THE EFFECTS OF FISH OIL SUPPLEMENTATION DURING EARLY INFANCY ON NEURODEVELOPMENT AT SIX YEARS OF AGE

THE UNIVERSITY OF WESTERN AUSTRALIA

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I dedicate this thesis to Megan.

Friends since we were six.
STATEMENT OF CANDIDATE CONTRIBUTION

The work presented in this thesis was performed by Alexandra Heaton between April 2011 and April 2015 at the School of Paediatrics and Child Health, University of Western Australia (UWA) under the supervision of Professor Karen Simmer, Dr Suzanne Meldrum, Dr Jonathan Foster and Professor Susan Prescott.

Alexandra Heaton completed the DNA extractions, and was responsible for participant recruitment, appointment bookings and 95% of the neuropsychometric assessments i.e. n = 320 appointments. For consistency, Miss Heaton hand-scored all assessments and questionnaires, provided written feedback to parents (i.e. neuropsychometric reports and any referral information), entered data, analysed results, in addition to writing, drafting and editing the following manuscript.

Recognition and appreciation goes to several collaborators. Fatty acid analysis and lipid profiling was conducted by Dr Mori and colleagues from the School of Medicine and Pharmacology, UWA. Genotyping was performed in Germany in the Munich laboratory headed by Professor Koletzko. Thanks also go to Catherine Frogley (Institute of Psychiatry, Kings College London), who performed 16 (i.e. 5%) neuropsychometric assessments while gaining work-experience in 2012.

This dissertation does not contain material that has been accepted for the award of any other degree or diploma at any university or other tertiary institution. To the best of my knowledge, the following academic research has not been previously published or written by another person, except where explicit reference is made. All authors have approved this dissertation and endorse its submission.

Alexandra Heaton
(PhD Candidate)

Winthrop Professor Karen Simmer
(Coordinating Supervisor)
This thesis investigates the effects of fish oil supplementation in healthy neonates on neurocognitive and behavioural outcomes after 6 years.

Fish oil contains omega-3 (ω-3) long-chain polyunsaturated fatty acids (LC-PUFAs) including docosahexaenoic acid (DHA). DHA rapidly accumulates within the brain from the third trimester to ~18 months of age. Here, it plays various structural and functional roles, including anti-oxidative, anti-inflammatory and anti-apoptotic effects in addition to enhancing neuroplasticity, synaptogenesis and neural signalling.

The postnatal diet directly modulates the DHA concentration within the developing human brain. However this diet can be variable, whereby the concentration of DHA in breast milk can vary 20-fold between individuals (Brenna et al., 2007); moreover, some formulas contain no DHA at all. Humans can manufacture LC-PUFAs via the metabolism of shorter chain precursors; however, the rate of metabolism varies between individuals, largely due to genetics. While dietary requirements are ill-defined, it is quite possible that many Australian infants are born and raised in a state of DHA insufficiency that will in-turn lower the fatty acid composition of their brains.

Assuming their DHA requirements are insufficient, it is hypothesised that infants could receive significant benefits from ω-3 LC-PUFA supplementation. There is a need for well-designed randomised controlled trials to critically evaluate whether ω-3 LC-PUFA supplementation during infancy leads to better neurocognitive/behavioural development in childhood.

The work presented here is the 6 year follow-up of a larger multidisciplinary study named the Infant Fish Oil Study (IFOS). This randomised controlled trial began in 2005 to investigate the immunological and neurodevelopmental effects of direct fish oil supplementation during infancy. IFOS enrolled (n = 420) pregnant women with a history of allergic disease and randomised their infants to receive fish oil (250 – 280 mg DHA + 60 – 110 mg EPA) or placebo (olive oil), daily, independent of feeding methods, from birth to 6 months.
The research question addressed by IFOS is distinctly different from the previous randomised controlled trials comparing LC-PUFA enriched formula versus unsupplemented formula. The study intended to justify the need – or otherwise – for fish oil *supplementation* in healthy Western infants.

Conducting a 6 year follow-up using this pre-existing IFOS cohort was appealing since the children had already been recruited, the intervention completed and blood samples; in addition to a wealth of other information had been collected at earlier follow-ups. Further strength of the 6 year follow-up is due to the knowledge and expertise within the research team, access to leading neuropsychometric tests and infrastructure - in addition to the pivotal fact that participants had reached an age whereby neurocognition can be more reliably assessed.

The 6 year follow-up evaluated four key areas of child development; language, executive functioning, global IQ and behaviour. At the time of planning the 6 year follow-up, we anticipated that language and communication at 6 years would be enhanced in the children from the treatment group, given our previous positive findings pertaining to gestural skills at 18 months (Meldrum et al., 2012). Analysis of language outcomes at 6 years was completed using the Clinical Evaluation of Language Fundamentals (CELF-4), the Renfrew Bus Story, and Children’s Communication Checklist (CCC-2). Investigation of executive functioning was warranted – considering how DHA accretes within the hippocampus and frontal cortex – key regions of the brain for memory. The CELF-4 provided a core score of Working Memory, which was performed in addition to several other stand-alone tests tapping into more specific mental processes / executive functioning (including Rapid Autonomic Naming and Word Associations). We also predicted higher global intelligence in the treatment group as it has been purported that children who receive more DHA during development have higher IQ (Hibbeln et al., 2007). Subsequently, we included the Wechsler Abbreviated Scale of Intelligence (WASI) and the Gifted Rating Scale (GRS). We were also keen to reassess these participants for a range of behavioural outcomes since controversial and conflicting reports exist as to whether ω-3 LC-PUFA affects behavioural outcomes in a positive or negative fashion. Parents completed the Child Behaviour Checklist (CBCL), and the Autism Spectrum Quotient: Children’s Version
(AQ-Child); indicating internalising- and externalising behaviours, and frequency of autistic-like-traits (respectively). Schoolteachers’ also completed a teachers version of the CBCL.

Foremost, we report that infant fish oil supplementation did not enhance the main outcomes of language, executive functioning, IQ or behaviour at 6 years. The treatment group scored significantly higher on one sub-test of working memory. However, the validity of this is questioned by a contrary finding in another subtest of working memory where the control group scored significantly higher than the treatment group. Surprisingly, the treatment group displayed higher externalising behaviours, specifically in the realm of oppositional defiance, compared to controls.

To help elucidate whether there is a causal link between supplementary LC-PUFA and neurocognitive / behavioural development; correlational analyses were performed with LC-PUFA concentrations at birth, 6 months and near 5 years. We found only DHA status at birth was correlated with less externalising behaviours and reduced hyperactivity. Concentrations of AA and total ω-6 LC-PUFA were correlated with several adverse neurocognitive outcomes including language.

Based upon the knowledge that an individuals’ capacity for endogenous LC-PUFA synthesis is dependent on genetic variants, we expected that there may be certain genetic subgroups who are more responsive to fish oil supplementation. We investigated 21 Single Nucleotide Polymorphisms (SNPs) and 39 haplotypes within the Fatty Acid Desaturase (FADS) gene cluster. By including FADS gene variants within our analysis of fish oil supplementation, we sought to illuminate a gene-diet interaction modulating an individual’s dietary requirements. Yet, contrary to our hypothesis, we found no evidence that a gene-diet interaction had occurred. Albeit, major FADS allele carriers (associated with high endogenous LC-PUFA synthesis) displayed more internalising behavioural problems compared to minor allele carriers.

Such findings were expected to be encouraging of a more targeted approach to fish oil supplementation dependant on personalised requirements. However, we conclude that – out of the 21 SNPs we investigated – neither a targeted, nor a complete
approach to fish oil supplementation appears to enhance the neurocognitive or behavioural development of healthy Western infants’.

In conclusion, the present randomised controlled trial demonstrated that, after 6 years, ω-3 LC-PUFA supplementation during early infancy provides little to no benefit to the neurocognitive or behavioural development of healthy full-term, predominantly breastfed, Western infants from well-educated, relatively high-income, urban Australia. As such, fish oil supplementation is not recommended in this healthy population. This conclusion was further confirmed by the fact that fish oil supplementation was associated with behavioural adversities at 6 years. More research is required to elucidate the link between long-term behavioural outcomes and infant fish oil supplementation.

This research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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Many thanks go to the parents and children who volunteered for this study. Their time and effort is greatly appreciated.

Many thanks to my four supervisors, Karen Simmer, Suzanne Meldrum, Jonathan Foster and Susan Prescott, for conceptualising this project and offering me this wonderful opportunity. Their mentorship and guidance over the past three and a half years has been invaluable and their knowledge and passion for research has inspired me. Without their help and guidance, the work that constitutes this thesis would not have been possible.

Staff and students (both past and present) within this laboratory group are to be thanked for initial participant enrolment in addition to the information and blood samples collected at earlier follow-up appointments.

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Aaron who supported me through the final tough months of writing this PhD.

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Last but not least, endless love and thanks go to my family; Mum, Dad, Will and James who joined me on this ride, supporting, nurturing and loving me. I’m very fortunate to have you and there’s no way I could have done it without you.

I look forward to enjoyable times beyond the PhD.
PUBLICATIONS ARISING FROM THESIS

This thesis constitutes published academic writing by Miss Alexandra Heaton, and co-authors, Dr SJ. Meldrum, Dr JK. Foster, Professor SL. Prescott and Professor K. Simmer. Bibliographical information provided below:

Heaton, AE., Meldrum, SJ., Foster, JK., Prescott, SL., Simmer, K., (2013). *Does docosahexaenoic acid supplementation in term infants enhance neurocognitive functioning in infancy?* Published in: Frontiers of Human Neuroscience. This publication constitutes Chapter 1, the Literature Review. Alexandra Heaton drafted the manuscript and S. Meldrum and K. Simmer assisted with editing and structure of ideas. J. Foster and S. Prescott provided help in proofreading and editing of the final draft.

Heaton, AE., Meldrum, SJ., Foster, JK., Prescott, SL., Simmer, K., (2014). *Fish oil supplementation in infants during the first 6 months of life has no effect on neurocognitive development at 6 years: A randomised control trial.* Pending Submission. This paper constitutes Chapter 4. Alexandra Heaton was responsible for managing all aspects of 6 year assessments, data collection and statistical analysis. All authors contributed to editing of the final manuscript.

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Heaton, AE., Meldrum, SJ., Simmer, K., (2014). *Genetic factors modulating individual dietary requirements for ω-3 LC-PUFA.* Pending Submission. This paper constitutes Chapter 6. All authors conceptualised the project and contributed to editing of the final manuscript. Alexandra Heaton was responsible for DNA extraction, genetic analysis and drafting and editing of the manuscript.
PRESENTATIONS/POSTERS ARISING FROM THIS PROJECT


Heaton, AE., Meldrum, SJ., Foster, JK., Prescott, SL., Simmer, K., (2015). Fish oil supplementation of healthy term infants during the first 6 months of life has no effect on neurocognitive development at 6 years: A randomised control trial. Plenary session / seminar – (top 10 highest scoring abstracts) at the European Society for Paediatric Gastroenterology, Hepatology & Nutrition, Amsterdam.

Heaton, AE., (2012). The effects of fish oil supplementation during early infancy on neurodevelopment at six years of age. Seminar presented at the School of Paediatrics & Child Health, University of Western Australia, Perth.

Heaton, AE., Meldrum, SJ., Foster, JK., Prescott, SL. Simmer, K., (2012). The effects of high-dose fish oil supplementation on neurodevelopmental outcomes during childhood depend on FADS polymorphisms. Poster presentation at the Combined Biological Sciences Meeting (CBSM), Perth.


ABBREVIATIONS

AQ-Child = Autism Spectrum Quotient Children’s Version

ADHD = Attention Deficit Hyperactivity Disorder

BSID-II = Bayley Scales of Infant Development – 2nd Edition

CBCL = Child Behaviour Checklist

CELF-4 = The Children’s Communication Checklist 4th Edition

∆5D & ∆6D = delta-5 (& -6) Desaturase (enzymes)

DSM = Diagnostic and Statistical Manual

FADS = Fatty acid Desaturases

FSIQ = Full Scale IQ

GRS = Gifted Rating Scale

IFOS = Infant Fish Oil Study

LC-PUFA = Long-Chain Polyunsaturated Fatty Acid

ω-3 / n-3 = Omega-3

ω-6 / n-6 = Omega-6

PIQ = Performance IQ

SNP = Single Nucleotide Polymorphism

SOPT = Self Ordered Pointing Test

TRF = Teacher Report Form (of the CBCL)

VIQ = Verbal IQ

WASI = Wechsler Abbreviated Scale of Intelligence
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CHAPTER 1

LITERATURE REVIEW

Does docosahexaenoic acid supplementation in term infants enhance neurocognitive functioning in infancy?

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Chapter 1

**ABSTRACT**

The particular role of docosahexaenoic acid (DHA), the omega-3 (ω-3) long-chain polyunsaturated fatty acid (LC-PUFA), in healthy neurocognitive development has been a topic of interest for several decades. Theoretical evidence, laboratory research and human epidemiological studies have convincingly demonstrated that DHA deficiency can negatively impact neurocognitive development. However, the results from randomized controlled trials of DHA supplementation in human term-born infants have been inconsistent. Subsequently, the proposal that dietary DHA enhances neurocognitive functioning in term infants is controversial. Particular debate surrounds whether healthy term-born infants are obtaining sufficient DHA to meet their requirements. It is important that the current evidence concerning DHA supplementation is carefully evaluated and all proper considerations addressed so that appropriate recommendations can be made.

This article will:

1) Provide an overview the ω-3 and ω-6 polyunsaturated fatty acids, principally DHA

2) Discuss the availability of DHA in the human diet and human capacity for endogenous synthesis

3) Explore the physiological mechanisms by which DHA plausibly influences neurocognitive capacity, focusing on the critical period during human infancy

4) Review the major observational studies and randomised control trials that have examined dietary DHA in human infants and animals and evaluate the current evidence for LC-PUFA supplementation in term infants

5) Characterize the optimal intake of DHA during infancy for neurocognitive functioning, based on existing research that has been undertaken in developed countries (specifically, within Australia).

