 108 | 104 | 6 | 107 | 62 | 32 | 101 |
|---|---|---|---|---|---|---|---|
| Difference in growth measurement of LCPUFA supplemented infants compared with control group; | | | | | | | |
| - Weight | ND | ↓ z-scores from term to 92 wk PMA | ↓ z-scores at 66 & 79wk PMA | ↓ boys at 19wk PMA | ND | ND | ND |
| - Length | ND | ↓ z-scores from term to 92 wk PMA | ND | ↓ boys at 19wk PMA | ND | ND | ND |
| - HC | ND | ↓ z-scores from term to 92 wk PMA | ↓ z-scores at 79wk PMA | ↓ boys at 11, 19wk PMA | ND | ND | ND |
| - Other | | ↓ weight-for-length at 79 and 92 wk PMA | ↓ weight-for-length at 48, 66, 79 and 92 wk PMA | ↓ gains in weight, length & HC in boys | | ↑ gain in HC over intervention period | ↑ gain in weight and length |

* number of infants in control and LCPUFA groups not reported separately

† Commencement of intervention unclear

$n_C$ = control group, $n_T$ = treatment group

AGA, appropriate for gestational age; BM, breast milk; CA, corrected age; EI, enteral intake; HC, head circumference; ITT, intention-to-treat; LCPUFA, long chain polyunsaturated fat; mo, months; PMA post menstrual age; ND, no difference between control and supplemented infants; NR, Not Reported; VLBW, very low birth weight

was performed on infants that received >90% of the trial diet, which was a low 54% (n = 28) of those enrolled. No sample size estimates to evaluate effects on growth were reported.

Innis *et al* has reported no difference in weight, length or head circumference of preterm infants after supplementation with n-3 LCPUFA [62]. In a double blind RCT, infants were fed a preterm formula with no LCPUFA (control formula), or one of two different test formulas (Tables 1.4 and 1.5). The first test formula was enriched with 0.3% of fat as DHA and the second formula contained both 0.3% DHA and 0.6% AA. The formulas were fed to preterm infants for a minimum of 28 days, until the infants were discharged from hospital, after which they received a term formula containing no LCPUFA. Pre-planned sample size estimates for measuring effects on infants' growth were met. No differences in weight, length and head circumference measurements were found between the control group and n-3 LCPUFA supplemented infants at EDD, 2 or 4 months CA, which could be attributed to the short duration of the intervention. However, weight at 2 months CA was significantly lower in the control and n-3 LCPUFA supplemented infants when compared with infants that received both n-6 and n-3 LCPUFA. These differences may indicate that growth was not affected by the n-3 LCPUFA but was enhanced when a source of n-6 LCPUFA was included in the formula. There were no significant differences in weight, length or weight-for-length between the randomised formula groups at 4 months CA. Many more trials that assess growth of preterm infants in response to a formula containing both n-3 and n-6 fatty acids have been published. These are discussed further in the following section.

Interestingly, the largest and therefore best powered trial supplementing preterm infants with 0.6% n-3 LCPUFA (0.5% DHA, 0.1% EPA) has reported some improvements in growth [101].. Although this formula had no n-6 LCPUFA, it did contain a relatively high concentration (0.9%) of the n-6 PUFA gamma-linolenic acid which may have supported endogenous synthesis of AA. The formula was fed to infants until 9 months CA and no differences in weight, length or head circumference were observed between

control and treatment groups at 9 or 18 months CA. In fact, greater weight gains from enrolment to 9 months CA were reported in the supplemented compared with the control group. In pre-planned subgroup comparisons, LCPUFA supplemented boys experienced greater weight gain from enrolment to 9 months and length gain from enrolment to 18 months.

Although it has been theorised that a balanced addition of n-6 LCPUFA can help to reduce a negative effect of n-3 LCPUFA on preterm infant growth [106], no differences in growth outcomes have been detected in all n-3 LCPUFA trials and one trial has suggested enhanced growth with supplementation. The implementation of the trial, sample sizes, age at assessment, the dose and duration of supplementation may have contributed to the apparent disparity in outcomes (Table 1.4). However, important factors relating to trial quality may have also influenced findings. Not all trials were designed at the outset with sufficient statistical power to detect differences in growth, others trials were not performed in an intention-to-treat manner or had large dropouts. Furthermore details such as the concealment of allocation are not clearly reported. Many trials have not adequately addressed the issue of birth size as an important predictor of later size. This emphasises the need for balanced sizes between groups at enrolment, which can be addressed in part by stratifying randomisation by birth weight. The trials also differ in their manner of reporting growth data. One trial has reported growth velocity [32], others present data from girls and boys combined [6,104,107], or reported data as Z - scores[6,62,104,108]. Z-scores overcome gender imbalances by comparing infant growth to a gender norm, but not all trials also published the mean and SD of data, which prevents the reader from conducting calculations and comparing the data to infants in other trials.

The discrepancies found in LCPUFA trials reporting growth outcomes has complicated interpretation and hindered decision-making. In an attempt to address differences in trial design, data from many trials were combined in a meta-analysis [68]. The outcome of the analysis indicated that LCPUFA

supplemented infants were heavier at 2 months CA, and longer at term and 2 months CA compared with infants fed formula with no LCPUFA. However, the analysis combined data from trials investigating n-3 LCPUFA in formula with trials supplementing infants with both n-3 and n-6 LCPUFA to reach this conclusion. This prevents separating the effects of n-3 LCPUFA in formula and determining the efficacy of including n-6 LCPUFA. An international collaborative effort examining growth modelling involving over 1600 preterm infants is currently underway [109]. This analysis is attempting to compare infants that received formula with only n-3 LCPUFA, with those fed formula with both n-3 and n-6 LPCUFA and those fed formula with no LCPUFA. This analysis will provide valuable information regarding separate contributions of n-3 and n-6 LCPUFA to infant growth.

### *1.6.2 Growth of Preterm Infants Fed Formulas Enriched with n-3 LCPUFA and n-6 LCPUFA*

Since the theory of 'balanced' n-6:n-3 LCPUFA ratio in formula, there has been a series of other RCT investigating the effects on growth after supplementing infants with formulas containing both n-3 and n-6 LCPUFA. Table 1.5 has been prepared to aid comparisons between trials by compiling factors relating to trial quality, details regarding the intervention and a summary of the findings. As with the n-3 LCPUFA trials, inconsistent results have been found after supplementing infants with both n-3 and n-6 LCPUFA. Some reported enhanced growth [62,102], no difference in growth [47,60,110] or poorer growth [100] when supplemented infants were compared to infants that received no LCPUFA in formula.

Increased growth has been reported in two RCT in preterm infants that included n-3 and n-6 LCPUFA[62,102]. The first trial discussed in the previous section, reported increased weight in preterm infants that received a formula containing 0.3% DHA and 0.6% AA for approximately 28 days [62]. Clandinin *et al* has compared growth of two different groups of infants fed formulas containing LCPUFA with a control group that were fed formula with no LCPUFA [102]. One treatment group received 0.4% n-3 LCPUFA (0.3% DHA and 0.1% EPA) from fish oil and 0.7% AA from fungal oil (FF group) and the other

received 0.3% DHA from algal oil and 0.7% AA from fungal oil until 12 months CA (AF group). Compared with control group, the infants in the AF group were significantly heavier from 6 to 18 months CA and longer at 2, 9 and 12 months CA. The AF group were also heavier than the FF group at 18 months CA and longer at 4, 9 and 12 months CA. Although the trial randomisation was stratified for birth weight, the mean birth weight of the FF group was significantly lower than the control and AF group, and a higher proportion of infants from the FF (n = 31, 34%) were born <1000g compared with the control (n = 21, 25%) and AF groups (n = 16, 22%). The smaller infants in the FF group probably affected the growth achievements of this cohort in later measurements, particularly as growth measurements were not adjusted with birth size as a covariate. The FF group were not significantly different from the control group in any growth measurements during the trial, indicating that growth of the infants supplemented with DHA from fish oil were comparable with the infants fed formula with no LCPUFA throughout the trial.

Two further trials that included sources of both n-3 and n-6 LCPUFA in formula have found no effect on growth outcomes [60,111]. In one trial preterm infants were fed a formula containing 0.35% of fat as DHA and 0.5% as AA until reaching 48 wk PCA and then weaned onto a formula containing no LCPUFA [111]. These infants were followed until reaching 1 year CA and no differences in growth were found between the two groups of infants. The second trial tested similar levels of LCPUFA, (0.3% of fat as DHA and 0.4% as AA), until 40 weeks PMA and then 0.2% DHA and 0.4% AA until reaching 1 year CA [60]. Repeated measures analysis revealed that at term age, control infants had lower gains in weight and length than LCPUFA supplemented infants. Small differences in gender and birth weight subgroups were found at various times, but none were consistent over the duration of the trial and considered inconsequential.

Table 1.5: Growth Performance of Preterm Infants Enrolled in RCT of n-3 and n-6 LCPUFA Formula Supplementation

| Reference | 62 | 111 | 60 | 100 | 47 | 102 | 45 | 79 | 112 |
|---|---|---|---|---|---|---|---|---|---|
| DHA | 0.34% | 0.35% | 0.26% to term | 0.17% | 0.24– 0.76% | 0.3% | 0.6% | 0.3% | 0.2% |
| EPA | | | | | | 0.1%Fish group | 0.1% | 0.1% | |
| Source | Algal Oil | Algal | EggTG or fish | Egg PL | Algal | Algal or Fish | Fish | Egg PL | Egg PL |
| AA | 0.6% | 0.5% | 0.42% | 0.31% | 03 – 1.1% | 0.7% | 0.1% | 0.35% | 0.35% |
| Source | Fungal | Fungal | Fungal | Egg PL | Fungal | Fungal | Fish | Egg PL | Egg PL |
| Eligibility | VLBW, AGA, EI=50kcal/kg/d | 750 – 2000 g, AGA | 750 – 1800 g | <1750 g, <37 weeks GA | AGA,<2300g, full EI at d 14 | <35 week GA | <34 week GA, AGA | <33 week GA, AGA | <2000 g |
| Intervention Period | ≥28 days | d2 of EI to 48 wk PMA | d4 of EI to 92wk PMA | <10 days to discharge | Birth to 6wk | d10 of EI to 12 mo CA | d2 of EI to 36wk PMA | d10 to 52 wk PMA | 3wk during hospital |
| Infants- enrolled | $n_C$=59 $n_T$=66 | $n_C$=78 $n_T$ =77 | $n_C$=144 $n_T$ =283 | $n_C$=100 $n_T$ =95 | $n_C$=22 $n_T$ =62 | $n_C$=119 $n_T$=242 | $n_C$=11 $n_T$=14 | $n_C$=26 $n_T$ =23 | $n_C$=10 $n_T$ =10 |
| - followed up | $n_C$=47 $n_T$=55 | $n_C$=50 $n_T$ =48 | $n_C$=91 $n_T$ =180 | $n_C$=84 $n_T$=74 | $n_C$=18 $n_T$ =48 | $n_C$=62 $n_T$=117 | $n_C$=9 $n_T$ =13 | $n_C$=25 $n_T$ =21 | $n_C$=10 $n_T$ =10 |
| Assessment age (PMA) | 40, 48, 57 | 40, 48, 92 | 40 to 92 | 79, 118 | ≈34, 38 | 40 to 118 | Intervention | 40, 52 | Intervention |
| Trial Details; | | | | | | | | | |
| Allocation | Computer | Computer | Computer | Random | NR | Random | Random | Random | Random |
| Concealment | Adequate | Adequate | Adequate | Adequate | Unclear | Adequate | Unclear | Unclear | Unclear |
| Blinding | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Sample size estimate | 50 per group | 60 per group | Not for growth | 100 per group | No | 58 per group | No | No | No |
| Not followed up | 26 (20%) | 57 (37%) | 156 (36%) | 37 (19%) | 18 (21%) | 182 (50%) | 3 (12%) | 3 (6%) | 0 (0%) |
| ITT | Yes | No | Yes | Yes | Yes | No | Yes | No | Yes |

| Reference | 62 | 111 | 60 | 100 | 47 | 102 | 45 | 79 | 112 |
|---|---|---|---|---|---|---|---|---|---|
| Difference in growth measurement of LCPUFA supplemented infants compared with control group; | | | | | | | | | |
| Weight | ↑ at 48 & 57 wkCA | ND | ↑ in eggTG group at term | ↓ at discharge & 118 wkPMA | ND | ↑ from 66 to 118 wkPMA in algal group | NR | NR | ND |
| Length | ND | ND | ↑ in eggTG group at term | ↓at 118wkPMA, ↓ z-score | ND | ↑ at 48, 79 & 92 wkPMA in algal group | NR | NR | ND |
| HC | NR | ND | ND | ND | ND | ND | NR | NR | ND |
| Other measurements | Weight-for-length ratio ↑at 48 wkPMA | ND in MUAC | ↓ gain in length & HC in girls at term | ND in weight, length, HC gains from enrolment to discharge | ND in weight or length gains over intervention period | | ND in weight, length or HC gains over intervention period | ND in weight, length or HC gains to 52 wkPMA | |

$n_C$ = control group, $n_T$ = treatment group

AGA, appropriate for gestational age; CA, corrected age; EI, enteral intake; HC, head circumference; ITT, intention-to-treat; LCPUFA, long chain polyunsaturated fat; MUAC, mid-upper arm circumference; ND, no difference between control and supplemented infants; NR, Not Reported; PMA post menstrual age; VLBW, very low birth weight; wkCA, weeks corrected

A number of smaller LCPUFA intervention trials have reported no difference growth performance of preterm infants [45,47,79,112]. These trials tested between 0.2 – 0.8% DHA and 0.1 – 1.1% AA in formulas fed to preterm infants for between 3 weeks to 3 months. Infants were followed only to the end of the intervention period. Gains in weight, length and head circumference during the intervention were the only growth assessments reported in two of the trials and weight, length and head circumference at study onset and completion were reported in the third trial. None of these trials reported differences in any growth measurements between infants fed control formulas compared with infants fed LCPUFA formula. Gender balance of trial groups at enrolment and follow up are not reported in two of the trials. Due to the small sample sizes, the trials lack sufficient power to make conclusions regarding the supplementation regime on the growth of preterm infants.

Fewtrell *et al,* reported reduced growth in infants fed formula supplemented with both n-3 and n-6 LCPUFA [100]. In this trial, exclusively formula fed infants born <37 weeks GA were randomised to receive a formula containing 0.2% DHA and 0.3% AA. The infants were fed the trial formulas for just over 30 days and assessments were performed at 9 and 18 months CA. Compared with the control group, the LCPUFA supplemented infants were 99 g lighter at discharge and 370 g lighter at 18 months CA, and were 1.5 cm shorter at 18 months CA. This outcome is surprising considering the low dose of DHA, the short duration of supplementation and the inclusion of n-6 LCPUFA in trial formula.

Since including n-6 and n-3 LCPUFA in formula trials, the Fewtrell *et al* trial has been the only to report poorer growth during follow up. The results of the Fewtrell *et al* trial pose a curious point of difference to other trials that suggested the EPA in fish oils and absence of AA were responsible for the effects on infant growth. The LCPUFA was sourced from egg phospholipids and consequently had only residual levels of EPA (0.04%) and a relatively large proportion of AA (0.3%). All other RCTs that supplemented infants with n-3 and n-6 LCPUFA from algal or egg triglyceride have found no differences in intention to

treat comparisons of growth (Table 1.5). Interestingly, the two trials that fed preterm infants formula supplemented with n-3 LCPUFA from fish oil and n-6 LCPUFA have not reported any deficits in growth when compared to infants that received the formula containing no LCPUFA [60,102]. This disparity in outcome between trials highlights the paucity of evidence to implicate n-3 LCPUFA, particularly EPA from fish oil, as the sole source of poorer growth in preterm infants.

## 1.7 Clinical Measures of Safety from LCPUFA Trials in Preterm Infants

The safety of adding LCPUFA to preterm infant formula has been monitored primarily by assessing infant growth, but also through reporting disease rates and adverse events collected during trials. In many RCT the collection of these data is a secondary outcome. This section briefly reviews other 'clinical' safety data collected from RCTs in preterm infants fed formulas containing n-3 LCPUFA. A great diversity of data has been reported, which spans many physiological functions and a broad range of diseases.

Many of the diseases described here are commonly associated with prematurity. Poor respiratory function is frequently present in preterm infants, due to the immaturity of the lungs at birth. A number of trials have reported data on the incidence of respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD) and other measures of respiratory therapy such as duration of intubation or oxygen supplementation. No difference in duration of ventilation or oxygen therapy has been observed between infants receiving control or LCPUFA supplemented formula [60,113], and no significant difference in incidence of BPD between control and supplemented infants [6,102]. A longer duration of ventilation and more days in a high oxygen environment was found in preterm infants that received LCPUFA supplemented formula when compared with infants that received no LCPUFA [101]. The authors suggest the infants receiving the LCPUFA formula were sicker before randomisation. However, the time

between birth and assignment to formulas is not clear and evidence for the onset or development of respiratory symptoms before assignment is not well described.

Longer duration of oxygen therapy, lighter birth weight [114] and formula feeding [115] has been associated with increased risk of retinopathy of prematurity (ROP) in observational studies in preterm infants. Three RCT have reported the incidence of ROP in preterm infants fed formula containing no LCPUFA compared with infants fed formulas ranging in LCPUFA from 0.1 – 0.3% DHA and 0.4 – 0.7% AA from 3 weeks to 12 months CA [62,102,113]. No differences in the incidence of ROP have been found between infants given control or LCPUFA enriched formulas. Although these 3 trials provide ROP information from over 300 preterm infants, given the low rate of severe eye disease larger sample sizes are necessary to demonstrate any change to the incidence ROP.

Due to the immaturity of their immune systems, preterm infants are at an increased risk of infection. Impaired innate immunity is largely due to deficits in neutrophil production [116] and impaired function [117]. These deficits take a number of weeks to improve and usually do not reach levels of adults by full term age [118]. LCPUFA are known mediators of the immune system. Studies of n-3 LCPUFA supplementation in adults have revealed suppression of both innate [119] and acquired immune responses [120-122]. Effects such as these may have the potential to place preterm infants receiving n-3 LCPUFA at an increased risk of infection. Only one RCT in preterm infants has attempted to identify some immunological changes after receiving a formula containing LCPUFA [123]. The infants involved were a subset from a larger trial [111] that were randomly allocated to a formula with no DHA (n=12) or formula with 0.35% of fat as DHA and 0.49% AA (n=15) for 4 weeks. A breast fed reference group was assessed in parallel (n=17) with the formula fed infants. White blood cell counts, lymphocyte subpopulations (including markers of maturity) and cytokine release were all measured. The control group had significantly higher percentages of T-helper lymphocytes, higher expression of antigen-naïve

phenotype on T-helper lymphocytes and lower lymphocyte IL-10 production than infants fed LCPUFA supplemented formula or breast milk. By comparison, infants fed LCPUFA enriched formula had an immunological profile similar to the breastfed reference group suggesting an improvement in the immune parameters measured in the LCPUFA supplemented infants. However, this trial was designed with a relatively short duration of supplementation and only investigated the effects on lymphocyte function. Given the large variability in immune responses and small number of infants evaluated, further studies will need to be conducted to better understand the influence of dietary LCPUFA on immune responses of preterm infants.

We can attempt to measure the outcome of LCPUFA supplementation on immune function by identifying related clinical outcomes. One important measure that has been frequently reported in intervention trials in preterm infants is the incidence of sepsis [60,62,101,102,111,113]. Trials reporting sepsis after supplementing infants with LCPUFA, range between 0.1 – 0.4% DHA and 0 - 0.7% AA and from 28 days until 1yr CA. All trials that assess incidence of sepsis report no difference between control and supplemented infants. These trials report data from over 1000 infants. Individually each trial is underpowered for detecting small differences in sepsis incidence. If combined, one thousand infants would be required to demonstrate a reduction in incidence of equal or greater than 6% from the current rate of 29% [124] (with 80% power and 95% confidence). Many more infants would be necessary to demonstrate more subtle changes in sepsis rates.

One of the most feared diseases of prematurity is necrotising enterocolitis (NEC). The underlying mechanisms of the disease are poorly understood however, it is thought that NEC may arise from restricted blood flow to the gastrointestinal (GI) tract. This may result in hypoxia and ischemia, followed by necrosis of the mucosa that impairs barrier function and increases the risk of infection associated with progression of the disease. NEC commonly results in surgical resection of the GI tract and it is a

major cause of perinatal death in preterm infants. Infants who survive NEC often have sustained hospital stays and significant long-term morbidity.

A number of LCPUFA supplementation trials have collected data on the incidence of NEC in their study populations [60,62,101,102,111]. Most trials have found no significant differences in suspected or confirmed NEC between infants fed formula with no LCPUFA compared with infants that fed n-3 LCPUFA supplemented formulas. One publication has reported a reduced incidence of NEC in infants fed formula containing 0.1% DHA and 0.4% AA until discharge compared with infants fed no LCPUFA [113]. However differences in formula composition other than the fatty acid may have contributed to the reduction in NEC. As with the other diseases of prematurity with low overall incidence rates, further evaluation of any role for n-3 LCPUFA in on NEC would require substantially more infants.

During development it is essential that the vascular system grows sufficiently well to deliver nutrients to growing tissues. It is also important that the vasculature grows optimally for long-term health and there is some suggestion that preterm infants are at increased risk of cardiovascular problems, such as high blood pressure later in life. In an observational study, Doyle *et al* demonstrated higher blood pressure in young adults born <1500 g than those born at term [125]. Blood pressure has also been investigated in a RCT that supplied LCPUFA to term infants [126]. Infants were randomised to receive a formula with no LCPUFA or a formula containing DHA (at 0.2 - 0.3% of total fat) and AA (at 0.3 to 0.4% of total fat) for 4 months. Follow up of approximately 60% of infants at 6 years of age found that infants that received the LCPUFA enriched formula had significantly lower mean blood pressure and lower diastolic blood pressure than infants that received the control formula. When compared to a reference group of breast fed infants, the control group had significantly higher blood pressure but the blood pressure of the LCPUFA group was not different. More research is needed to ascertain if preterm infant blood pressure

is responsive to dietary LCPUFA and if the effect on blood pressure offers other long-term health benefits.

Data from RCTs that report clinical outcomes suggest that there is little effect of LCPUFA on respiratory outcomes, the incidence of ROP or sepsis in preterm infants. There is preliminary evidence that LCPUFA in preterm infant formula could enhance lymphocyte maturity and possibly influence the incidence of NEC. Substantially larger trials are necessary to confirm other potential benefits of LCPUFA in formula and identify the optimal dose to improve short and long term prognosis for preterm infants.

## 1.8 Rationale for Thesis

In Australia and many other countries, an absence of DHA in the diet of preterm infants is now considered unacceptable as most recognise the importance of supplying at least some DHA. Despite much research over many years, the dose and duration of DHA required for optimal growth, development and long-term health of preterm infants is not known. Larger, well-designed and controlled RCT are required to determine if early DHA supplementation confers further improvements to development at higher doses.

While the benefits of supplementing formula fed preterm infants with DHA are studied, the effect of supplementing breast fed preterm infants has not been addressed. Given that breast milk DHA concentration is proportional to the DHA content of the mother's diet and that this (in Australia) is less than that transported across the placenta during fetal development, it is necessary to consider if the DHA intakes of the breast fed preterm infant are optimal.

The information contained herein describes the design, implementation, safety and efficacy of a randomised controlled trial of DHA supplementation in preterm infants. Infants randomised to the control group received the standard level of DHA in current clinical practice that is, the level present in preterm formula (0.35% of total fat) or in maternal milk (approximately 0.2% of total fat). The exact percentage of DHA depends on the ratio of breast milk to formula in the infants' diet. Treatment group infants received DHA at 1% of total fat, through breast milk via supplementation of lactating mothers or by addition to preterm infant formula. This level of DHA is modelled to meet the estimated rate of n-3 LCPUFA accretion during the last trimester of pregnancy. Supplementation began at five days or less from the commencement of enteral feeds through to the infant reaching their estimated due date (EDD). Infants were followed up until reaching 4 months CA. The trial attempted to mimic the n-3 LCPUFA intake of term infants fed breast milk after birth. During the intervention and follow up phases, families were encouraged to breastfeed infants, however, those that chose to formula feed their infants were encouraged to use a commercial formula containing LCPUFA.

The success of the intervention was determined by infant erythrocyte fatty acid status at the end of the intervention period. Compliance with the supplementation was determined by fatty acid analysis of maternal breast milk. Primary efficacy was assessed by sweep VEP acuity at 4 months CA. VEP acuity at 2 months CA and VEP latency at 2 and 4 months CA were recorded as secondary outcomes of visual development. The primary safety assessment was size at 4 months CA. Secondary safety outcomes included growth measures at EDD and rates of diseases of prematurity and adverse events.

# CHAPTER 2: DEVELOPMENT OF A SWEEP VEP ACUITY TECHNIQUE AND EVALUATION OF LATENCY RESPONSES

## 2.1 Introduction

Two techniques, based on behavioural or physiological responses to visual stimuli have been used to measure visual acuity of infants. Behavioural techniques are cheaper, portable and have been extensively used in LCPUFA trials. However, due to greater variability in behavioural acuity measurements, a larger sample size is required to investigate dietary treatments. Electrophysiological techniques require expensive equipment and expertise to perform but are advantageous in that they demonstrate lower variability, permitting investigation of fewer infants or smaller effect sizes. A range of electrophysiological techniques has been used to study visual acuity in infants.

Our research group has previously developed an electrophysiological technique for measuring acuity of infants [42,127,128]. The technique employs a transient checkerboard stimulus to elicit visual evoked potential (VEP) responses to a range of spatial frequencies. The stimulus has a slow reversal rate and is shown to infants for 30 seconds. This permits full examination of all components of the VEP waveform. The amplitude of the response is recorded and plotted against the log of the visual angle subtended by the stimulus. A log-linear relationship between amplitude and check size is exhibited near the acuity limit and best-fit regression line is extrapolated through 0 volts to estimate the acuity threshold. This estimate represents a theoretical point where the infant no longer responds to the stimulus, the limit of their visual acuity. The technique depends on collecting data at several spatial frequencies near the acuity limit to have sufficient points to extrapolate an acuity estimate. The time required to collect amplitude data to many different sized stimuli is a difficulty with the technique as infants often become unsettled.

An alternative to the transient method is the steady-state VEP. The stimulus is a black and white striped (grating) pattern that reverses rapidly. The timing of the reversal rate evokes the stimulation and recovery of the infant's neurones to resemble a sine wave; this rapid neuronal stimulation is called 'steady-state'. The amplitude responses from a range of stimuli are plotted against the spatial frequency and an acuity estimate is extrapolated. The advantage of the technique is that the rapidity of the pattern reversal reduces the time required to collect amplitude data to as little as 10 seconds for each different stimulus.

Further enhancement of steady-state VEP is to 'sweep' through a range of spatial frequencies over a 10-second presentation. Steady state is achieved through the rapid reversal rate. Commonly, each spatial frequency is shown for half or one second period before changing. Ten different spatial frequencies can be shown to an infant in a 10-second sweep that changes spatial frequency every second. As with all VEP techniques, the amplitude of the infant's response decreases with increasing spatial frequency and acuity is estimated from amplitude versus spatial frequency function. The sheer speed of the technique is a great advantage when assessing infants and a number of research groups have applied this technique to studies of infant acuity. However, a wide range of acuities has been reported from sweep VEP studies. For example, acuity of 4-month-old infants varies from 7 cycles per degree (cpd) [129,130] to as high as 12 cpd [70]. The methodological differences between studies may have contributed to the wide range of acuities reported. Sweep VEP acuity data from 10 separate studies have been compiled in Table 2.1 to systematically compare the stimulus, recording and analysis conditions, and determine the extent of methodological differences among studies.

Beginning with the stimulus conditions, different patterns, reversal rates and spatial frequencies have been used to study infant acuity. The two types of patterns used in sweep acuity studies are sine and square wave. Sine wave gratings are preferred as they exhibit simpler spatial frequencies which are not

as affected by refractive errors [59]. Reversal rates vary between 10 and 14 reversals per second, the rapidity of which is suitable for achieving neuronal depolarisation in 'steady-state' [131]. The spatial frequencies of the stimulus must be of a sufficient range to attract and retain infant's attention and to reach beyond the infants acuity limit. Spatial frequencies shown to 4-month-old infants vary from 0.5 - 11.5 cpd to 1 - 20 cpd. The spatial frequencies must also step down in size in roughly linear increments to prevent over or under estimation of acuity and without flicker, which could evoke a response based on luminance. Specific details such as these are infrequently reported.

The recording of quality VEP data is dependant upon the placement of electrodes and many different approaches have been described. The simplest array requires an active electrode placed over the occipital cortex and two additional electrodes to act as referencing and earthing electrodes. Some trials have used two active electrodes referenced to two different sites. This increases the chance of recording data suitable for extrapolating an acuity value, and an alternative reference site may offer the advantage of reduced background noise. Some trials have placed a reference electrode on the ear, however the scalp is preferred as it is more appropriate for sampling background EEG noise.

There is substantial disparity in the criteria applied to the analysis of sweep data. The analysis components of the reports in Table 2.1 have been separated into 4 general areas for further discussion; background noise, signal-to-noise ratio (SNR), phase and the number of sweeps used in the acuity calculation.