6) Consider the dual influence of genes and nutrition on brain structure and postulate that DHA requirements vary between individuals, depending on their genetic profile.
The omega-3 (ω-3) long chain polyunsaturated fatty acid (LC-PUFA), docosahexaenoic acid (DHA; 22:6 ω-3), is involved in several critical brain functions (Bradbury, 2011). Adequate supply of this fatty acid is particularly important throughout the period of rapid brain growth that occurs between the last trimester and the first year of life (Innis, 2008). This period of early development is known as the ‘brain growth spurt’ (Martinez & Mougan, 1998) and during this time, DHA and arachidonic acid (AA; 20:4 ω-6) are deposited within the cerebral cortex at a rapid rate (Clandinin et al., 1980; Martinez, Conde, & Ballabriga, 1974).

The amount of DHA that accretes within the foetal brain during gestation depends on a range of factors including, maternal diet, DHA stores, placental transport genetics and the availability of certain enzymes (McCann & Ames, 2010). After birth, the developing brain continues to accrete DHA – usually obtained via breast milk or infant formula. However, the amount of dietary DHA supplied during infancy can range considerably between individuals.

Formula-fed infants in many countries, including Australia, may be at risk of receiving insufficient dietary DHA; since the inclusion of pre-formed ω-3 and ω-6 LC-PUFAs is not mandatory (Australia New Zealand Food Authority, 2013). Breastfed infants are not necessarily impervious to DHA deficiency either since the concentration of DHA within breast milk depends on maternal DHA stores and diet (Innis, 2008). It is disconcerting that the average DHA intake of Australian women during pregnancy and lactation is below global recommendations (Meyer, 2011, Bourre, 2007). It is therefore possible that many Australian infants are born and raised on DHA deficient diets that will in turn affect the fatty acid composition of their brains.

Unanimous concern that infant diets low in DHA causes functional neurophysiological consequences is somewhat abated by the fact that infants are capable of synthesising ω-3 and ω-6 LC-PUFAs endogenously from their essential fatty acid precursors (Guesnet & Alessandri, 2011). The conversion of ω-3 and ω-6 essential fatty acids, alpha linolenic acid (α-LA) and linoleic acid (LA) into their respective LC-PUFA products entails a desaturation and elongation reaction cascade (Figure 1.1.). These enzymes
have been shown to be present from as early as 26 weeks gestation (Uauy, Mena, Wegher, Niento, & Salem Jr, 2000). However, there is considerable debate as to whether the amount of DHA generated endogenously meets infant requirements to facilitate optimal neurophysiological development (Guesnet & Alessandri, 2011).

**Figure 1.1. The metabolic pathways involved in synthesising ω-3 and ω-6 long chain polyunsaturated fatty acids from their respective precursors.**

The process of converting α-LA into EPA and DHA is well recognised to be a variable and inefficient process. Stable-isotope tracer studies have shown that dietary α-LA accounts for between 0.2% and 8% of EPA and <0.05 and 4% of DHA (Burdge & Calder, 2005; Plourde & Cunnane, 2007). Similarly, the ω-6 LC-PUFA metabolism is also inefficient, with less than 0.1% of LA converted into AA. The efficacy with which endogenous LC-PUFA synthesis occurs is moderated by the availability of the rate-limiting enzymes, delta-6 (Δ6D) and delta-5 (Δ5D) desaturase. Due to the importance of the fatty acid desaturation pathway for LC-PUFA synthesis, research has identified the genomic regions encoding fatty acid desaturase (FADS) activity, mapped to chromosome 11q12-13.1 of the human genome in the year 2000; (FADS1 for Δ5D and FADS2 for Δ6D).
Subsequent research has confirmed that single nucleotide polymorphisms (SNPs) in the FADS genes moderate individual capacity for LC-PUFA synthesis (Glaser, Lattka, Rzehak, Steer, & Koletzko, 2011). So, it is increasingly being recognized that dietary requirements for DHA and other LC-PUFAs may vary across the population and are probably dependent on individual genetic profile. Specifically, it is understood that an individual’s dietary requirements for DHA and other LC-PUFAs depends on their FADS genetic profile (Koletzko et al., 2008).

Another factor that can influence the rate of DHA synthesis is imbalanced consumption of ω-6 and ω-3 fatty acids. This is because both families compete for access to the same enzymes, (desaturases and elongases) and transport systems (Salem, Wegher, Mena, & Uauy, 1996; Uauy, Mena, Wegher, Nieto, & Salem, 2000). Consequently, an excess in one can inhibit the endogenous metabolism of the other (Schmitz & Ecker, 2008). Notably, increased consumption of ω-6 fatty acids has occurred over the course of the past several decades (Calder, 2012). As such, modern diets are in stark contrast to those of our paleolithic ancestors, who are thought to have consumed roughly equal ratios of ω-3 : ω-6 fatty acids (Simopoulos, 2001). Today, Western diets typically contain approximately 16 times more ω-6 than ω-3 fatty acids. This shift has occurred for a number of reasons including, but not limited to the fact that most cooking oils, foods containing wheat and red meats are all rich in ω-6 fatty acids and popularly consumed (Table 1.1). In contrast, rich dietary sources of DHA and eicosapentaenoic acid (EPA) are less prevalent, (i.e. found in cold-water fish, fish oil supplements, breast milk and supplemented infant formula).

The neurophysiological concerns that arise from low dietary DHA with concurrent high dietary ω-6 fatty acid intake are obviously compounded by the fact that humans are notoriously inefficient at endogenously synthesising DHA. This article evaluates the current level of evidence for LC-PUFA supplementation in term infants by summarising the major animal studies and clinical trials and randomised control trials in human infants concerning the neurocognitive effects of fatty acid supplementation.
Table 1.1. Common dietary sources of ω-3 and ω-6 short chain essential fatty acids (α-LA & LA) and long chain polyunsaturated fatty acids (LC-PUFA [DHA and AA]).

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<th>Dietary Sources</th>
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<tr>
<td>Essential fatty</td>
<td>α-LA</td>
<td>LA</td>
</tr>
<tr>
<td>acids</td>
<td>Linseed &amp; canola, Flaxseed, walnut, herring,</td>
<td>Corn, sunflower, safflower, walnut, peanut, wheat, pork, herring,</td>
</tr>
<tr>
<td></td>
<td>salmon, tuna &amp; green leaves</td>
<td>salmon &amp; tuna</td>
</tr>
<tr>
<td>LC-PUFA</td>
<td>DHA &amp; EPA</td>
<td>AA</td>
</tr>
<tr>
<td></td>
<td>Herring, salmon, trout, tuna, fish oil supplements, eggs &amp; breast milk</td>
<td>Beef, pork, poultry, whole-grain wheat</td>
</tr>
</tbody>
</table>

There are several lines of evidence as to why low levels of DHA are cause for concern. The concentration of DHA and other PUFAs within the brain can alter the neuronal membrane fluidity and physical structure of neurons (Youdim, Martin, & Joseph, 2000). ω-3 and ω-6 LC-PUFAs are also involved in the production and activity of several neurotransmitters such as dopamine and serotonin (Aid, Vancassel, Linard, Lavialle, & Guesnet, 2005; Chalon, 2006; Zimmer et al., 2002), affecting synaptic transmission and substrate binding to membrane receptors (Horrocks & Farooqui, 2004). Furthermore, DHA has been shown to affect neural functioning via its influence on gene expression in mammalian brain tissue (de Urquiza et al., 2000; Kitajka et al., 2004).

The process of neurite outgrowth in hippocampal neurons is enhanced by DHA, which may in turn promote learning (Calderon & Kim, 2004) as the hippocampus is a critical brain region for memory formation (Berger et al., 2012; Rolls, 2008). DHA may also improve learning and memory through its role in the development of pre- and post-synaptic proteins which enable synaptic transmission and long-term potentiation (Cao et al., 2009). Certainly, it is apparent that DHA plays numerous important biophysical roles in brain structure and function, and has the potential to influence neurocognitive development and subsequent performance (McNamara & Carlson, 2006; Parletta, Milte, & Meyer, 2013).

**Dietary DHA During Pregnancy and Throughout the Neonatal Period**

Considering the rapid accretion of DHA into the brain during the last trimester of pregnancy and into the first year of life, it is important to consider whether optimal DHA intake is occurring during this period. Numerous expert and government
authorities worldwide agree that dietary DHA requirements are increased during pregnancy and lactation when a minimum of 200 mg of DHA per day is recommended (Van Elswyk & Kuratko, 2009). This dose can be achieved by eating 1 – 2 portions of fish per week or taking fish oil supplements (Koletzko et al., 2008). However, recent figures indicate that 91% of Australian women are failing to meet DHA recommendations during pregnancy and lactation (Cosatto, Else, & Meyer, 2010), with similar trends noted in many other Western countries (Meyer, 2011).

In Australia, the median daily intake of DHA during pregnancy is approximately 96 mg per day, ranging from 8 – 632 mg per day across individuals (Cosatto et al., 2010). Since the dietary intake and maternal stores of DHA during pregnancy are known to be key determinants of infant blood DHA concentrations at birth (Bourre, 2007), low DHA consumption by women eating Western diets has prompted some concern for the neurological and neurocognitive development of their offspring (Rogers, Valentine, & Keim, 2013).

With respect to intake of DHA via breastfeeding, findings from the 2010 Australian National Infant Feeding Survey have revealed that around 96% of Australian women initiate breastfeeding after giving birth (Australian Institute of Health and Welfare, 2011). However, this figure rapidly declines during the weeks and months following birth (Burns, Schmied, Fenwick, & Sheehan, 2012). This despite international recommendations for exclusive breastfeeding for the first 6 months postnaturally (Kramer & Kakuma, 2001), in Australia an average of only 56% of women breastfeed for this recommended amount of time.

A comprehensive analysis of human breast milk LC-PUFA compositions have revealed the current worldwide average DHA concentration is approximately 0.32% of total FAs (Brenna et al., 2007). This corresponds to approximately 60 mg of DHA per day for the first 6 months postnaturally, assuming an average breast milk intake of 750 mL per day (Cunnane, Francescutti, Brenna, & Crawford, 2000).

Analysis of Australian breast milk concentrations in 1981 and 1993 found that the amount of DHA decreased by 27% over this time period while concentrations of AA remained the same (Makrides, Simmer, Neumann, & Gibson, 1995). A similar decline in
human milk DHA has been reported in a Canadian population (Innis, 2003). The decline in breast milk DHA may plausibly be explained by dietary shifts over time, the use of different fatty acid analytical techniques (Makrides et al., 1995) or through differences in maternal FADS genotype across studies (Moltó-Puigmarti et al., 2010; Xie & Innis, 2008) – an issue that will be discussed further below.

The concentration of DHA accreted by the developing brain is proportional to the amount of dietary DHA infants receive. Human autopsy studies conducted in Australia (Makrides, Neumann, Byard, Simmer, & Gibson, 1994) and the United Kingdom (Farquharson et al., 1995) both report that the brain tissue of breastfed infants contained higher concentrations of DHA compared to those fed DHA-free formula. These findings are consistent with animal studies, where dietary restriction of ω-3 PUFA decreased the amount of DHA within the brain (Brenna, 2011; Diau et al., 2005; Luchtman & Song, 2013). One of the compelling arguments for including DHA and other LC-PUFAs in infant formula is to render its composition more similar to breast milk, which is commonly cited as the ‘gold standard’ for infant nutrition (Burns et al., 2012). DHA enriched formula can enable formula-fed infants to attain DHA levels that are equivalent to their human milk receiving counterparts (Cunnane et al., 2000). However, clear functional benefits to infant development need to be demonstrated before DHA enriched formula can be unequivocally recommended.

**DHA FOR NEUROCOGNITION: EVIDENCE FROM ANIMAL RESEARCH**

The evidence that DHA deficiency is detrimental to neurocognitive development is well established in animal models. The functional consequences of lower DHA concentrations within the central nervous system in baboons include visual (Neuringer, Connor, Lin, Barstad, & Luck, 1986) and motor deficits (Champoux et al., 2002). DHA deficient rodents exhibit a range of neurocognitive impairments, including problems with learning and memory (Catalan et al., 2002) and can have a heightened stress response (Fedorova & Salem, 2006). Studies have found that ω-3 PUFA deficient rodents exhibit poorer performance in the Morris water maze test compared to their ω-3 PUFA-sufficient counterparts (Lamptey & Walker, 1978; Lim, Hoshiba, & Salem, 2005; Moriguchi, Greiner, & Salem, 2000; Sheaff, Moriguchi, Hutton, Slotnick, & Salem,
Animal studies allow more flexibility with respect to study design and potentially offer greater insight into the neurological mechanisms influenced by DHA deficiency in human infants (Romijn, Hofman, & Gramsbergen, 1991). However, there are limitations in the extrapolation of findings obtained in animal studies to the study of nutrition in humans. Research undertaken in non-human primates offers certain advantages over rodent studies in terms of transferability to humans, related to similarities in relative brain size, retinal microarchitecture and other anatomical, physiological and genealogical homologies (Brenna, 2011). However, human neurocognitive functioning is more complex and relies to a greater degree on higher cognitive capacities such as language and executive functioning (Luchtman & Song, 2013). A degree of caution should be applied when generalizing findings from animals to humans (Innis, 2007). Nonetheless, there is a strong and consistent body of evidence obtained from animal studies linking ω-3 LC-PUFA deficiency to impaired neurocognitive functions. These links warrant further investigation through clinical research in humans.

**MATERNAL DIETARY DHA INTAKE AND SUPPLEMENTATION**

Epidemiological studies of maternal DHA intake provide insight into the potential value of DHA on neurocognitive outcomes. A very large observational study (n = 11,875) in pregnancy found a significant association between low maternal seafood consumption (<340 g per week) and suboptimal neurocognitive outcomes in childhood (Hibbeln et al., 2007). Children aged 6 months to 8 years whose mothers consumed low seafood diets during pregnancy had lower verbal IQ, displayed less pro-social behaviour (defined as voluntary behaviour intended to benefit another) and had poorer social and communication skills compared to those whose mothers consumed high seafood diets (Hibbeln et al., 2007).

Furthermore, in a study of Canadian Inuit people (who typically consume a DHA-rich diet) DHA concentrations in umbilical cord plasma were positively associated with longer gestation, better visual acuity, higher scores of novelty preference on the Fagan
test at 6 months and higher cognitive scores on the BSID-II of mental and psychomotor performance at 11 months (Jacobson et al., 2008).