Table 2.1: Comparison of Sweep VEP Acuity Techniques

| Paper | Stimulus | Rever-sal Rate | Infant | Stimulus Range (cpd) | Response Details | Sweep Criteria | Noise Criteria | Phase Criteria | SNR & Regression Line | Acuity at 4 months |
|---|---|---|---|---|---|---|---|---|---|---|
| 132 | Vertical Square Wave | 6.6Hz | FT | 17wk; 1 – 15 | $O_1$ & $O_2$ (active), $O_z$ (reference). AFT | Vector averaged min 3 trials, usually 5 | 2 adjacent frequencies | Specific details NR; "phase coherence" | SNR details NR | 10.6 cpd |
| 60 | Horizontal Square Wave | 7.5Hz | PT | NR | NR | Vector averaged | NR | NR if or how applied to acuity. | Interpolation of 3 points where 1>SNR<noise | ≈8 cpd * |
| 130 | Vertical Square Wave | 6Hz | FT | 8, 17wk; 0.5 - 11.5 | $O_z$ active, $C_z$ reference, $P_z$ ground | Vector average min of 3sweeps. Single sweep used when average not scored | 10 and 14 Hz | NR how applied to acuity | SNR≥3 | ≈7 cpd * (SD ≈1) |
| 43 | Vertical Sine Wave | 6 Hz | FT | 2mo; 0.6 - 10 4mo; 1 - 16 | 5 sites, $O_1$ & $O_2$ referenced to $O_z$. $O_1$ & $O_2$ ref. to $C_z$. AFT | Mean of all successful extrapolations | NR | NR | NR | ≈7 cpd |
| 133 | Vertical Sine Wave | NR | FT | 1mo; 0.5 – 8 2mo; 0.6 - 10 4mo; 1 - 16 | $O_1$ & $O_2$ referenced to $O_z$. $O_1$ & $O_2$ referenced to $C_z$. AFT | Mean of all successful extrapolations | 11 and 13 Hz | Constant or lagging | SNR >3 for 2 points. Computer scored, adjusted manually poor fit | 4mo data NR separately |
| 70 | Vertical Sine Wave | 6.6Hz | FT | 1-16 | 5, sites NR AFT | 5 sweeps, highest taken | Ref. Jorgensen *et al* 1998 | Ref. Jorgensen *et al* 1998 | Ref. Jorgensen *et al* 1998 | 12.8 cpd |
| 134 | Vertical Sine Wave | 6.6 Hz | FT | NR | $O_1$ & $O_2$ referenced to $O_z$. $O_1$ & $O_2$ referenced to $C_z$. AFT | Highest score in all 5 channels | Details NR in this paper | Details NR in this paper | Details NR in this paper | ≈12 cpd* |
| 135 | Vertical (sine/ square NR) | 6Hz | FT | 0.5 - 15 for a 15wk infant | $O_z$ and 3cm right ear, ground on left ear | As per Norcia 1985 | 14 Hz | NR | Regression line from highest peak to SNR = 1.5 | NR |
| 57 | Vertical Sine Wave | 6Hz | FT | 1 - 20 | $O_z$ and 3cm right | Highest individual sweep or averaged | 14Hz | Constant or lagging | SNR = 3 | 9.0 ± 1.3 cpd |
| 129 | Vertical Sine Wave | 6Hz | FT | 30:1 range | $O_1$ & $O_2$ (active), and $O_z$ reference | Best single sweep or vector average | 14 Hz | Constant or not lagging > $90^o$ | SNR 3:1 or better | 7 ± 1.0 cpd |

AFT, adaptive filter technique; FT, full term; NR, not reported; PT, preterm; SNR, signal to noise ratio

* Indicates mean acuity at 4 months of age has been estimated from graph

Background noise data are an essential component of VEP acuity analysis. It is necessary to discriminate random EEG noise (and other changes in electrical potential such as muscle contraction, electrode and equipment artefacts) from signal data. Three main differences exist in the noise frequencies used in sweep acuity estimates. Some studies use average noise criteria derived from the next closest frequencies to the signal (eg 11 and 13 Hz) [43], others average two noise frequencies further from the signal (10 and 14 Hz) [130] and finally just one frequency (at 14 Hz) [57,129,135] has also been used. Frequencies closest to the signal may at first seem appropriate to use for background noise however, Norcia *et al* suggests that the noise frequency should not be closer than 1 Hz to prevent contamination of noise with signal data [57].

To select strong signal responses a SNR is applied to the amplitude versus spatial frequency regression lines. Most sweep VEP acuity studies have set minimum SNR criteria of ≥3 to apply to at least one data point included in the regression line used to extrapolate acuity [57,129,130,133]. Some studies have not cited a specific SNR and others have used SNR ratios other than ≥3 [60,135].

Data related to the timing of an infant's VEP response (called phase) is sometimes collected in sweep VEP studies. Both signal and background data are susceptible to artifactual changes in electrical potential [57] and it is the amplitude of these responses that are used to estimate acuity. As phase is more stable than amplitude, phase criteria can help identify increases in amplitude that are not related to the stimulus. The use of phase criteria in the analysis of sweep acuity studies varies. Some articles mention phase [60,130] or 'phase convergence' [132] in their methods, or report constant or lagging phase [133] but most have not reported any specific phase limits, restrictions or guidelines used for the analysis of acuity data. Recently a newer method of extracting phase information called the Adaptive Filter Method [136] has been used in sweep VEP studies.

Finally, differences between studies exist in the manner in which a sweep or sweeps are deemed suitable for estimating acuity. In early acuity work Norcia *et al* reported the highest of any individual sweep or an averaged performance best reflected the threshold of acuity [57]. In recent years Lauritzen *et al* have argued that because the acuity extrapolations for an individual are normally distributed, the most accurate acuity estimation is an average of all successful extrapolations [133]. Other groups have used a vector average from a minimum specified number of trials [132] and some have reported using the values from a single successful sweep if the average was not successful in generating an acuity extrapolation [130].

Any or all of these differences in the design and analysis of sweep VEP acuity data may influence the estimation of acuity. Further investigation of the analysis criteria is necessary to determine if this may account for some of the discrepancies in acuity between studies.

In this chapter, I describe a prospectively collected observational study of VEP responses of infants. I investigated the effect of applying different analysis criteria on the sweep data to optimise the technique. VEP latency responses were collected and used to establish a range of checkerboard stimuli best suited for testing infants. Based on the information generated from this study, the most suitable VEP techniques for use in a DHA intervention trial are described.

## 2.2 Prospective, Observational Study of VEP Responses of Infants

Infants were recruited from the Flinders Medical Centre during January 2001 through to October 2001. Sweep VEP acuity and transient VEP latency measurements were collected at 2 and 4 months (corrected age (CA) for preterm infants) from the same infants. Term and preterm infants were enrolled to collect data from a potentially wide range of VEP responses. The study protocol was submitted and approved by the Flinders Clinical Research Ethics Committee (approval number 137/00).

*Eligibility and Recruitment of Term Infants*

Infants born at term (>37 weeks gestation) with a birth weight >2500 g were eligible for inclusion to the full term group. The Flinders Medical Centre Birth Register was scanned for eligible babies. Any infants suffering serious complications during pregnancy, delivery, or during the perinatal period were not invited to participate. A covering letter from the nursery unit head, a study Information Sheet, questionnaire and reply paid envelope was sent approximately 1 month after birth to the mothers last known address listed on the birth register. All respondents were contacted by telephone to discuss the study and if willing to participate, an appointment was organised for within one week of the infant reaching 2 months of age. For privacy reasons non-respondents were not called or contacted further.

*Eligibility and Recruitment of Preterm Infants*

Infants born <36 weeks gestation were eligible for the preterm group. The Director of the Neonatal Intensive Care Unit (NICU) identified eligible infants. A covering letter signed by the Director of the NICU, an Information Sheet, questionnaire and reply-paid envelope was sent to the mothers address. Infants were excluded if they had major congenital or chromosomal abnormalities, were on a respirator for ≥7 days, retinopathy of prematurity ≥grade 2, intraventricular haemorrhages ≥grade 2, or eye pathologies (such as strabismus or nystagmus) noted by a paediatrician. Respondents were contacted by telephone and if willing to participate, an appointment was organised within one week of the infant reaching 2 months old.

*Consent*

Appointments were conducted ±1 week of the infant turning 2 and 4 months CA. At the first appointment the purpose of the study and the procedure for recording VEP measurements was discussed with the parents before obtaining signed consent. Background data including details of

socio-economic and lifestyle factors (maternal age, maternal and paternal years of education and employment, maternal alcohol consumption and cigarette smoking) was also obtained.

*VEP Recordings*

The order of the sweep or transient presentation was determined by a randomisation schedule. A staff member not involved with the study prepared the schedule in sealed opaque envelopes, each stating whether the sweep or transient recording should be performed first. Each sealed envelope was opened just prior to testing. The randomisation of the tests was to ensure that systematic errors such as infant fatigue did not contribute to the collection of VEP data.

For all VEP recordings the infant sat on their parents lap at 50 cm from the monitor. The infant's scalp was wiped with alcohol swabs (70 % Isopropyl alcohol, BRI 5530/200 Briemar Nominees Pty Ltd, Australia) and five Grass 5 mm gold cup electrodes (Cat No F-E6GH, Grass Instruments Division, Astro-Med Inc., W. Warwick RI 02893) were applied to the infants scalp using Grass Electrode Cream (EC2 Grass Instruments Division, Astro-Med Inc., W Warwick RI 02893). All VEPs were conducted in a darkened room (50 cd/$m^2$) illuminated only by the testers' computer monitor (facing away from the infant) and the monitor projecting the patterned image toward the infant (Figure 2.1). If required, infants' attention was directed to the screen by a small musical toy or pencil tapped at the top of the screen. Time was given for the infants to recover if irritable or sleepy and infants were fed if hungry.

**Figure 2.1**: An Infant and Her Mother are Photographed after a Transient VEP Test. No room lighting is on during the test.



***Description of Subjects***

A total of 11 preterm infants were enrolled and all attended both appointments at 2 and 4 month CA. Of the 32 full term infants enrolled, 30 attended appointments at 2 months of age. Two families replied late to the postal invitation to attend, resulting in their infants being too old to attend the 2-month appointment. At the 2-month appointment, one infant fell asleep and was unable to be aroused for VEP recordings. VEP data were collected from the remaining 29 term infants at 2 months of age. At 4 months of age, one infant was lost to follow-up and two were unavailable due to parental work commitments, resulting in data collected from 29 term infants. The collection of VEP data was attempted from all infants attending appointments.

The distribution of girls and boys in the full term and preterm groups was not even, with 68% girls in the full term group and 17% girls in the preterm group ($p = 0.001$) (**Table 2.2**). As expected, the gestational

age at birth and birth weight were both significantly lower in the preterm group ($p < 0.001$). Other characteristics such as maternal age and years of education of either parent were not different across the groups. However, there were differences in smoking and alcohol intake between mothers of the preterm and full term infants. Mothers of preterm infants did not smoke during pregnancy compared with 5 mothers (25%) of full term infants ($p < 0.0005$). Baseline data show that more mothers of preterm infants (n = 5 / 11, 45%) drank alcohol during pregnancy than mothers of full term infants (n = 3 / 32, 13%), ($p<0.0005$). For those mothers that drank alcohol during pregnancy, the mean ($\pm$ SD) intake per week was reported to be $0.85 \pm 0.8$ in the preterm group and $3.3 \pm 0.6$ in the term group.

Table 2.2: Description of Infants Enrolled in Observational Study of VEP

| | Term Infants<br>n = 32 | Preterm Infants<br>n = 11 |
|---|---|---|
| Male (%) | 10 (31) | 8 (73) † |
| Birth gestational age (wk)^ | $39 \pm 1$ | $32 \pm 2$† |
| Birth weight (grams)^ | $3524 \pm 477$ | $1908 \pm 361$† |
| Mothers' age (yr)^ | $27 \pm 5$ | $31 \pm 5$ |
| Mothers' education (yr)^ | $12 \pm 2$ | $13 \pm 2$ |
| Parity* | 1 (0 – 3) | 0 (0 – 2) |
| Smoked in pregnancy | 5 (16) | 0 (0) |
| Smokers in family home* | 0 (0 – 2) | 0 (0 – 1) |
| Alcohol in pregnancy* (drinks/day) | 0 (0 – 4) | 0 (0 – 2) |
| Fathers' education (yr)^ | $12.3 \pm 2.5$ | $13.0 \pm 2.0$ |

Values are number of infants (and percentage) and ^mean ($\pm$ SD), or *median (and range) when data not normally distributed

† Significantly different between term and preterm groups, $p<0.05$

## 2.3 Sweep VEP Protocol

Dr Brett Jeffrey (Portland, Oregon) designed the protocol for sweep acuity testing, which included the type and size of stimulus, the electrode montage and the recording of VEP data. The protocol required the placing of four electrodes on the infants scalp; one reference electrode placed on the sagittal plane at 1cm above the inion ($O_z$), two active electrodes placed at 3cm left ($O_1$) and 3cm right ($O_2$) of the reference electrode and an earth electrode at the central vertex ($C_z$). The active electrode detects the change in electrical potential from each eye, where $O_1$ measures the change at the occipital cortex in response to the image seen with the right eye and $O_2$ records the response from the left eye.

The infants were presented with a horizontal sinusoidal grating pattern of constant luminance, cycling 6 times per second (reverses at 12 Hz). Each 10-second stimulus swept linearly through 10 different spatial frequencies, from low to high (ranging from 0.51 to 8.18 cpd. Frequencies from 1 – 100 Hz were recorded simultaneously, with frequencies at 50 Hz removed with a notch filter. The responses were amplified 10,000 times on two Grass model PC511 AC Amplifiers (an amplifier for each active electrode). Impedance was matched in both channels, usually at less than 5 microvolts. Eight or more sweeps were presented to infants depending on infant fatigue.

### *2.3.1 Development of Analysis Software for Sweep VEP Acuity Data*

A program for analysing acuity from the sweep VEP data was developed using Matlab computer software (Version 6.0.0.88 Release 12, by The Mathworks Inc). A goal in developing the software was to reduce the amount of tester manipulation of the data. Ms. Sherry Randhawa at the Department of Engineering, Flinders University, wrote the software in consultation with Dr Brett Jeffrey and myself. The analysis was developed and modified from the description of sweep analysis published by Norcia *et al* [57].

The collection of sweep data began with the simultaneous recording of data from predominately each eye, through two active electrodes $O_1$ and $O_2$. The signal of interest occurs at the stimulus reversal rate of 12 Hz. To analyse data, the program selected the infants' response at 12 Hz and averaged each section of 50 data points. This is equivalent to the infants' response at every $1/6^{th}$ of a second. This was then used to calculate the average amplitude at each of the 10 spatial frequencies tested.

The software compiled the signal data from all sweep recordings into matrix format; left eye, right eye and the average of both channels. From these data, six line graphs of the amplitude versus spatial frequency were generated. They showed the left, right and combined data for each single sweep and the vector average of the left, right and combined data from all sweep recordings during the testing session. Each sweep added another row into the matrix and there was no limit on the number of sweeps permitted. The tester was able to exclude a sweep from the matrix. This was necessary to adjust for the infants' state of arousal during the testing procedure. If the infant was looking away, moving, crying or sleeping it would affect the response signal resulting in artifactual changes that were not suitable for estimating acuity.

Background noise data were entered into the matrix at each spatial frequency, in the same fashion as the response frequencies. When data from 2 frequencies were selected as background noise, the average of the frequencies was entered into the matrix. The average noise across the entire sweep was used to calculate a signal-to-noise ratio (SNR) and plotted on the amplitude versus spatial frequency graph, along with the noise at each spatial frequency.

Criteria describing the phase component of the response were written into the software. To calculate phase, the raw data were multiplied by the sine and cosine components of the stimulus at the reversal

rate. Phase values were plotted for visual scrutiny underneath the amplitude graph (in radians) –180 to +180 degrees.

The software analysed the data at the highest spatial frequency and determined if each data point met the SNR and phase criteria described later. If it did, the software screened data at the next lowest spatial frequency to determine if it met the criteria. This continued with decreasing spatial frequencies until all data points were scanned. The software automatically drew a regression line through points that met the predefined noise, SNR and phase criteria. When this occurred a clearly identifiable black regression line appears. The regression equation was solved and the point where the regression line passed through 0 μV was generated from the equation. Small asterisk-shaped icons in black identified the points included in the regression line. If the criteria were not met, no regression line was drawn and no acuity value was calculated. This completed the analysis for one sweep. The data from each sweep recording was then averaged with all other sweep recordings and the analysis criteria were applied to the vector-averaged data.

The program was designed to analyse the data from each channel ($O_1$, $O_2$ and the average of both channels) individually so that the infant's best acuity performance in either right, left or both eyes combined could be identified and recorded.

### *2.3.2 Evaluation of Sweep, SNR and Phase Analysis Criterion on Mean Acuity*

Data collected from 4 month-old infants (n = 40) were used to evaluate the effect of changing the analysis criterion on the resultant acuity. Acuity data generated from the analysis criteria were compared using paired T tests, with probability of $<0.05$ considered significant.

The following analysis criteria were evaluated;

- Assessing four different criteria used to select a sweep for the acuity calculation which included;
    - All data or as many sweeps as possible to generate an acuity estimate (referred to as 'all'), which maximised use of data collected,
    - The best performance in a single sweep (called 'best single'),
    - The best acuity performance in a vector average of a minimum of 3 sweeps ('best vector'),
    - Analysis of data using the infants best performance in either the vector average or the single sweep ('best of either'),
- A comparison of two different SNRs;
    - SNR of >3 for at least one point on a regression line and 2 other points having SNR >1.5, or two points with a SNR >3.0
    - SNR of >2 for at least one point on a regression line and 2 other points having SNR >1.5, or two points with a SNR >2.0
- Comparison of phase criteria;
    - Including data points in a regression line that lead by no more than 20 and lag by no more than 90 degrees,
    - Including data points in a regression line that lead by no more than 30 degrees but permit a constant lag,
    - Reassessing data with no phase restrictions.

These analyses were performed on the average of 11 and 13 Hz frequencies as background noise. Dr Makrides, Associate Professor Gibson and myself, together with Associate Professor Algis Vingrys and Dr Bang Bui from the University of Melbourne, Department of Optometry and Visual Science met and reviewed the outcome of these analyses. During this review, the possibility that side-lobe leakage of the signal into the 11 and 13 Hz noise frequencies may be resulting in increased background noise was discussed. It was decided that to complete the optimisation, the analysis of the sweeps required further investigation using a different noise frequency. The frequency had to be further than 1 Hz from the signal, it could not be a harmonic of the signal and it could not be a frequency spontaneously present, as it would add to the noise. A frequency of 14 Hz was chosen as it met these conditions.

- Acuity data from optimised SNR, phase and sweep selection data were used to compare two different background noise frequencies;
    - The average of signals at 11 and 13 Hz,
    - The signal at 14 Hz.

### *2.3.3 Results of Comparisons of Acuity Analysis Criteria*

Not all analysis criteria applied to the acuity data resulted in a successful acuity extrapolation. The success rate varied with the sweep criterion applied, Table 2.3.1 shows that using SNR≥3 with strictest phase for analysing sweeps resulted in only 20 (50%) successful single sweep acuity extrapolations from 40 infants. As the SNR or phase was relaxed, more sweeps met the analysis criteria resulting in more acuity extrapolations. The single sweep category had the least successful rate of acuity extrapolations, limiting the usefulness of this category for analysing sweep data. The best acuity performance had the highest rate of successful acuity extrapolations as it drew on the best performance in a single sweep with best vector average categories.

Comparisons of mean acuities derived from the various sweep criteria resulted in many significant differences (Table 2.3). When as many sweeps as possible were used to calculate acuity ('all' group) there was a blunting of acuity values. This could be because the data consists of all responses, including those that were not optimal. By selecting the best sweeps the mean acuity increased. The 'best acuity' category reflected the best performance of the infant under the testing circumstances and is closest to the highest acuity achievable and permits comparisons with more reports from a diverse range of studies.

The number of sweeps used in the calculation of the vector average was compared to see if there were any differences between the analysis criteria. It was found that the number of sweeps used to generate a vector-averaged acuity value was approximately 5 across all comparisons (data not shown). Subtle

differences between groups were small and probably immaterial as the average number of sweeps used was well above the minimum of 3.

Applying a SNR of 2 or 3 clearly had an impact on the acuity outcome (Table 2.3). All comparisons of the SNR data were significantly different, irrespective of other phase or sweep criteria and in all cases the mean acuity of the SNR2 were greater than SNR3. The argument put forth by Norcia *et al* for choosing SNR3 over a SNR2 is to reduce the chance of false positives [57]. Although acuity values are lower with SNR3, the importance of collecting clear strong VEP signal must be emphasised, as this is a key characteristic of the sweep technique.

There was a general trend for increased acuity as the phase criteria was relaxed. Comparison of the means showed some significant differences between acuities calculated with different phase criteria. The largest differences in acuity were found between the most extreme phase cases; strict and no phase.

Table 2.3: Successful Extrapolations and Acuity (in cpd) from Different Analysis Criterion at 4 Months of Age

| | SNR 3 | | SNR 2 | | |
|---|---|---|---|---|---|
| | Success Rate | Acuity* | Success Rate | Acuity* | p (SNR2 vs SNR3) |
| Strict Phase; | | | | | |
| All | 21 / 40 | $5.0 \pm 1.2^{ab}$ | 32 / 40 | $6.3 \pm 3.1^{ab}$ | 0.047 |
| Best Single | 20 / 40 | $5.8 \pm 2.9^{1}$ | 26 / 40 | $6.0 \pm 3.0^{cd\ 7}$ | 0.044 |
| Best Vector | 23 / 40 | $5.6 \pm 2.0^{a\ 2,3}$ | 32 / 40 | $7.3 \pm 3.0^{ac\ 9}$ | <0.0005 |
| Best of either | 26 / 40 | $5.9 \pm 2.3^{b\ 4,5}$ | 35 / 40 | $7.2 \pm 3.1^{bd\ 10}$ | <0.0005 |
| Relaxed Phase; | | | | | |
| All | 28 / 40 | $5.6 \pm 2.6^{ab}$ | 34 / 40 | $6.3 \pm 3.1^{ab}$ | 0.03 |
| Best Single | 23 / 40 | $5.7 \pm 2.6^{cd}$ | 30 / 40 | $6.1 \pm 2.7^{cd\ 6}$ | 0.011 |
| Best Vector | 28 / 40 | $6.4 \pm 2.7^{ac\ 2}$ | 34 / 40 | $7.3 \pm 3.1^{ac\ 8}$ | <0.0005 |
| Best of either | 31 / 40 | $6.4 \pm 2.7^{bd\ 4}$ | 38 / 40 | $7.2 \pm 3.1^{bc\ 10}$ | <0.0005 |
| No Phase; | | | | | |
| All | | ND | 38 / 40 | $6.1 \pm 2.9^{ab}$ | |
| Best Single | 32 / 40 | $5.9 \pm 2.9^{a\ 1}$ | 40 / 40 | $6.4 \pm 2.9^{cd\ 6,7}$ | 0.005 |
| Best Vector | 33 / 40 | $6.5 \pm 2.9^{b\ 3}$ | 38 / 40 | $7.5 \pm 3.5^{ace\ 8,9}$ | 0.015 |
| Best of either | 35 / 40 | $6.7 \pm 3.1^{ab\ 5}$ | 39 / 40 | $7.8 \pm 3.3^{bde\ 10}$ | 0.011 |

* Mean $\pm$ SD

$^{a, b, c}$ Same superscript letters indicate significant differences ($p < 0.05$) between sweep criteria (all acceptable, single, best vector and best of either) within each phase and SNR comparison

$^{1, 2, 3\ etc}$ Superscript numbers indicate a significant difference ($p < 0.05$) between phase

ND, not done

Phase criteria provide an additional safeguard by locking into the timing of the infant's response to the stimulus adding reliability to acuity measurements. The timing of an infants' response naturally lags with increased spatial frequency and the strict phase (permitting only 90$^{o}$ lag) might unnecessarily limit the number of acuity extrapolations. Studies that apply this strict analysis to their data describe the collection of VEP data into 1-second bins that include a 0.5 second portion of data from the previous epoch [57]. This type of analysis would result in some smoothing or grading of amplitude and phase data as spatial frequency increases, as there are averaged data between each spatial frequency. This may be particularly important when applying strict phase restrictions, as an additional data point between each spatial frequency would result in a more modest phase lag from data point to point. This difference in phase between each 0.5-second point may be small enough to evade the strict 90$^{o}$ lag phase criteria. In this study, the criterion is applied to the phase at each spatial frequency. The difference between points is likely to be greater as it has not been averaged with the previous 0.5 second of data. Applying strict phase criteria to this data resulted in less successful acuity extrapolations and lower mean acuity. In these analyses, the relaxed phase criterion allows for constant or lagging phase with increasing spatial frequency and does not unduly restrict the number of acuity extrapolations.

The 4-month-old infant data were reanalysed using the 14 Hz noise frequency. A comparison of the best acuity performance between the different noise criteria is presented in Table 2.4. The 14 Hz noise frequency resulted in a reduction of background noise and made the distinction between signal and noise greater. This then allowed more data points to be included at higher spatial frequencies resulting in significantly higher acuity with the 14 Hz noise criteria. An improved success rate of acuity extrapolations was also observed with the 14 Hz noise criteria.

Table 2.4: Successful Extrapolations and Acuity (in cpd) from 14 Hz Noise Frequency at 4 Months of Age

| | Success Rate (n = 40) | Acuity* |
|---|---|---|
| Relaxed Phase / SNR 3 / 14 Hz noise | 37 | 8.47 ± 3.26** |
| Relaxed Phase / SNR 3 / 11,13 Hz noise | 31 | 6.40 ± 2.70** |

* Mean ± SD

** Significant difference in mean acuity ($p < 0.0005$)

The optimised sweep VEP analysis criteria was; 14 Hz noise frequency, SNR 3, relaxed phase and an acuity extrapolation that reflects the infant's best performance in either a single sweep or a vector average. The acuities of 4-month-old infants analysed with this optimised technique were normally distributed and ranged from 4.2 to 20 cpd. Eight infants demonstrated acuities considerably farther than the highest spatial frequency of the stimulus, indicating that the visual acuity of these infants was beyond the testing range. The data from these infants at 4 months CA were not included in further analysis. The mean (± SD) acuity of the 29 infants successfully tested at 4 months of age is 9.3 ± 3.0 cpd.

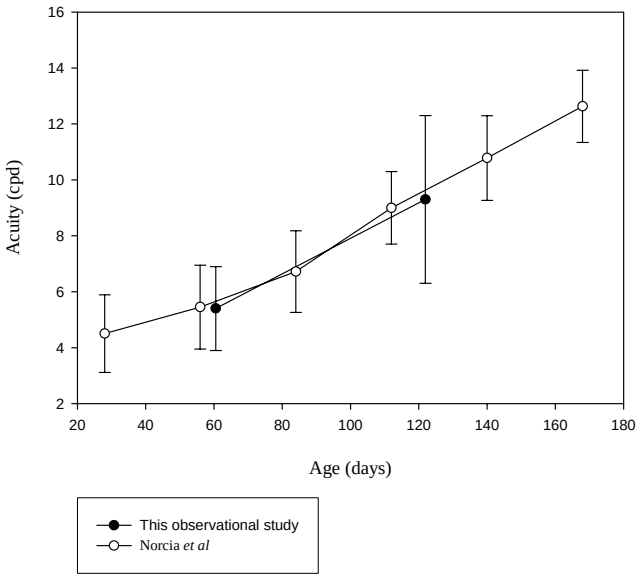
*Sweep VEP Acuity at 2 Months Compared With 4 Months of Age*

Sweep VEP data were collected from 39 infants at 2 months of age. Thirty-eight successful acuity extrapolations were performed using the optimised analysis criteria. Sweep acuities were normally distributed and ranged from 2.5 to 10.8 cpd (n = 38 / 39) at 2 months of age. The mean (± SD) acuity at 2 months of age was 6.3 ± 2.1 cpd. Compared with the data at 4 months of age a significant difference in acuity was observed ($p = 0.001$). This indicates that the sweep VEP acuity technique is sensitive enough to detect maturational change in a wide range of acuities of young infants.

*Comparison of Acuity Data with Norcia et al*

The acuities of the infants from my study at 2 and 4 calendar months of age were potted with the data of Norcia *et al* as reported from 1 to 6 lunar months of age [57] to investigate the external validity of the data (see Figure 2.2). Although differences in standard deviations exist between this study and Norcia *et al*, a good correlation was observed. Some of the variation in responses could be due to the heterogeneity of the population in this cohort. Other differences that may exist between the studies such as neonatal (birth weight) and lifestyle characteristics (eg exposure to smoking) are unable to be compared as Norcia *et al* did not report these.

Figure 2.2: Comparison of Acuity Data at 2 and 4 Months of Age with Norcia *et al*




*Comparison of Sweep VEP Acuity at 2 and 4 Months with Other Studies*

The acuity at 2 months of age is similar to Norcia *et al* but higher than another publication reporting acuities at 2 months of age. Auestad *et al* reported sweep VEP acuity at 8 weeks of age in term infants of approximately 4 ± 2 cpd [130]. Similar standard deviations are also observed between this study and

that of Auestad *et al* at 2 months of age. Other data to compare sweep VEP acuity in 2-month-old infants has not been found.

In 4-month-old term infants, Auestad *et al* reported acuity of approximately 7 ± 1 cpd [130]. In preterm infants O'Connor *et al* reported mean acuities near 8 cpd [60]. Higher acuities, near 12 cpd are reported by other groups that use the adaptive filter technique (AFT) to extract signal and noise data from the VEP response [70,132-134]. AFT is able to extract and discriminate signal and phase data from background EEG resulting in a reduction of overall background EEG noise and enhancing the gap between signal and noise [136]. This would result in more points at higher spatial frequencies meeting the SNR criteria and acuities at higher spatial frequencies would be extrapolated. Compared with the data reported here, AFT also improves the number of successful acuity extrapolations from a testing session and the high acuities have been reported using this technique [70,132,134].

Using the adaptive filtering technique, Lauritzen *et al* compared the average of acuities extrapolated from single sweeps with a signal average similar to the method described in this study. There was no selection of sweeps that reflected the infant's best performance in the averaged comparison, which would potentially reduce the acuity estimate as demonstrated in the 'all' group in this study. Interestingly, the enhanced extraction of the signal and the dampened use of all successful acuity extrapolations resulted in a mean (± SD) acuity of 9.1 ± 3.2 cpd, which compared well with mean and SD acuities of 4 month-old infants reported here and by Norcia *et al.*

A possible criticism of the sweep technique used in this study is the limited range of spatial frequencies shown to infants, which was most noticeable at 4 months of age. A wider range of stimulus may have increased the mean acuity at this age. The spatial frequencies can be readily adjusted to accommodate infants with well-developed acuity for the intervention trial.

*Summary of Optimisation of the Sweep VEP Acuity Technique*

This section has described the development and optimisation of sweep VEP method for measuring acuity of infants using computer software to assist analysis. The process of investigating different analysis criterion has highlighted both paucity of information and disparities in methodology between studies. Comparisons of VEP data from this study has demonstrated that small changes to analysis criteria can significantly alter mean acuity. The criteria determined as most optimal for the analysis of sweep VEP data is;

- SNR3
- Background noise frequency 14Hz
- Phase cannot lead >30$^{o}$ but can remain constant or lag continuously
- And to use the infant's best acuity performance from a single sweep or the vector average of a minimum of 3 sweeps.

To accommodate the finer acuity of 4 month-old infants, the range of stimulus is widened to 13.6 cpd. This is the sweep VEP acuity method used later in a DHA intervention trial in preterm infants (Chapter 4).

## 2.4 VEP Latency Responses to Transient Checkerboard Stimuli

VEP recordings in response to transient checkerboard stimuli are a well-established technique for measuring the integrity of the visual-neural pathway. Latency responses to checkerboard stimuli have been used in studies of neural toxicity where slower or impaired responses are demonstrated in infants exposed to smoking, alcohol and other illicit substances *in utero* [137]. Our laboratory has previously reported latencies to transient checkerboard stimuli in term infants [42]. The main purpose of this study was to evaluate the stimulus size most appropriate for testing VEP latency responses of 2- and 4-month-old infants. This information could then be applied in choosing one or more check sizes to evaluate latency responses of preterm infants in a DHA intervention trial. VEP latency recordings were collected from the same infants enrolled in the observational pilot study used develop the sweep acuity technique (Section 2.2).

### *2.4.1 Method for Recording VEP Latency Responses*

Three electrodes were used to detect responses to transient VEP recordings. The active electrode was placed at 1cm above the inion ($O_z$), a reference electrode at the midline on the hairline of the forehead ($F_z$) and an earth electrode at the central vertex ($C_z$), as defined by the International 10-20 System for electrode placement.

A checkerboard pattern (of constant luminance) reversing at 2 Hz for 30 seconds was generated by the Enfant 4010 program (Neuroscientific Corp, Farmingdale NY). Checks ranging in size between 10 to 96 min of arc were presented on a monitor (32 x 21 cm) in the following order, 48, 20, 96, 34, 17, 10 then 69 min of arc. The checks were presented in this order to reduce the infants tiring before collecting responses to both large and small checks. If the arousal state of the infant allowed, this sequence was repeated. The transient recording was stopped during the presentation if the infant looked away. Response frequencies from 1 – 100 Hz were amplified 10,000 times by Grass model PC511 AC

amplifier (Grass Instrument Division, Astro-Med Inc., W. Warwick RI 02893) and recorded using the Enfant 4010 software. Latency was determined as the time to the appearance of the first positive peak (P1) of the response. The latency of the P1 response was used for data analysis, the mean was used when duplicate measurements were available.

### *2.4.2 Results of VEP Latencies to Transient Checkerboard Images*

Latency responses to checkerboard stimuli varied depending on the stimulus size and infant age. As not all infants responded to every check presented, the mean latencies reported in Table 2.5 include only the respondents to the stimulus. At 2 months of age, all infants responded to the first check shown (48 min of arc) however, in the course of the testing session, two larger checks (96 and 69 minutes of arc) were shown to the infants with a 93% and 85% response rate. This may suggest that infant fatigue, disinterest or boredom affect the success of testing at 2 months CA. By 4 months of age the overall response rate to the different sized checks was fairly consistent, with 98% of infants responding to both the first and last checks shown (48 and 69 min of arc).

Latency responses ranged from 125 to 230 milliseconds (msec) to the 96 minute check, and 175 to 230 msec to the 10 minute check at 2 months of age. By 4 months of age the responses ranged from 105 to 233 msec, and 127 msec to 243 msec to the 96 and 10 minute checks, respectively. The typical increase in latency with decreasing size of the stimulus was clearly present in all infants at both ages.

Table 2.5: Latency (msec) of Transient VEP Responses at 2 and 4 Months of Age

| Check size (minutes of arc) | 2 month old n = 40 | 4 month old n = 40 |
|---|---|---|
| 96 | 163 ± 27<br>n = 37 (93%) | 125 ± 24*<br>n = 40 (100%) |
| 69 | 176 ± 24<br>n = 34 (85%) | 126 ± 22*<br>n = 39 (98%) |
| 48 | 179 ± 24<br>n = 40 (100%) | 134 ± 24*<br>n = 39 (98%) |
| 34 | 190 ± 25<br>n = 33 (83%) | 140 ± 26*<br>n = 39 (98%) |
| 20 | 200 ± 33<br>n = 29 (73%) | 152 ± 32*<br>n = 38 (95%) |
| 17 | 210 ± 33<br>n = 22 (55%) | 149 ± 18*<br>n = 29 (73%) |
| 10 | 207 ± 23<br>n = 9 (23%) | 162 ± 28<br>n = 21 (53%) |

Results expressed as mean ± SD

* Indicates significant difference ($p<0.0005$) between 2 and 4 months of age

To determine if infant fatigue had affected the latency of the response, infants shown the transient stimulus first were compared with those shown the sweep stimulus first (data not shown). No significant differences in latencies were found. As expected the speed of the response decreased in the time from 2 to 4 months in all infants (Table 2.5). A paired sample T test was used to evaluate the maturity of each infant's response with highly significant results ($p <0.0005$) for all checks except for 10 min of arc ($p = 0.43$).

*Post hoc* comparisons of latencies between preterm and term infants showed no differences at 2 months of age. At 4 months of age, there was a general trend for preterm infants to have longer

latencies than the term group (data not shown). For most checks this did not reach statistical significance due to small sample sizes and large standard deviations. However, latencies to checks 69, 34 and 10 were significantly longer in preterm infants compared to term infants (check 69, mean ± SD preterm infants 137 ± 35, term infants 121 ± 12, $p < 0.05$; check 34 preterm infants 158 ± 29, term infants 132 ± 21, $p < 0.05$; check 10 preterm infants 193 ± 64, term infants 153 ± 11, $p < 0.05$).

### *2.4.3 Discussion of Latency Responses*

Robust and typical P1 latency responses to transient stimuli were demonstrated in both preterm and term infants. As the check sizes were reduced, the time to reach the peak (P1) of the response (data reported here as latency) was increased, and the proportion of respondents decreased. A significant maturation of latency responses occurred between 2 and 4 months of age as evidenced by the shortening of response times and an improvement in response rate, particularly to the smaller checks.

A compliant state of arousal is pivotal to the collection of VEP data. All 2-month-old infants demonstrated the ability to resolve the first (48 minutes of arc) check shown. Surprisingly, a poorer success rate was exhibited later in the testing session to larger checks (96 and 69 minutes of arc, 93% and 85% success rate, respectively). This is in part due to lack of compliance, demonstrating only a short duration of time is available to collect VEP responses in 2-month-old infants. Response rates in 4-month-old infants were not similarly affected. The number of stimuli shown to infants in the DHA intervention trial will need to be reduced to accommodate the attention spans of 2-month-old infants.

Similar response rates and latencies to transient checkerboard stimuli have been reported in term infants [42,138,139]. In the study by McCulloch and Skarf [138] the VEP latencies of term infants at 2 and 4 months are reported to checks of 120, 60, 30, 15 and 7.5 minutes of arc. At 2 months of age, only 4 of the 10 infants tested were reported to resolve checks at 15 and none at 7.5 minutes of arc and the

latency of 9 two-month-old infants measured with the 60 minute check was 163 ± 20 milliseconds. Comparable data were published by Moskowitz and Sokol, where data from two-month-old infants shown checks at 60 and 48 minutes (data were pooled) had latency of 178 ± 24 milliseconds. In the present study, two-month-old infants exhibited similar latencies to a 48 minute check (179 ± 24 milliseconds). Latency responses to a check subtending 48 minutes of arc at 4 months of age were 113 ± 5 [138], 119.1 ± 7 [139] compared with 134 ± 24 found in this study. Previous latency responses performed on healthy, fully breast fed term infants of approximately 138 and 155 milliseconds were reported to 20 and 10 minute checks (respectively) from an intervention trial conducted by this department [42].

At 4 months of age there was a trend for latency responses to be longer in the preterm group and responses to some checks reached statistical significance. A comparison of transient checkerboard latency responses in 24 term and 24 preterm infants from 1 to 6 months of age, revealed no significant differences between the infants based on their corrected age [78]. It is tempting to speculate that differences in latencies found between term and preterm infants found in this study may be indicative of a wider or more diverse range of neural development in preterm infants. An effect of gestational age at birth on latency to checkerboard stimulus in infants has been demonstrated in infants born between 37 and 42 weeks gestation [140]. However, further sufficiently powered studies with larger sample sizes would be necessary to determine differences in latency responses between preterm and term infants.

### *2.4.4 Summary of Transient VEP Data*

The trend for increased latencies to transient stimulus in the preterm group offers an interesting area of investigation in a DHA intervention trial. To reduce the burden of collecting data to many different stimuli and improve the chance of a successful test, it was concluded that fewer checkerboard stimuli must be shown to infants. Large checks of 96 and 69 minutes of arc at 2 months and 69 and 48 at 4 months of age were selected.

# CHAPTER 3: DESIGN AND IMPLEMENTATION OF DOUBLE BLIND RANDOMISED CONTROLLED DHA INTERVENTION TRIAL IN PRETERM INFANTS

## 3.1 Trial Design and Implementation

### *3.1.1 Introduction*

This chapter describes the design and implementation of a double blind randomised controlled DHA intervention trial in preterm infants. The trial outcome compares preterm infants that receive approximately 50 mg/kg/d of DHA with the level of DHA received through current clinical practice (estimated to be between 12 and 20 mg/kg/day). The high dose of DHA is designed to meet the estimated *in utero* accrual rate of n-3 LCPUFA and is achieved by increasing the DHA content in milk fat to 1%. The trial was designed to accommodate any mode of feeding (ie breast, formula or combination of both) without affecting maternal choice and allows medical staff to support and encourage breastfeeding. In order to mimic the DHA exposure of a fetus carried to term, the duration of the intervention was from within 5 days of commencing any enteral feeds until the infant reached their estimated due date (EDD). The trial protocol was submitted and approved by the Research Ethics Committee at the Women's and Children's Hospital (approval number REC 473/00).

### *3.1.2 Aims*

The strategy was to increase the concentration of DHA in breast milk and formula given to preterm infants by supplementing the mother (in the case of breastfeeding) or by direct addition to formula (for all formula feeds). Maternal compliance was determined by the concentration of DHA in maternal breast milk and formula, and the success of the intervention was determined by infant erythrocyte membrane phospholipid fatty acid DHA at the end of the intervention period.

### *3.1.3 Hypothesis*

Infants supplemented from enrolment to 40 weeks postmenstrual age (PMA) with DHA at 1% of total fatty acids will have:

- Improved VEP acuity at 2 and 4 months CA,
- No difference in weight at the end of the intervention period or at 4 months corrected age (CA),

when compared with infants fed DHA at the standard clinical practice level (0.2 to 0.35% of total fat as DHA).

### *3.1.4 Primary Outcomes*

The primary objective of the trial was to compare the efficacy and safety of the high-dose DHA (treatment group) compared with usual DHA intake (control group) of preterm infants. Efficacy of the treatment was assessed by visual acuity at 4 months CA using the sweep VEP acuity technique. The primary safety measurement was infant growth at 4 months CA.

### *3.1.5 Secondary Outcomes*

VEP acuity data at 2 months CA and VEP latency data at 2 and 4 months CA were collected as secondary outcome measures of visual performance. Secondary growth data included weight, length and head circumference at the end of the intervention period, growth velocity and z-scores for intervention and follow up phases. Other measures of safety included feeding and tolerance data, incidence of diseases of prematurity and factors relating to clinical morbidity. A detailed description of the data collected is provided later in this chapter. Briefly, the measurements included; tolerance and gastrointestinal data, incidence of necrotising enterocolitis (NEC), duration of hospitalisation, number of septic events and number of blood transfusions, length of stay, respiratory data including the days of respiratory support or oxygen therapy, incidence of hyalin membrane disease (HMD), development of

retinopathy of prematurity (ROP), intraventricular haemorrhage (IVH) and periventricular leukomalacia (PVL).

### *3.1.6 Eligibility, Inclusion and Exclusion Criteria*

#### *Inclusion Criteria*

Infants born <33 weeks gestational age were eligible for the trial. Enrolment was within 5 days of commencing any enteral feeds. This was to prevent accumulation and use of unsupplemented breast milk and to commence the intervention as quickly as possible.

#### *Exclusion Criteria*

Infants with major congenital or chromosomal abnormalities were excluded. Lactating mothers were not allowed to participate if they had a contraindication to consuming fish oil capsules (eg bleeding disorders or taking regular anticoagulant therapy).

The Women's and Children's Hospital services a large rural network with some patients as far away as 3 hours by air. Due to the impracticalities of follow up, infants who resided out of the Adelaide metropolitan area after discharge from hospital were also excluded.

### *3.1.7 Randomisation Schedule*

To ensure balanced enrolments across weight ranges the randomisation schedule was stratified for birth weight (<1250g and ≥1250g). The schedule was also stratified for gender, as the primary safety outcome (weight) is known to be influenced by gender.

Prior to the study commencement a computer generated randomisation schedule containing the dietary assignment was sealed in opaque envelopes by administrative staff not involved in the trial. Each

pregnancy comprised one unit of randomisation. Multiple births were randomised to the same group based on the birth weight and gender of the firstborn infant.

### *3.1.8 Masking of Treatment*

The trial was planned with 4 colour-coded groups, two treatments and two controls. The double-group strategy was to assist in masking the treatment and control groups. Capsules for the control and treatment were identical in colour, size and shape. The capsules were packed at Flinders Medical Centre by staff from the Fatty Acid Laboratory that was not involved in the conduct of the trial. All medical, nursing and clinical trial staff were unaware of which of the colour-coded groups were control or treatment.

### *3.1.9 Enrolment Procedure*

A neonatologist approached the parents of eligible infants to talk about the trial and provide an Information Sheet for their consideration. The parents were encouraged to discuss the trial with their doctor, nursing staff and other family members. A research midwife or a neonatologist usually enrolled study subjects, but occasionally I undertook this role. If the parents agreed to participate in the study, they were then asked to read and sign a consent form.

### *3.1.10 Allocation of Intervention*

Once consent was obtained I was contacted to allocate the intervention according to the randomisation schedule. The schedule was protected in a locked office at the Child Nutrition Research Centre (CNRC) and not at the nursery. Each new enrolment was sequentially assigned their unique study number and allocated to one of the four coloured groups. Lactating mothers were issued with capsules according to their allocated colour. A card was placed on the infants' isolet in the nursery and their medical notes

were tagged with a sticker that identified their participation and their colour-code in the trial. Any formula feeds required during the intervention period were matched according to the colour allocated.

### *3.1.11 Implementation of Trial*

Breastfeeding mothers were asked to consume six capsules per day containing 500 mg of oil, from enrolment until the infants estimated due date (EDD). The placebo capsules contained soy oil, which has no DHA and does not alter the DHA content of the breast milk [36]. The treatment capsules contained tuna oil and the full 3g daily dose of tuna oil was designed to increase breast milk DHA content to approximately 1% of total fat. The fatty acid composition of the soy and DHA capsules were tested by capillary gas chromatography at Fatty Acid Laboratories (Flinders Medical Centre, South Australia). The composition of the major fatty acids is presented in Table 3.1. Clover Corporation donated all capsules for the trial. As per hospital policy, families were encouraged to continue breastfeeding for as long as the mothers chose.

Table 3.1: Fatty Acid Composition (% Total) of One 500 mg Capsule

| | Soy oil capsules | Tuna oil capsules |
|---|---|---|
| Total Saturates | 16.0 | 24.4 |
| Total Monounsaturates | 24.5 | 28.4 |
| Oleic 18:1 n-9 | 22.7 | 19.6 |
| Total n-6 PUFA | 53.4 | 6.9 |
| Linoleic 18:2 n-6 | 53.4 | 2.7 |
| Arachidonic 20:4 n-6 | 0.0 | 1.8 |
| Total n-3 PUFA | 5.9 | 39.9 |
| α-Linolenic 18:3 n-3 | 5.9 | 0.4 |
| Eicosapentaenoic 20:5 n-3 | 0.0 | 6.5 |
| Docosapentaenoic 22:5 n-3 | 0.0 | 1.2 |
| Docosahexaenoic 22:6 n-3 | 0.0 | 29.5 |

Throughout the trial and as per hospital policy, only the mothers' own milk was used for her infant and when that was not supplied a preterm formula was used. During hospitalisation all trial formula was prepared by blinded nursery staff in a dedicated milk preparation room. To adjust the formula DHA content, two drops of oil from the infant's allocated coloured group was added to each 60mL bottle of preterm infant formula. The standard preterm infant formula used at the Women's and Children's Hospital was a ready-to-feed liquid formula, Nutriprem (see Table 3.2 for details of the formula composition). Infants were given Nutriprem from the onset of enteral feeds through to reaching EDD unless otherwise prescribed by a doctor. All Nutriprem required for infants after discharge from hospital but before the end of the treatment phase was donated by Nutricia Australia. The fatty acid composition of the formula was regularly checked during the trial, these data are presented in Chapter 4.

Table 3.2: Composition of Preterm Infant Formula

| | |
|---|---|
| Energy (kJ/L) | 3390 |
| Protein (g/L) | 24 |
| Total Fat (g/L) | 44 |
| Comprising Fatty acids; | |
| - Total Saturated (g/L) | 18 |
| - Total Monounsaturated (g/L) | 11 |
| - Total Polyunsaturated (g/L) | 6.8 |
| - Linoleic Acid (g/L) | 5.7 |
| - α-linolenic acid (g/L) | 0.6 |
| - Arachidonic acid (g/L) | 0.3 |
| - Docosahexaenoic acid (g/L) | 0.2 |
| Carbohydrate (g/L) | 78 |
| Sodium (mmol/L) | 18 |
| Potassium (mmol/L) | 21 |
| Calcium (g/L) | 1 |
| Iron (mg/L) | 9 |

At discharge, I issued families with sufficient capsules and formula (if formula feeding) to continue the feeding regime until the infants EDD. At this time, I demonstrated how to prepare the formula and gave parents written instructions to take home. If formula feeding, families were encouraged to use a formula containing DHA after the end of the intervention period.

### *3.1.12 Data Collection During Hospitalisation*

Shortly after enrolment demographic data were collected from the infant's mother. Feeding and growth data were collected daily during the infant's hospitalisation. Research midwives usually collected the data daily, although during holidays and other busy times I provided additional support for data collection. At the end of hospitalisation all clinical measurements and diagnoses were reviewed by the Director of the Women's and Children's Hospital Neonatal Intensive Care Unit (NICU) except when infants were transferred to other hospitals.

### *3.1.13 Follow-up of Infants Transferred to Other Hospitals*

Infants were frequently transferred to other hospitals in the metropolitan area. The Women's and Children's Hospital is one of only 2 hospitals providing tertiary care for preterm infants in South Australia and infants were transferred to downstream level 2 or level 1 units for ongoing care. When infants were transferred a portfolio containing a letter of introduction to the attending doctor, a trial folder containing contact details, formula recipe, a trial synopsis and a copy of the signed consent form travelled with the infant, along with a small stock of capsules and formula.

I maintained regular contact with downstream hospitals through daily phone calls and weekly visits for the duration of each infant's hospitalisation. I regularly presented in-services at these hospitals, which were well attended by nursing staff. The contact with hospitals staff was necessary not just to ensure compliance, but also to deliver trial stocks, collect breast milk samples and collect feeding and clinical

data. During the course of the trial, infants were discharged to 6 different local hospitals. With permission from the nursery unit head, I reviewed the medical records of each infant at discharge from downstream hospitals. At this time growth measurements, enteral intakes, medications and other clinical data were documented and cross-checked against information recorded in the infants Case Report Form (CRF).

### *3.1.14 Data Collection at the Estimated Due Date*

Appointments at ± 1 week of the infants EDD took place at the Child Nutrition Research Centre (CNRC) clinic rooms at the Women's and Children's Hospital or at the family home. At this time I recorded a brief feeding and medical history from the mother and collected infants weight, length and head circumference (HC) measurements according to the description in Section 3.3. A midwife collected a heel prick blood sample for fatty acid analysis. Frozen expressed breast milk samples collected over the preceding 7 days were also collected.

### *3.1.15 Data Collection at 2 Months Corrected Age*

Appointments at ± 1 week of the infant reaching 2 months CA took place at the CNRC clinic rooms. At this time I recorded a brief feeding and medical history from the mother and collected infants weight, length, HC, mid upper arm circumference (MUAC) and abdominal girth measurements according to the description in Section 3.3. I also collected and analysed VEP latency and acuity data according to the description in Chapter 2. Briefly, latency measurements were taken in response to two high-contrast reversing checkerboard stimuli with visual angles of 96 and 69 minutes of arc and acuity measurements were recorded in response to a horizontal sinusoidal grating pattern that swept linearly through spatial frequencies of 0.26 to 8.43 cpd. The order of the VEP latency and VEP acuity tests varied. A randomisation schedule was prepared by administrative staff not involved in the trial and sealed in opaque envelopes. At each appointment the next envelope was opened and the VEP test to be

conducted first was identified. The randomisation of the tests was to ensure that infant fatigue did not consistently contribute to either VEP test.

### *3.1.16 Data Collection at 4 Months Corrected Age*

Appointments at ± 1 week of the infant reaching 4 months CA took place at the CNRC clinic rooms. Infant feeding information, medical history and growth data were collected as per the 2 month CA appointment. VEP data were collected and analysed according to the description in Chapter 2 except that the stimulus was adjusted to reflect the improved visual performance of older infants. Latency responses were recorded from checkerboard stimulus with visual angles of 69 and 48 minutes of arc. Sweep acuity was recorded to horizontal sinusoidal grating stimulus at 1.0 to 13.6 cpd. The order of the VEP latency and VEP acuity tests were randomised as reported for the appointment at 2 months CA (3.1.15).

### *3.1.17 Compliance and the Success of the Intervention*

Compliance with the capsules regime and fortification of infant formula was assessed by fatty acid analysis and by maternal report. Lactating mothers were asked to provide a sample of expressed breast milk at fortnightly intervals during the hospitalisation period and everyday for the 7 days leading up to the EDD appointment. Prior to the 7-day collection, mothers were instructed to express the daily sample at the same time of the day and the same place in the feed. At the EDD appointment, mothers were asked to estimate the number of capsules missed taking (if providing breast milk for their infants) and the number of formula feeds given without the oil added (if providing formula for their infants). Spot checks of formula fatty acid composition were taken at random throughout the trial. Infant plasma and erythrocyte cell membrane fatty acid composition from the heel prick blood sample collected at EDD was used to determine the success of the intervention. Analysis of fatty acids is described in Section

3.2 and the outcome of these analyses is reported in Chapter 4. The laboratory held results of fatty acid analysis until data collection and blinded analyses were completed.

### *3.1.18 Data Management and Maintaining the Trial Blinding*

After completion of follow up for all infants, the data were entered and screened for errors. The three highest and lowest values for every variable entered into the database were checked for errors. Two errors were found and corrected. A random selection of 10 clinical research files were cross checked against the database, no errors were found. Further checking was not performed.

The data in the format of the four treatment groups were combined into the control and treatment interventions without unblinding. This was performed by a Research Officer at the Flinders Medical Centre (FMC) Fatty Acid Laboratory that was aware of the treatment allocation. Each infant's unique study number and colour-group allocation were removed and the data were recoded into the control and treatment groups. However, the data were identified only with an arbitrary letter 'A' or 'B', the control or treatment groups were not identified. I then performed the statistical analyses on the 'A' and 'B' groups, which included baseline variables and comparisons of all primary and secondary outcome data. Specific details regarding the statistical analyses are described in the text associated with each results chapter (Chapters 4 and 5).

The majority of this thesis (Chapters 1 through 5) was written without knowledge of the control and treatment groups, the only exception being the fatty acid information (only Figure 4.2 and Table 4.5, Chapter 4) which was withheld until completion of the main statistical comparisons. This was necessary as the treatment group could be identified by the higher DHA content in breast milk, formula and erythrocyte phospholipids. As infant and coloured-group identifying information was removed from the dataset, the study investigators and I remain masked to the intervention groups.

### *3.1.19 Sample Size Estimates*

Sample size calculations (Table 3.3) are based on two-sided estimates with 80% power and Type 1 error of 0.05. The sample size estimates are separated into birth weight categories. The expected differences are larger in the smaller infants as it is thought that these infants have the most to gain from the intervention. The smaller infants also have a wider range of responses, which is why the expected standard deviations are larger. The acuity standard deviations are based on pilot data. The minimum difference in weight that could be detected at 4 months CA is based on differences in growth between n-3 LCPUFA supplemented compared with unsupplemented formula fed infants reported in the literature [100,104]. Infants born <1250 g represent approximately 40% of infants hospitalised in NICU. A target of 75 infants per group was set to reach sample size estimates for all infants and to accommodate a 20% loss to follow-up.

Table 3.3: Sample Size Estimates for Primary Outcomes at 4 Months CA

| Primary Outcome | Expected Difference | Expected SD | Estimate of Sample Size |
|---|---|---|---|
| VEP Acuity infants born <1250g | 3 cpd | 2.5 cpd | 25 |
| VEP Acuity infants born ≥1250g | 2.5 cpd | 1.5 cpd | 46 |
| Weight of infants <1250g | 500 g | 950 g | 58 |
| Weight of infants ≥1250g | 400 g | 825 g | 44 |

### *3.1.20 Analysis of Trial Data*

Statistical analyses were performed using SPSS for Windows (Version 11.0.0, Chicago Il, USA), with probability <0.05 considered significant. Primary analyses were conducted on intention-to-treat group comparisons followed by covariate adjustment for birth weight and gender. Subgroup analyses were limited to randomisation strata; gender and birth weight (<1250 and ≥1250 g) subgroups. Categorical variables were compared by Chi-squared tests; continuous normally distributed variables by independent samples T tests and non-parametric continuous variables by Mann-Whitney U tests. In Chi

squared comparisons, the Yates correction for continuity statistic was used to prevent overestimation of the significance and Fischer's exact probability test was applied when examining variables of low incidence. Further details pertaining to analysis of specific variables are described in each results chapter (Chapters 4 and 5).

The use of regression modelling to investigate the effect of LCPUFA on infant size or visual acuity has been a relatively common secondary analyses reported from previous trials. This type of analysis has not been reported in my thesis. The remainder of this section discusses the complexities associated with relating dietary fatty acids to preterm infant growth and the rationale for not performing these analyses.

There are a number of conceptual reasons suggesting the assumptions underlying regression analysis may not be appropriate to use on data from randomised controlled trials, such as in my trial. Usually in LCPUFA trials the fatty acid status of the treatment and control groups are compared to evaluate success of an intervention and compliance with the trial protocol. Compliance may be good in both groups, but the effect on fatty acid status can only be seen in the infants that received the intervention. There is uncertainty regarding whether a parent that complies better with the trial protocol will have an influence on the outcome. Other regression related factors such as collinearity between fatty acids might also contribute to unstable analyses. Correlations exist between fatty acids as they were measured as a percentage of the total; as one increases others decrease. Furthermore, as my trial intended to alter fatty acid status, the assumption that the fatty acid profile of infants in the control and treatment groups are part of the same population is inappropriate.

Infant growth is dependent on the energy and protein content of the infants' diet. Both protein and energy intake are influenced by the proportion of breast feeding in the infants diet, the introduction and

cessation of breast milk fortification, the commencement of sucking breast feeds and the use of modular supplements [141-144]. Accommodating any combination of breast and formula feeding was a deliberate and valuable feature of my trial. This permitted investigating the outcome of increasing dietary DHA in context of common feeding practices and not only the small proportion of infants exclusively fed breast milk or infant formula. This feeding strategy did not permit control of protein and energy content of the infants' diet.

The fatty acid profile of milk in the infants' diet strongly influences the erythrocyte fatty acid content. Compared with formula, breast milk contains many differences in fatty acid composition including a lower concentration of LA and a wider range of fatty acids. In addition, the types of fatty acids in breast milk vary with maternal diet. As a result, there is a wide variation in erythrocyte fatty acid profile observed in infants receiving a diet of breast milk, formula milk or a combination of both.