Most published observational epidemiological studies have recognized a positive association between maternal intake of ω-3 LC-PUFA rich foods during pregnancy and neurocognitive development of offspring (Boucher et al., 2011; Hibbeln et al., 2007; Mendez et al., 2009; Oken et al., 2008; Oken et al., 2005). However, the observational study by (Gale et al., 2008), detected no association between the frequency with which mothers ate fish in pregnancy and full-scale or performance IQ of their offspring at 9 years of age. Nevertheless, these researchers did find that children whose mothers had eaten oily fish had higher verbal IQ and a reduced risk of hyperactivity compared to those children whose mothers did not eat oily fish, after adjustment for potential confounding factors. However, the credibility of these findings might be somewhat weakened due to un-accountable confounding variables that could independently affect neurodevelopmental outcomes in childhood (Boyd et al., 2013; Drane & Logemann, 2000).

It is well established that maternal fish oil supplementation during pregnancy substantially increases foetal DHA concentration at the time of birth (Larqué, Gil-Sánchez, Prieto-Sánchez, & Koletzko, 2012). Furthermore, two randomised control trials have shown that DHA supplementation during pregnancy offers significant benefit to infant neurocognitive development (Dunstan, Simmer, Dixon, & Prescott, 2008; Judge, Harel, & Lammi-Keefe, 2007). However, the randomised control trials in this area are not easily comparable and positive effects have not been identified in all studies (Lo et al., 2012).

One long-term follow-up found that maternal supplementation enhanced neurocognitive outcomes up to 4 years later (I. Helland, Smith, Saarem, Saugstad, & Drevon, 2003) but the effect did not persist after 7 years (I. Helland et al., 2008). A Bangladeshi randomised control trial found that maternal DHA supplementation from 25 weeks gestation until delivery had no effect on infant BSID-II mental and psychomotor performance outcomes at 10 months (Tofail et al., 2006). However, it is possible that the control oil used by Tofail et al., (2006); (which contained α-LA: 2700
mg and LA: 2250 mg per day) may have inadvertently promoted infant neurocognitive status, thereby attenuating any treatment effect from the intervention.

A meta-analysis and systematic review of maternal ω-3 LC-PUFA supplementation undertaken by (Gould, Smithers, & Makrides, 2013) highlighted numerous potential areas of bias in the current literature and remarked on the relatively poor quality of most randomised control trials in this field. Of major concern was the consideration that publications seldom reported the randomization process and/or method(s) used to conceal treatment allocation from participants. According to Gould et al. (2013), the only randomised control trial of maternal DHA supplementation and infant neurocognitive outcomes that was considered to be genuinely free from bias was conducted in Australia. Makrides et al. (2010) found no significant association between moderate DHA supplementation (800 mg per day) during pregnancy and BSID-II scores of language or cognitive development at 2½ years (n = 2399). However, revealed that children in the treatment group manifested significantly lower incidence of cognitive delay compared to their un-supplemented counterparts.

In summary, randomised control trials and epidemiological studies evaluating the potential neurocognitive impact of maternal DHA supplementation during pregnancy have revealed somewhat heterogeneous results in healthy term infants. Consequently, it may be premature to make unequivocal recommendations about any neurocognitive benefits of DHA supplementation in healthy term infants based on the currently available research findings.

**CORRELATIONS BETWEEN INFANT NEUROCOGNITION AND BREASTFEEDING**

Over the years, many prospective observational studies have indicated that breastfed infants have a significant neurocognitive advantage over their formula fed counterparts (Agostoni et al., 2001; Anderson, Johnstone, & Remley, 1999; Kramer et al., 2008; Oddy et al., 2003; Oddy, Li, Whitehouse, Zubrick, & Malacova, 2011). It has been theorized that this is due to the higher presence of DHA in breast milk, relative to formula milks. Some studies have found positive associations between DHA concentrations within breast milk and/or infant blood levels and better outcomes of visual acuity (Innis, Gilley, & Werker, 2001; Jørgensen, Hernel, Hughes, & Michaelsen,
However, it is likely that breastfeeding enhances infant development due to a number of inter-related factors as reviewed in (Jain, Concato, & Leventhal, 2002).

Observational studies are potentially confounded by the heterogeneous composition of breast milk (both within and between lactating individuals), environmental factors such as maternal/infant bonding and other influences (Jain et al., 2002). One particularly significant potentially confounding factor is social economic status (SES), which is positively associated with both maternal and infant IQ, along with the decision to breastfeed (de Jager, Skouteris, Broadbent, Amir, & Mellor) (Der, Batty, & Deary, 2006). Furthermore, the breastfeeding act itself may be indicative of maternal attentiveness and nurturing which may independently foster long-term positive effects on infant neurocognitive outcomes (Morley, Cole, Powell, & Lucas, 1988). Despite appropriate statistical techniques used to try to control for the influence of confounders, observational studies can be subject to systematic bias and results should therefore be interpreted with some caution.

A study by (Caspi et al., 2007) tested the association between breastfeeding and child IQ with respect to FADS2 genetic profile, specifically in terms of the SNP rs174575. An interaction effect was reported, whereby, children who were breastfed as infants and rs174575 major allele carriers achieved higher scores on standardized IQ tests compared to the major allele carriers who were not breastfed. Meanwhile, those homozygous for the minor allele were found to have similar IQs, irrespective of feeding method. These findings remained statistically significant after accounting for potential confounding variables including intrauterine growth, family social economic status, and maternal cognitive ability. While potentially important in terms of the possible interaction between breastfeeding and the genetic status of the infant, these findings have yet to be replicated by other research studies.

**Correlations Between DHA Status and Infant Neurocognition**

Many trials have found that higher plasma or erythrocyte DHA concentrations (often as a result of LC-PUFA supplementation) are positively correlated with infant neurocognitive outcomes (Agostoni et al., 1997; Agostoni, Trojan, Bellu, Riva, & Giovannini, 1995; E. Birch, Garfield, Hoffman, Uauy, & Birch, 2000; Drover et al., 2011;
Gibson, Neumann, & Makrides, 1997; I. Helland et al., 2003; Innis, 2003; Innis et al., 2001; Jensen et al., 2005). Some studies, on the other hand, have found no significant relationships (Auestad et al., 2001; Lucas et al., 1999; Makrides, Neumann, Simmer, & Gibson, 2000), while conversely two studies have found that higher infant DHA blood concentration has a negative neurocognitive effect (Lauritzen, Jorgensen, Olsen, Straarup, & Michaelsen, 2005; Scott et al., 1998). It should be cautioned that associations between DHA status and infant neurocognitive status do not necessarily demonstrate causality, nor do they demonstrate the effectiveness of the intervention alone – as this may be confounded by other nutrients (Innis, 2003).

**RANDOMISED CONTROL TRIALS OF LC-PUFA IN TERM AND PRETERM INFANTS**

In order to confirm whether dietary LC-PUFA is responsible for the enhanced neurocognitive outcomes associated with breastfeeding and higher DHA status, randomised control trials of LC-PUFA supplementation are necessary. Several of these trials have been conducted, usually in formula-fed infants. The most common methodology involves comparing the neurodevelopmental outcomes of infants randomized to receive infant formula with DHA (either alone or in combination with AA and/or other PUFAs) or placebo (un-supplemented formula).

The majority of trials in healthy term infants have shown little or no consistent, beneficial effects on neurocognitive outcomes as a result of dietary LC-PUFA supplementation. However, infant LC-PUFA supplementation has resulted in no negative effects on growth, development or morbidity (Koletzko et al., 2005). There is, therefore, currently no compelling argument either for or against LC-PUFA supplementation in term infants with respect to neurocognitive outcomes.

This conclusion has been re-iterated in three consecutive versions of the Cochrane review (Simmer, 2001; Simmer, Patole, & Rao, 2008, 2011) that have evaluated 9, 14 and 15 relevant randomised control trials, respectively. Interestingly, in the authors’ conclusions for both the (2008) and (2011) Cochrane reviews, Simmer et al. refers to the positive results found by the Dallas group (E. Birch et al., 2005) and state that these results need to be replicated in other settings. These authors also propose that in the future randomised control trials should explore the use of DHA derived from single cell
microalgae and supply higher doses of DHA, in line with typical human milk concentrations throughout the world (DHA 0.32%).

(Hoffman, Boettcher, & Diersen-Schade, 2009) reviewed 20 randomised control trials within this field including several studies not included in the Simmer et al. (2008) Cochrane review and elaborated on some methodological factors including dosage, source and duration of supplementation. (Hoffman et al., 2009) concluded that trials which supplied term infants with DHA in concentrations greater than 0.3% total FAs (in addition to AA>0.3% total FAs) were more likely to identify a significantly positive effect on neurocognitive and visual outcomes.

In another meta-analysis utilizing individual patient data with a considerable sample size (n = 870), (Beyerlein et al., 2010) combined the raw scores from four methodologically similar randomised control trials (Bouwstra et al., 2005; Fewtrell et al., 2004; Fewtrell et al., 2002; Lucas et al., 1999) which each assessed BSID-II outcomes at 18 months. The analysis concluded that LC-PUFA supplemented formula conveys no significant benefit on neurodevelopment at 18 months, as assessed using the BSID-II. However, this meta-analysis was unable to access individual patient data from all relevant trials (E. Birch et al., 2000; Clandinin et al., 2005).

Evidence that infant DHA supplementation conveys significant benefit on visual acuity is derived from a systematic review of 12 clinical studies from the Harvard School of Public Health (SanGiovanni, Berkey, Dwyer, & Colditz, 2000). (SanGiovanni et al., 2000) incorporated the results from both randomized and non-randomized studies of DHA supplemented formula and concluded that increased dietary DHA improved visual acuity in term infants at 2 and 4 months of age. It should be cautioned that analyses that combine findings from both randomized and non-randomized trials have a higher risk of incorporating selection bias in the recruitment for the trial (Szajewska, 2011).

More recent reviews in this field suggest that more research is required before definitive recommendations can be made concerning whether term infants would benefit from ω-3 LC-PUFA supplemented formula (Agostoni, 2008; Belkind-Gerson, Carreón-Rodríguez, Contreras-Ochoa, Estrada-Mondaca, & Parra-Cabrera, 2008;
The majority of randomised control trials of infant DHA supplementation (as described above) use infant formula and are constrained by the consideration that their study samples have chosen not to breastfeed, thereby reducing external validity of the findings (Gibson & Makrides, 1998; Smithers, Gibson, McPhee, & Makrides, 2008a). There have been a small number of studies which have supplemented infants with DHA directly, thereby bypassing the need to employ formula-based supplementation. To the best of our knowledge, only one such direct supplementation randomised control trial has addressed the effect of DHA on infant neurocognitive functioning (Meldrum et al., 2012).

In the recent double-blinded, randomised control trial conducted in Australia (Meldrum et al., 2012), healthy term infants (n = 287) were randomized to either very high dose fish oil (incorporating 250 – 280 mg DHA plus 60 – 110 mg EPA) or placebo (olive oil) per day from birth to 6 months. The study determined that while infants within the fish oil group had significantly higher DHA concentrations in erythrocyte membranes and plasma phospholipids at 6 months of age relative to the placebo group, there was no significant difference between standard or composite scores of the BSID-III (third edition) at 18 months or on outcomes from the Child Behavior Checklist.

In a subtest which explored the development of infant communication skills (n = 185), Meldrum et al., (2012) found that scores for later developing gestures and total number of gestures were significantly higher in the fish oil group compared to the placebo group at both 12 and 18 months. This finding is interesting since gestural skills in infancy are associated with visual recognition memory, deferred imitation and turn-taking skills (Heimann et al., 2006). Furthermore, these skills are understood to predict language and communicative ability in later life (Acredolo & Goodwyn, 1988).

Direct supplementation of the oil emulsion allowed participation of breast- and/or formula-fed infants alike. However, a potential criticism of this study is that the odour of the fish oil may not have been adequately masked, and therefore parents were
frequently able determine which treatment their child was receiving. This, in turn, may have affected parents’ ratings of their child’s gestural abilities.

While the focus of the current paper is on term infant neurocognitive response to DHA supplementation, valuable information can be gleaned from investigation into the preterm population. Preterm infants are especially vulnerable to DHA deficiency as they have not had access to maternal lipid stores for the normal period of gestation (Haggarty, 2002). Similar to full term infants, preterm infants fed formula milk without DHA have lower DHA status compared to those fed human milk (S. Carlson, Rhodes, & Ferguson, 1986) or LC-PUFA supplemented formula (Koletzko, Schmidt, Bremer, Haug, & Harzer, 1989; Lapillonne et al., 2000).

It has been identified that children born preterm have higher rates of learning disabilities, language impairment, attention deficits, hyperactivity and reduced cognitive test scores compared to (gender- and age-matched) children born at term (Bhutta, Cleves, Casey, Cradock, & Anand, 2002; Perricone & Morales, 2011). Although there are many factors associated with preterm delivery, it is possible that lower DHA status during the critical period of brain growth may contribute towards impaired neurocognitive development (McNamara & Carlson, 2006).

The DINO (DHA infant neurodevelopmental outcomes) double-blind randomised control trial provided supplementation to lactating mothers (n = 657) in order to increase breast milk DHA (Makrides et al., 2009). Lactating women were either supplied 6 × 500 mg DHA-rich tuna oil capsules per day or placebo soy oil capsules. Mothers were encouraged to breastfeed; however, if the mother chose not to provide breast milk or if additional milk was required, infant formula was provided. The DHA concentrations of the formula matched the typical milk DHA concentrations of the two groups.

The DINO study found that preterm infants in the DHA group had better visual development – as determined through sweep visual evoked potential acuity at 4 months corrected age (i.e. chronological age corrected for the degree of prematurity); (Smithers, Gibson, McPhee, & Makrides, 2008b). A higher mean mental development index of children in the DHA supplemented group (as assessed using the BSID-II) was
found, however this benefit was not statistically significant. Yet the number of children in the DHA group with low cognitive scores (indicative of mildly delayed development) was significantly smaller in the treated group compared with the control group.