The wide variation in dietary energy intake, fatty acid status and preterm infant growth, combined with other trial related factors have the potential to result in unreliable or flawed analyses. Therefore, exploring the relationship between fatty acids and growth of preterm infants using regression analysis has considerable limitations and beyond the scope of this thesis.

## 3.2 Method for Fatty Acid Analysis of DHA Intervention Trial Samples

### *3.2.1 Introduction*

Fatty acid analyses were performed on samples of maternal breast milk, formula, and infant erythrocyte phospholipids generated from the trial. So that I could remain masked to the intervention group of each infant, all analyses were performed off site at the Fatty Acid Laboratories, Flinders Medical Centre, Bedford Park, South Australia. The fatty acid analyses were performed by a Laboratory Assistant that was blind to the intervention group. The techniques for fatty acid analysis are well established and have

been previously published [36,42]. The following section contains details of the extraction of the lipid phase from each of the specimens, the separation of phospholipid fatty acids by Thin-Layer Chromatography (TLC) conversion to fatty acid methyl esters (FAMEs) and subsequent analysis by GC chromatography.

### *3.2.2 Extraction of Total Fatty Acids*

Plasma was separated from erythrocytes by centrifugation. Erythrocytes were washed 3 times in cold isotonic saline before extraction of fatty acids. To extract all fatty acids, a 150 μL sample of maternal breast milk or washed erythrocytes, were diluted with 1.5 mL of cold 0.9% isotonic saline. For breast milk samples, this was added to 2mL of AR methanol and 3mL AR chloroform. The washed erythrocyte was added to 2 mL of AR isopropanol and 3 mL AR chloroform. The samples were mixed well by vortex and allowed to stand 5 minutes before centrifuging at 1500 g for 10 minutes. The lower chloroform layer was used for further analysis.

### *3.2.3 Separation of Erythrocyte Phospholipids*

A 250 μL aliquot of fatty acids extracted from erythrocytes were spotted onto 0.3 mm Silica Gel Plates (Merck, Germany) and placed in TLC tank containing a 3:1 mixture of petroleum spirit:acetone. The phospholipid fraction remains at the spot site and was visualised under UV light before scraping into 2 mL of 1% sulphuric acid in methanol.

### *3.2.4 Methylation of Extracted Fatty Acids*

Total fatty acids from breast milk and erythrocyte phospholipid fatty acids fractions were methylated for 3 hours at 70 $^{o}$C before extraction in 0.25 mL of distilled water and 0.5 mL of Heptane. The upper heptane layer containing the methylated fatty acids were removed and added to a vial containing

anhydrous sodium sulphate. The fatty acid methyl esters (FAMEs) were evaporated under nitrogen to approximately 100 μL ready for GC analysis.

### *3.2.5 Gas Chromatograph Analysis of FAME*

Without transferring any sodium sulphate, a 50 μL volume of FAMEs was injected into the gas chromatograph (Hewlett Packard 6890) and separated by a 0.25 μm column coated with BPX70 (SGE Pty Ltd, Australia). The temperature rose by 5 $^{o}$C per minute to 220 $^{o}$C with flame ionisation set at 300 $^{o}$C. The FAME samples were mobilised by Helium gas at 35 cm per second along the 50 m column and the methyl esters were identified by comparison to retention times of standards (GLC – 463 Nu-check Prep Inc, MN 56028, USA).

## 3.3 Measuring Growth of Infants

### *3.3.1 Weight*

An electronic balance was used to determine infant weight to the nearest 5 g. The scales were calibrated annually with standard weights. The infant was undressed and weighed without nappy.

### *3.3.2 Length*

While undressed, the infant laid upon a length board (O'Leary, Ellard Instruments, CA). The head of the infant was held in the Frankfort plane with the vertex touching the headboard. The infants' legs were gently extended keeping hips aligned and toes pointing upward. The footboard was moved up and length was determined as the point where the infants' heels just touched the footboard. Length measurements were recorded to the nearest 1 mm. If the infant moved or resisted extending the legs, a second and sometimes a third measurement was taken. The average of two measurements that did not differ by more than 3 mm was recorded as the length. A preemie length board was purchased for use in the NICU unit and nursing staff performed length measurements according to this standard procedure.

### *3.3.3 Head Circumference*

Head circumference was measured to the nearest 1 mm using non-stretchable paper tape. The paper tape was wrapped around the largest part of the head covering the occipital to frontal areas. The average of two measurements was taken. If these differed by more than 2 mm, a third measurement was taken and the average of the two closest head circumference measurements was recorded.

### *3.3.4 Mid Upper Arm Circumference*

The mid point of the acromiale to radiale distance of the infant's right arm was measured using a non-stretchable paper tape and the mid point determined from the average of the measurements. The tape was repositioned at the mid point and the measurement was recorded to nearest 1mm with the arm relaxed at the infant's side.

### *3.3.5 Abdominal Girth*

The infant's central abdomen was located by measuring the mid point between the lower costal (rib) border and anterior most part of the iliac crest. The paper tape was positioned at the central abdomen and abdominal girth measured to the nearest 1mm using non-stretchable paper tape.

## 3.4 Descriptions and Definitions of Clinical Measurements

Where possible, the clinical measurements and diagnoses are consistent with the definitions by the Australian and New Zealand Neonatal Network (ANZNN) [124].

### *3.4.1 Sepsis*

Sepsis was diagnosed from haematological results indicating infection in conjunction with a strong and consistent clinical picture. This was confirmed by isolation of a pathogenic bacterial, fungal or viral organism in infant blood or cerebrospinal fluid.

### *3.4.2 Feeding and Tolerance*

The number of days of parenteral nutrition, days to reach full enteral feeds and days of intravenous lipids were recorded. Daily enteral feeding information including volumes of breast milk, whether breast milk was fortified, the number of feeds directly at the breast, the daily volume and name of formula and the caloric density of the formula given were collected during hospitalisation. The number of days feeds were interrupted were recorded, partial days were included as 1.

### *3.4.3 Necrotising Enterocolitis*

NEC was indicated by clinical symptoms resulting in cessation of enteral feeds and commencement of antibiotics. In accordance with the ANZNN description, NEC is diagnosed with a total of 4 of the following symptoms including at least one systemic and one intestinal symptom. Systemic symptoms include; apnoea, temperature instability, lethargy, bradycardia. Intestinal symptoms include; a residual volume of $\geq$25% on 2 or more occasions, vomiting, faecal blood, abdominal distension, palpable abdominal mass. Other symptoms may include; portal vein gas, persistent dilated loop, abdominal cellulitis. Serial x-rays, surgical or post mortem examination may confirm the diagnosis of NEC.

### *3.4.4 Respiratory Data*

The number of days the infants were ventilated, the time spent on continuous positive airway pressure (CPAP) and duration of oxygen therapy were recorded. Infants that developed with hyalin membrane disease (HMD) or bronchopulmonary dysplasia (BPD) were identified by oxygen dependency after reaching 36 weeks PMA.

### *3.4.5 Retinopathy of Prematurity*

Infants were routinely screened for Retinopathy of Prematurity (ROP) by a paediatric ophthalmologist during their hospitalisation. The ANZNN classifications are reproduced below;

*Stage 1* Demarcation line

Stage 2 Ridge

*Stage 3* Ridge with extra-retinal fibrovascular proliferation

*Stage 4* Retinal detachment.

The most severe stage in either eye was documented. Infants requiring laser or cryotherapy for ROP were also identified.

### *3.4.6 Neural Injury*

Intraventricular haemorrhages (IVH) were categorised by brain hemisphere, identified by cranial ultrasound and classified according to ANZNN definitions. The ANZNN criteria are graded by severity from subependymal germinal matrix haemorrhage (Grade 1) through to intraparenchymal haemorrhage (Grade 4). The presence of periventricular leukomalacia (PVL) injuries were identified by ultrasound and categorised by brain hemisphere and region (occipital, parietal and frontal).

### *3.4.7 Other Clinical Data*

The infant's entire length of stay (LOS) was recorded. This included all days from birth through to discharge, even if the infant was discharged after the end of the intervention. LOS data were also followed through to downstream hospital transfers. The number of days at each level of care was determined; part days were included as one. Days where the infant moved from high to lower level of care were recorded as the highest level of care.

Other measurements include the number of transfusions and the number of readmissions in the time between discharge and 4 months CA. The number and type of surgical procedures conducted for clinical purposes were also documented.

# CHAPTER 4: EFFICACY OF HIGH DOSE DHA (1%) ON PRETERM INFANT VISUAL ACUITY AND LATENCY

## 4.1 Abstract

In this chapter, the efficacy of feeding preterm infants milk containing a high concentration of docosahexaenoic acid (DHA) was assessed. In a double blind randomised controlled trial (RCT), preterm infants born <33 weeks gestation received breast milk or formula containing 1% DHA (treatment group) or fed DHA according to current feeding practices (approximately 0.3% DHA, control group). Efficacy was assessed by comparing visual evoked potential (VEP) acuity and latency responses at 2 and 4 months corrected age (CA).

The success of DHA supplementation was evident with infants in the treatment group having a significantly higher erythrocyte phospholipid DHA ($p < 0.0005$) compared with control the group. Acuity between the control and treatment groups was not different at 2 months CA (VEP acuity in cpd (mean ± SD) control group, 5.6 ± 2.4, treatment group 5.6 ± 2.4, $p = 0.96$). However, at 4 months CA the infants in the treatment group exhibited a significantly higher acuity (control group, 8.2 ± 1.8, treatment group 9.6 ± 3.7, $p = 0.03$). This effect remained after controlling for gender and birth weight. No significant differences were found between control and treatment groups in VEP latencies at 2 or 4 months CA.

Acuity at 4 months CA may be modestly improved by increasing the DHA concentration in milk fed to preterm infants. It is not known if higher concentrations of DHA in milk may further improve acuity. Further studies are necessary to determine if this intervention has long-term effects on visual development or if it is capable of improving other aspects of neurodevelopment of preterm infants.

## 4.2 Introduction

It is widely accepted that preterm infants fed formula containing DHA develop higher acuity than infants that do not receive DHA [5-7,60]. As a result, DHA is now added to preterm infant formula. Most trials that have measured acuity of preterm infants tested DHA concentrations similar to that observed in breast milk of Western women (approximately 0.3% of total fat) [6,7,60-62]. Yet, as hypothesised (Chapter 1), preterm infants may benefit from a supply of DHA which more closely matches the *in utero* rate of DHA accretion. In this chapter I present the results of a double-blind randomised controlled intervention trial in preterm infants. Infants who received DHA through breast milk or formula according to current clinical practices (0.2 to 0.35% DHA) were compared with infants who received milk containing 1% DHA. The primary measure of efficacy was sweep VEP acuity. Unique to this trial was the ability to accommodate breast and formula feeding, through supplementation of lactating mothers and fortification of preterm formula.

## 4.3 Method

The methods for recording sweep VEP acuity and latency data are described in Chapter 2. Details of the design and conduct of the trial are described in Chapter 3.

### *Statistical Analyses*

All statistical analyses were performed using SPSS for Windows (Version 11.0.0, Chicago Il, USA), with probability <0.05 considered significant. Primary analyses were conducted on intention-to-treat group comparisons followed by covariate adjustment for birth weight and gender. Subgroup analyses were limited to randomisation strata; gender and birth weight (<1250 and ≥1250 g) subgroups. Exploratory analyses were performed on infants predominantly fed breast milk at the end of the intervention period (≥80% of diet). Categorical variables were compared by Chi-squared tests; continuous normally distributed variables by independent samples T tests and non-parametric continuous variables by Mann-

Whitney U tests. In Chi squared comparisons, the Yates correction for continuity statistic was used for the comparison of two categorical variables to prevent overestimation of the significance and Fischer's exact probability test was applied when examining variables of low incidence.

## 4.4 Results

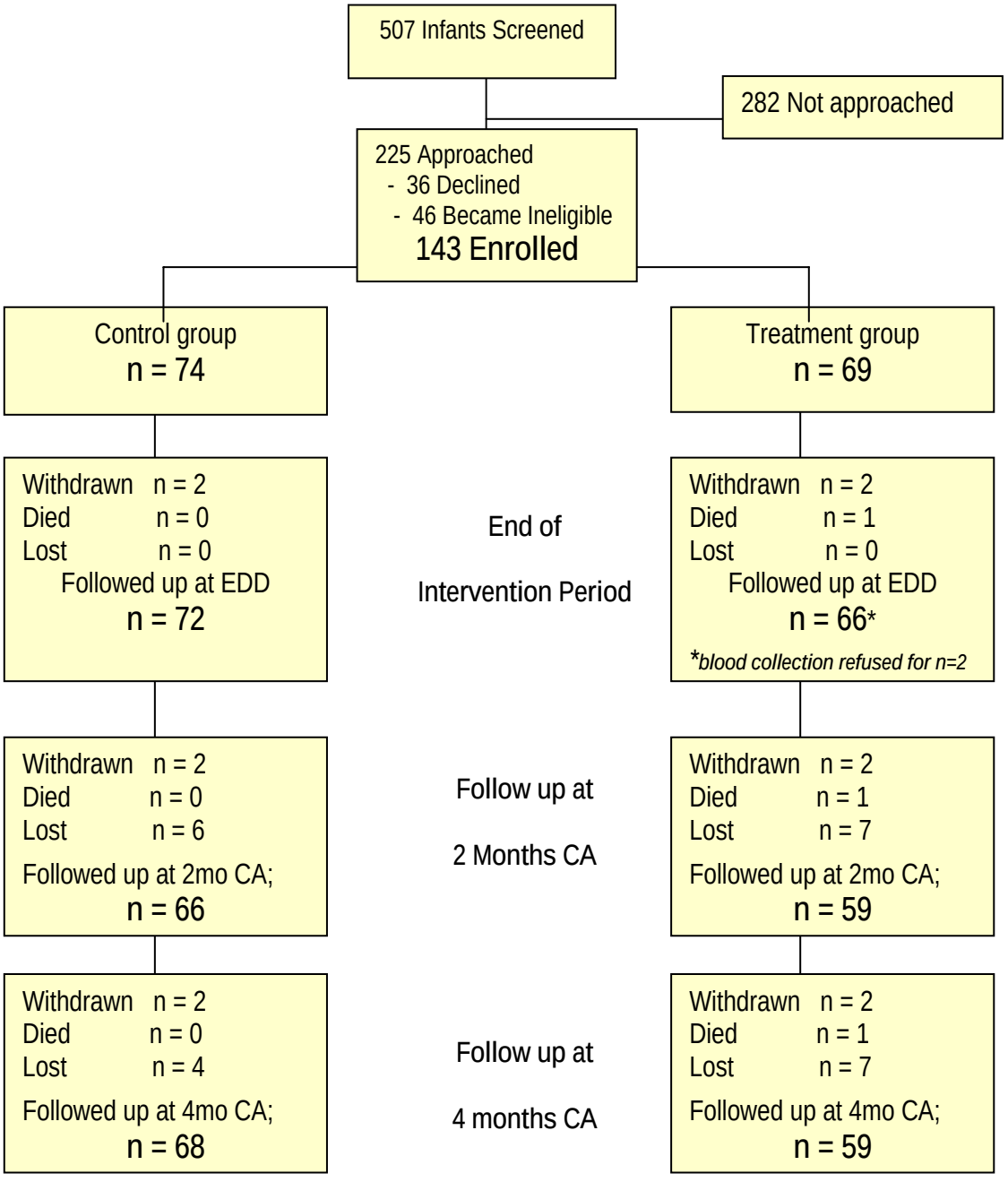
### *Enrolment and Flow of Participants through the Trial*

Five hundred and seven preterm infants admitted to the neonatal nurseries at the Women's and Children's Hospital, Adelaide were screened for eligibility. Many of these infants were not approached for participation in the trial due to ineligibility. This included infants with congenital abnormalities (n = 9), non–English speaking (n = 7), residing in country areas (n = 163), mothers taking anti-coagulant medication (n = 4) and a further 10 infants died. The parents of some infants (n = 89) were not approached for other reasons including; transfer to another hospital before approaching (n = 15), periods of research staff leave (n = 4), temporary shortage of trial formula (n = 4), parents not approached at the request of the Neonatal Intensive Care Unit (NICU) staff (n = 55), infants involved in other research trials (n = 6) and for medical reasons (n = 5). The medical reasons included; one infant with exophthalmos, two infants with ineligible twin, one infant with a serious IV burn and another with severe IVH. One of the eligibility criteria was that infants were to be enrolled within 5 days of commencing enteral feeds. Unfortunately, a further 46 infants achieved five days of full enteral feeds in the time between approaching the parents and allowing the parents time to consider participation in the trial, rendering the infant ineligible. Only 36 parents approached for the trial declined to participate resulting in a total of 143 infants enrolled in the trial.

The infants enrolled in the trial were randomised to receive one of the two interventions (Figure 4.1). Seventy-four infants were randomised to the control and 69 to the treatment intervention. During the course of the trial, four infants were withdrawn at the parents' request (two from each group) and one

from the treatment group died. At 2 months CA, six infants from the control group and seven from the treatment group did not attend follow up appointments. All families that did not attend this appointment were contacted and invited to attend when their infant reached 4 months CA. Three infants from the control group and two from the treatment group who did not attend the 2 month appointment returned at 4 months CA. A total of 4 infants from the control and 7 from the treatment group did not attend the 4 month CA follow up appointment.

Figure 4.1: Flow of Participants through the DHA Intervention Trial



*Baseline Characteristics of Trial Participants*

Infants randomised to the treatment group were comparable with infants in the control group (Table 4.1). There were no differences in the proportion of boys, infants born <1250 g or small for gestational age (SGA) and multiple births (all of which were twins) between groups. The postnatal age at randomisation did not differ between groups. Two infants (from the control group) were conceived as twins but were born as singletons due to a late *in utero* death of the sibling. These infants were considered twins for all analyses. Birth weight ranged between 640 g to 2620 g in the control group and 530 g to 2280 g in the treatment group.

Table 4.1: Enrolment Characteristics of Trial Participants

| | Control Group<br>n = 74 | Treatment Group<br>n = 69 |
|---|---|---|
| Male‡ (%) | 35 (47) | 35 (51) |
| Infants born <1250 g‡ (%) | 34 (46) | 33 (48) |
| Infants born SGA‡ (%) | 13 (18) | 11 (16) |
| Singletons‡ (%) | 50 (68) | 51 (74) |
| Age at randomisation (days)* | 5 ± 3 | 6 ± 3 |
| Enrolment – weight (g)*<br>- length (cm)*<br>- HC (cm)* | 1277 ± 403<br>38.8 ± 3.8<br>27.5 ± 2.6 | 1234 ± 408<br>38.9 ± 4.0<br>27.0 ± 2.7 |
| Withdrawals‡ (%) | 2 (3) | 2 (3) |

Values are ‡incidence and (percentage) or *mean ± SD as indicated

Family, lifestyle characteristics and socioeconomic status (SES) of infants enrolled in the trial were not different between the control and treatment groups (Table 4.2). Maternal gravida ranged from one to nine pregnancies in the control group and one to eight in the treatment group. As twins were enrolled in

the trial there were not as many mothers as infants. Mothers of infants totalled 61 in the control and 60 in the treatment group. Many mothers took dietary supplements during pregnancy (47 / 61 (77%) and 52 / 60 (87%) in the control and treatment groups, respectively). The majority of mothers took iron with or without folate during pregnancy (19 / 61 (31%) and 24 / 60 (40%) in the control and treatment groups, respectively). The numbers of mothers consuming a supplement containing n-3 LCPUFA during pregnancy did not differ between groups. SES status was based on the occupation of the parents and did not differ between groups. Paternal, education and employment data was not available from one of the four infants (control group) withdrawn from the trial as withdrawal occurred later on the day of randomisation and before all family data could be collected. For all variables, the infants withdrawn from the trial were similar to infants that were not withdrawn.

Table 4.2: Family and lifestyle details of trial participants by intervention group

| | Control Group n = 74 | Treatment Group n = 69 |
|---|---|---|
| Number of Mothers in each group‡ | 61 | 60 |
| Primiparous ‡ | 25 (34) | 32 (46) |
| Maternal gravida† | 2 (1 – 9) | 2 (1 – 7) |
| Maternal age (yr)* | 31 ± 6 | 29 ± 6 |
| Maternal education (yr)* | 12 ± 3 | 12 ± 2 |
| Maternal SES †^ | 5.3 (2.3 – 6.6) | 4.9 (2.6 – 6.6) |
| Took supplements containing n-3 LCPUFA during pregnancy‡ | 5 (7) | 4 (6) |
| Number of Fathers in each group‡ | 59 | 58 |
| Paternal education (yr)* | 13 ± 3 | 12 ± 2 |
| Paternal SES †^ | 4.9 (1.5 – 6.4) | 4.9 (2.3 – 6.6) |

Values are ‡incidence and (percentage), †median and (range) or *mean ± SD as indicated

^ SES calculation based on occupation [145]

Antenatal medical history, delivery and clinical picture at birth were not different between the control and treatment groups (Table 4.3). Many mothers received steroid therapy in the antenatal period, with the majority completing at least one dose (53 / 61 (87%) and 54 / 69 (78%) in the control and treatment groups, respectively). Most infants were born by Caesarean section and the majority required resuscitation at birth. Many infants were resuscitated with mask oxygen (28 / 74 (38%) and 28 / 69 (41%) in control and treatment groups, respectively) or with mask intermittent positive pressure ventilation (17 / 74 (22%) and 15 / 69 (22%) control and treatment infants, respectively). A proportion of infants were intubated (26 / 74 (32%) and 19 / 69 (28%) of control and treatment infants, respectively). Only one infant (1 / 69 (1%)) from the treatment group required external cardiac massage at birth. A small proportion of infants had Apgar scores less than five at one minute after birth (8 / 74 (11%) and 5 / 69 (7%) in the control and treatment groups, respectively). No infant had Apgar scores less than 5 at five minutes after birth.

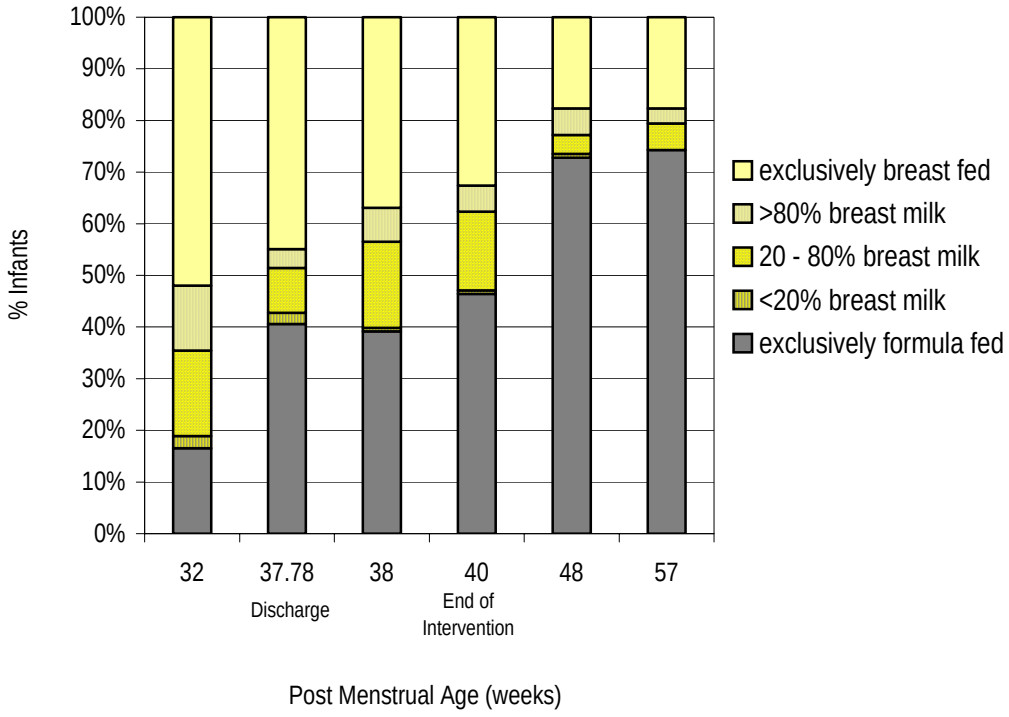
Table 4.3: Clinical Picture of Trial Participants at Birth

| | Control Group<br>n = 74 | Treatment Group<br>n = 69 |
|---|---|---|
| Birth weight* (g) | 1355 ± 438 | 1304 ± 442 |
| GA at birth* (wk) | 29.5 ± 2.2 | 29.0 ± 2.6 |
| IVF conception‡ (%) | 7 (10) | 9 (13) |
| Received Antenatal Steroids ‡ (%) | 67 (93) | 60 (90) |
| Caesarean Birth‡ (%) | 55 (76) | 50 (75) |
| Apgar* – one minute<br>– five minutes | 6.7 ± 1.7<br>8.6 ± 1.1 | 6.8 ± 1.8<br>8.6 ± 1.1 |
| Received Resuscitation‡ (%) | 69 (96) | 60 (90) |

Values are ‡incidence and (percentage) or *mean ± SD as indicated

An aim of the trial was to accommodate both breast and formula feedings. This was important as many preterm infants receive combination feeds in the neonatal period. Figure 4.2 shows the percentage breast milk in the infants' diet during the intervention period through to 4 months CA (57 weeks post menstrual age). The majority of infants received some breast milk early in the intervention period. By the end of the intervention period the proportion of infants fed breast milk had reduced but was still slightly more than those fed infant formula. This trend continued and by 4 months CA the majority of infants were fed a formula.

Figure 4.2: Proportion of Breast Milk in Infants' Diet



*Compliance with Trial Intervention*

At the end of the intervention period, mothers estimated how many capsules they had missed taking (if breast feeding their infants) and the number of formula feeds their infant received that were not trial formula (if formula feeding). There was no difference in the reported number of capsules missed between the two groups ($p = 0.36$). Of the 61 mothers in the control group, 54 initiated (88%) initiated breast milk feeding and 36 / 61 (59%) reported never missing any capsules during the intervention period. In the treatment group, 49 of the 60 (82%) mothers initiated breast milk feeding and 36 of these women (60%) reported never missing any capsules. Only three extreme non-compliers were identified; two mothers from the control group who estimated not taking between 90 to 100 capsules and one mother from the treatment group who estimated not taking approximately 80 capsules (equivalent to not complying with the protocol for 14 to 17 days). The two non-compliant mothers in the control did not report experiencing any side effects of consuming the capsules. However, the non-compliant mother from the treatment group reported burping and vomiting after consuming the capsules, which may have contributed to her failure to take the capsules according to trial protocol.

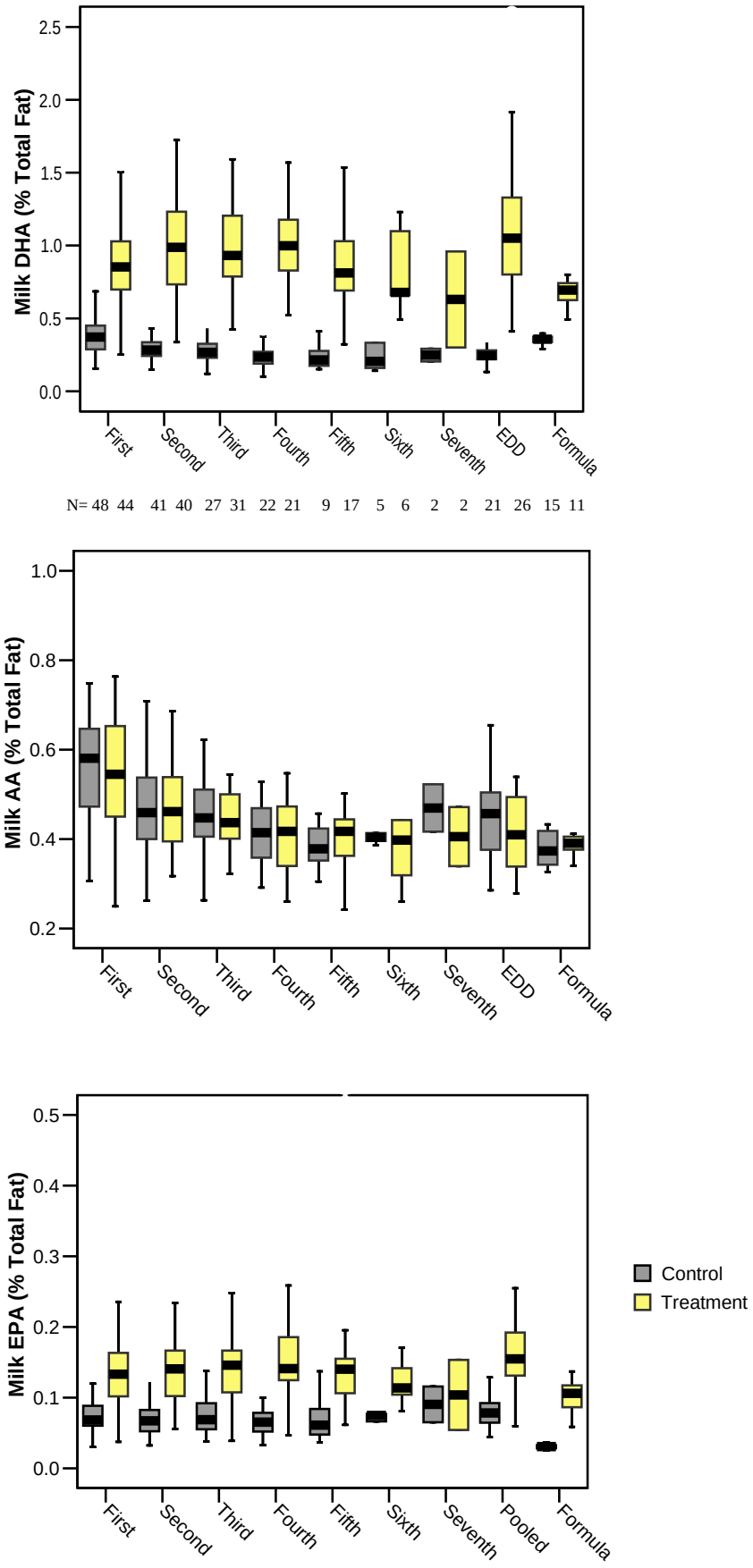
Breast milk was collected at least once after the commencement of the intervention from 48 / 61 (79%) mothers in the control and 44 / 60 (73%) in the treatment group. At the end of the intervention period 34 / 61 (56%) mothers from the control group and 27 / 60 (45%) mothers from the treatment group were continuing to breastfeed. Seven-day pooled consecutive breast milk samples were collected at the end of the intervention period from 21 / 61 (34%) mothers in the control and 26 / 60 (43%) in the treatment group.