In pre-planned secondary analyses, the DINO study found DHA supplementation had a significantly positive effect on cognitive outcomes in girls compared to boys. The reason for differences in response to DHA as a function of gender remains unclear but may be related to the higher rate of LC-PUFA synthesis that has been identified in females (Burdge & Nagara, 2002). The authors proposed that the dose of DHA chosen for this study may not have been sufficient to elicit equivalent neurocognitive benefits in males and they considered whether enhancing DHA concentrations may evoke neurocognitive advantages (Makrides et al., 2009). Furthermore, the authors concluded that the DHA concentration in standard human breast milk of Australian women is sub-optimal for the visual development of preterm infants (Smithers et al., 2008b).

There have been numerous LC-PUFA supplementation trials in preterm infants which have reported greater visual acuity following enhanced dietary DHA relative to placebo (D. Birch, Birch, Hoffman, & Uauy, 1992; S. Carlson, Werkman, Rhodes, & Tolley, 1993; Smithers et al., 2008b). Similarly, numerous randomised control trials in preterm populations have found that DHA supplementation positively affects neurocognitive outcomes including language comprehension (O'Connor et al., 2001), memory (Henriksen et al., 2008) and mental and psychomotor development (Clandinin et al., 2005).

However, the review by (Qawasmi, Landeros-Weisenberger, Leckman, & Bloch, 2012) points out that there are also many randomised control trials which have consistently shown no neurocognitive effect, both in preterm and term infant populations. Similarly, (Schulzke, Patole, & Simmer, 2011) concluded that there is insufficient evidence to recommend DHA supplementation with respect to routine neonatal care in preterm infants. The most recent Cochrane review of LC-PUFA supplementation of preterm formula concludes that there are no clear long-term benefits on visual or intellectual development after pooling data across studies of preterm infants (Schulzke et al., 2011).
Finally, two recently published reviews reflect uncertainty as to whether preterm infants should be supplemented with dietary DHA with respect to potential long-term neurocognitive or developmental benefits (Lapillonne, Groh, Lozano Gonzalez, & Uauy, 2013; Molloy, Doyle, Makrides, & Anderson, 2012). Further investigation is clearly warranted concerning the role of DHA supplementation in modulating optimal neurocognitive outcomes in preterm infant populations.

**FADS1 and FADS2 Polymorphisms Modulate Dietary Requirements**

A relatively new area of investigation concerns how genetic differences may modulate individual LC-PUFA requirements. Such research has focused on the genes FADS1 and FADS2. These genes are known to act upon the enzymes delta-5 desaturase (Δ5D) and delta-6 desaturase (Δ6D) and influence the efficiency with which shorter chain ω-3 and ω-6 PUFAs are converted into LC-PUFA products (Bokor et al., 2010; Glaser et al., 2011; Nakamura & Nara, 2004; Rzehak et al., 2009; Schaeffer et al., 2006).

SNPs within FADS1 and FADS2 have been associated with the ratio of desaturation precursors (i.e. α-LA, LA, eicosadienoic acid (EDA; 20:2 ω-6) and dihomogamma linolenic acid (DGLA; 20:3 ω-6) to desaturation products, including the LC-PUFAs (EPA, DPA and AA) (Gillingham et al., 2013; Schaeffer et al., 2006). In a very large German study conducted in an adult population, the authors found that up to 28% of AA variability was associated with 11 common SNPs (and 5 SNPs of reconstructed haplotypes) from the FADS1 and FADS2 gene clusters (Schaeffer et al., 2006). Yet, the FADS polymorphisms tested by Schaeffer et al. (2006) could only explain approximately 7% of EPA and 3% of DHA levels.

Few subsequent studies have been able to identify a significant association between FADS polymorphisms and DHA concentrations in human populations (Koletzko, Lattka, Zeilinger, Illig, & Steer, 2011; Lattka et al., 2012). However, Koletzko et al. (2011) identified significant associations between foetal and maternal FADS genotypes and DHA concentrations in foetal circulation, irrespective of maternal diet. While it currently appears that DHA concentrations are primarily modulated through dietary supply, several FADS genetic polymorphisms are still under investigation.
INTERNATIONAL INCONSISTENCY

Inconsistent findings within this literature have been the subject of much consideration. Suggestions as to why discrepancies may have occurred are outlined in the review published by (Meldrum, Smith, Prescott, Hird, & Simmer, 2011). Previous studies were identified as having considerable variability in inadequate sample sizes, doses of DHA utilized, source of DHA used (e.g. from fish, algae, krill or egg), age at which supplementation was initiated, duration of supplementation, type of neurodevelopmental assessments used to evaluate neurocognitive functioning and variability in participant compliance across studies. It was also speculated in this review - that genetic polymorphisms might represent a potentially relevant factor affecting the outcomes of these randomised control trials. It is now recognized that dietary requirements for DHA and other LC-PUFAs may be somewhat heterogeneous across individuals; i.e. dependent on an individual’s genetic profile (Koletzko et al., 2011).

In addition to FADS genetic considerations, the lack of consistency across randomised control trials of DHA supplementation may also be due, at least in part, to gender based differences in LC-PUFA metabolism from shorter chain precursors (Guesnet & Alessandri, 2011). It is known that the capacity to convert α-LA into ω-3 LC-PUFAs including DHA is significantly greater in females than in males (Burdge, 2004), resulting in higher DHA circulating plasma concentration in females (Giltay, Gooren, Toorians, Katan, & Zock, 2004). This is thought to be due to the influence of oestrogen and other hormones on the activity and expression of ∆5D and ∆6D in the liver (Decsi & Kennedy, 2011; Extier et al., 2010).

Evidence from the Western Australian pregnancy cohort (RAINE) study (n = 1038) has also found long term neurocognitive benefits from breastfeeding to be gender specific (Oddy et al., 2011). This study found that breastfeeding had a more pronounced neurocognitive benefit on male infants, as evident until 10 years of age (Oddy et al., 2011). A gender specific response to DHA enriched human breast milk was also

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observed by (Makrides et al., 2009) in the aforementioned DINO study of preterm infants. Females manifest a greater capacity for LC-PUFA synthesis (Burdge & Nagura, 2002), such that males may have higher dietary DHA requirements during infancy (Makrides et al., 2009).

**CONCLUSIONS AND FUTURE DIRECTIONS**

DHA is known to play a critical role in the developing human brain and there is evidence of its neurobiological importance during the brain growth-spurt (McCann & Ames, 2005). DHA deficiencies in animals during this window of development cause deleterious consequences on a range of neurodevelopmental outcomes, particularly in the realm of problem behaviours, Attention Deficit Hyperactivity Disorder (ADHD) (McNamara & Carlson, 2006). High intake of oily fish during pregnancy appears to benefit children’s neurocognitive development (Hibbeln et al., 2007). Additionally, several studies have demonstrated positive associations between infant blood concentrations of DHA and neurocognitive status (Agostoni, Riva, Trojan, Bellu, & Giovannini, 1995; Boucher et al., 2011; Innis et al., 2001; Jacobson et al., 2008; Krabbendam, Bakker, Hornstra, & van Os, 2007; Ryan & Nelson, 2008).

Yet, previous randomised control trials undertaken in this field provide conflicting evidence concerning the putative neurocognitive benefits of ω-3 LC-PUFA supplementation in healthy full term infants. Despite the consideration that the majority of randomised control trials report little or no effect from supplementation (as cited in several Cochrane reviews and meta-analyses), many researchers suggest that further work should be undertaken in order to better define optimal DHA intakes before and during infancy.

It is possible that DHA deficiency during critical periods of brain growth and structural organization may render the brain vulnerable to neurological or neurodegenerative diseases later in life (Farquharson et al., 1995). The development of the brain’s architecture prenatally and during infancy lays down the foundation on which the structure of the adult brain is based. It is therefore possible that early modification of LC-PUFA levels will have long-term structural and functional consequences which may
be too long-term and/or subtle to detect in healthy infants and children (McNamara & Carlson, 2006). However, more noticeable effects may emerge in older individuals.

While randomised control trials are generally thought to provide the most robust indexes of causal mechanisms in human clinical research (Szajewska, 2011), the results from observational studies in humans and intervention studies in laboratory animals also deserve serious consideration. In future, larger and higher dose randomised control trials should be undertaken using more sensitive measures of infant and child neurocognitive capacity. A more careful approach to the design and analysis of observational epidemiological studies is also likely to yield tangible benefit (for example, through undertaking larger studies which are able to consider a wider range of potentially confound variables).

It would be of far-reaching benefit for more international collaboration to be undertaken within this area of inquiry. Data access facilitated through computational platform/s that enable sharing of de-identified, individual patient data (IPD) could provide researchers with the means to carry out statistically novel methods of data interpretation on a very large scale. Meta-analysis of individual patient data allows researchers to analyse the raw data of relevant original studies (using similar covariates) and thereby derive potentially more reliable conclusions (Stewart & Parmar, 1993).

To the best of our knowledge, only one individual patient data meta-analysis on the neurocognitive effects of LC-PUFA supplementation during infancy has been conducted (Beyerlein et al., 2010). Beyerlein et al., (2010) were able to access the raw data from 4 out of the 6 relevant randomised control trials they identified. One of this biggest barriers to performing individual patient data meta-analyses is obtaining (usually not-publically available) raw data from relevant randomised control trials. It would be invaluable if guidelines and policies are implemented within a collaborative international scientific framework that is more sensitive to ethico-legal and data-ownership issues.

Despite the absence of a scientific consensus with regard to putative benefits (as identified in this review), most manufacturers of infant formula offer DHA and AA in
certain formulations and market them as superior products that can enhance neurocognitive growth and development (Simmer et al., 2011). This shaping of public opinion through retail is controversial, considering that there is little concrete scientific evidence to support these claims. Australia is one of the leading markets for ω-3 products and supplements (McManus, Merga, & Newton, 2011). Consequently, consumers are at increased risk of being misled by current marketing approaches. It is important for future research to address these claims definitively, so as to either avoid unnecessary supplementation of infants’ diets (and the associated economic cost) and to ensure that infants are universally provided with adequate dietary DHA to prevent suboptimal neurocognitive development.
CHAPTER 2

THE 6 YEAR FOLLOW-UP: RATIONALE, HYPOTHESES AND AIMS
Rationale

Modern dietary habits, together with prevailing methods of food production have led to widespread decline in the consumption of dietary omega (ω)-3 fatty acids and concurrent increase in ω-6 fatty acids (Brenna & Carlson, 2014). While the extent and consequences of dietary ω-3 fatty acid deficiency are still not fully understood, it is widely presumed that ω-3 long-chain polyunsaturated fatty acids (LC-PUFA) deficiency contributes to the aetiology of “Western lifestyle” diseases, including some common neurological and mental health problems (Bradbury, 2011). Furthermore, there is reason to believe that modern human diets may provide insufficient amounts of docosahexaenoic acid (DHA) in order to support optimal neurocognitive development during infancy (Muskiet et al., 2006). In response to growing concerns by the public on these issues, fish oil supplements along with various ω-3 LC-PUFA fortified-foods are popular in Australia (McManus et al., 2011; Mellentin, 2008) and marketed widely for their ability to improve early neurocognitive health (Adarme-Vega, Thomas-Hall, & Schenk, 2014; Pushpangadan et al., 2014).

Evidence linking ω-3 LC-PUFA with neurocognitive/behavioural development comes from a range of testimonies. Several large observational studies have reported positive associations between fish intake during pregnancy/lactation and long-term offspring intelligence (Hibbeln et al., 2007; Oken et al., 2008). Furthermore, positive associations between blood DHA status and better cognitive and visual function in healthy children born to mothers with perinatal fish oil supplementation have been observed (for review see: (Muskiet, Fokkema, Schaafsma, Boersma, & Crawford, 2004). Moreover, observational research has consistently reported breastfed individuals display enhanced neurocognitive development compared to their unsupplemented-formula fed counterparts who did not receive dietary ω-3 LC-PUFA (Marszalek & Lodish, 2005). It should be noted that, while such association studies cannot demonstrate causality, further support of this relationship comes from reports that the neurocognitive benefit associated with breast milk is modulated by a gene-diet interaction – specifically involving the fatty acid desaturase (FADS) genes regulating endogenous LC-PUFA synthesis (Caspi et al., 2007; Steer, Smith, Emmett, Hibbeln, & Golding, 2010). However, while it is possible that some infants could receive significant benefits from
increased dietary supply of ω-3 LC-PUFA, the efficacy of supplementation overall has not yet been ascertained.

While there appears to be an intrinsic link between ω-3 LC-PUFA and neurocognitive development, numerous randomised controlled trials comparing preterm and term infant formulas with and without LC-PUFA have been unable to demonstrate the effectiveness of the intervention. Some have found evidence in favour of the inclusion of LC-PUFA within infant formula, whereas others have found no significant group differences regarding neurocognitive or behavioural outcomes (Simmer et al., 2011). These randomised controlled trials were mostly restricted to formula fed infants and do not allow for the direct supplementation of healthy term infants receiving breast milk. They may therefore have limited external validity with regard to the well-educated, high-income population who are less likely to use infant formula exclusively for the first 6 months. This is because well-educated, high-income parents are generally more informed about the benefits of breastfeeding and/or may be in a better position to continue breastfeeding for the first 6 months (i.e. in terms of employment type, work environment and access to resources). Studies incorporating this population subset are most relevant as they are more likely to purchase fish oil supplements.

The present study embodies a novel design, enabling high dose ω-3 LC-PUFA or placebo supplementation of healthy term born infants as an adjunct to their usual milk diets from birth to 6 months. In an earlier follow-up of the Infant Fish Oil Study (IFOS), this research team previously reported that fish oil supplementation had no significant effect on global infant neurocognitive development at 18 months when compared to control infants, as assessed through the Bayley Scales of Infant and Toddler Development – 3rd Edition (Meldrum et al., 2012). However, the Macarthur–Bates Communicative Development Inventory indicated some positive effects in terms of the complexity of developing gestures and the total number of gestures. In addition, we found higher anxiety and depression in the fish oil group – as reported via the Achenbach Child Behavior Checklist (CBCL).

For the 6 year follow-up of the IFOS cohort, presented in this thesis, we were interested in evaluating whether these encouraging effects on communication at 18
months of age persisted and/or whether the preliminary behavioural outcomes at this age were still contraindicated by fish oil supplementation. Also, based on the evidence from previous literature as well as the inerrant social interest in cognitive-enhancement, we aimed to include an evaluation of IQ and several other neurocognitive and behavioural outcomes using the best tests/assessments available.