The DHA concentration of breast milk samples collected fortnightly during the intervention period varied between 0.1 to 1.1% (of total fat) in the control group and 0.3 to 2.6% in the treatment group. Breast milk of mothers taking the treatment capsules was significantly higher in DHA compared with mothers

taking the control capsules (% total fatty acids; treatment 1.0 ± 0.4 n=187, control 0.3 ± 0.1 n=175, p<0.0005). Figure 4.3 shows selected LCPUFA concentrations of the fortnightly samples and pooled 7-day breast milk sample collected during the intervention period. In this figure, the horizontal bold line represents the median LCPUFA value, edges of the box represent the interquartile range and whiskers represent the highest and lowest values. Breast milk EPA content was marginally higher in the treatment group compared with the control group (% total fatty acids; treatment 0.22 ± 0.07, control 0.19 ± 0.04, p<0.0005). Breast milk AA was not significantly different between the groups (% total fatty acids; treatment 0.47 ± 0.13, control 0.48 ± 0.12, p= 0.56).

At EDD, a total of 61 / 74 (82%) infants in the control and 56 / 70 (80%) in the treatment groups had received some formula. Whilst hospitalised, infants were fed formula prepared by nursing staff according to the trial protocol. Parents prepared the formula in the time from discharge until the end of the intervention period. In this time, parents estimated feeding their infants an alternative formula on 0 to 24 occasions (both groups). Formula DHA content (Figure 4.3) was significantly higher in the treatment group compared with the control group (DHA as % of total fatty acids (mean ± SD), treatment group 0.70 ± 0.16, control group 0.35 ± 0.03, p<0.0005).

Figure 4.3: Breast Milk and Formula Fatty Acid Content of Control and Treatment Groups




**Samples are consecutive fortnightly breast milk, 7-day pooled EDD breast milk and formula**

*Success of the Intervention*

At the end of the intervention period blood sample collection was attempted from all infants that attended the appointment, except for one mother of twins (treatment group) who declined to have blood taken. Insufficient sample and technical problems resulted in a loss of erythrocytes for analysis in three control (3 / 74 (4%)) and one treatment group (1 / 70 (2%)) specimens. This resulted in erythrocyte membrane phospholipid fatty acid analysis of 68 / 74 (92%) of the control group and 63 / 70 (90%) of the treatment group infants.

The percentage of erythrocyte membrane phospholipid ALA was below the limit of detection in 11 (15%) control and 8 (12%) treatment samples. For statistical analyses, half the assay limit of detection (0.025% of total fat) was substituted as the concentration of ALA for these samples. Compared with the control group, erythrocyte membrane phospholipid DHA was significantly higher in the treatment group ($p<0.0005$) (Table 4.4). Numerous other differences in erythrocyte membrane phospholipids fatty acids were present between the control and treatment groups; the treatment group had significantly lower erythrocyte LA ($p=0.02$), AA ($p<0.0005$) and total n-6 PUFA ($p<0.0005$). Higher EPA ($p<0.0005$) and total n-3 PUFA ($p<0.0005$) were found in the treatment compared with control group. There was no significant difference in erythrocyte phospholipid ALA or docosapentaenoic acid (DPA) between the control and treatment groups ($p = 0.18$ and $p = 0.52$ respectively).

Table 4.4: Infant Erythrocyte Membrane Phospholipid Fatty Acid (%total) at the End of the Intervention Period

| | Control Group n = 74 | Treatment Group n = 70 |
|---|---|---|
| Number of samples assessed for erythrocyte phospholipid fatty acids | n = 68 (92%) | n = 63 (90%) |
| Total Saturates | 41.5 ± 1.3 | 42.2 ± 2.7 |
| Total Monounsaturates | 18.1 ± 1.6 | 17.9 ± 1.6 |
| Linoleic Acid | 8.8 ± 0.9 | 8.3 ± 1.2* |
| Arachidonic Acid | 16.0 ± 1.1 | 14.9 ± 1.3** |
| Total n-6 PUFA | 32.0 ± 1.2 | 29.6 ± 2.5** |
| Alpha – Linolenic Acid | 0.07 ± 0.03 | 0.08 ± 0.04 |
| Eicospentaenoic Acid | 0.3 ± 0.1 | 0.5 ± 0.3** |
| Docosapentaenoic Acid | 1.5 ± 0.4 | 1.4 ± 0.4 |
| Docosahexaenoic Acid | 5.2 ± 0.7 | 6.8 ± 1.2** |
| Total n-3 PUFA | 7.1 ± 0.9 | 8.8 ± 1.7** |

Values reported as mean ± SD

Data are significantly different (*$p < 0.02$, **$p<0.0005$)

*Masking of Treatment*

At the end of the intervention period, mothers were asked which treatment they believed they and their infant were randomised to receive. The choices offered were; control, treatment or don't know (Table 4.5). The pattern of response to each category were different between the control and treatment groups, with mothers in the control group selecting evenly between control, treatment or don't know and 76% of mothers of infants in the treatment group correctly choosing their allocation to the treatment group.

Table 4.5: Maternal Guess of Treatment Allocation

| | Control Group n = 74 | Treatment Group n = 70 |
|---|---|---|
| Number of Responses | 72 (97) | 67 (96) |
| Treatment Group (%) | 21 (28) | 52 (74) |
| Control Group (%) | 24 (32) | 4 (6) |
| Don't know (%) | 27 (36) | 11 (16) |

Data expressed as incidence and percentage in (parentheses)

*Primary Visual Outcome: Sweep VEP Acuity at 2 and 4 Months CA*

As discussed in Chapter 2, the spatial frequency of the stimulus was improved to reflect the finer acuity of 4-month-old infants. This adjustment was finalised after the commencement of the trial. The first sixteen infants from control group and fifteen infants from the treatment group were tested with the lower spatial frequency at 4 months CA. The acuity data collected from these infants at 4 months CA has not been included, as the stimulus is considered unsuitable for testing older infants (refer to Chapter 2, page 70). The infants excluded at 4 months CA did not differ in gender, birth weight, gestational age or randomisation group from those tested with the appropriate stimulus ($p = 0.5$, $p = 0.22$, $p = 0.92$ and 0.75, respectively). However, compared with infants tested using the appropriate stimulus, the excluded infants had a higher proportion of singletons (26 / 31 (84%) compared with 70 / 96 (73%), $p = 0.02$) and infants born AGA (26 / 31 (84%) compared with 60 / 96 (63%), $p = 0.02$).

Recording of sweep VEP data was unsuccessful in 10 infants at 2 months CA (5 control, 5 treatment infants) and 1 infant at 4 months CA (control group). At 2 months CA, one infant was asleep and could not be aroused for testing (treatment group) all other unsuccessful tests had VEP recordings that did not meet the SNR ≥3 criteria required for an acuity extrapolation. No significant differences between the control and treatment groups were found in the channel from which acuity was collected at 2 months CA ($p = 0.92$) or at 4 months CA ($p = 0.50$). There were also no significant differences in the total number of sweeps recorded or the number used in a vector averaged acuity estimate between the control and treatment groups (number of sweeps collected; at 2 months CA, control group (mean $\pm$ SD) $11 \pm 2$, treatment group $11 \pm 2$, $p = 0.30$; at 4 months CA, control group $11 \pm 2$, treatment group $11 \pm 2$, $p = 0.4$; number of sweeps used to generate a vector averaged acuity at 2 months CA, control group $5 \pm 2$, treatment group $4 \pm 2$, $p = 0.52$; at 4 months CA, control group $5 \pm 2$, treatment group $5 \pm 2$, $p = 0.83$). The infants' best acuity performance was derived from a single sweep in eighteen infants from each group at 2 months of age and 10 infants from the control group and 13 infants from the treatment group

at 4 months of age. The proportion of acuity extrapolations from a single sweep was not significantly different between the control and treatment groups at 2 or 4 months CA ($p = 0.7$ and $p = 0.4$, respectively). Overall, the recording conditions did not differ between the control and treatment groups at 2 or at 4 months CA.

The acuity data were normally distributed and ranged from 1.2 to 11.9 cpd in the control group and 2.0 to 12.0 cpd in the treatment group at 2 months CA. At 4 months CA, acuities ranged from 3.1 to 16.4 cpd in the control group and 4.0 to 21.9 cpd in the treatment group.

Table 4.6: Intention-to-treat Comparisons of Sweep VEP Acuity (in cpd)

| | Assessment Age | Control Group n = 74 | Treatment Group n = 69 |
|---|---|---|---|
| Unadjusted Data | 2 months CA | n = 61 (82%) $5.6 \pm 2.4$ | n = 54 (78%) $5.6 \pm 2.4$ |
| | 4 months CA | n = 51 (69%) $8.2 \pm 1.8$ | n = 44 (64%) $9.6 \pm 3.7$ * |
| Covariate Adjusted Data | 2 months CA | $5.5 \pm 3.1$ | $5.1 \pm 3.6$ |
| | 4 months CA | $8.2 \pm 2.9$ | $9.7 \pm 2.7$ * |

Acuity reported as mean $\pm$ SD, *$p<0.05$

A highly significant maturation of acuity was found between 2 and 4 months CA in both groups ($p<0.0005$ for both groups). No significant differences in acuity were found between the control and treatment groups at 2 months CA ($p = 0.96$), (Table 4.6). However, at 4 months CA acuity was significantly higher in the treatment group compared with the control group ($p = 0.025$).

A weak but significant negative correlation between birth order (singleton, first born twin and second born twin) and acuity, and consumption of alcohol during pregnancy and acuity occurred at 2 months

CA (r = -0.196 p = 0.036 and r = -0.197 p = 0.035, respectively). No significant differences in acuity were found between the control and treatment groups after covariate adjustment for birth order, alcohol consumption, birth size and gender at 2 months CA (p = 0.62). No significant correlations occurred between any of the pre-randomisation variables and acuity at 4 months CA. After covariate adjustment for birth size and gender, acuity at 4 months CA remained significantly higher in the treatment group than in the control group (p = 0.017).

*Sweep VEP Acuity; Gender and Birth Weight Subgroup Comparisons*

Acuities of infants were separated into gender (Table 4.7) and birth weight (Table 4.8) subgroups for further analysis. At 2 months CA, no differences in acuity were found between the control and treatment infants in the subgroup of boys (p = 0.3), girls (p = 0.4), infants born <1250g (p = 0.8) or infants born ≥1250g (p = 0.8). No change to these outcomes were found after adjusting for birth weight in gender subgroup comparisons (p = 0.4 for both male and female subgroups), or after adjusting for gender in birth weight subgroups (<1250 subgroup p = 0.5, ≥1250g subgroup p = 0.8).

At 4 months CA there was a general trend for the treatment group to exhibit higher acuity than the control group. This was not significant in the subgroup of girls (p = 0.3) or infants born <1250 g (p = 0.2), but neared significance in the infants born ≥1250g (p = 0.07) and was significant in the boys subgroup (p = 0.03). After adjustment for birth weight in male and female subgroups, girls in the treatment group were not different from the control group (p = 0.34) and boys in the treatment group were significantly higher than control group (p = 0.02). Acuity of the treatment group was not significantly different from the control group in the subgroup of infants born <1250 g after adjusting for gender (p = 0.09). However, after adjustment for gender in the ≥1250g subgroup, the treatment group had a significantly higher acuity than the control group (p = 0.05).

Table 4.7: Acuity (cpd) at 2 and 4 Months CA by Gender Subgroup

| | Assess-ment Age | Male Subgroup n = 70 | | Female Subgroup n = 73 | |
|---|---|---|---|---|---|
| | | Control n = 35 | Treatment n = 35 | Control n = 39 | Treatment n = 34 |
| Un-adjusted Data | 2 months CA | n = 26 (74%) 5.3 ± 2.1 | n = 26 (74%) 6.0 ± 2.5 | n = 35 (90%) 5.9 ± 2.6 | n = 28 (82%) 5.3 ± 2.3 |
| | 4 months CA | n = 24 (69%) 8.0 ± 2.0 | n = 22 (63%) 10.1 ± 3.7 * | n = 27 (69%) 8.4 ± 1.5 | n = 22 (65%) 9.2 ± 3.7 |
| Covariate Adjusted Data | 2 months CA | 5.3 ± 2.3 | 6.0 ± 2.3 | 5.9 ± 2.5 | 5.3 ± 2.5 |
| | 4 months CA | 8.0 ± 3.0 | 10.1 ± 3.0 * | 8.4 ± 2.8 | 9.2 ± 2.7 |

Acuity reported as mean ± SD, *significantly higher in treatment group p<0.05

Table 4.8: Acuity (cpd) at 2 and 4 Months CA by Birth Weight subgroups

| | Assess-ment Age | <1250g Subgroup n = 73 | | ≥1250g Subgroup n = 70 | |
|---|---|---|---|---|---|
| | | Control n = 38 | Treatment n = 35 | Control n = 36 | Treatment n = 34 |
| Un-adjusted Data | 2 months CA | n = 30 (79%) 5.5 ± 2.6 | n = 25 (71%) 5.7 ± 2.3 | n = 31 (86%) 5.7 ± 2.2 | n = 29 (85%) 5.6 ± 2.4 |
| | 4 months CA | n = 26 (68%) 8.2 ± 1.9 | n = 22 (63%) 9.1 ± 2.8 | n = 25 (69%) 8.3 ± 1.7 | n = 22 (65%) 10.2 ± 4.4 |
| Covariate Adjusted Data | 2 months CA | 5.3 ± 2.6 | 5.7 ± 2.5 | 5.7 ± 2.4 | 5.6 ± 2.4 |
| | 4 months CA | 7.9 ± 2.5 | 9.1 ± 2.3 | 8.2 ± 3.4 | 10.1 ± 3.3 * |

Acuity reported as mean ± SD, * significantly higher in treatment group p<0.05

*Exploratory Acuity Analysis of Infants Primarily Fed Breast Milk*

The proportion of infants fed both breast milk and formula during the intervention period was large (Figure 4.2). At the end of the intervention period approximately 40% of infants were predominantly fed

breast milk (≥80% of diet). Exploratory acuity analyses were performed on this subgroup of infants (Table 4.9). No significant differences in acuity were found at 2 months. At 4 months CA acuity was higher in the treatment compared with the control group but this did not reach significance. The power of this subgroup analysis is limited by the small sample size (*post hoc* power calculation is approximately 70%).

Table 4.9: Sweep VEP Acuity (cpd) at 2 and 4 Months CA of Infants Primarily Fed Breast Milk at the End of the Intervention Period

| | Control Group n = 74 | Treatment Group n = 69 | Statistical significance |
|---|---|---|---|
| 2 months CA | n = 19 (26%) 5.6 ± 2.0 | n = 24 (35%) 6.3 ± 2.4 | p = 0.29 |
| 4 months CA | n = 16 (22%) 8.4 ± 2.0 | n = 21 (30%) 10.1 ± 3.8 | p = 0.08 |

Acuity reported as mean ± SD

*Secondary Visual Outcome: VEP Latency at 2 and 4 months CA*

VEP latency data were successfully collected from all infants that attended follow up appointments, except for one 2 month-old girl that could not be aroused from sleep (treatment group). Two 4-month-old girls were tested with only the 69 min of arc stimulus (1 control and 1 treatment group). The latency data were normally distributed and at 2 months CA ranged from 132 to 238 and 135 to 278 milliseconds in the control group and 148 to 232 and 132 to 245 milliseconds in the treatment group (to the 96 and 69 minutes of arc stimulus, respectively). At 4 months CA, latencies were normally distributed and ranged from 107 to 217 and 108 to 203 milliseconds in the control group and 103 to 197 and 107 to 218 milliseconds in the treatment group (to the 69 and 48 minutes of arc stimulus, respectively). Compared with the larger stimulus, latencies were increased with the smaller stimulus in both groups of infants at 2

and 4 months CA. Maturation of the latency responses was observed between 2 and 4 months CA ($p<0.0005$) and was demonstrated in all infants.

Table 4.10: Comparisons of VEP Latency (in milliseconds) at 2 and 4 months CA

| | | Control Group n = 74 | Treatment Group n = 69 | P |
|---|---|---|---|---|
| Unadjusted Data | 2 months CA; | n = 66 (92%) | n = 58 (88%) | |
| | 96 min of arc | 188 ± 27 | 182 ± 24 | 0.2 |
| | 69 min of arc | 200 ± 29 | 193 ± 27 | 0.2 |
| | 4 months CA; | n = 67 (93%) | n = 58 (84%) | |
| | 69 min of arc | 131 ± 21 | 129 ± 20 | 0.8 |
| | 48 min of arc | 138 ± 23 | 135 ± 23 | 0.5 |
| Covariate Adjusted Data | 2 months CA; | | | |
| | 96 min of arc | 189 ± 25 | 182 ± 25 | 0.1 |
| | 69 min of arc | 200 ± 27 | 193 ± 27 | 0.1 |
| | 4 months CA; | | | |
| | 69 min of arc | 131 ± 20 | 129 ± 20 | 0.2 |
| | 48 min of arc | 138 ± 22 | 134 ± 22 | 0.3 |

Latency reported as mean ± SD

Although there was a trend for shorter latencies in the treatment group, there were never any significant differences between the control and treatment groups at 2 or 4 months CA (Table 4.10). A proportion of the variation in latency measurements is attributed to head circumference with larger head circumferences associated with longer latencies [140]. Covariate adjusted data using both gender and birth weight (shown in Table 4.10), or gender and head circumference (data not shown) resulted in no significant differences between the control and treatment groups. There were no differences in latencies between the control and treatment group in any gender (Table 4.11) or birth weight subgroups (Tables 4.12).

Table 4.11: Latency (milliseconds) at 2 and 4 Months CA by Gender subgroups

| | Male Subgroup n = 70 | | | Female Subgroup n = 73 | | |
|---|---|---|---|---|---|---|
| | Control n = 35 | Treatment n = 35 | P | Control n = 39 | Treatment n = 34 | p |
| 2 months CA; | n = 30 (86%) | n = 28 (80%) | | n = 36 (92%) | n = 30 (85%) | |
| 96 min of arc | 194 ± 28 | 185 ± 28 | 0.2 | 184 ± 22 | 179 ± 22 | 0.4 |
| 69 min of arc | 206 ± 28 | 200 ± 28 | 0.4 | 196 ± 25 | 186 ± 25 | 0.1 |
| 4 months CA; | n = 31 (89%) | n = 27 (77%) | | n = 36 (95%) | n = 31 (91%) | |
| 69 min of arc | 137 ± 22 | 127 ± 22 | 0.1 | 126 ± 17 | 130 ± 17 | 0.3 |
| 48 min of arc | 141 ± 25 | 133 ± 25 | 0.2 | 135 ± 20 | 136 ± 20 | 0.9 |

Latency reported as mean ± SD

Table 4.12: Latency (milliseconds) at 2 and 4 Months CA by Birth Weight Subgroups

| | <1250g Subgroup n = 67 | | | ≥1250g Subgroup n = 76 | | |
|---|---|---|---|---|---|---|
| | Control n = 34 | Treatment n = 33 | p | Control n = 40 | Treatment n = 36 | p |
| 2 months CA; | n = 31(91%) | n = 28(82%) | | n = 35 (92%) | n = 30(88%) | |
| 96 min of arc | 200± 25 | 191 ± 24 | 0.2 | 181 ± 25 | 175 ± 25 | 0.3 |
| 69 min of arc | 212 ± 26 | 203 ± 25 | 0.2 | 192 ± 27 | 185 ± 27 | 0.3 |
| 4 months CA; | n = 33(97%) | n = 28(85%) | | n = 34 (92%) | n = 30 (88%) | |
| 69 min of arc | 140 ± 22 | 131 ± 21 | 0.1 | 124 ± 17 | 127 ± 17 | 0.5 |
| 48min of arc | 149 ± 25 | 138 ± 24 | 0.09 | 129 ± 20 | 131 ± 30 | 0.6 |

Latency reported as mean ± SD

## 4.5 Discussion

This unique RCT has demonstrated that preterm infants fed milk containing DHA at levels designed to meet *in utero* accretion rate have higher visual acuity at 4 months CA compared with infants who received milk containing approximately 0.3% DHA. The improvement in acuity is modest 1.4 cpd, or 20 / 75 compared with 20 / 60 Snellen equivalents. Enhanced acuity is consistent with two other n-3 LCPUFA trials in preterm infants that have measured acuity using a VEP technique [5,60]. Birch *et al* showed that infants fed formula containing 1% of total fat as n-3 LCPUFA (0.4% DHA and 0.5% EPA) to 4 months CA had an improvement of approximately 5 cpd. O'Connor *et al* reported that infants fed formula containing 0.25% DHA to term and 0.16% DHA thereafter exhibited a 3 cpd improvement in VEP acuity at 6 months CA. Differences in feeding regime between my trial and those of Birch *et al* and O'Connor *et al* may have contributed to the larger improvements in acuity observed in other studies. Both Birch *et al* and O'Connor *et al* compared infants fed some LCPUFA in formula with infants fed formula containing no LCPUFA. Furthermore, both trials continued the feeding regime to the time of VEP testing. By comparison, my trial compared a low dose of DHA with a high dose and the intervention period was only until the infants reached their EDD. Differences in the VEP technique may have also contributed to size of improvement in acuity. In both my trial and O'Connor *et al*, a sweep VEP grating was used to assess acuity, whereas Birch *et al* used a reversing checkerboard. As maturation of visual responses differ with the type of stimulus [58,135], the reversing checkerboard may have contributed to the large improvement seen in the Birch *et al* trial. Irrespective of these differences, a significant change in acuity was found in my trial, demonstrating that there is room to improve acuity of preterm infants by increasing dietary DHA. Interestingly, no effect of the treatment was found at 2 months CA and the acuities of the control and treatment groups were remarkably similar at this time. The absence of an effect on VEP acuity at one assessment, followed by a subsequent improvement was reported in the trial by O'Connor *et al* [60]. As with other aspects of development, this may indicate

that a supply of DHA early in the neonatal period may be necessary to support optimal acuity development at later ages.

The sweep VEP technique used for assessing acuity in this trial is supported with data from similar infants. The control group infants from my trial and treatment group infants reported by O'Connor *et al* received milk containing similar levels of n-3 LCPUFA until reaching their EDD. At 4 months CA the acuity of these infants was very similar, even with small differences in trial design (such as duration of supplementation) or with the VEP technique (such as square wave gratings and reversal rates). This demonstrates that the VEP technique used in this trial is comparable with the work of others and defends any criticisms for improving the stimulus to accommodate higher acuities of 4-month-old infants. Furthermore, the similarity in acuity shows remarkable external validity within the preterm population.

In secondary analyses, the comparisons of acuity in gender and birth weight subgroups showed that infants from the treatment group had consistently higher acuity than the control group infants; however this reached significance only in boys, or after adjusting for gender in the subgroup of infants born ≥1250g. Previous trials in preterm infants have not described gender subgroup analyses, consequently it is not known if the improvements in acuity described in other trials have been more pronounced in boys than girls. In term infants, male gender has been negatively associated with visual acuity when assessed with transient checkerboards [66]. Other factors such as birth weight, ROP or exposure to cigarette smoke may also influence visual acuity [66]. No differences in ROP, exposure to cigarette smoke and birth weight were observed between the control and treatment groups in either gender or birth weight subgroups. Although boys receiving the high dose DHA show more rapid maturation of acuity, any mechanisms for such an effect remain unclear and the possibility that the significant effect could be due to random error must be considered.

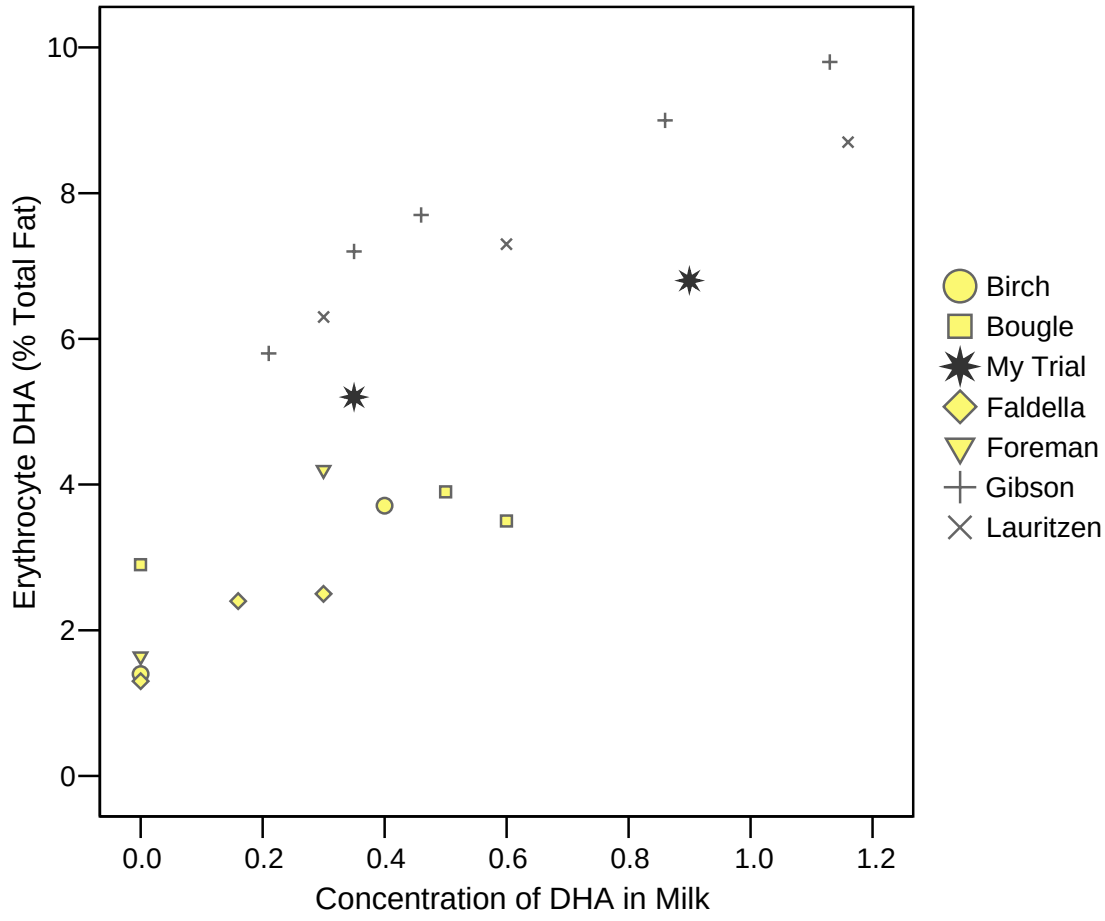
In the present trial, the modest improvement in acuity at 4 months CA may have been affected by the concentration of DHA in formula, which was lower in the treatment group than the desired 1% of total fat. Formula milk took slightly longer time to prepare with the addition of the oil and it is not known if less care or time taken to prepare formula milk may have contributed to the lower DHA content. Training of staff and regular feedback might have improved the DHA content of the formula. In future studies, the DHA concentration of infant formula may be better controlled by using a formula manufactured to a specified DHA concentration. Exploratory comparisons of acuity between the treatment and control groups infants primarily fed breast milk throughout the intervention period showed an improvement of 1.7 cpd in the treatment group. However, the number of infants is small and the outcome of this analysis is not significant. It is tempting to speculate that treatment group infants might have achieved even higher acuities if the DHA content of infant formula had consistently reached the 1% of total fat.

Feedback from mothers attending the infants EDD appointment indicated that there was poor masking within the treatment group. In anticipation of the difficulty of disguising fish oil capsules, the trial was designed with two treatment and two control groups. Data from these groups were not combined until all data was collected, entered and cleaned. In addition, this particular statistic was not performed until all primary efficacy and safety outcomes were analysed. Investigators remained blinded to this finding throughout data collection and analysis of primary outcomes; as a result the trial quality remains high.

Supplementation of lactating mothers with fish oil resulted in wide variations in breast milk DHA. This probably reflects natural variation in breast milk DHA with fish oil supplementation as the median remained steady at 1% and the variance is consistent with previous studies in lactating women [42]. The concentration of DHA in milk for treatment infants was successful in achieving significantly higher erythrocyte phospholipid DHA compared with the control group. It is noted that the DHA in red cell

membranes of preterm infants fed milk containing 1% DHA is similar to term infants fed breast milk near 0.3% DHA[42,43](breast fed term infants are shown as '+' and 'x' symbols in Figure 4.4). Compared with term infants, the lower erythrocyte DHA in preterm infants is not likely to be a result of poor compliance as maternal report and breast milk DHA were excellent. It cannot be explained as differences in absorption from infant formula or breast milk as DHA is readily absorbed from both types of milk [37]. Emerging literature indicates that current feeding practices may not be meeting all the energy requirements for preterm infants [146]. Therefore it is possible that circulating LCPUFA may have been used in fat oxidation for energy. In term infants, higher erythrocyte DHA is achieved with higher dietary DHA and this is associated with increased acuity [43]. This indicates that in these preterm infants, there may be further capacity to improve DHA status through dietary means.

Figure 4.4: Increase in Erythrocyte DHA in Response to Milk DHA



As expected, EPA concentration was mildly increased in treatment group breast milk and formula as a result of the low level of EPA in the fish oil supplement. The increased milk EPA was sufficient to elevate the infants' erythrocyte phospholipid EPA from 0.3 to 0.5% of total fatty acids in the control compared with the treatment groups, respectively. The size of the increase in EPA is relatively minor compared with the fluctuations between the groups in this trial (Figure 4.2), in other trials [36,43] and from observations in the wider population [31,34,35,42]. The control group infants demonstrated significantly higher erythrocyte phospholipid AA compared with the treatment infants. This effect was anticipated, as increased dietary DHA in formula or breast milk tends to displace some AA from erythrocyte membrane phospholipids [36,105]. Nevertheless erythrocyte AA was similar to infants exclusively breast fed for 3 months [31,42] and remained higher than preterm infants fed formula containing no LCPUFA [32,33,44,45,79].

A secondary aim of this trial was to describe latencies of preterm infants to checkerboard stimuli after n-3 LCPUFA supplementation. All infants demonstrated the typical shortening of latency responses with larger stimulus and with age. At 2 months CA the latencies of the infants in this trial are nearly 20 milliseconds longer than that observed in Chapter 2, which may be due to the larger number of preterm infants and severity of their illness compared with the observational cohort. By 4 months CA there is little difference in latencies between the pilot study and the RCT infants, and the latencies are consistent with reports using similar techniques [138,139]. The absence of differences at 4 months CA indicates that similar levels of neural myelination may have been reached in the control and treatment groups.

No other n-3 LCPUFA intervention trials in preterm infans have assessed latency responses to checkerboard stimuli, however, three other n-3 LCPUFA trials have reported latencies to flash VEP stimuli [45,61,79]. The findings of the present trial are consistent with two of these reports [45,61]. In these trials, no significant differences in latencies were found after feeding formulas containing 0.3 – 0.6% n-3 LCPUFA [45,61]. Faldella *et al* reported enhanced N4 and P4 latencies after feeding formula containing

0.3% DHA and 0.4% AA until 3 months CA compared with infants that received formula with no LCPUFA [79]. However, this trial was not performed in an intention-to-treat manner and although *post hoc* power calculations are close to 80% power, the small sample sizes reduce the validity of the observation (n = 12 and n = 17 in control and treatment groups, respectively).

The clinical relevance of the improvement in acuity after the high DHA intervention in the present trial is not clear as most infants from both groups achieved an acuity considered to be normal. Evidence from infants born at term show that stereo-acuity and letter matching in childhood is related to poorer n-3 LCPUFA status in infancy [147] and DHA status at birth has also been related to development of attention [148]. These trials suggest that the effects of early LCPUFA nutrition may persist over time. Similar trials, where follow up of visual development in childhood after LCPUFA supplementation have not been reported for preterm infants. Just as evidence from this trial suggests current practices in n-3 LCPUFA nutrition may be inadequate to support optimal visual development, other aspects of development of preterm infants may also be vulnerable to DHA nutrition. Research interest is now moving towards investigating whether supplementing preterm infants with n-3 LCPUFA enhance other areas of infant development such as intelligence quotient (IQ), attention or behaviour. Further assessment of infants at various ages and in multiple areas of development are necessary to confirm the optimal level of dietary DHA necessary to support all facets of development. Whether other aspects of infant development can also be improved though this intervention is beyond the scope of this thesis and are the subject of future work.

Irrespective of future studies, my trial has demonstrated the feasibility of increasing DHA status of preterm infants. Most importantly, the DHA intervention was inclusive of all feeding regimes and was easily and readily accepted by lactating mothers. This trial was been performed in infants from a wide range of gestational ages in which many of the typical diseases of prematurity were present, offering

excellent generalisability to preterm infants. My trial indicates that the amount of DHA fed to preterm infants in breast milk and formula may not be sufficient for optimal visual development.

# CHAPTER 5: GROWTH AND SAFETY OF FEEDING PRETERM INFANTS 1% DHA IN MILK; OUTCOMES OF A RCT

## 5.1 Abstract

The safety of feeding preterm infants milk containing a high concentration of DHA (1% of total fat, treatment group) compared with current feeding practices (approximately 0.3% DHA, control group) was assessed by comparing weight, length, head circumference and other clinical measures.

The control and treatment groups did not differ in weight, length or head circumference (HC) at the end of the intervention period (weight (mean ± SD) control group 3133 ± 478, treatment group 3175 ± 453, p = 0.60; length, control group 48.1 ± 3.0, treatment group 48.5 ± 2.3, p = 0.39; HC, control group 35.1 ± 1.5, treatment group 35.1 ± 1.1, p = 0.82) or at 4 months corrected age (CA) (weight, control group 6205 ± 1023, treatment group 6282 ± 894, p = 0.67, length, control group 60.8 ± 3.4, treatment group 61.3 ±2.6, p = 0.31; HC, control group 41.9 ± 1.6, treatment group 41.8 ± 1.2, p = 0.73). No differences in weight, length or HC were found between control and treatment groups according to birth weight subgroups (<1250 g or ≥1250 g). In gender subgroup analyses, growth did not differ in boys, but girls in the treatment group were heavier (control group 5693 ± 815, treatment group 6139 ± 873, p = 0.032) and longer (control group 59.3 ± 2.8, treatment group 60.8 ± 2.6, p=0.025) than the control group at 4 months CA. The rate of increase in weight, length or HC and weight or length z-scores did not differ in intention-to-treat comparisons of the control and treatment groups.

Clinical data collected from infants in this randomised controlled trial (RCT) were comparable with other Australian preterm infants. No differences were found between control and treatment groups in any of the intention-to-treat comparisons including; number of times feeds were interrupted, necrotising enterocolitis (NEC), respiratory outcomes, incidence or severity of retinopathy of prematurity (ROP),

intraventricular haemorrhage (IVH), periventricular leukomalacia (PVL), sepsis, length of stay, number of transfusions, surgery or rehospitalisation after discharge. A meta-analysis to assess the risk of LCPUFA in milk for preterm infants was performed using data from my trial and other published trials. The Relative Risk (RR) for NEC, bronchopulmonary dysplasia (BPD), ROP, IVH and sepsis revealed no significant change in risk with increased n-3 LCPUFA in milk. The low disease incidence limited the power of these analyses and further studies are needed to confirm these findings.

The RCT described in this chapter demonstrates that feeding infants milk containing 1% DHA until reaching EDD appears to be safe: high dose DHA supplementation of preterm infants does not alter the weight, length or head circumference to 4 months CA

## 5.2 Introduction

n-3 long-chain polyunsaturated fatty acid (LCPUFA) supplementation has been implicated as a factor related to poor growth of preterm infants [6,100,104,107]. There is continuing debate regarding the cause of growth deficits in some infants fed formula containing supplemental n-3 LCPUFA. Low levels of circulating arachidonic acid (AA) (as a result of increased eicosapentaenoic acid (EPA)) in preterm infants have been associated with poorer growth. This has led to the hypothesis that poor AA status may be causally related to growth outcomes [106]. This has resulted in recommendations to include AA in preterm formulas [149]. Since these early findings, no randomised controlled trial (RCT) has reported poorer growth in preterm infants fed fish oil enriched formula compared with infants that receive no LCPUFA in formula. However, poorer growth was reported in preterm infants given LCPUFA enriched formula from egg phospholipids containing both DHA and AA [100]. The disparity between trials makes implications for current practice confusing. Thus it was important to monitor growth of infants in my trial, where the level of dietary AA remained constant (at approximately 0.5% of total fat) and DHA was varied.

Many LCPUFA intervention trials also report morbidity outcomes of infants fed trial formulas. Although some trials have not been statistically powered to detect differences, these data provide a means to monitor the progress of infants given formulas containing LCPUFA and contribute to the growing body of knowledge regarding the safety and benefits of enriched feeds. Some benefits of LCPUFA supplementation have been demonstrated, with improvements in markers of immune function [123] and reductions in the number of infants with NEC [113]. In most cases, LCPUFA in formula has not been associated with any change in neonatal health outcomes or the incidence of diseases of prematurity.

It was hypothesised that increasing the level of DHA in milk for preterm infants would not alter growth outcome or the health of preterm infants. Weight, length and HC at 4 months CA formed the primary assessment of safety and clinical events comprised secondary outcome data.

## 5.3 Method

The method of collecting anthropometric measurements and clinical definitions are described in Chapter 2. Measurements were recorded throughout the intervention period, at discharge, at the infants EDD, at 2 and at 4 months CA according to the research plan and methods previously described in Chapter 3.

### *Statistical Analyses*

For all analyses, intention-to-treat group comparisons were performed using SPSS for Windows (Version 11.0) with probability <0.05 considered significant. Continuous normally distributed variables were compared by independent sample T tests and non-parametric continuous variables by Mann-Whitney U tests. Categorical variables were compared by Chi-squared tests, where Fischer's exact probability test was applied when comparing variables of low incidences.

The primary safety outcome of weight, length and HC at 4 months CA was first analysed unadjusted. Between-groups analysis of covariance was then determined to adjust for birth size and gender. Subgroup analyses at 4 months CA were limited to the stratification subgroups; gender and birth weight (<1250 g and ≥1250) and restricted to only the primary safety outcome at 4 months CA. The weight, length and HC of the control and treatment groups were also compared at the end of the intervention period, firstly unadjusted then after adjustment for birth size and gender.

Secondary growth analyses included comparisons of growth velocity and z-scores between the control and treatment groups. Growth velocity was separated into the intervention period (from enrolment to end of the intervention at infants EDD) and follow up period (from EDD through to 4 months CA). The raw weight and length data collected at the end of the intervention period and at the 4 month follow up were converted to z-scores using the data and software at Centre for Disease Control (CDC) website (http://www.cdc.gov, accessed July 2005). To convert raw data to z-scores, the mean of a gender and age matched reference population is taken away from the anthropometric measurement of each infant and divided by the standard deviation of the reference population. As the reference data are standardised for gender, the z-score from boys and girls can be combined to enhance statistical power of the analyses.

The CDC publishes the most recent infant growth modelling data, according to current medical and social practices. These data do not include infants born <1500 g and consequently might not reflect the growth of preterm infants. However, in conducting these analyses, it was deemed appropriate to compare the growth of preterm infants from my study with their peers.

Clinical diseases associated with prematurity were monitored throughout the trial. The low incidence of some of these diseases limits the power of comparisons. Many previous LCPUFA trials in preterm infants have also reported incidence of clinical events. Data from these trials were combined with my trial data in a meta-analysis. The analysis was separated into 2 subgroups, depending on the treatment. The first subgroup analysis compared infants that received no LCPUFA in formula with those that received formula with LCPUFA. The data from my trial comprised a second subgroup as it compared infants fed breast milk and formula containing a low dose of DHA with infants fed a high dose of DHA. The analysis was performed using software available from the Cochrane Collaboration (www.cochrane-collaboration.org, downloaded March 2006). The analysis was limited to selected

clinical diseases; ROP, proven NEC, bronchopulmonary dysplasia ((BPD) (defined by requirement for oxygen after 36 weeks post menstrual age), IVH and sepsis (confirmed by blood culture). Only trials that applied the same clinical definition or symptoms as described in Chapter 3 were included in the analysis. For ROP and IVH, severity of disease was considered important and where data were available separate analyses were performed on incidence of severe ROP and severe IVH (grade ≥3). Trials not reporting grade or severity of disease were retained in separate analyses. The relative risk (or risk ratio (RR)) statistic was used to compare the risk of the clinical event in the treatment group compared with the control group. As no statistical heterogeneity between trials was identified, fixed effects analyses were performed.

## 5.4 Results of Primary Safety Outcome: Infant Growth Data

The following section describes the anthropometric data collected at EDD and 4 months CA. Data collected at enrolment, discharge and at 2 months CA can be found in Appendix A1.

Growth data were collected from 71 / 74 (96%) control group and 66 / 69 (96%) treatment group infants at EDD and 68 / 74 (92%) control group and 59 / 69 (86%) treatment group infants at 4 months CA (Figure 4.1, Chapter 4 for flow of participants through trial). Data were normally distributed. At 4 months CA, the weight of girls in the control group ranged from 4240 to 7510 g and the treatment group ranged from 4760 to 8290 g. For boys, weight varied from 4750 to 8730 g in the control group and 4560 to 8600 g in the treatment group.

No significant differences in any anthropometric measures were found in unadjusted intention-to-treat comparisons of control versus treatment infants at discharge, EDD, 2 and 4 months CA (Table 5.4.1 and Appendix A1). Covariate adjustment for birth weight and gender did not alter this result (Table 5.4.1). A small to moderate interaction effect of group and gender occurred for head circumference at

EDD (p = 0.004, effect size 6%) and weight at 4 months CA (p = 0.039, effect size 3%). At 4 months CA, interaction effects between group and gender approached significance for length (p = 0.07, effect size 3%), head circumference (p = 0.07, effect size 2.6%), mid upper arm circumference (p = 0.08, effect size 2.5%) and abdominal girth (p = 0.055, effect size 3%). The interaction effect indicates that there may be small differential effects of LCPUFA on growth of girls compared with boys.

Data collected at the primary endpoint of 4 months CA were separated into gender subgroups for further analysis. No differences were found in weight, length or HC between the subgroup of boys randomised to the control compared with the treatment group, either before or after adjusting for birth size (Table 5.4.2). At 4 months CA the weight, length and mid upper arm circumference (MUAC) of girls in the treatment group were significantly larger than the control group (p = 0.032, p = 0.025 and p = 0.014, respectively). After controlling for birth size, small but significant differences remained; girls in the treatment group had significantly larger weight, length and MUAC at 4 months CA (p = 0.052, p = 0.037 and p = 0.029, respectively) than the control group.

Table 5.4.1: Anthropometry of control and treatment groups at the end of the intervention period (EDD) and at 4 months CA

| | | Control group n = 74 | Treatment group n = 69 |
|---|---|---|---|
| Unadjusted Data | At EDD: | n = 71 (96%) | n = 66 (96%) |
| | Weight (g) | 3133 ± 478 | 3175 ± 453 |
| | Length (cm) | 48.1 ± 3.0 | 48.5 ± 2.3 |
| | HC (cm) | 35.1 ± 1.5 | 35.1 ± 1.1 |
| | At 4 months CA: | n = 68 (92%) | n = 59 (86%) |
| | Weight (g) | 6205 ± 1023 | 6282 ± 894 |
| | Length (cm) | 60.8 ± 3.4 | 61.3 ± 2.6 |
| | HC (cm) | 41.9 ± 1.6 | 41.8 ± 1.2 |
| | MUAC (cm) | 13.6 ± 1.3 | 13.9 ± 1.3 |
| | Abdominal Girth (cm) | 41.9 ± 2.8 | 42.0 ± 2.8 |
| Covariate Adjusted Data | At EDD: | | |
| | Weight (g) | 3137 ± 371 | 3187 ± 373 |
| | Length (cm) | 48.1 ± 1.7 | 48.5 ± 1.6 |
| | HC (cm) | 35.1 ± 0.8 | 35.2 ± 0.8 |
| | At 4 months CA: | | |
| | Weight (g) | 6229 ± 767 | 6310 ± 768 |
| | Length (cm) | 60.9 ± 2.5 | 61.4 ± 2.3 |
| | HC (cm) | 41.9 ± 0.8 | 41.9 ± 1.5 |
| | MUAC (cm) | 13.7 ± 0.8 | 13.9 ± 1.5 |
| | Abdominal Girth (cm) | 42.0 ± 2.5 | 42.0 ± 2.3 |

Results expressed as mean ± SD

Table 5.4.2: Anthropometry of the control and treatment infants at 4 months CA by gender subgroup

| | Boys n = 70 | | Girls n = 73 | |
|---|---|---|---|---|
| | Control Group n = 35 | Treatment group n = 35 | Control Group n = 39 | Treatment group n = 34 |
| Unadjusted Data: | n=31(89%) | n=27(77%) | n=37(95%) | n=32(94%) |
| Weight (g) | 6817 ± 910 | 6452 ± 904 | 5693± 815 | 6139± 873† |
| Length (cm) | 65.6 ± 3.2 | 62.0 ± 2.4 | 59.3 ± 2.8 | 60.8 ± 2.6† |
| HC (cm) | 42.8 ± 1.6 | 42.1 ± 1.3 | 41.1 ± 1.0 | 41.5 ± 1.0 |
| MUAC (cm) | 14.3 ± 1.2 | 13.9 ± 1.3 | 13.1 ± 1.1 | 13.9 ± 1.3† |
| Abdominal Girth (cm) | 45.5 ± 2.4 | 42.4 ± 2.9 | 40.6 ± 2.4 | 41.5 ± 2.7 |
| Covariate Adjusted Data: | | | | |
| Weight (g) | 6743 ± 828 | 6539 ± 782 | 5721 ± 760 | 6089 ± 774† |
| Length (cm) | 62.4 ± 2.5 | 62.2 ± 2.4 | 59.4 ± 2.4 | 60.6 ± 2.5† |
| HC (cm) | 42.7 ± 1.4 | 42.3 ± 1.4 | 41.1 ± 1.0 | 41.4 ± 1.0 |
| MUAC (cm) | 14.2 ± 1.3 | 14.0 ± 1.2 | 13.2 ± 1.0 | 14.0 ± 1.1† |
| Abdominal Girth (cm) | 43.4 ± 2.7 | 42.6 ± 2.5 | 40.6 ± 2.4 | 41.5 ± 2.5 |

Results expressed as mean ± SD

† Significantly different between control and treatment group for gender ($p < 0.05$)

There were no significant differences in weight, length, HC, MUAC or abdominal girth between control and treatment groups in both birth weight subgroups, either before or after adjustment for gender (Table 5.4.3). Significant interactions between group and gender occurred in the ≥1250 g subgroup of infants for all measurements (weight, $p = 0.004$ effect size 13%; length, $p = 0.012$ effect size 10%; head circumference, $p = 0.008$ effect size 11%; MUAC, $p = 0.002$ effect size 14%; abdominal girth, $p = 0.005$ effect size 12%).

Table 5.4.3: Anthropometry of control and treatment groups at 4 months CA by birth weight subgroups

| | <1250 g birth weight n = 67 | | ≥1250 g birth weight n = 76 | |
|---|---|---|---|---|
| | Control Group n = 34 | Treatment Group n = 33 | Control Group n = 40 | Treatment Group n = 36 |
| Unadjusted Data: | n =33 (97%) | n =28 (85%) | n = 35 (88%) | n = 31 (86%) |
| Weight (g) | 5810 ± 808 | 5859 ± 690 | 6578 ± 1074 | 6665 ± 893 |
| Length (cm) | 59.5 ± 3.0 | 59.9 ± 2.4 | 62.0 ± 3.4 | 62.6 ± 2.0 |
| HC (cm) | 41.4 ± 1.3 | 41.6 ± 1.2 | 42.3 ± 1.7 | 42.0 ± 1.2 |
| MUAC (cm) | 13.2 ± 1.2 | 13.4 ± 1.3 | 14.1 ± 1.2 | 14.9 ± 1.2 |
| Abdominal Girth (cm) | 41.2 ± 2.5 | 41.1 ± 2.5 | 42.6 ± 2.9 | 42.7 ± 2.9 |
| Covariate Adjusted Data: | | | | |
| Weight (g) | 5889 ± 744 | 5878 ± 718 | 6517 ± 862 | 6667 ± 860 |
| Length (cm) | 59.7 ± 2.8 | 60.0 ± 2.7 | 61.8 ± 2.4 | 62.7 ± 2.4 |
| HC (cm) | 41.5 ± 1.3 | 41.6 ± 1.2 | 42.2 ± 1.2 | 42.0 ± 1.2 |
| MUAC (cm) | 13.3 ± 1.2 | 13.4 ± 1.2 | 14.0 ± 1.1 | 14.3 ± 1.1 |
| Abdominal Girth (cm) | 41.4 ± 2.5 | 41.1 ± 2.4 | 42.4 ± 2.6 | 42.7 ± 2.6 |

Results expressed as mean ± SD

*Growth Velocity*

The growth rate varied from 18 to 45 g/day for weight, 0.6 to 1.5 cm/wk for length and 0.5 to 1.1 cm/wk for head circumference during the intervention period. During the follow up period the growth rate varied between 13 to 46 g/day, 0.4 to 1.0 cm/wk and 0.3 to 0.5 cm/wk for weight, length and HC, respectively. No significant differences were found in growth velocity between control and treatment group infants during the intervention or follow up phases, either before (Table 5.4.4) or after adjustment for birth weight and gender (data not shown). Nor were any differences in growth velocity found between control and treatment infants in birth weight and gender subgroups (Appendices A2 and A3).

Table 5.4.4: Growth velocity from enrolment to EDD and EDD to 4 months CA by intervention group

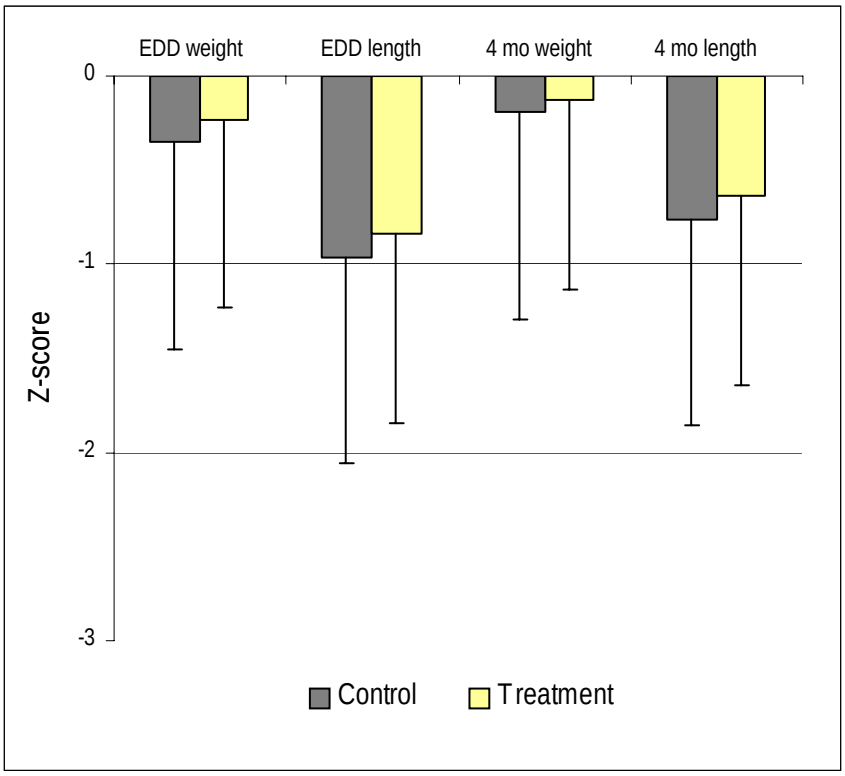
| | Control Group<br>n = 74 | Treatment Group<br>n = 69 |
|---|---|---|
| Enrolment to EDD;<br>Weight gain (g/day)<br>Length gain (cm/wk)<br>HC gain (cm/wk) | n = 70 (96%)<br>28 ± 5<br>1.0 ± 0.2<br>0.8 ± 0.1 | n = 66 (96%)<br>29 ± 6<br>1.0 ± 0.2<br>0.8 ± 0.1 |
| EDD to 4 Months CA;<br>Weight gain (g/day)<br>Length gain (cm/wk)<br>HC gain (cm/wk) | n = 68 (92%)<br>25 ± 5<br>0.7 ± 0.1<br>0.4 ± 0.1 | n = 59 (86%)<br>26 ± 5<br>0.7 ± 0.1<br>0.4 ± 0.1 |

Results expressed as mean ± SD

*Z-score*

The z-scores of the control and treatment groups at EDD and 4 months CA are depicted in Figure 5.1. As indicated by the negative z-score, infants in this study were below the average size of infants born at term. This was expected, as smaller size in preterm infants has been well described [103]. There were no significant differences in weight or length Z-scores between the control and treatment groups. The large standard deviations associated with the Z-scores are indicative of the diversity of growth performance in preterm infants.

Figure 5.1: Comparison of Z-scores at EDD and 4 Months CA



## 5.5 Discussion of Primary Safety Outcome

Intention-to-treat analyses demonstrated no anthropometric differences between infants fed milk containing high dose DHA and those fed according to current practices. The subgroup of girls demonstrated differences between the control and treatment infants, with a trend for slightly larger infants in the treatment group. The increased size cannot be attributed to a faster rate of growth as no differences in growth velocity between control and treatment infants were found. Although the number of subgroup analyses was limited to the randomisation strata, the multiplicity of statistical comparisons increases the risk of finding a significant outcome through chance alone. Therefore, the possibility that the significant difference present in the subgroup of girls is due to chance cannot be excluded.

Consistent with the data from my trial, many other LCPUFA intervention trials have not reported differences in anthropometry between preterm infants fed formulas containing no LCPUFA and infants fed n-3 LCPUFA (and no n-6 LCPUFA) [32,62,101,108], or n-3 LCPUFA and n-6 LCPUFA [46,60,62,102,111,112]. Data from five of these trials were combined in a meta-analysis from which it was concluded that formula containing both n-3 and n-6 LCPUFA did not impair growth of preterm infants [68]. As observed in the subgroup of girls in my trial, the authors reported that LCPUFA in formula resulted in increased length at term, and increased weight and length at 2 months CA [68].

Regardless of these observations there has been some reluctance to test higher doses of DHA in preterm infant formula. This hesitation appears to stem from three trials that have demonstrated poorer growth after supplementation with 0.5% and 0.3% n-3 LCPUFA from fish oil [6,104,107] and 0.2% DHA and 0.3% AA from egg phospholipid [100]. Early work by Carlson *et al* suggested that the increased n-3 LCPUFA in milk displaced n-6 LCPUFA and that the lower n-6 LCPUFA was associated with poorer growth [106]. In the present trial although the milk AA was similar in both groups, erythrocyte phospholipid

AA was significantly lower in the treatment group (Chapter 4 Table 4.4) infants and growth was not affected.

The cautious approach to testing milk with high concentrations of n-3 LCPUFA has resulted in only two trials in preterm infants testing levels of DHA at or near 1% of total fat and without altering AA. Uauy *et al* compared growth of preterm infants fed formula containing 1% n-3 LCPUFA (0.4% DHA and 0.6% EPA) from fish oil until 4 months CA with infants fed formula with no LCPUFA [108]. No significant differences were found between supplemented and control infants in weight, length or HC. However, the sample size was small (n = 27) and probably insufficient for detecting differences in growth. In a larger trial involving nearly 200 preterm infants, Fewtrell *et al* found the size of infants fed formula containing 0.6% n-3 LCPUFA from fish oil until 9 months CA was not different from infants fed formula with no LCPUFA. Small increases in rate of weight and length gain were observed in n-3 LCPUFA supplemented infants [101]. Together with the data reported in the present trial, there does not appear to be a consistent relationship between n-3 LCPUFA in milk and growth of preterm infants.

Unlike previous LCPUFA trials, the inclusion of breast and formula fed infants is unique to my trial. This is a strength of the trial as it better represents the mixed feeding (breast milk and formula) that Australian preterm infants frequently receive. However, this feeding regime did add complexity to the relationship between LCPUFA status and growth. This was mainly due to two factors. Firstly, the variations in growth as a result of energy balance. Preterm formula has an energy density of 24 cal/oz and formula fed infants received preterm formula from enrolment until reaching their EDD. Breast fed infants received 24 cal/oz milk only when human milk fortifier was added to expressed breast milk. Fortifier is added to breast milk once enteral feeds are established, but it cannot be added during feeding at the breast. Therefore on average, breast fed infants received milk of lower energy density than formula fed infants during the intervention period. Secondly, the fatty acid profiles of breast milk

and preterm formula are quite different, and the proportion of each type of milk in the infants' diet modifies the LCPUFA status of the infant. Compared with breast milk, preterm formula has lower saturated fats, higher monounsaturated fatty acids and a preponderance of linoleic acid. Furthermore, breast milk has a much wider range of long chain fatty acids, a higher proportion of medium chain length fatty acids and the fatty acid composition varies with the diet of the mother. Although including breast fed infants resulted in some limitations in evaluating the relationship between fatty acids and growth, it does offer extraordinary generalisability to the wider population of infants born <32 weeks gestation.