IFOS commenced in 2005 with the goal of investigating the early immunological and neurocognitive effects of high-dose fish oil. Organisation of the present 6 year follow-up was favourable, by virtue of the fact that the IFOS cohort had previously been recruited, intervention completed and a wealth of information (and most notably, blood samples) already collected on these participants. Further support for the present study comes from the availability of specialised knowledge and expertise already within the research team, good communication with compliant families in the cohort, access to the required research tools and infrastructure in addition to the pivotal fact that the participating children had now reached an age whereby neurocognitive assessment is more reliable (Cheatham, Colombo, & Carlson, 2006; Makrides et al., 2014). Moreover, few trials of infant ω-3 LC-PUFA supplementation have explored the long-term effects beyond approximately 4 years of age.

The 6 year follow-up has been designed to overcome some of the important limitations of previous trials by choosing specific neurodevelopmental tests to maximise the likelihood of detecting significant differences between the groups. As the healthy, term-born participants of the present study are expected to perform within the normal range, the battery of neurocognitive assessments and behavioural questionnaires were suited to detect subtle differences in skills rather than just the identification of deficit. The tests included have a high ceiling level of function such as the gifted rating scale (GRS). The study also includes several non-standardised tests to examine high-level executive function and cognitive skill (in addition to well established tests of child development such as the Wechsler Abbreviated Scale of Intelligence, i.e. WASI). Unlike most standardised psychometric assessments, these non-standardised tests were not developed for the purpose of diagnosing mental delay. It is predicted that the assessments that comprise the battery of tests used in
this 6 year follow-up will detect subtle neurocognitive developmental differences with statistical significance.

Specifically, we aimed to evaluate four primary areas of neurocognitive development: language, executive functioning, global IQ and behaviour. At the time of planning the 6 year follow-up, we expected that language and communication at 6 years would be better amongst the children from the fish oil group, given our previous positive findings pertaining to gestural skills at 18 months (Meldrum et al., 2012) – since early gestural skills are associated with child vocabulary (Iverson & Goldin-Meadow, 2005). Evaluation of executive functioning, including working memory was based on our understanding of how DHA interacts within neural tissue (i.e. affecting physicochemical processes that underlie hippocampal- and frontal cortex-based cognition). We also assessed global measures of intelligence since it has been proposed that children who receive higher DHA during development display higher IQ (Hibbeln et al., 2007). We were also keen to assess these participants for a range of behavioural outcomes since controversial and conflicting reports exist as to whether ω-3 LC-PUFA affects behavioural outcomes.

The availability of blood samples enabled us to extend our area of study enquiry beyond just the intervention, which was providing ω-3 LC-PUFA supplementation during infancy and evaluating the effects in childhood. Obtaining a biological marker of fatty acid status was ideal for this study, since DHA status in peripheral blood may serve as a proxy for the DHA status in the brain (Létondor et al., 2014). Fatty acid analysis of peripheral blood collected longitudinally (at birth, 6 months and around 6 years) offers insight as to the bioavailability of DHA in the brain during these key time points of early development. Together, this information was useful to assess whether or not there is a causal link between fish oil supplementation during infancy and neurocognitive / behavioural development. This research strategy should overcome discrepancies of previous research.

Also by virtue of previous blood collections, we were able to perform DNA extraction from available samples. This way each child was genotyped for polymorphisms within the FADS gene cluster. Based on previously published literature we expected that there may be certain subgroups who would be more responsive to ω-3 LC-PUFA
supplementation (Al-Hilal et al., 2013). Thus, the IFOS 6 year follow-up study aimed to consider an important aspect thus far missing in the debate regarding the utility of ω-3 LC-PUFA supplementation during infancy; that is the gene-diet interaction modulating an individual’s dietary requirements. By including FADS gene variants within our analysis, we sought to provide additional insights into the differential effects of genetic subgroups on dietary modification of LC-PUFA and in turn, the role of ω-3 LC-PUFA in neurocognition and behaviour. Such findings may be encouraging of a more targeted approach to fish oil supplementation in line with personalised requirements. Furthermore, this could justify the need (or otherwise) for blanket fish oil supplementation in healthy Western infants’ diets.
Hypotheses

The IFOS randomised controlled trial was originally designed to test the hypothesis that high dose ω-3 LC-PUFA supplementation during infancy will increase the systemic concentrations of DHA and EPA and therefore reduce the risk of allergic disease and improve neurocognitive developmental outcomes.

The 6 year follow up meets the need for well-designed randomised controlled trials to critically evaluate whether supplementation of healthy term-born infants with ω-3 LC-PUFA affects later neurocognitive- and behavioural development.

In the current 6 year follow-up study of IFOS participants, we propose three overarching hypotheses:

I. Children who received dietary fish oil supplementation during infancy will display enhanced language, executive functioning and global IQ at 6 years compared to children within the control group. This will be addressed in Chapter 4.

II. Circulating ω-3 LC-PUFA (specifically, DHA) status at birth, 6 months and 5 years will be positively correlated with language, executive functioning, global IQ and behaviour at 6 years of age. This will be addressed in Chapter 5.

III. Major FADS allele carriers (associated with high endogenous LC-PUFA synthesis) are expected to achieve better neurocognitive developmental outcomes, i.e. language, executive functioning, global IQ and behaviour at 6 years compared to minor allele carriers. This effect may be further enhanced by fish oil supplementation due to a gene-diet interaction. If the high LC-PUFA status of major allele carriers is boosted further by fish oil supplementation, a significant gene-diet effect may immerge favouring neurocognitive/behavioural development. Conversely, we expect poorer neurocognitive/behavioural scores from minor allele carriers in the placebo group (as these participants are more likely to have sustained DHA deficiency. This will be addressed in Chapter 6.
Chapter 2

Aims

I. To examine the effects of ω-3 LC-PUFA supplementation during infancy (i.e. from birth to 6 months) on language, executive functioning, global IQ and behaviour at 6 years of age.

II. To examine whether ω-3 LC-PUFA status at birth, 6 months or 5 years is associated with language, executive functioning, global IQ and behaviour at 6 years.

III. To examine the effect of FADS1 and FADS2 polymorphisms on language, executive functioning, global IQ and behaviour at 6 years and to examine whether the variations within the FADS gene cluster modulate the effect of fish oil supplementation.
CHAPTER 3

MATERIALS AND METHODS
STUDY POPULATION

A total of 420 pregnant women were recruited during their third trimester (between 28 and 40 weeks gestation) for the Infant Fish Oil Supplementation Study (IFOS). Recruitment occurred between June, 2005 and October, 2008. The women were recruited from public and private obstetric/antenatal services in the Perth metropolitan area in Western Australia. Approval was obtained from the Princess Margaret Hospital Research and Ethics Committee as well as the University of Western Australia Human Research Ethics Committee.

CRITERIA FOR ELIGIBILITY

Eligible participants had a history of asthma, allergic rhinitis or eczema, in addition to a confirmed-positive skin prick test (wheal diameter >2 mm) to at least one allergen extract (cat, rye, cow’s milk, egg, house dust mite, grasses, moulds, peanut, histamine, dog, cockroach, feathers; Hollister-Stier Laboratories). In the absence of maternal allergy, women were also eligible if they had another child formally diagnosed (by a medical professional) with an allergic disease. Women were ineligible for the study if they consumed fish oil during pregnancy (≥ 1000 mg per day), typically ate more than three fish-meals per week, or, if they engaged in smoking or illicit drug use during pregnancy. Infants diagnosed with major congenital or neonatal health abnormalities, requiring medical and/or surgical treatment were not eligible for continuation within the trial.

RANDOMISATION

Infants who remained eligible were randomised to either placebo or fish oil supplementation from birth until 6 months of age. The randomisation schedule was prepared by an independent biostatistician, and stratified according to maternal allergy (asthma versus other allergy), parity (first child versus two or more children) and paternal allergic disease (allergic versus non allergic). Two groups were created; a control group (n = 202) and a fish oil treatment group (n = 218); see Figure 3.1.
**Figure 3.1. IFOS design, data collection and participation from recruitment to the 6 year follow-up.**

**SUPPLEMENT AND DOSAGE INFORMATION**

Participants were randomised to receive either fish oil (650 mg), containing 250 – 280 mg of DHA and 60 – 110 mg of EPA or placebo oil capsules. The placebo capsules were image matched (identical in size, shape and colour) to the fish oil capsules and contained (650 mg) olive oil (432.9 mg oleic acid).

The concentration of oleic acid within placebo capsules was deemed insignificant considering the amount of oleic acid naturally occurring in breast milk (~1100 mg oleic acid per day) (CSIRO Division of Human Nutrition, 1996). On the other hand, the
amount of DHA provided by the fish oil capsules (~265 mg) was more than twice the amount of DHA typically found in breast milk (105 mg*).

It should be noted that breast milk samples from a small subgroup of IFOS mothers (Figure 3.1) were analysed for fatty acid composition. We were able to determine that the DHA levels in the breast milk of IFOS participants (111 mg†) was slightly higher than world wide levels. Based on our calculations, the breastfed infants in the treatment group therefore received ~376 mg of DHA per day (111 mg from breast milk + 265 mg from fish oil). However, due to the fact that capsule compliance ranged from 0 - 100 %, the actual intake of DHA amongst breastfed infants in the treatment group ranged from 111 mg to 390 mg. Breastfed infants in the placebo group received approximately 111 mg of DHA per day.

**Oral Administration Procedure**

Participants were instructed to give the capsules to the infants in the morning, prior to/during feeding. Capsules were designed to be punctured and the oil administered to the infant orally; either directly into the infants mouth or incorporated within the milk if bottle-feeding.

It should be noted that this specific methodology of direct oral administration is key and differentiates IFOS from the majority of other randomised control trials of postnatal ω-3 LC-PUFA supplementation. Thus, parents could choose to feed their infants any combination of breast milk and/or infant formula (be it standard and/or gold formulation) – without interrupting the study intervention (Figure 3.2.).

* Calculated based on the world wide average DHA (0.32% of total fatty acids in breast milk); (Brenna et al., 2007) assuming breast milk intake of 780 mL/day, containing 4.2% fat.
† Calculated from IFOS breast milk DHA at 3 months (0.34 ± 0.15) and 6 months (0.34 ± 0.22) assuming breast milk intake of 780 mL/day, containing 4.2% fat.
**Chapter 3**

**Figure 3.2. Participants diet during the first 6 months**

**Supplementation Period**

Treatment commenced at birth and ceased after 6 months. The duration of treatment coincided with the average age whereby milk (either formula or breast milk) functions as the primary energy source.

**Capsule Source**

The majority of infants enrolled in the study (n = 377) received capsules supplied by Ocean Nutrition, Canada Ltd., purchased in 2005. However, Ocean Nutrition discontinued their product before the trial’s recruitment phase had concluded. Subsequently, Nu-mega, Australia donated the capsules provided to the final 43 infants enrolled in the study. Ethical approval regarding the substitution of the capsule supplier was obtained from the Princess Margaret Hospital Ethics Committee.

The Ocean Nutrition capsules and the Nu-mega capsules contained comparable doses of DHA (280 mg, 250 mg) and EPA (110 mg, 60 mg respectively). The DHA and EPA were formulated as ethyl esters in the Ocean Nutrition capsules and triglycerides in the Nu-mega capsules. Similarly, the placebo capsules both contained 650 mg olive oil (66.6% oleic acid). There was no significant difference between the brands in terms of the mean erythrocyte DHA concentrations ($P = 0.732$) at 6 months of age or allergy
prevalence. Subsequently, all children, irrespective capsule supplier were included in the final analyses.

**PURITY AND QUALITY OF OIL CAPSULES**

The fish oil had been molecularly distilled to remove heavy metals, dioxins, PCBs and other toxins to below detectable levels that are deemed safe for use in infants. Furthermore, capsules were tested for quality at regular intervals throughout the trial to confirm that the fatty acid composition remained constant and the percentage of rancid oil (peroxides and acid bi-products) remained within Australian health standards (Therapeutic Goods Administration, 2012). The analysis of capsule purity was conducted by an independent laboratory approved by the Therapeutic Goods Administration.

**TREATMENT BLINDING PROTOCOL**

The study was designed as a double blind randomised controlled trial, where both participants and staff were unaware of group allocation. As such, group allocation was concealed from both the participants and researchers until data collection for early immunological and language/development had been completed at 2 ½ years (this time point was specified at the commencement of the trial, and made known to participants).

Following this, unblinding of the group allocations occurred and participants were informed (by phone-call or letter) of their treatment allocation by a research assistant not involved in the study process. Participants were asked not to discuss their group allocation with other research staff. Subsequent follow-up studies of this cohort – (including the present study) have employed blind researcher(s) to conduct all follow-up assessments and manage the respective data.

Prior to the unblinding of participants at 2 ½ years, parents were asked to guess which group they believed they were in. Of the people who attended the 6 year follow-up; 92.8% of participants in the fish oil treatment group had correctly guessed their allocation, compared to 59.6% of the control group. Despite the use of peppermint flavouring in both oils, their guesses were primarily based on smell.
Chapter 3

After all 6 year assessments were completed, preliminary data analysis was conducted in a semi-blinded fashion whereby the groups were known as group A and group B, without knowing which group was the LC-PUFA supplemented group. Importantly, the researchers directly responsible for the 6 year follow-up remained blinded to the treatment groups until the final stage of data analysis.

**IFOS Cohort Data Collected Prior to 6 Years**

The IFOS study participants have been followed-up at regular intervals since their enrolment, for the purpose of investigating the effect of infant fish oil supplementation on numerous immunological, cardiovascular and neurocognitive/behavioural outcomes. The clinical assessments were conducted when children were three months, 6 months, 12 months, 18 months, 2 ½ years, 5 years and 6 years of age. Lists of the clinical information and biological samples collected at each visit are provided in Table 3.1 and information pertaining to neurocognitive and behavioural outcomes collected at earlier time points are illustrated in Figure 3.3. More information can be found in Appendix A.

![Flow of data collected about neurocognitive and behavioural outcomes prior to the 6 year follow-up.](image)

**Figure 3.3. Flow of data collected about neurocognitive and behavioural outcomes prior to the 6 year follow-up.**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment Group</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bayley Scales of Infant Development</td>
<td>138</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maccarther Language Assessment</td>
<td>125</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child Behaviour Checklist</td>
<td>91</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maccarther Language Assessment</td>
<td>94</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bayley Scales of Infant Development</td>
<td>149</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maccarther Language Assessment</td>
<td>144</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3

**Table 3.1. Infant Biological Samples Collected Previously**

<table>
<thead>
<tr>
<th>Group</th>
<th>Birth</th>
<th>3M</th>
<th>6M</th>
<th>12M</th>
<th>18M</th>
<th>2.5Y</th>
<th>5Y</th>
<th>6Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Umbilical cord blood</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast milk samples collected</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood collected</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine samples collected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saliva samples collected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**COMPLIANCE/ADHERENCE TO CAPSULES**

Parents were asked to record their daily capsule administration in a logbook and return any unused capsules to the pharmacy for counting purposes. The number of capsules consumed over the course of the trial was calculated based on an average of these measures.

**BIOLOGICAL INDICATOR OF COMPLIANCE**

Baseline levels of DHA in erythrocyte membranes were ascertained from samples of umbilical cord blood (n = 109). Venous blood was collected at 6 months of age (after supplementation period had concluded); and DHA levels were re-analysed. While there was no difference between the baseline DHA status of the two groups at birth, the treatment group showed significantly higher DHA after 6 months of fish oil supplementation (**Table 3.2.**). Comparison of fatty acid status between the two groups functioned as a biological indicator of compliance following supplementation. Additional data pertaining to ω-3 and ω-6 PUFA levels is available in **Appendix B**.

**Table 3.2. Comparison of DHA levels of control and treatment groups at birth, 6 months and 5 years: IFOS cohort**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CB -Erythrocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>57</td>
<td>7.44</td>
<td>1.44</td>
<td>.748</td>
</tr>
<tr>
<td>Treatment</td>
<td>52</td>
<td>7.36</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td><strong>6m -Erythrocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>71</td>
<td>6.22</td>
<td>1.59</td>
<td>.033</td>
</tr>
<tr>
<td>Treatment</td>
<td>68</td>
<td>6.83</td>
<td>1.74</td>
<td></td>
</tr>
<tr>
<td><strong>6m – Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>80</td>
<td>4.49</td>
<td>1.33</td>
<td>.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>65</td>
<td>5.35</td>
<td>1.60</td>
<td></td>
</tr>
</tbody>
</table>

* DHA levels given as a % of total fatty acids
PARTICIPANT RECRUITMENT

An information sheet inviting participants to attend the 6 year follow up was provided at the 5 year visit. For participants who did not attend the 5 year visit, the information sheet was mailed. Participants were contacted via telephone, text message, email and postal mail, according to their preference. In an attempt to maximize the trials validity, all participants – with current contact details available – were contacted between 5 and 6 years and invited to partake in the current neurodevelopmental follow-up. This gave those previously disengaged from the study the opportunity to clarify whether they intentionally withdrew from all components of the trial or had only wanted to quit the (fish oil/placebo) intervention. Subsequently, the total number of withdrawn participants went from 65 to 50. Once contact was made, most participants were willing to attend the 6 year follow-up appointment. The majority of parents were interested in receiving their child’s neurocognitive-developmental report. Thus, provision of each child’s neurodevelopmental report (post-assessment) likely incentivised participation and contributed to the good 6 year follow-up.

NEUROCOGNITIVE ASSESSMENTS

Two trained assessors administrated the battery of tests. Alexandra Heaton administered the majority of assessments (n = 300), initially assisted by Catherine Frogley, Bachelor of Psychology (Honours); (n = 15). All assessments and questionnaires were scored by Alexandra Heaton to ensure inter-rater reliability. Training in neurodevelopmental testing was provided by a team of accredited professionals, including a Neuropsychologist, a Clinical Psychologist and a Speech Pathologist (Jonathan Foster, Catherine Campbell & Suzanne Meldrum, respectively).

The present study aimed to assess the participating children using a battery of neurocognitive tests and questionnaires in order to evaluate various aspects of their neurocognitive functioning. The tests were chosen with a particular focus towards language and communication as these processes were identified in the investigation at 12 and 18 months. Meldrum et al., (2012) found children within the fish oil group
performed better with their later developing gestures ($P = 0.007; P = 0.002$, respectively) and the total number of gestures ($P = 0.023; P = 0.006$, respectively) compared with the placebo group. In keeping with other research in this field, various other facets of child neurocognitive development were also explored through the tests chosen: including IQ, working memory, behaviour, attention, and executive functioning (S. Carlson, 2009; Eilander, Hundscheid, Osendarp, Transler, & Zock, 2007; Innis, 2007).

**CONDITIONS OF 6 YEAR FOLLOW-up**

The assessments took place in a quiet clinic room at the Children’s Clinical Research Facility at Princess Margaret Hospital. The tests were delivered in the same order for each participant (in the order listed above), with a short break between the WASI and CELF administration. Stickers were used to reward the child at the end of each test.

**ASSESSMENT DURATION**

While there was some variation in the amount of time required to perform the assessment, the majority of assessments were completed in approximately 2 hours. At half-time, participants were encouraged to take a break, jump around, stretch their muscles or engage in some other form of light physical exercise within the facility. Where possible, the assessments were carried out at the same time of the day (approximately 9.30 am). However, if parents stipulated another time for their convenience, flexibility ensued.

**ETHICAL APPROVAL AND PARTICIPANT CONSENT**

The 6 year follow-up study was approved by the University of Western Australia and the Princess Margaret Hospital Ethics Committee. Informed consent was obtained from parents regarding parent questionnaires their child’s participation in the neurocognitive assessment. Approval to include two questionnaires for the school teacher was granted from the Western Australian Education Department on the basis of additional consent from the child, the child’s parents, the school principal and the school teacher.
STUDY PARTICIPATION AND WITHDRAWAL

At the 6 year visit, 335 infants were assessed for neurocognitive development, 156 from control group and 179 from fish oil group (see Table 3.2). Thus, out of the 420 participants initially recruited to IFOS, there was an 80% retention rate at the 6 year follow-up. Eighty-five participants did not participate in the 6 year follow-up due to reasons outlined in Table 3.3. Several participants could not be contacted to discuss the 6 year follow-up, despite approximately three phone call attempts, three text messages, a posted letter and two emails.

Table 3.2. Number of participants attending the 6 year follow-up from the fish oil treatment group and the control group.

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Treatment Group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attended</td>
<td>156</td>
<td>179</td>
<td>335</td>
</tr>
<tr>
<td>Did Not Attend</td>
<td>46</td>
<td>39</td>
<td>85</td>
</tr>
<tr>
<td>Total</td>
<td>202</td>
<td>218</td>
<td>420</td>
</tr>
</tbody>
</table>
Table 3.3. Number of participants who did not attend the 6 year follow-up and reasons for non-attendance across the fish oil treatment and the control groups.

<table>
<thead>
<tr>
<th>Reason</th>
<th>Control Group</th>
<th>Treatment Group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Busy with Family</td>
<td>7</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Health</td>
<td>4</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Moved</td>
<td>9</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Withdrawn/uncontactable</td>
<td>26</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
<td>39</td>
<td>85</td>
</tr>
</tbody>
</table>

**BATTERY OF ASSESSMENTS**

**Clinical Evaluation of Language Fundamental (CELF)**

The Clinical Evaluation of Language Fundamentals 4\textsuperscript{th} Edition (CELF-4) was designed to detect language disorders or developmental delay in students; aged 5 to 21 years (Semel, Wiig, & Secord, 2003). Two composite scores were obtained, a Core Language Score and a Working Memory Score, both of which were standardised according to age (mean: 100, SD: 15). The Core Language score was derived from 4 subtests; Concepts and Following Directions, Word Structure, Recalling Sentences and Formulating Sentences. Working memory was evaluated through the subtests, Number Repetition Forwards (assessing memory), Number Repetition Backwards (assessing high level executing thinking) and Familiar Sequences. Additional subtests that were not standardised according to age included Word Association (of the three categories; animals, foods and jobs) and Rapid Autonomic Naming (RAN). Administration of these nine CELF-4 subtests took approximately 50 minutes.

**Wechsler Abbreviated Scale of Intelligence (WASI)**

The Wechsler Abbreviated Scale of Intelligence (WASI) was designed as a short and reliable measure of intelligence for those aged between 6 and 89 years (Wechsler, 1999). It was administered in full at the 6 year follow-up. The WASI comprised of four subtests: Vocabulary, Similarities, Block Design and Matrix Reasoning. WASI raw scores for each subtest were converted into age adjusted T-scores to derive three main scores; Performance IQ, Verbal IQ and Full Scale IQ. These scores indicated how the individual performed in comparison to a normative population, 6 years and above (Axelrod, 2002). Administration of the WASI took approximately 50 minutes per individual.
**THE FRUIT STROOP TEST**

The Fruit Stroop test was designed to assess executive function, inhibition of response and working memory. Stroop tests typically provide individuals with words written in different colours. However, to overcome the limited reading ability of our 6 year old participants, the Fruit Stroop test was developed by the current investigators, (Appendix C) using previously established protocol (Archibald & Kerns, 1999). The test took the form of a 4 page booklet containing shapes and pictures in 4 colours. Across all pages, the pseudorandom order of colours was kept the same. Participants were required to correctly identify as many colours as they could within the given time period (45 seconds).

The first page contained rows of coloured rectangles. The second page contained the same colour-sequence; appropriated as pictures of fruit. The third page contained the same pictures of fruit except they were depicted in black and white; so participants needed to recall the colours accordingly. The final page contained the same sequence of fruits however, this time they were incorrectly coloured (e.g. purple banana) so participants needed to inhibit their automatic response to name the correct colour. The final score was calculated with the equation: 

\[
\frac{(Page 1 \times Page 3)}{(Page 1 + Page 3)} - Page 4.
\]

**THE SELF ORDERED POINTING TEST (SOPT)**

The SOPT was developed by the current investigators; based on previously established protocol (Cragg & Nation, 2007). The test was designed to assess memory; specifically, non-spatial and complex working memory in children aged 5 to 11 years of age. The test was made up of 4 separate items of increasing complexity. Items were comprised of a set of pictures of familiar objects neatly arranged in grids (Appendix D).

Participants were required to point to a different picture on each page so that by the end of the item, all pictures had been selected once. The relative position of pictures were pseudorandomly modified so that spatial location was not informative with consecutive pages. The task employed working memory because participants needed to remember which pictures in each item set they had already touched. The number of errors made (i.e. selecting a particular picture more than once) was subtracted from
There were no time restrictions, yet all participants completed the task in approximately 10 minutes.

**The Renfrew Bus Story Narrative Test**
The Renfrew Bus Story Test was designed to assess narrative or story-telling skills in children aged 3 – 9 years of age. Participants were told a narrative story by the examiner, accompanied with pictures and then required to immediately re-tell the story in conjunction with the same picture prompts. It obtained three scores based on: sentence length, sentence complexity and the quality of information corresponding to the original story line. The Renfrew Bus Story has been shown to be predictive of later language development and academic performance (Pankratz, Plante, Vance, & Insalaco, 2007; Stothard, Snowling, Bishop, Chipchase, & Kaplan, 1998). The test required approximately 7 minutes to administer.

**Questionnaires**
The three parent questionnaires and two teacher questionnaires were mailed to participants approximately one week prior to the appointment. Parents were asked to complete their questionnaires and bring them on the day of the assessment. Parents were also asked to give the teacher-questionnaires to the principal of their child’s school once they had explained the purpose of the teacher-questionnaires to their child and obtained their consent. The school principal and the school teacher were informed about the study and invited to sign their appropriate consent forms. The teacher was responsible for returning the two questionnaires and consent documents using supplied pre-paid envelope, addressed to the PhD student at the School of Paediatrics and Child health.

**Autism Spectrum Quotient: Children’s Version (AQ-Child)**
The parent-questionnaire, Autism Spectrum Quotient: Children’s Version (AQ-Child) evaluated autism-spectrum-like traits in children 4 to 11 years of age (Auyeung, Baron-Cohen, Wheelwright, & Allison, 2008). The three-page questionnaire contained 50 statements concentrating on areas such as social interaction, communication, behavioural flexibility, attention switching, attention-to-detail, communication, and imagination. Parents were able to quantify how well each statement characterised
their child using a four-point Likert scale ranging from “definitely agree” to “definitely disagree.” AQ-Child scores below 70 were considered to be within the normal range. The AQ-Child questionnaire took approximately 10 minutes for parents to complete.

**THE CHILDREN’S COMMUNICATION CHECKLIST (CCC-2)**

The CCC-2 questionnaire identifies language impairment, pragmatic impairment, communication problems, and indicates whether autistic-like traits may warrant further investigation. It is applicable to children aged 4 – 16 years of age. At the 6 year follow-up, parents completed the CCC-2 questionnaire. General Communication Composite (GCC) scores were calculated, which identified children likely to have clinically significant language problems. The GCC composite scores were derived from 10 sub-tests; (A: Speech, B: Syntax, C: Semantic, D: Coherence, E: Inappropriate initiation, F: Stereotyped language, G: Use of context, H: Use of non-verbal communication, I: Social relations and J: Interests). The highest score possible is 160 and lowest is 40. Scores between 85 and 115 are considered average, however scores below 59 are considered to be below the normal range.

The CCC-2 questionnaire also provided a Social Interaction Deviance Composite (SIDC) – identifying children with a communicative profile similar to autism. The SIDC was calculated by summing the scales E - J, and then subtracting scales A - D, to give an index of discrepancy between overall language abilities and pragmatic/social skills. The SIDC was not reported to the parents as this score is more relevant in a clinical setting and unless the individuals GCC score was also outside the normal range, the SIDC has little practical relevance for non-autistic children of average communicative ability. The CCC-2 questionnaire took approximately 10 to 15 minutes to complete.