The anthropometric data collected from the infants in my study are comparable with reports of other preterm infants [150,151] and with other LCPUFA trials [6,44,60,62,102,111]. Despite this consistency, a number of differences exist between my trial and previous LCPUFA trials. In almost all previous formula trials infants were primarily fed 24 cal/oz formula [44,60,62,102,111], (in one trial the energy density of the formula is unclear [6]). Where possible, the infants in my trial were breast fed therefore the energy density of the infants diet might have been lower than in other LCPUFA trials. The infants in my trial began the intervention shortly after the commencement of enteral feeds; unlike some LCPUFA trials where the intervention began after enteral feeds were established [6,44,60,62,99,100,105,109,110]. Furthermore, my study included infants with a wide range of illnesses compared with some LCPUFA trials that have focussed on 'healthier' infants by excluding those with many diseases common to preterm infants [6,44,60,62,102,111]. The energy density of milk, the early commencement of the intervention and the health profiles of infants have the potential to impact on later growth of infants. Therefore, the comparability in size between formula fed preterm infants involved in previous LCPUFA trials and the infants fed 1% DHA from breast milk and formula in my trial adds further strength to the suggestion that the increased DHA in milk does not negatively affect infant growth in the presence of some AA.

Growth of preterm infants fed breast milk or preterm infant formula containing approximately 1% DHA and 0.5% AA until their EDD exhibit growth that is not different to infants that receive 0.3% DHA and 0.5% AA.

## 5.6 Results of Secondary Safety Outcome: Clinical Data

*Gastrointestinal Data and Incidence of Necrotising Colitis*

The duration of total parenteral nutrition (TPN) varied from 0 through to 51 days and the time to reach full enteral feeds from 2 to 40 days (Table 5.6.1). Three infants (4%) from the control group and two (3%) infants from the treatment group contracted NEC. During the trial, one infant (treatment group) died as a result of the NEC. This infant never reached full enteral feeds, consuming a total of 612 mL of breast milk over 13 days of partial enteral feeds. Overall, there were no statistically significant differences in NEC (p = 0.942), the duration of TPN (p = 0.587), duration of IV lipids (p = 0.562), or the days to reach full enteral feeds (p = 0.392) between infants in the control and treatment groups. No significant differences were found between control and treatment groups in the number of days that feeds were interrupted.

Table 5.6.1: Gastrointestinal Data and Incidence of NEC by Intervention Group

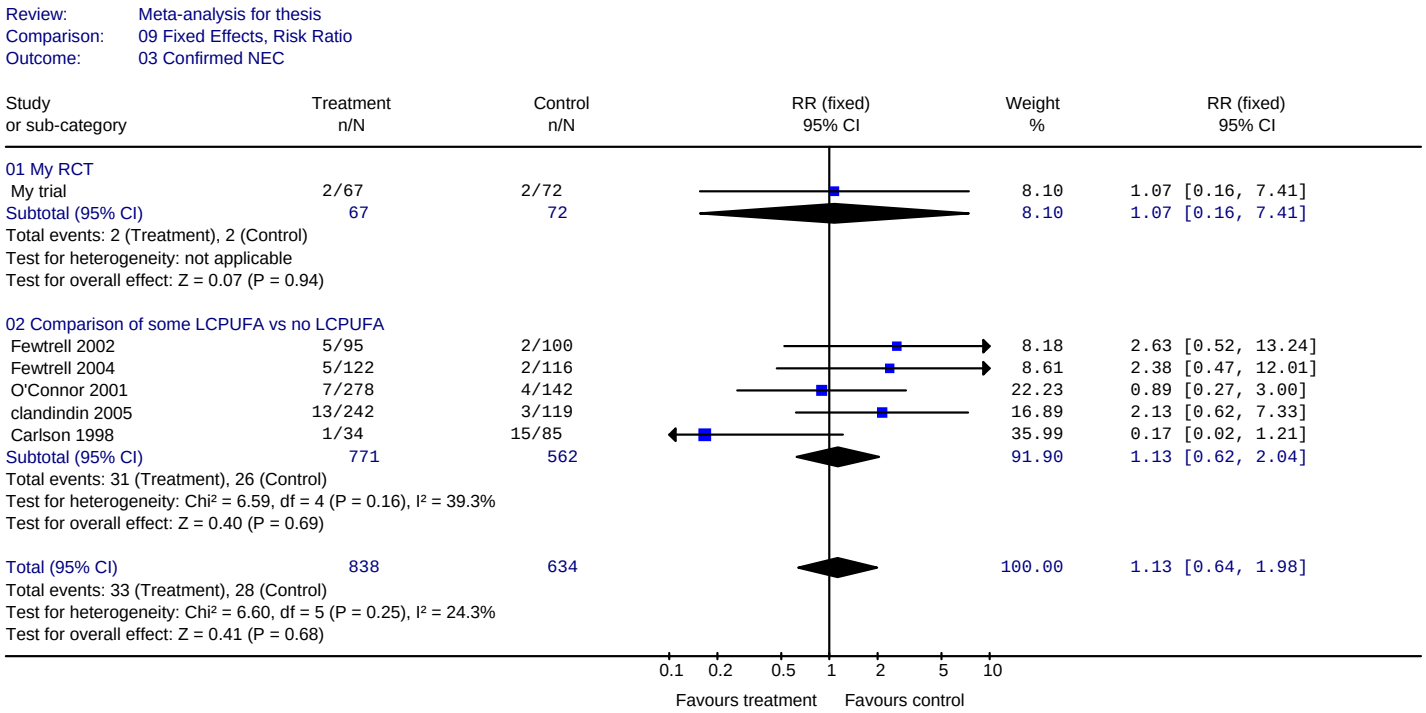
| | Control Group n = 74 | Treatment Group n = 69 |
|---|---|---|
| Days to reach full enteral feeds | 9 (2 – 40) | 9 (3 – 36) |
| Days of IV lipids | 6 (0 – 36) | 7 (0 – 36) |
| Number of infants with 1 or more confirmed NEC events* | 2 (3) | 2 (3) |
| Infants having feeds interrupted* | 42 (57) | 43 (63) |
| Number of days of feeds interrupted during intervention period | 1 (0 – 26) | 2 (0 – 24) |

Values are medians (range), except for *number of infants (percentage)

NEC data were extracted from previous LCPUFA trials in preterm infants [60,100-102,113] and combined with the data from the present trial in meta-analysis (Figure 5.2). The analysis includes over 1400 infants

and indicates that there is no significant change in Relative Risk of NEC with the addition of LCPUFA to preterm infant formula (RR = 1.13, 95% CI 0.64 – 1.98, p = 0.68).

Figure 5.2: Meta-analysis of the Incidence of Necrotising Enterocolitis (NEC) in Preterm Infants in LCPUFA Intervention Trials



*Respiratory Data*

The requirement for respiratory system support was assessed from birth until removal of respiratory support (including home oxygen therapy). The duration of respiratory support varied widely between infants (range; 0 - 104 days ventilated, 0 - 39 days of continuous positive airway pressure (CPAP) and 0 - 115 days of oxygen therapy) (Table 5.6.2). There were no differences in the duration of ventilation ($p = 0.33$), the duration of CPAP ($p = 0.16$) or the duration of oxygen supplementation ($p = 0.81$) between the control and treatment groups. The number of infants that received surfactant, required oxygen at 36 wk postmenstrual age (PMA), oxygen at discharge, or had hyalin membrane disease (HMD) was not significantly different between the control and treatment groups ($p = 0.99$, $p = 0.719$, $p = 0.705$ and $p = 0.858$, respectively).

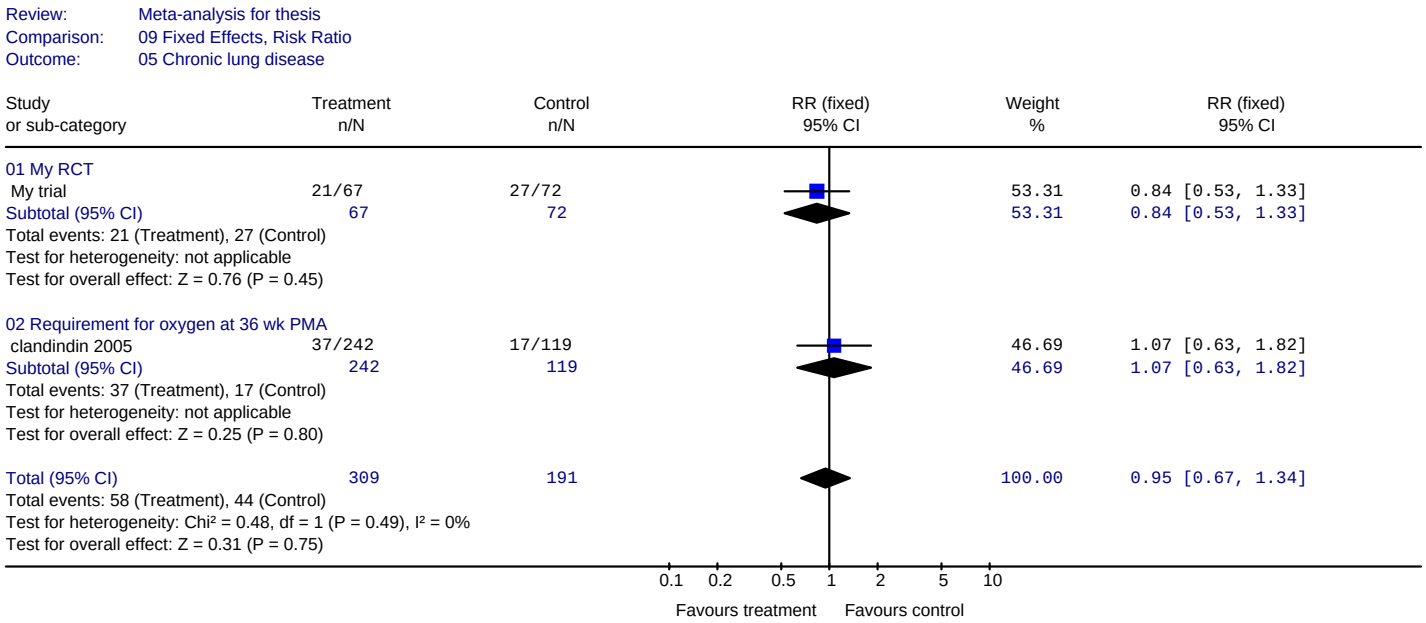
Table 5.6.2: Respiratory Characteristics of Trial Participants by Intervention Group

| | Control Group n = 74 | Treatment Group n = 69 |
|---|---|---|
| Days of ventilation* | 2 (0 – 56) | 2 (0 – 104) |
| Days of CPAP* | 1 (0 – 39) | 2 (0 – 32) |
| Days on oxygen* | 9 (0 – 107) | 11 (0 – 115) |
| Infants that Received Surfactant (%) | 43 (58) | 37 (54) |
| Required oxygen at 36 wk PMA(%) | 28 (38) | 21 (30) |
| Required oxygen at discharge (%) | 22 (30) | 17 (25) |
| Infants Diagnosed With HMD (%) | 49 (66) | 44 (64) |

Values are medians (range), except for *number of infants (percentage)

Only one LCPUFA trial has used the more modern definition of BPD as the requirement for oxygen at 36 weeks PMA and these data were used for the meta-analysis [102]. No significant change in relative risk of BPD with LCPUFA in milk was observed when data from this trial was combined with data from my study (RR = 0.95, 95% CI 0.67 – 1.34, $p = 0.75$) (Figure 5.3).

**Figure 5.3:** Meta-analysis of the Incidence of Bronchopulmonary Dysplasia (BPD) Defined as Requirement for Oxygen at 36 Weeks PMA in LCPUFA Intervention Trials



*Retinopathy of Prematurity*

The National Health and Medical Research Council (NHMRC) of Australia recommends routine ophthalmological examinations for retinopathy in all preterm infants born <32 weeks gestation or <1500 g [152]. Retinopathy data were not collected for a total of 18 infants (11 from control group, 7 from treatment group). In the control group nine infants born at 32 completed weeks of gestation and two born <32 weeks gestation were not assessed for retinopathy. In the treatment group six infants born at 32 weeks gestation and two born at 31 weeks were not assessed for retinopathy.

Table 5.6.3: Retinopathy of Trial Infants by Intervention Group, Categorised According to Most Severely Affected Eye

| | Control Group n = 74 | Treatment Group n = 69 |
|---|---|---|
| Total Number of infants assessed | 63 (85) | 62 (90) |
| Total Number with retinopathy | 13 (18) | 17 (25) |
| Stage 1 | 7 (9) | 8 (12) |
| Stage 2 | 4 (5) | 7 (10) |
| Stage 3 | 2 (3) | 2 (3) |
| Stage 4 | 0 (0) | 0 (0) |

Values are incidence rates and (percentages) for infants assessed for retinopathy

Of the 120 infants that had retinopathy examinations 15 (12%) had stage 1, ten (8%) had stage 2 and two (3%) had stage 3 retinopathy in the left eye. Fifteen (12%), 11 (9%) and two (3%) had retinopathy at stages 1, 2 and 3 respectively, in their right eye (Table 5.6.3). One male infant from the control group had only one eye affected and another male also from the control group had different stages of retinopathy between left and right eyes. All other infants had the same degree of retinopathy in both eyes. Only one infant was prescribed laser therapy for treatment of retinopathy at stage 3.

No difference in the incidence of any retinopathy was found between the control and treatment groups (p = 0.741). Further, the severity of retinopathy was not significantly different between the control and treatment groups (p = 0.313). Statistical analysis comparing the number of infants in the treatment and control groups that required therapy for retinopathy was not performed, as there was only one infant from the control group prescribed laser therapy.

Retinopathy data from two previous LCPUFA trials were combined in meta-analyses with data from the present trial [102,113] (Figure 5.4 and 5.5). In the first analysis, the effect of LCPUFA on the incidence of any ROP was calculated. This analysis included over 600 infants and demonstrated that there is no significant change in relative risk ROP with LCPUFA (RR = 1.28, 95% CI 0.99 – 1.66, p = 0.06) (Figure 5.4). The RR statistic is of borderline significance and favours the control formula. The second meta-analysis included the number of infants with severe retinopathy (≥grade 3), as this is associated with significant long-term morbidity and poorer visual outcome in preterm infants. This analysis included 260 infants and demonstrated no change in relative risk of ROP with LCPUFA (RR = 0.84, 95% CI 0.20 – 3.51, p = 0.81) (Figure 5.5).

Figure 5.4: Meta-analysis of the Incidence of Any Retinopathy of Prematurity (ROP) in Preterm Infants in LCPUFA Intervention Trials

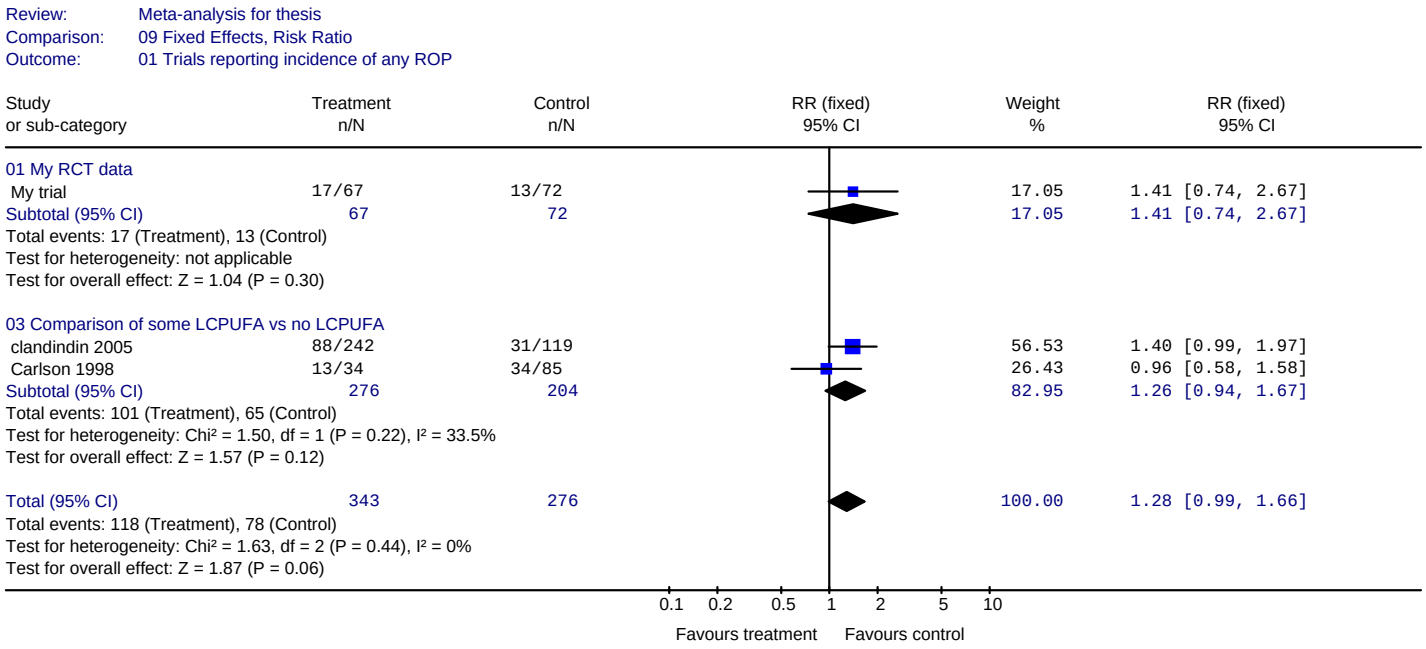
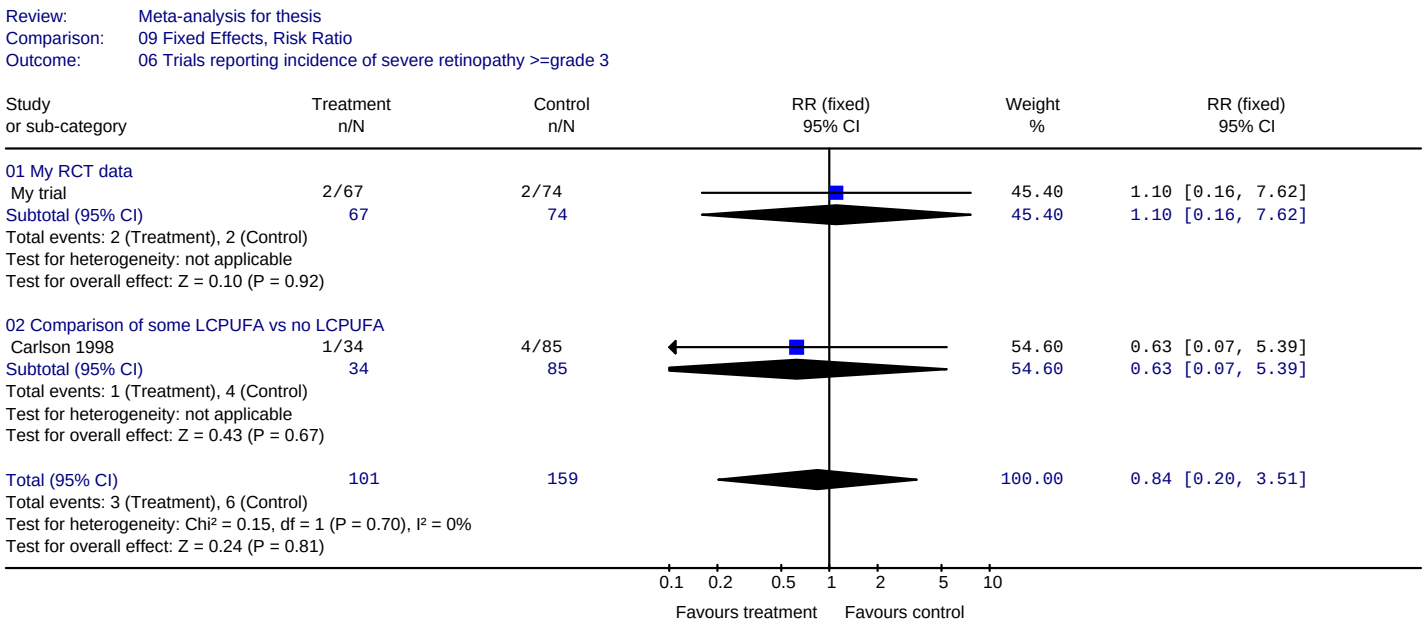


**Figure 5.5:** Meta-analysis of the Incidence of Severe Retinopathy of Prematurity (Defined as Grade ≥3) in Preterm Infants in LCPUFA Intervention Trials



*Brain Injury*

There were nine (12%) infants from the control group and six (9%) infants from the treatment group that had a brain injury (IVH or PVL) (Table 5.6.4). There were equal numbers of infants with IVH between the control and treatment groups, with three infants having bleeds in one brain hemisphere and three infants having bleeds across both hemispheres in both groups. The severity of all IVH did not exceed grade two. Only one infant (control group) had both IVH and PVL. No statistically significant differences in the number of infants with brain injuries were found, although the number of infants with PVL approached significance ($p = 0.053$). This was a result of no infants in the treatment group ever developing PVL in any region of the brain.

Table 5.6.4: Incidence of Neural Injuries by Intervention Group

| | Control Group<br>n = 74 | Treatment Group<br>n = 69 |
|---|---|---|
| Brain injuries (%) | 9 (12) | 6 (9) |
| IVH (%) | 6 (8) | 6 (9) |
| PVL (%) | 4 (5) | 0 (0) |

Values are incidence rates (percentages in parentheses)

Figures 5.6 and 5.7 show two meta-analysis forest plots of IVH data from four LCPUFA intervention trials in preterm infants [100-102]. In the first meta-analysis, trials reporting IVH were combined to investigate the effect of LCPUFA on the overall incidence of IVH (Figure 5.6). The analysis includes data from over 900 preterm infants and demonstrates no significant change in relative risk of IVH with LCPUFA supplementation (RR = 0.89, 95% CI 0.64 – 1.23, p = 0.49). Severe IVH (≥grade 3) is associated with poorer long-term outcomes in preterm infants. The second meta-analysis included trials that reported the grade of IVH separately from overall incidence of IVH (Figure 5.7). This permitted investigation of the effect of LCPUFA on the severity of IVH. The analysis suggested no significant

change in severity of IVH with LCPUFA supplementation (RR = 0.91, 95% CI 0.35 – 2.34, p = 0.85), however fewer infants were included in this analysis consequently reducing power.

Figure 5.6: Meta-analysis of the Incidence of Any Intraventricular Haemorrhage (IVH) in Preterm Infants in LCPUFA Intervention Trials

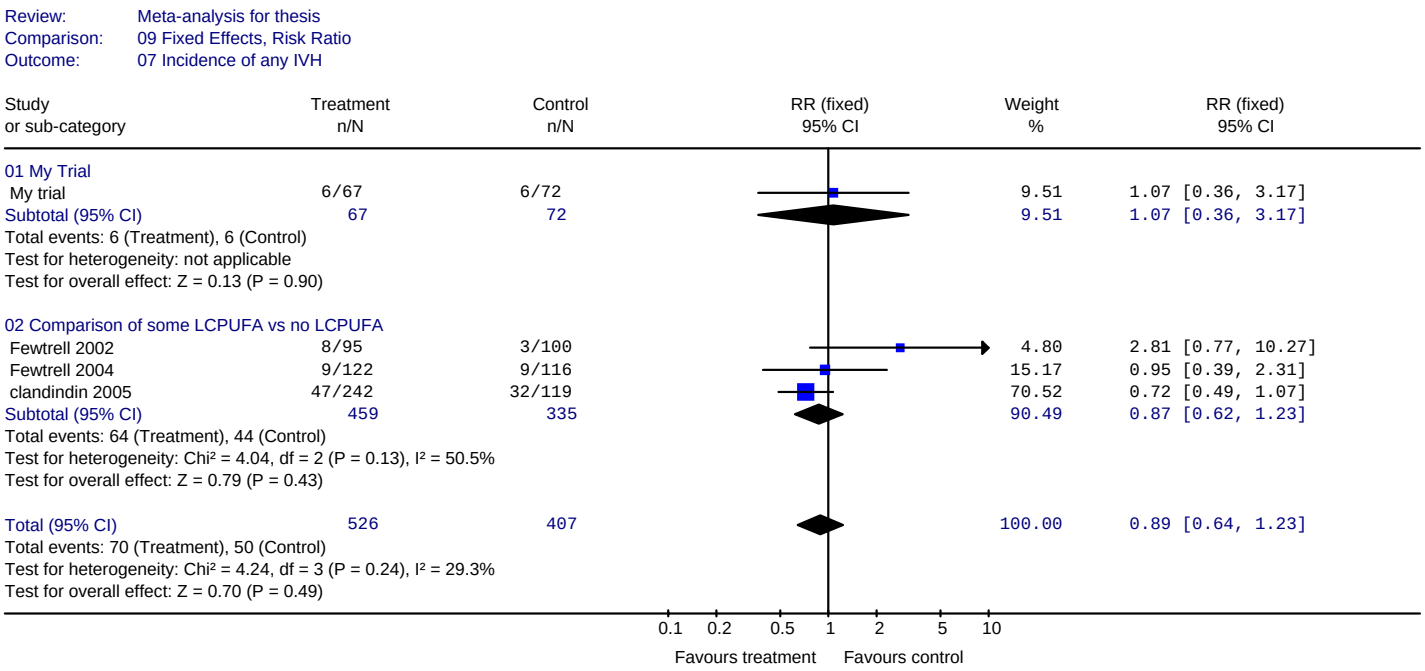
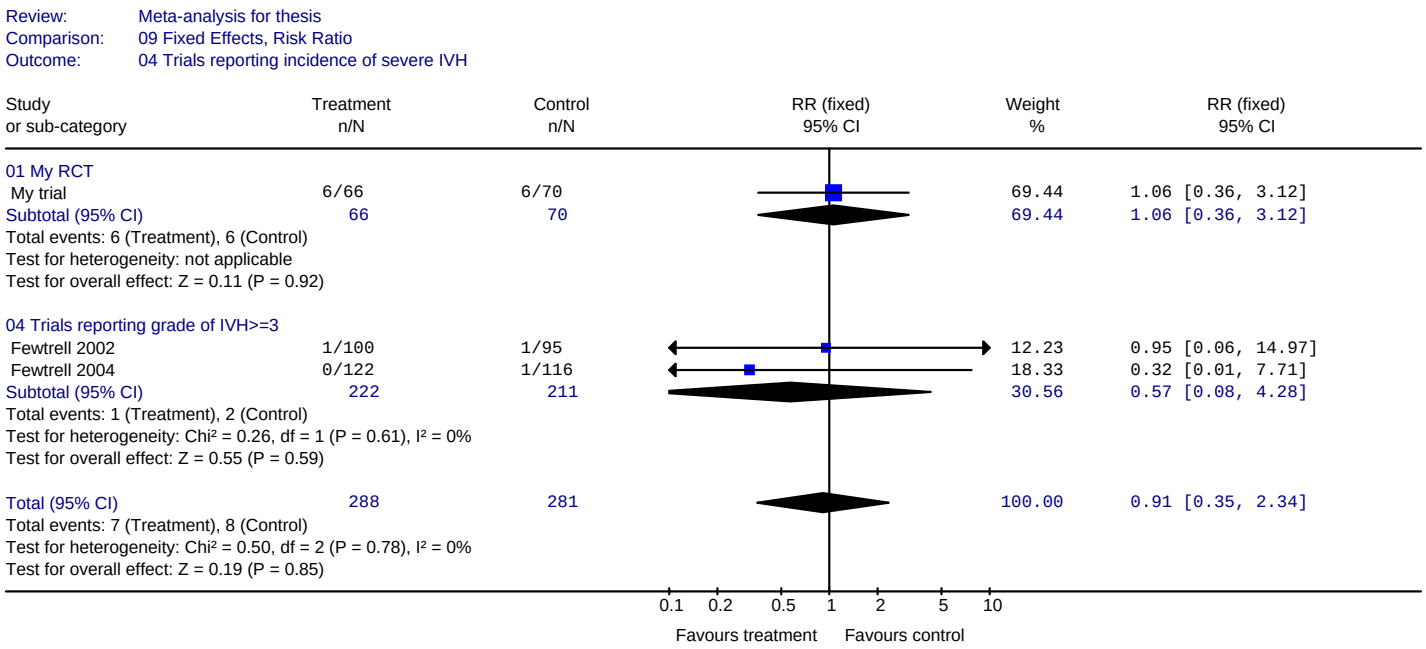


Figure 5.7: Meta-analysis of the Incidence of Severe Intraventricular Haemorrhage (Defined as Grade ≥3) in Preterm Infants in LCPUFA Intervention Trials



*Sepsis*

A small proportion of infants in the control group (10%) and treatment groups (15%) contracted sepsis on one or more occasions (Table 5.6.5). One infant from the treatment group had 2 cases of sepsis during the hospitalisation period. Despite this, there were no statistically significant differences in the number of infants diagnosed with sepsis between the treatment and control groups (p = 0.27).