**THE CHILD BEHAVIOR CHECKLIST (CBCL) 6 – 18**

The CBCL was completed by parents in order to assess the competencies and problems of the child. It derived a Total Competence Scores based on parent reports of the child’s capacity and willingness to engage in various activities in social- and school settings. The Total Competence Score was normatively scaled to provide a Competence Scale T-score that indicating the degree to that the child is functioning, in comparison to others aged 6 – 18 years. The second component of the CBCL a series of
112 statements to respond to about their child on a three-point Likert scale (0 = Not True, 1 = Somewhat/Sometimes True, 2 = Very True/Often True). Each statement relates to one of eight Syndromes: I) Anxious/Depressed, II) Withdrawn/Depressed, III) Somatic Complaints, IV) Social Problem, V) Thought Problems, VI) Attention Problem, VII) Rule-Breaking Behaviour and VIII) Aggressive Behaviour. These scores are further computed to determine an Internalising Score (pertaining to problems within the self), Externalising score (pertaining to conflicts with others) and a Total Behavioural Score (for all problems reported). DSM-orientated profiles are also provided that can suggest a particular problem warranting further investigation, such as: Affective, Anxiety, Somatic, Attention Deficit/Hyperactivity, Oppositional Defiant and Conduct Problems. Raw scores from each of the syndrome scale and DSM-orientated score were converted into T-scores of which, scores below 65 were considered to be within the normal range. The CBCL questionnaire took approximately 15 to 20 minutes to complete.

**Teachers Report Form (TRF) 6 – 18**

With the consent of the parent, child, WA Education Department, school principal, and teacher; the teacher was invited to complete two questionnaires including the Teachers Report Form (TRF). The TRF derived 5 DSM-orientated profile scores indicative of the child’s ‘Adaptive Functioning’ i.e. Academic Performance, Working Hard, Behaving Appropriately, Learning and Happy. Additionally, the TRF provided the same 8 syndrome scores as the parent CBCL. The TRF provided insight into the child’s functioning at school as seen by their classroom teacher. Used in conjunction with the parent report form of the CBCL, the TRF helped provide a comprehensive assessment of the child’s functioning, highlighting areas of strength and/or weakness. Raw scores from each of the syndrome scale and DSM-orientated score were converted into T-scores – of which, scores below 65 were considered to be within the normal range. The TRF questionnaire took approximately 15 minutes for teachers to complete.

**Gifted Rating Scale (GRS)**

The school teacher was also invited to complete the Gifted Rating Scale (GRS). The questionnaire was applicable for children aged 6 to 12 years of age. It consisted of 72 items, grouped into 6 scales, i.e. intellectual ability, academic ability, creativity, artistic
talents, leadership ability and motivation. Raw Scores for each scale were converted into T-scores (mean: 50, SD: 10). The GRS questionnaire took approximately 10 to 15 minutes for teachers to complete.

**REPORTING NEURODEVELOPMENTAL RESULTS TO PARENTS**

The final report stating the participants results was posted to parents once approved and signed by the assessor, and co-signed by either a speech therapist, or neuropsychologist supervising the project. At the parent’s request and when appropriate, referrals to developmental specialists were provided.

**LABORATORY TECHNIQUES**

**DNA EXTRACTION**

All DNA extractions were performed by the PhD candidate at the Children’s Clinical Research Facility laboratory (n = 277). The majority of DNA (n = 269) were extracted from blood pellets (0.3 – 2 ml) collected at an earlier clinical assessment (either 5 years, 6 months or if neither were available, cord blood samples were used) using QIAamp DNA Midi Kits (Qiagen). However, a sub-group of saliva samples (n = 8) were obtained from those who did not agree to blood sample collection, yet consented to DNA extraction for genetic research. Saliva samples were gathered at 5 years, utilizing Oragene® oral DNA collection kits.

The methods used were in-line with the manufacturer’s instructions (QIAamp DNA, 2000). As represented in Figure 3.4, blood samples were treated with protease and buffer solutions before being vigorously shaken to facilitate maximum cell lyses. Samples were incubated at 70°C for ≥10 minutes using an electric heat block. Ethanol (96%) precipitated the DNA and samples were then centrifuged within Midi anion exchange columns at 1850 × G-force (3000 RPM). Buffers designed to further denature proteins, remove salts and purify the DNA were added. It was recommended that the samples were centrifuged for one minute at 4500 × G-force (5000 RPM); however the calibre of machines in the Children’s Clinical Research Facility reached a maximum speed of 4000 RPM. To compensate for the sub-optimal centrifugal speed, Qiagen recommended an additional incubation period (10 minutes at 70°C). DNA was elucidated in a low-salt buffer with neutral pH (Tris•Cl, pH 7.0). To maximize nucleic
acid concentration, the final step was repeated twice: samples were incubated at room temperature for five minutes before they were centrifuged for 2 minutes at maximal speed (4000 RPM). DNA samples were transferred into correspondingly labelled Eppendorf tubes.

Saliva samples were collected in Oragene® oral DNA collection kits at 5 years from participants (n = 8) who did not agree to blood sample collection yet consented to genetic research. DNA was extracted from these samples using the prepIT® Genomic DNA Mini Prep Kit according to manufacturer’s instructions (DNA Genotek). Briefly, samples were incubated at 50°C using an electric heat block for 2 hours. Buffer was added and samples vortexed at maximum speed for 1 minute before they were centrifuged within Mini anion exchange columns at 8000 × G-force for 1 minute. Multiple high speed centrifugal cycles allowed buffers to wash and purify DNA. Finally, elution buffer was added and sample incubated for 1 minute at room temperature before centrifuging at 12000 × G-force for 1 minute. In-line with DNA from blood pellets, the DNA from saliva samples were elucidated in (low salt) buffer and stored in Eppendorf tubes.

DNA QUALIFICATION AND QUANTIFICATION

Nucleic acid concentrations were determined using a Nanodrop™ spectrophotometer (Thermo Scientific, 2008) that exposed each sample to ultraviolet light at a wavelength of 260 nanometres (nm), while measuring the amount of light absorbed.

Spectrophotometric analysis measured the samples protein concentration (proteins absorb light at 280 nm) and the ratio of nucleic acid/protein (OD260/OD280) was used to assess sample purity. Optimal DNA sample purity was generally considered (OD260/OD280) 1.8ng/µL. Readings significantly lower than 1.8ng/µL suggested contamination with protein (left over from the nucleic acid isolation process) and

Figure 3.4. Schematic representation of DNA extraction process from blood pellet
flagged potential overestimation of DNA concentration (Gabriel, Ziaugra, & Tabbaa, 2009).

A secondary measure of sample purity compared the absorption at 260 nm (i.e. nucleic acid concentration) to the absorption at 230 nm. Optimal purity of OD260/OD230 was considered approximately 2.2ng/μL (Thermo Scientific, 2011). Ratios outside this range suggested a problem with the sample or with the extraction procedure as absorption at 230 nm reflects contamination of the sample by substances such as carbohydrates, peptides, phenols or aromatic compounds.

Agarose gels were run for a subset of samples (n = 15) with low nucleic acid concentrations (<2000ng per 100μL). 10 μL of DNA was run on a 1% Agarose gel electrophoresis (Invitrogen™) at 100V for 80 minutes to confirm the quality and molecular weight of samples.

**HOMOGENIZING DNA CONCENTRATIONS**

The nucleic acid concentrations of each DNA sample (as measured by the Nanodrop™) were diluted with low-salt, pH neutral buffer (Tris•Cl, pH 7.0) to a constant value. The ratio of nucleic acid to buffer was 2000 ng per 100 μL. Diluted DNA samples were stored in microtubes and shipped to Munich, Germany on dry ice for SNP analysis (Figure 3.5.).

**Figure 3.5.** Isolated DNA samples from 277 participants were then transported to Germany where FADS genotyping was performed and then genotype data sent back to Perth Laboratory via email communication.
Chapter 3

**GENOTYPING**

International collaboration was arranged with Professor of Paediatrics Berthold Koletzko, in Munich, Germany. Prof Koletzko and his research team have published extensively with regards to FADS polymorphisms and LC-PUFA levels in humans (Koletzko et al., 2011). The process involved in SNP analysis is described briefly below:

DNA was quantitated and then amplified using (locus-specific) PCR, utilising primers devised using MassARRAY®- an Assay Design program (Sequenom®). In order to check for accuracy and to eliminate false results from genotyping repeat regions, primers were aligned to the gene clusters (http://genome.ucsc.edu/cgi-bin/hgBlat). With the application of iPLEX® Gold reaction products, single base extensions were initiated, using mass-modified dideoxynucleotide terminators of oligonucleotide primers. Such primers anneal immediately upstream of the polymorphic site of interest. The products of the iPLEX® Gold reaction were transferred to SpectroCHIP® arrays that were then read by the MassARRAY® analyser compact. This facilitated the detection of; insertions, deletions, substitutions, and other polymorphisms in amplified DNA.

Spectra data was ascertained and the identification of SNP alleles was achieved with iPLEX® matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS, used as per the manufacturer’s instructions (Sequenom®) detected allele-specific primer extension products (Ragoussis, Elvidge, Kaur, & Colella, 2006). Employing a suite of software tools, including TyperAnalyzer software (Sequenom®), analysis of assays in multiplex reactions was performed. Visualization of genotype analysis was facilitated through computational models of physical microplates, with their wells color-coded according the strength of the genotype (relative to the optimal threshold for a conservative to moderate call). Finally, standard quality control was assured through the inclusion of (10%) duplicate and negative samples.

**SINGLE NUCLEOTIDE POLYMORPHISM SELECTION**

Twenty one FADS SNPs were chosen; five were selected from the FADS1 gene cluster, fourteen from the FADS2 gene cluster, two from the FADS3 gene cluster and two were located within the intergenic region, between the FADS1 and FADS2 genes (see Table
SNP selection was primarily based on findings from previous research pertaining to essential fatty acid metabolism and LC-PUFA concentrations in humans (Koletzko et al., 2011; Schaeffer et al., 2006). It was also provisional that the minor allele frequencies (MAF) of the chosen SNPs were high (≥ 10%) to enhance the probability of detection within the sample population. Information on linkage disequilibrium was retrieved through the Hap Map database, (http://hapmap.ncbi.nlm.nih.gov).

**Table 3.4. Characteristics of Single Nucleotide Polymorphisms within the FADS gene cluster**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Functional Region</th>
<th>Position(^a)</th>
<th>Alleles (Major/Minor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs174545</td>
<td>FADS1</td>
<td>61569306</td>
<td>C/G</td>
</tr>
<tr>
<td>rs174546</td>
<td>FADS1</td>
<td>6159830</td>
<td>C/T</td>
</tr>
<tr>
<td>rs174548</td>
<td>FADS1</td>
<td>61571348</td>
<td>C/G</td>
</tr>
<tr>
<td>rs174553</td>
<td>FADS1</td>
<td>61575158</td>
<td>A/G</td>
</tr>
<tr>
<td>rs174556</td>
<td>FADS1</td>
<td>61580635</td>
<td>C/T</td>
</tr>
<tr>
<td>rs174448</td>
<td>Intergenic(^b)</td>
<td>61639573</td>
<td>T/C</td>
</tr>
<tr>
<td>rs174449</td>
<td>Intergenic</td>
<td>61640379</td>
<td>A/G</td>
</tr>
<tr>
<td>rs99780</td>
<td>FADS2</td>
<td>61596633</td>
<td>C/T</td>
</tr>
<tr>
<td>rs174570</td>
<td>FADS2</td>
<td>61597212</td>
<td>C/T</td>
</tr>
<tr>
<td>rs174574</td>
<td>FADS2</td>
<td>61600342</td>
<td>C/A</td>
</tr>
<tr>
<td>rs174575</td>
<td>FADS2</td>
<td>61602003</td>
<td>C/G</td>
</tr>
<tr>
<td>rs174576</td>
<td>FADS2</td>
<td>61603510</td>
<td>C/A</td>
</tr>
<tr>
<td>rs174578</td>
<td>FADS2</td>
<td>61605499</td>
<td>T/A</td>
</tr>
<tr>
<td>rs174579</td>
<td>FADS2</td>
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<tr>
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</tr>
<tr>
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<td>61624705</td>
<td>G/A</td>
</tr>
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<td>61624885</td>
<td>C/G</td>
</tr>
<tr>
<td>rs968567</td>
<td>FADS2</td>
<td>61595564</td>
<td>G/A</td>
</tr>
<tr>
<td>rs2727271</td>
<td>FADS2</td>
<td>61603358</td>
<td>A/T</td>
</tr>
<tr>
<td>rs174455</td>
<td>FADS3</td>
<td>61656117</td>
<td>A/G</td>
</tr>
</tbody>
</table>

\(^a\) Position in base pairs was derived from dbSNP

\(^b\) Intergenic regions between FADS1/FADS2

**STATISTICS**

Where applicable, the scores from the neurodevelopmental tests were converted into age-standardized scores and percentile ranks using the applicable tables provided by the manufacturers.

The characteristics of the treatment group were compared to those of the control group. However, imbalanced characteristics between the fish oil and control groups...
was not expected \textit{a priori} since the process of randomisation generally ensures that the participants’ characteristics are equally distributed between the groups. Similarly, the characteristics of those who attended the 6 year follow-up were compared to those who did not participate.

For continuous, normally distributed (or normalised by natural log transformation) data, group comparisons were conducted using independent T-tests. The normality of each sub-test was assessed visually with any abnormalities confirmed using Q-Q plots and Kolmogorov-Smirnov tests. Where normality was not achieved and could not be improved by natural log transformation, non-parametric Mann-Whitney U tests were performed and/or Friedman two-way analysis of variance of ranks applied. Non-parametric data was analysed using Pearson’s Chi square (\(\chi^2\)) tests. Multiple linear regression analyses were performed to evaluate the effect of the treatment variable while adjusting for confounders. Differences between the two groups were denoted by \(P\)-values (unless stated otherwise, statistical significance set to \(P < 0.05\)).

Statistical analysis was performed using the IBM statistical software, Statistical Package for the Social Sciences (SPSS) Version 21 for PC.

\textbf{Statistical Power}

For our power calculations, we assumed an \([\alpha]\) level of 0.05 to reach a statistical power of 0.8. Subsequently, there is an 80% chance that the study will detect a relationship between the independent and the dependent variables if the true change in the dependent variables falls outside one standard deviation change in the independent variable.