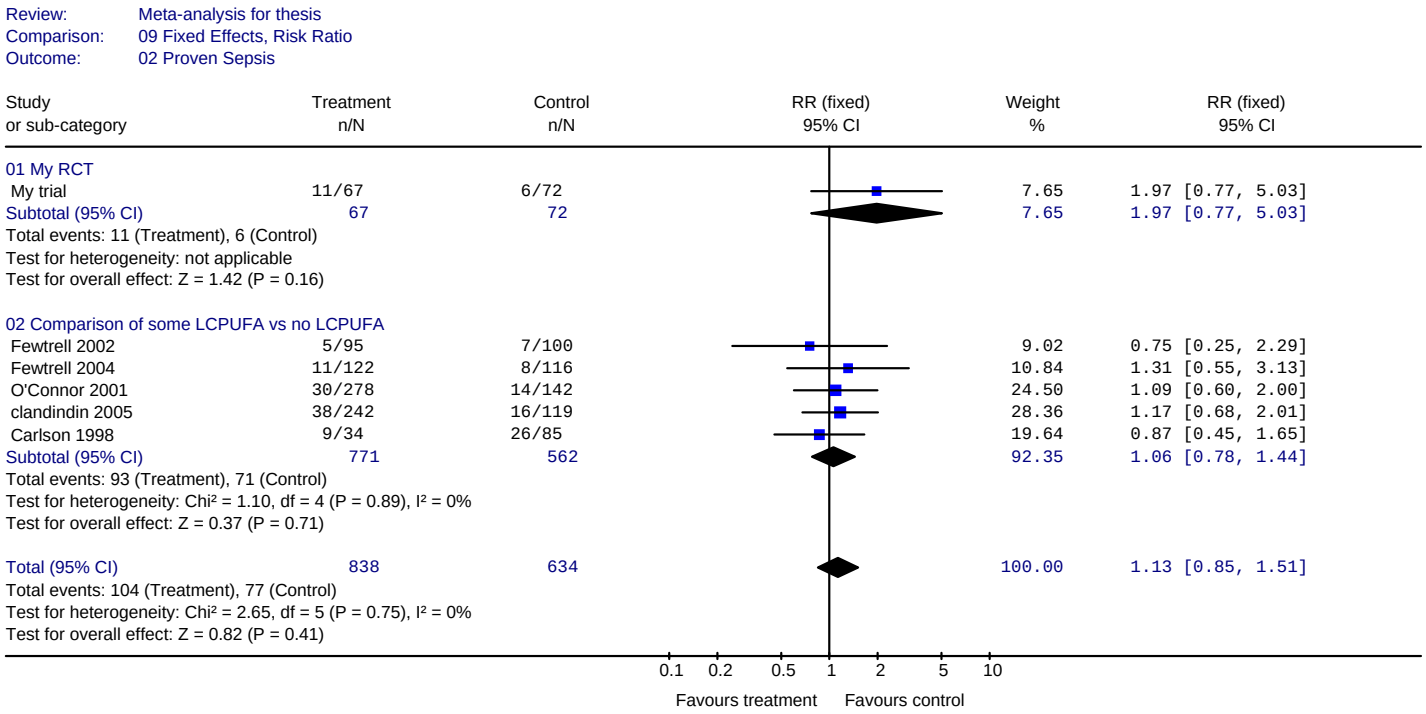
Table 5.6.5: Sepsis Data by Trial Intervention Group

| | Control Group<br>n = 74 | Treatment Group<br>n = 69 |
|---|---|---|
| No sepsis events | 67 (91) | 58 (81) |
| 1 sepsis event | 6 (8) | 10 (15) |
| 2 sepsis events | 0 (0) | 1 (2) |

Values are incidence rates (percentages in parentheses)

Incidence of sepsis (confirmed by blood culture) has been reported in five LCPUFA trials [60,100-102,113]. As a result, data from over 1300 infants were included in the meta-analysis. The analysis indicates that there is no significant change in relative risk of sepsis with LCPUFA supplementation (RR = 1.13, 95% CI 0.85 – 1.51, p = 0.41) (Figure 5.8).

Figure 5.8: Meta-analysis of the Incidence of Sepsis in Preterm Infants in LCPUFA Intervention Trials



*General Morbidity*

Other variables that reflect the clinical course of the infants during the intervention and follow-up periods were also collected. These variables included the number of infants requiring blood transfusions, length of admission, number of infants requiring surgery and number readmitted to hospital.

Morbidity of the infants varied widely across both groups and the healthiest infants received no blood transfusions, did not undergo surgery and were not readmitted to hospital in the period from discharge to 4 months CA (Table 5.6.6). However, 14 infants (10%) received one transfusion and a further 52 infants (36%) received more than one transfusion. Between the control and treatment groups, there were no significant differences in the number of infants that required one or more transfusions ($p = 0.24$) or the number of transfusions infants received ($p = 0.31$).

The length of stay in each level of care was comparable between the groups. Thirty-nine infants (30%) were readmitted to hospital at least once in the period from the first discharge from hospital to reaching 4 months CA, with 12 infants readmitted more than once. There was no significant difference between control and treatment infants in the number of infants re-admitted to hospital ($p = 0.09$).

There were eight infants (11%) in the control group and 16 (24%) infants in the treatment group that underwent surgery one or more times. Four infants (three in the control group and one in the treatment group) required surgery twice. The most commonly performed surgeries included patent ductus arteriosus (PDA) ligation in four control and five treatment group infants, hernia surgery in four control and seven treatment group infants, and laparotomies in one control and three treatment group infants. One infant from each group had a skin graft for intravenous extravasation, one control group infant had surgery for pyloromyotomy and one treatment group infant had a bronchoscopy. The number of infants

that received surgery in the control compared with the treatment group was not significant, although the probability borders on significance (p = 0.06).

One infant from the treatment group died during the treatment phase of the trial. Conclusions from post mortem examination of this infant determined that the death was a result of complications of NEC and it was unlikely to be related to the trial intervention. There was no significant difference in the number of infants that died between control and treatment groups (p = 0.97).

Table 5.6.6: Morbidity and Mortality Data According to Intervention Group

| | Control Group n = 74 | Treatment Group n = 69 |
|---|---|---|
| Number of transfusions | 0 (0 – 11) | 1 (0 – 14) |
| Number of infants requiring surgery* | 8 (11) | 16 (23) |
| Days in NICU | 9 (0 – 74) | 12 (0 – 110) |
| Days in level 2 care | 13 (0 – 41) | 11 (0 – 33) |
| Days in level 1 care | 28 (3 – 59) | 26 (0 – 57) |
| Discharge age (weeks PMA)^ | 38 ± 1 | 38 ± 2 |
| Number of children re-hospitalisations at 4 months CA* | 24 (32) | 13 (19) |
| Neonatal Deaths (Total)* | 0 (0) | 1 (2) |

Values are medians (and range), except for *number of infants (percentage) and ^mean ± SD

## 5.7 Discussion of Clinical Data

*Gastrointestinal Data and Incidence of NEC*

The incidence of NEC varies from year to year, but over years of reporting Australian and New Zealand neonatal data the incidence of NEC in preterm infants is relatively stable at approximately 4% [153]. In my trial, a similar percentage of infants developed NEC. No differences were found in the incidence of NEC or any feeding or tolerance data between the treatment and control groups.

Comparable rates of NEC have been published for preterm infants enrolled in other LCPUFA intervention trials [60,62,100-102,111]. Most trials have reported no difference in incidence of NEC between preterm infants that receive formula with no LCPUFA and those that receive formula with some LCPUFA [60,62,100-102,111]. One report suggests a reduction in the incidence of NEC with LCPUFA in formula [113]. Unfortunately in addition to LCPUFA, there was also a high level of choline in the treatment formulas, which complicates the interpretation of these findings. The combining of published NEC data in meta-analysis showed no clear association of LCPUFA in milk and incidence of NEC. The low incidence of NEC means that the further studies are necessary to support this observation with sufficient sample size to exclude the possibility of random error.

Data from my trial add further evidence in support of the concept that there is little difference in tolerance after feeding preterm infants milk containing up to 1% of total fat compared with infants fed according to current practices. Further trials are necessary to evaluate any relationship between LCPUFA in milk for preterm infants and NEC.

*Respiratory Data*

In Australia and New Zealand approximately 60% of infants born <32 weeks gestation are intubated at birth. Exogenous surfactant is given to around 85% of infants as routine preventative therapy, despite this, approximately 18% of infants born <32 weeks gestation develop HMD [153]. Compared with Australian neonates born <32 weeks gestation, fewer infants enrolled in my trial required ventilation at birth and fewer infants received surfactant. A higher percentage of infants enrolled in my study developed HMD compared with other Australian neonates, which may be related to the lower use of surfactants.

Although there were subtle differences between the respiratory characteristics of the study infants and the wider preterm population there were no differences in respiratory variables between the control and treatment groups. More specifically, there were no significant differences in their early requirement for respiratory support (such as days of ventilation, or duration of oxygen), nor were then any differences in the development of chronic respiratory symptoms.

Only one LCPUFA trial in preterm infants has reported differences in respiratory outcomes in control compared with LCPUFA supplemented infants. It was reported that infants fed formula with 0.6% n-3 LCPUFA spent a longer time ventilated compared with control infants [101]. The authors of this study stated that the LCPUFA supplemented infants were sicker at birth and since ventilation preceded randomisation this finding may not have been related to the intervention. By comparison, infants in my trial were supplemented with twice the level of DHA and no difference in the duration ventilation was found. Other LCPUFA trials have also reported incidence of BPD, however there is little consistency in the criteria applied to define the disease [60,100,102,113]. It is thought that the clinical characteristics of BPD have changed since the introduction of surfactant therapy and that the modern definition of oxygen at 36 weeks PMA is more appropriate than the original (that is, requirement for oxygen at 28 postnatal

days)[154]. Only one LCPUFA trial has reported BPD according to this modern definition. Although meta-analysis of this trial with my trial data suggested no change in risk of BPD, the small number of trials included in the analysis limits the power of this observation. Further studies are required to address this issue with sufficient statistical power to exclude trial bias and random error.

*Retinopathy of Prematurity*

The recommendation to follow up preterm infants born <32 weeks gestation or <1500 g is due to their increased risk of developing retinopathy [152]. Despite these recommendations, retinopathy follow up is not performed on approximately 7% of Australian preterm infants and data are not available on a further 4% of infants [153]. The proportion of infants in my trial born <32 weeks gestation that did not receive ROP review was 3% in the control group and 1% in the treatment group. Follow up of trial infants born <32 weeks gestation was higher than national averages and the proportion of trial infants with no retinopathy and the proportion with significant vascularisation of the retina (grade III or grade IV) were comparable with national rates [153].

Some LCPUFA intervention trials set retinopathy as an exclusion criterion, particularly if retinopathy is severe (≥ grade 3) and a visual outcome is measured [44,61,104]. This is because severe ROP is associated with vision impairment. Of the five LCPUFA trials that have reported ROP data [6,62,101,102,113] only the two trials that described the clinical definition of retinopathy were included in the meta-analysis [102,113]. The incidence of retinopathy in these two trials (approximately 40% of their study sample) is substantially higher and birth weights are lower compared with infants in my study [102,113]. The infants in these trials were either exclusively formula fed from birth [113] or received ≥80% of diet as formula during hospitalisation [102]. Formula intake is associated with increased incidence of ROP [115]. Thus differences in trial participants or formula intake might have contributed to the increased incidence of ROP. The meta-analysis indicated no significant difference in incidence of any retinopathy or in incidence of

severe eye disease with formula containing LCPUFA. Although the RR statistic for the incidence of any retinopathy approached significance the relatively small sample size from few trials may introduce bias and random error. Furthermore, both of the trials included in the meta-analysis were not analysed in an intention-to-treat manner and the randomisation procedures and concealment of allocation were not clearly reported in one of the trials [113]. These factors may have introduced selection bias in the trial design. Therefore, it must be emphasised that further studies are necessary to confirm the findings of these analyses.

My trial provides evidence that suggests no change in incidence of retinopathy between preterm infants fed milk containing 1% of fat as DHA and infants fed according to current practices. There are few well described ROP data available from other LCPUFA trials in preterm infants. Therefore, further data are necessary to fully assess the effect of LCPUFA on incidence or severity of ROP.

*Brain Injury*

Cerebral ultrasounds are undertaken shortly after preterm birth to detect intraventricular haemorrhages as a standard program of clinical care. Follow up ultrasounds are conducted routinely in the ensuing weeks to monitor IVH, ventricle dilatation and for early detection of PVL. The number of Australian preterm infants suffering significant neural haemorrhages has steadily declined to approximately 6% of infants born <32 weeks gestation and 4% of infants are diagnosed with PVL [153].

Of the infants enrolled in my study 8% had a cerebral haemorrhage and 5% developed PVL. The incidence or severity of IVH and PVL was not different between the treatment and control groups, although the small proportion of infants developing these conditions limits this power of this analysis. With these limitations in mind, it was noted that in my trial no infants from the treatment group developed PVL. Larger groups of infants would be necessary to evaluate if the level of DHA in infant

feeds was able to protect the neonatal brain from PVL. This would be important to investigate further, given the severity of this disease.

As with retinopathy, many DHA intervention trials set IVH and PVL as exclusion criteria. This most commonly occurs when developmental assessments are a primary outcome of the trial. Infants who suffer neurological events in the neonatal period are more likely to have in developmental problems. This introduces variation in neurodevelopmental scores and as a result, detecting differences in neurodevelopment requires a larger sample size. However, excluding these infants does not reflect the true range of morbidity for infants born prematurely and these infants need to be included in DHA intervention trials to better represent the broad range of neurological outcomes of preterm infants. The IVH meta-analysis included data from three trials that did not exclude preterm infants with IVH and described the classification of the IVH [100-102]. In these trials, the dose of DHA varied from 0.2 to 0.5% of total fat in formula and this was fed to the infants for between 3 weeks through to 1 year CA. The meta-analysis suggests that the relative risk of any IVH or of severe IVH was not influenced by LCPUFA. However, the small number of trials means that the possibility of bias and random error cannot be excluded. Clearly further trials are necessary to elucidate any role for LCPUFA in neural diseases.

Another RCT has investigated the effect of DHA supplementation on neural structures [61]. Preterm infants with very little or no neural damage were randomised to receive formula with no LCPUFA or with 0.34% DHA and 0.7% AA until 6 months CA. Magnetic Resonance Imaging (MRI) examinations were performed at 3 and 12 months CA to measure the degree of myelination. No difference in myelination was found between infants fed formula containing no LCPUFA and those fed formula with LCPUFA. The dietary regime was unlikely to influence myelination as myelin is largely comprised of unsaturated or monounsaturated fatty acids [155]. However, this trial does demonstrate *in vivo* that LCPUFA does not appear to influence the myelination process in relatively 'healthy' preterm infants.

Although no change in incidence of IVH or PVL were found by increasing the DHA in milk to 1% of total fat, further studies are necessary to address this issue with sufficient rigour and statistical power.

*Sepsis*

Unlike retinopathy and brain haemorrhages, eligibility of preterm infants for DHA intervention trials cannot be limited by sepsis events as these often occur after randomisation. Since DHA has the capacity to modulate immune responses it was important to assess the effect of increasing an infant's DHA status on the performance of the immune system. Septicaemia may provide a rudimentary indication of susceptibility to infection.

The increased DHA in milk for treatment group infants did not result in changes to sepsis rates compared with infants fed DHA according to current practices. The absence of any relationship between sepsis and LCPUFA in formula for preterm infants has also been observed in each of the seven trials that have reported sepsis [33,60,62,100-102,111,113]. The meta-analysis includes over 1300 infants and suggests that there is little evidence of any change in risk of sepsis with increased LCPUFA in milk for preterm infants.

My trial provides further evidence that 1% DHA in milk for preterm infants does not change the incidence of sepsis. Further studies are necessary to confirm safety of this dose of DHA with sufficient statistical power.

*General Morbidity*

The monitoring of morbidity outcomes provides additional safety information to add to the pool of available knowledge, ultimately to enhance decision-making regarding LCPUFA supplementation in milk for preterm infants. The types of 'general morbidity' data collected in other n-3 LCPUFA intervention trials have varied. Specific reports include; no differences in length of stay [101,113], number of blood transfusions [6,108] and readmissions to hospital [101] between preterm infants fed formula with LCPUFA compared with infants fed no LCPUFA. Data published in other LCPUFA trials supports the findings from my trial in which no differences between control and treatment infants were found in all outcomes.

In my study the percentage of infants that required surgery come close to statistical significance, with higher rates in the treatment group. A mechanism for this difference is not clear, particularly as surgery was required for a diverse range of clinical condition from substantially different aetiologies, some of which were present prior to randomisation. To my knowledge, no other LCPUFA trials have reported the number of surgeries in their cohorts.

The clinical picture and morbidity of preterm infants fed milk containing 1% of fat, as DHA did not appear to differ from infants who received DHA in milk according to current clinical practices.

## 5.8 Summary of Safety Data

In summary, the data described in this chapter suggests no difference in safety outcomes between preterm infants fed milk containing 1% DHA and infants fed according to current practices (~0.3% DHA). Specifically, no differences were found in weight, length and head circumference measurements of control and treatment groups and enhanced growth in subgroup of girls at 4 months CA. Although all indicators of clinical morbidity were not different between the control and treatment groups, the incidence of many disease of prematurity reported in this chapter is low. Consequently, larger sample sizes are required to fully evaluate the high dose DHA intervention diseases of low incidence. In the light of the efficacious effects of this intervention described in Chapter 3 & 4, these safety data offer new and exciting information for the study of LCPUFA in milk for preterm infants.

# CHAPTER 6: GENERAL DISCUSSION

In Australia, infants born <32 weeks gestation represent approximately 2% of total births [153]. These infants require substantial medical care, are typically smaller and demonstrate poorer performance in many areas of development than infants born at term [103]. Feeding the preterm infant is a challenge due to the immaturity of their gastrointestinal system and their high demand for nutrients to support postnatal growth and development. Breast milk is the feed of choice for preterm infants as it offers many benefits and thus strategies to increase feeding of breast milk within the neonatal unit are actively encouraged. However, it is now widely accepted that breast milk does not meet all the nutritional needs (with regard to protein and some minerals) of the preterm infant, and this has led to fortification of expressed breast milk for preterm infants. In this context, the view of breast milk as the gold standard of LCPUFA intake is an assumption that until now had not been tested. My trial is unique, as it has addressed two important issues. Firstly, I developed a new approach that attempted to determine the optimal dose of DHA for preterm infants. This was accomplished by applying knowledge of gestational accretion of n-3 LCPUFA and estimating the concentration of DHA in milk necessary to supply a similar quantity during the neonatal period. Secondly, I tested this new dose of DHA on a representative group of preterm infants through maternal supplementation, so breast milk was naturally enriched with DHA. Formula with complementary DHA concentration was provided so that DHA intake was not dependent on mode of feeding.

My trial demonstrated that infants fed milk containing a high dose of DHA exhibited improved visual acuity at 4 months CA when compared with infants fed according to current practices. The mean increase in acuity was modest but was consistently higher across all subgroups of treated infants compared with the control group. This finding is important as early visual experience has the capacity to affect later function [59, 147]. In severe cases it is known that poorer visual input in infancy can result in

lasting visual impairment. However, it is not known if this is also true for subtle differences in sweep VEP acuity. In term infants, higher erythrocyte DHA status (up to 10% of total fat) has been associated with enhanced visual acuity [43]. The treated infants in my study had erythrocyte phospholipid DHA at approximately 7%; therefore it is tempting to speculate that an even higher DHA status might have resulted in further increases to visual acuity. This leads to the question of whether infants were supplemented with enough DHA to achieve a high DHA status.

The strategy of supplementing lactating mothers with tuna oil to increase breast milk DHA was particularly successful. The median DHA content of breast milk fed to treatment group infants was raised to 1% of total fat. Although the DHA concentration of the intervention formula was designed to match this level, it was found to only contain ~0.7%. During the intervention period infants were predominately fed breast milk hence the majority of infants in the intervention group were fed milk with an average DHA concentration close to 0.9%. As this intervention was designed to meet the *in utero* accretion of DHA, theoretically infants should have had a DHA status at the end of the intervention period similar to infants born at term. This was achieved, as the erythrocyte phospholipid DHA concentration of treated infants was comparable with levels reported for Australian term infants at day 5 of life [125]. Interestingly, term infants fed breast milk with a concentration of DHA between 0.8 – 1.3% have a higher percentage of DHA in erythrocyte membrane phospholipids [42,43], demonstrating that it may be possible to further increase the proportion of DHA in erythrocyte membrane phospholipids. A number of potential strategies exist for elevating the DHA status of preterm infants above that demonstrated in my trial. Increasing the concentration of DHA in breast milk and formula over 1.0% of total fat is the most direct method. However this approach might be problematic as infant erythrocyte DHA tends to plateau as the DHA concentration in breast milk increases beyond 1% of total fat [31]. This is due to competition with other fatty acids present in milk. Reducing the LA content of preterm formula

or the LA content of the maternal diet might help to increase DHA status by lessening competition with other dietary fatty acids.

As I have demonstrated that the maturation of visual acuity can be improved with increased dietary DHA, it is plausible that other visual functions or tissues which depend on high concentrations of DHA might also show enhanced or improved performance. For example, improving the quality of visual input in infancy might enhance of other aspects of visual development such as visual attention. There is some existing evidence that doses of 0.2% DHA in formula fed to preterm infants modulate attention behaviour [93,94]. With respect to other tissues, neural cortex contains a substantial quantity of DHA and preterm infants are known to have poorer performance in neurodevelopmental tests compared with infants born at term. My study showed a modest improvement in visual acuity, however a similarly modest increased in neurodevelopment would be welcomed. Consequently, neurodevelopmental outcomes will form an important extension of this work. In order to fully elucidate any role of DHA in neurodevelopment more sensitive tests that assess specific elements of cognition may need to be developed and long term cognitive outcomes are needed to substantiate the theory that improvements in early development are sustained. In future studies, it will be especially important to assess the effect of the energy density of the infants' diet, as this may have the potential to complicate or interfere with the effects of an intervention. If infants are not receiving adequate energy it is possible that a higher proportion of dietary DHA will be oxidised for energy and therefore would not be available for incorporation into retinal, neural or other tissues.

An important aspect of my trial was to evaluate the safety of increased LCPUFA in milk for preterm infants. Although clinical event data has been accumulating from a number of LCPUFA trials the meta-analyses indicated there are insufficient numbers of infants to confirm safety of increased LCPUFA in milk for incidences of ROP, IVH and BPD. The incidences of sepsis and NEC included data from over

1300 infants and indicated no change in risk with LCPUFA in milk. The primary safety outcome of weight at 4 months CA, showed no difference with increased dose of n-3 LCPUFA. This is supported with a growing body of data from good quality trials that have demonstrated no difference in size of preterm infants fed formula with no LCPUFA compared with infants fed formula with only n-3 LCPUFA, or both n-3 and n-6 LCPUFA.

The ratio of n-3:n-6 LCPUFA in milk for preterm infants is a controversial issue. The claim that formula must contain a higher level of AA than DHA to prevent overt reductions in infant growth is the subject of intense scientific debate [157,158]. There is a lack of evidence to support this recommendation and my trial is the first to have measured preterm infant growth after intervening with varying ratios of AA to DHA. AA is present in both breast milk and preterm formula and although all infants received some AA, the ratio of DHA:AA was not at the suggested ratio of 1:2 [149]. In my trial the ratio was reversed; that is infants were fed milk with the ratio of 1% DHA to 0.5% AA. The growth of infants fed milk with the high dose DHA was not different from the control group and were similar to other preterm infants. Therefore, evidence in support of the current recommendations for a ratio between DHA and AA in formula is lacking and indicates this ratio may be of questionable value.

My trial suggests that the DHA requirement of preterm infants may be higher than that present in preterm formula or breast milk of Australian women. The benefits of feeding preterm infants breast milk should be emphasised. To support optimal visual development of preterm infants, lactating mothers should be encouraged to increase the concentration of DHA in their breast milk. This intervention provides positive reinforcement, promotes breast feeding initiatives and supports lactating mothers in providing the best quality nutrition for their infant. The significance of this finding is that this simple and readily acceptable intervention to increase the concentration of DHA in milk has the opportunity to enhance early visual function of a large proportion of preterm infants. Further research will be

necessary to understand the full impact of this intervention on other aspects of preterm infant development.

APPENDIX A

Table A.1: Anthropometry of Control and Treatment Groups at Enrolment, Discharge and 2 Months CA

| | Control group<br>n = 74 | Treatment group<br>n = 69 |
|---|---|---|
| At enrolment: | n = 74 (100%) | n = 69 (100%) |
| Weight (g) | 1277 ± 403 | 1234 ± 408 |
| Length (cm) | 38.8 ± 3.8 | 38.9 ± 4.0 |
| HC (cm) | 27.5 ± 2.6 | 27.0 ± 2.7 |
| At discharge: | n = 72 (97%) | n = 66 (96%) |
| Weight (g) | 2722 ± 399 | 2689 ± 397 |
| Length (cm) | 46.0 ± 2.5 | 46.3 ± 2.4 |
| HC (cm) | 33.8 ± 1.3 | 33.7 ± 1.36 |
| At 2months CA: | n = 67 (91%) | n = 59 (86%) |
| Weight (g) | 4843 ± 789 | 4955 ± 702 |
| Length (cm) | 54.9 ± 3.2 | 55.4 ± 2.6 |
| HC (cm) | 39.3 ± 1.5 | 39.1 ± 1.2 |
| MUAC (cm) | 12.2 ± 1.1 | 12.4 ± 1.3 |
| Abdominal Girth (cm) | 39.2 ± 2.8 | 40.0 ± 2.7 |

Data reported as mean ± SD

APPENDIX A

Table A.2: Growth Velocity From Birth to EDD and From EDD to 4 Months CA by Gender

| | Boys n = 70 | | Girls n = 73 | |
|---|---|---|---|---|
| | Control n = 35 | Treatment n = 35 | Control n = 39 | Treatment n = 34 |
| Growth Velocity from Enrolment to EDD; | n = 33 (89%) | n = 33 (91%) | n =39 (100%) | n = 33 (97%) |
| Weight gain (g/day) | 31 ± 6 | 29 ± 6 | 27 ± 5 | 29 ± 6 |
| Length gain (cm/wk) | 1.0 ± 0.2 | 1.0 ± 0.2 | 1.0 ± 0.2 | 1.0 ± 0.2 |
| HC gain (cm/wk) | 0.8 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.1 |
| Growth Velocity from EDD to 4 Months CA; | n = 31 (89%) | n = 27 (77%) | n = 37 (95%) | n = 32 (94%) |
| Weight gain (g/day) | 28 ± 5 | 27 ± 6 | 23 ± 5 | 25 ± 5 |
| Length gain (cm/wk) | 0.8 ± 0.1 | 0.8 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 |
| HC gain (cm/wk) | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.1 |

Data reported as mean ± SD,

CA, corrected age; HC, head circumference

APPENDIX A

Table A3: Growth Velocity From Birth to EDD and From EDD to 4 Months CA by Birth Weight

| | <1250 g birth weight n = 67 | | ≥1250 g birth weight n = 76 | |
|---|---|---|---|---|
| | Control Group n = 34 | Treatment Group n = 33 | Control Group n = 40 | Treatment Group n = 36 |
| Growth Velocity from Enrolment to EDD; | n = 34 (100%) | n = 32 (97%) | n = 38 (95%) | n = 34 (94%) |
| Weight gain (g/day) | 26 ± 4 | 25 ± 3 | 31 ± 5 | 33 ± 6 |
| Length gain (cm/wk) | 1.0 ± 0.2 | 1.0 ± 0.1 | 1.0 ± 0.2 | 1.0 ± 0.2 |
| HC gain (cm/wk) | 0.8 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.1 |
| Growth Velocity from EDD to 4 Months CA; | n = 33 (97%) | n = 28 (85%) | n = 35 (88%) | n = 31 (86%) |
| Weight gain (g/day) | 24 ± 4 | 24 ± 4 | 26 ± 6 | 27 ± 6 |
| Length gain (cm/wk) | 0.8 ± 0.1 | 0.8 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 |
| HC gain (cm/wk) | 0.4 ± 0.04 | 0.4 ± 0.05 | 0.4 ± 0.05 | 0.4 ± 0.05 |

Data reported as mean ± SD

## APPENDIX B: PUBLICATIONS ARISING FROM THIS WORK

### I Published Abstracts

Smithers LG, Gibson RA, McPhee AM and Makrides M. (2005) *Asia Pacific Journal of Clinical Nutition,* 14, s99.

Smithers LG, Gibson RA, McPhee AM and Makrides M (2004) *Asia Pacific Journal of Clinical Nutrition,* 13, s50

Smithers LG, Gibson RA, McPhee AM and Makrides M (2005) *Asia Pacific Journal of Clinical Nutition,* 14, s99.

### II Oral Conference Presentations

Plenary Presentation

Smithers LG, Gibson RA, McPhee AM and Makrides M. LCPUFA status of preterm infants: a randomised trial comparing two doses of docosahexaenoic acid. Oral plenary presentation at International Society for Fatty Acids and Lipids (ISSFAL) conference in Cairns Australia, July 2006.

Smithers LG, Gibson RA, McPhee AM and Makrides M. Visual development of preterm infants fed high dose docosahexaenoic acid. Annual Scientific Conference, Nutrition Society of Australia, Brisbane, August 2004

Smithers LG, Gibson RA, McPhee AM and Makrides M. Feeding patterns of preterm infants born <33 weeks gestation. Annual Scientific Conference, Nutrition Society of Australia, Hobart, December 2003

Smithers LG, Gibson RA, McPhee AM and Makrides M. Very preterm infants: what are they fed? South Australian Branch meeting of the Australian Society for Medical Research, Adelaide, June 2003

### III Poster presentations

Smithers LG, Gibson RA, McPhee AM and Makrides M. Growth of preterm infants fed high dose docosahexaenoic acid. Annual Scientific Conference, Nutrition Society of Australia, Melbourne, December 2005

Smithers LG, Gibson RA, McPhee AM and Makrides M. Clinical events after LCPUFA supplementation of infant formula for preterm infants: a meta-analysis, accepted for poster presentation at ISSFAL conference in Cairns Australia, July 2006

### IV AWARDS

Award for Best Oral Presentation for the Clinical Sciences field at the ISSFAL conference, Cairns Australia, July 2006

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