We calculated that the data obtained from 320 children permits detection of a 2.36 point difference in primary dependent variable (core language composite score), per one SD change of the independent variable at a two-sided 0.05 significance level. This is based on the assumption that the SD of Core Language is 15 (accessed 23rd January 2014, from: http://hedwig.mgh.harvard.edu/sample_size/js/js_associative_quant.html).
CHAPTER 4

Fish oil supplementation of healthy term infants during the first six months of life has no effect on neurocognitive development at six years: A randomised trial

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To be submitted to Maternal and Child Nutrition
ABSTRACT

There has been widespread promotion of fish oil supplements for enhancing intelligence and other elements of cognitive functioning. However there is little peer-reviewed, empirical data concerning the utility of these products in healthy term infant populations, particularly with respect to lasting effects into early childhood. Using a variety of neurocognitive tests and assessments, we evaluated whether direct, oral fish oil supplementation during infancy leads to better neurocognitive/behavioural development at 6 years. 420 healthy, full-term infants enrolled in this randomised controlled trial. The intervention group received omega-3 (ω-3) long-chain polyunsaturated fatty acid supplementation (250 – 280 mg DHA and 60 – 110 mg EPA) from birth to 6 months. The control regime was olive oil. After the 6 month intervention period, erythrocyte and plasma ω-3 LC-PUFA levels were significantly elevated in the fish oil group ($P = 0.033$ and $P = 0.001$). 80% of the original cohort re-enrolled for the 6 year follow-up, (n = 335). No significant differences were observed between the fish oil and control groups for the main neurocognitive outcomes measured. However, fish oil treatment was associated with negative externalising and oppositional/defiant behaviour ($P = 0.035$, $P = 0.006$), particularly in boys ($P = 0.01$; $P = 0.004$). Our results suggest that in healthy, full-term infants, high dose fish oil supplementation provides no significant neurocognitive or behavioural benefit at 6 years of age.
INTRODUCTION

Dietary consumption of oily fish has been shown to benefit cognitive outcomes in children. Yet, it is unknown whether similar effects can be achieved by nutritional supplementation with fish oil during critical periods of brain development. Fish oil supplements contain the omega-3 long-chain polyunsaturated fatty acids (ω-3 LC-PUFAs) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). DHA is readily incorporated into the developing brain during the ‘brain growth spurt’ from the last trimester to ~18 months (Martinez & Mougan, 1998), with a peak occurring during the first 6 months of post-natal life (Guesnet & Alessandri, 2011).

Brain regions with the highest affinity for DHA are the frontal cortex (Galkina, Putilina, & Eshchenko, 2014) and the hippocampus (Calderon & Kim, 2004). These regions subserve higher-order cognitive processes, including executive functions, episodic memory and focused attention (Cheatham et al., 2006). These structures, including their interconnecting pathways via the limbic system, are also associated with the development of a child’s social, emotional and behavioural attributes (Barkley, 2001; Kuratko, Barrett, Nelson, & Salem, 2013).

Conceptually, fish oil supplementation during the critical periods of human brain development could affect neurocognitive development and behaviour, since cellular and animal models suggest that DHA is implicated in many neural processes. DHA concentrates within neural membranes, altering their physicochemical structure to improve membrane fluidity. By interacting with neural membrane proteins, DHA increases the speed of signal transduction (Connor, Neuringer, & Reisbick, 1991; Horrocks & Farooqui, 2004) and enhances neurite outgrowth in hippocampal neurons, which may in turn promote learning and memory (Calderon & Kim, 2004). DHA is also involved with the production and activity of several important neurotransmitters, including dopamine and serotonin (Kodas et al., 2004; Kodas, Vancassell, Lejeune, Guilloteau, & Chalon, 2002). Additionally, DHA and EPA have neuro-protective effects as they have anti-oxidative, anti-inflammatory and anti-apoptotic properties (Farooqui, 2009).
Undoubtedly, the brain must contain DHA to function properly. However, the notion that dietary DHA supplementation during infancy enhances neurocognitive functioning in young children remains controversial. Despite the strong theoretical rationale, the empirical evidence concerning the effect of dietary DHA on neurocognitive and behavioural outcomes in children is less clear. Numerous randomised control trials have been conducted - most of which have compared the cognitive and/or visual outcomes of children fed LC-PUFA enriched infant formula versus standard formula. The results of such trials have been thoroughly reviewed with no definitive conclusion as to the efficacy of LC-PUFA enriched infant formula, particularly with regards to the long term neurocognitive and behavioural effects into childhood (Qawasmi, Landeros-Weisenberger, & Bloch, 2013; Qawasmi et al., 2012; Ryan et al., 2010; Simmer et al., 2011). The lack of consistent evidence resulting from these studies may be due to a number of methodological factors (see review: (Meldrum, Smith, et al., 2011). However, the aim of the present trial does not attempt to add to the debate as to whether infant formula should contain LC-PUFA. Rather, the present study aims to justify the need – or otherwise – for fish oil supplementation in healthy, full-term, primarily breastfed infants from a typical Western society.

Some authorities recommend infants receive approximately 100 mg of dietary DHA per day [EFSA Panel on Dietetic Products, 2010 #1087](Health Council of the Netherlands, 2001 #1085](National Health and Medical Research Council Commonwealth of Australia, 2006 #1086). Prior to introduction of complimentary foods, preformed DHA is only provided through breast milk or infant formula. On average, infants who are exclusively breastfed will receive approximately 105 mg of DHA per day – assuming breast milk intake of 780 mL/day, containing 4.2% fatty acids of which DHA comprises 0.32% of total fatty acids (Brenna et al., 2007). It is putatively assumed that human breast milk should provide sufficient amounts of DHA for the developing infant. However, we cannot be sure that breast milk unequivocally provides adequate DHA.

Developing humans can obtain DHA through endogenous metabolism of shorter-chain precursors (Carnielli et al., 1996) from 26 weeks gestation (Uauy, Mena, Wegher, Nieto, et al., 2000). However, this is a variable and potentially inefficient process (Brenna & Diau, 2007; Innis, 2007; Joordens, Kuipers, & Muskiet, 2007; Simopoulos,
Given the limited capacity for endogenous DHA synthesis, infants receiving breast milk with low DHA content or un-supplemented infant formula might not be receiving sufficient DHA in their diet unless they receive supplementation (Koletzko et al., 2001; Muskiet et al., 2004).

Infants raised on formula diets devoid of DHA display lower concentrations of DHA within plasma, erythrocytes and post-mortem brain tissue than those receiving dietary DHA (Farquharson et al., 1995; Makrides et al., 1994). Whether lower concentrations of DHA in the brains of formula-fed term-infants impairs neurocognitive development has not yet been clearly established.

There is still prevailing uncertainty as to the dietary requirements of DHA for typically developing term born infants (Lauritzen, Hansen, Jorgensen, & Michelsen, 2001).

This large range (0.06 – 1.4% of total fatty acids) is primarily influenced by maternal DHA intake in the diet (Bourre, 2007). Pregnant and lactating women are encouraged to acquire no less than 200 mg of DHA per day (Van Elswyk & Kuratko, 2009). However, many women consuming western diets are not meeting these guidelines (Schuchardt, Huss, Stauss-Grabo, & Hahn, 2010).

The lack of consistent evidence resulting from these studies may be due to a number of methodological factors (see review: (Meldrum, Smith, et al., 2011). First, many previous trials employed standardised tests measuring global outcomes rather than assessing specific cognitive processes. These broad assessment measures may not be adequately sensitive to detect more subtle differences in brain function between groups of healthy children. Second, DHA doses and/or duration of supplementation may have been insufficient in these studies to confer significant neurocognitive benefit.
(i.e. DHA below modern breast milk concentrations). Third, few trials of infant ω-3 LC-PUFA supplementation have explored the long-term effects beyond 4 years of age, when neurocognitive development can be more reliably assessed (Cheatham et al., 2006; Makrides et al., 2014). Furthermore, it should be noted that many previous trials have been conducted in Western populations with presumably well-nourished participants. Therefore, the absence of effect may be due to adequate baseline maternal and infant nutrition, whereby participants are already operating at ‘ceiling’ in physiological terms.

Based on extant findings in the peer-reviewed literature, the necessity for dietary supplementation of infants with ω-3 LC-PUFA is unclear. Yet, there is undoubtedly large financial gain to be derived from the commercialisation of these products (Adarme-Vega et al., 2014). Fish oil supplements along with various ω-3 LC-PUFAs fortified-foods are widely marketed commodities, with global consumer spending on such products expected to reach US$34 billion by 2016 (Schultz, 2012, 2013). Indeed, Australia is one of the leading international markets (McManus et al., 2011; Mellentin, 2008). It is therefore important that the current evidence regarding DHA supplementation is carefully evaluated so that appropriate health recommendations can be made.

The work presented here is part of the Infant Fish Oil Study (IFOS) which commenced in 2005 with the goal of investigating the immunological and neurocognitive effects of high dose fish oil during infancy. We previously reported that fish oil supplementation had no significant effect on global infant neurocognitive development at 18 months, as assessed through the Bayley Scales of Infant and Toddler Development – 3rd Edition (Meldrum et al., 2012). However, the Macarthur–Bates Communicative Development Inventory (Words and Gestures) indicated some positive effects in terms of the complexity of developing gestures ($P = 0.007$; $P = 0.002$, respectively) and the total number of gestures ($P = 0.023$; $P = 0.006$, respectively). In addition, we found that there was higher reports of anxiety and depression in the fish oil group ($P = 0.018$).

In the present study we conducted a 6 year follow-up of the Infant Fish Oil Study (IFOS), utilising a variety of sensitive neurocognitive tests in children from a typical Western society. In order to maximise our likelihood of identifying a group effect at
Chapter 4

the 6 year follow-up we employed several psychometric tests, measuring both global and specific aspects of neurocognitive development. Specifically, we evaluated three primary areas of neurocognitive development, in addition to global measures of intelligence. First, in line with our previous findings, we expected that language and communication at 6 years would be better amongst the children from the fish oil group, and secondly that participants in the fish oil group may display more conspicuous behavioural traits associated with anxiety and/or depression (Meldrum et al., 2012)(Cheatham, Nerhammer, Asserhø, Michaelsen, & Lauritzen, 2011; Makrides et al., 2014). Third, based on our understanding of how DHA interacts within neural tissue (i.e. affecting physicochemical processes that underlie hippocampal and frontal-based cognition), we expected children who had taken fish oil as infants would score higher on tasks which called upon executive functions, including working memory.

MATERIALS AND METHODS

Study Participants

To summarise the original trial (Meldrum, D’Vaz, Dunstan, Mori, & Prescott, 2011), four hundred and twenty pregnant women were recruited during their third trimester for the Infant Fish Oil Study (IFOS). IFOS was designed to assess the effects of fish oil supplementation on infant allergy and neurocognitive development. Recruitment occurred from public and private antenatal services in the Perth metropolitan area in Western Australia between June 2005 and October 2008. Eligible participants had a history of asthma, allergic rhinitis or eczema, in addition to a confirmed-positive skin prick test to a panel of common allergens. Women were ineligible for the study if they consumed fish oil during pregnancy (≥1000 mg per day), typically ate more than three fish-meals per week or smoked during pregnancy. Infants diagnosed with major congenital or neonatal health abnormalities requiring medical and/or surgical treatment were not eligible for continuation within the trial.

Randomisation

Infants were randomised to receive either the treatment – high dose fish oil capsules (650 mg) containing 250 – 280 mg of DHA plus 60 – 110 mg of EPA (n = 218), or alternatively, allocated a 650 mg of placebo oil containing 66.6% (ω-9) oleic acid; (n = 202). The randomisation schedule was prepared by an independent biostatistician, and
stratified according to maternal and paternal allergy, in addition to parity (first child versus two or more children).

**Supplementation Treatment**

The intervention commenced at birth and ceased after 6 months. The duration of treatment coincided with the average age whereby milk (either formula or breast milk) functions as the primary energy source. The dose of DHA was more than twice the amount naturally provided in breastmilk and substantially higher than previous comparative trials studying the effects of ω-3 LC-PUFA supplementation during infancy. The amount of oleic acid (432.9 mg) within placebo capsules was deemed insignificant, considering the average daily oleic acid intake of breastfed infants (~1100 mg per day); (CSIRO Division of Human Nutrition, 1996).

The fish oil and placebo capsules were image matched and designed to be punctured so that the oil could be directly administered into the infants’ mouth or incorporated within milk if bottle-feeding. Participants were instructed to give the capsules to the infants in the morning, prior to/during feeding.

**Compliance**

Parents were asked to record their daily capsule administration in a log book and return any unused capsules to the pharmacy for counting. Participant compliance was ascertained based on a composite of these measures. Fatty acid levels were analysed at birth and after the supplementation period (at 6 months). Between group comparisons of fatty acid composition in erythrocyte membranes and plasma phospholipids at 6 months was used to assess the effectiveness of the intervention on infant fatty acid status.

**Fatty Acid Analysis**

The fatty acid analysis was conducted using previously published methods (Mori et al., 2000). Briefly, the phospholipid fraction of the blood plasma and the erythrocyte lipids were extracted from whole blood using methanol–chloroform (2:1). Lipid fractions were analysed using thin layer chromatography. Fatty acid methyl esters were analysed by gas-liquid chromatography (Agilent Technologies Australia Private Limited) using Supelco SP-2560 column’s (Sigma-Aldrich Private Limited) and hydrogen as the
carrier gas. Both cord and peripheral blood were processed by the same technique that enabled separation into plasma, mononuclear cell and erythrocyte fractions.

**BLINDING**

The study was designed as a double-blind randomised controlled trial, where both participants and staff were unaware of group allocation. As such, group allocation was concealed from both the participants and researchers until data collection for early immunological and neurological development had been completed at 18 months (this time point was specified at the commencement of the trial, and made known to participants). Participants were asked not to discuss their group allocation with other research staff or participants. The 6 year follow-up assessments and associated materials were exclusively managed by blind researcher(s). Once all 6 year assessments were completed, analysis of the data proceeded blinded to which children were in each group whereby treatment and placebo groups were masked as A or B (such that statistical analysis was undertaken with no knowledge of the identity of the groups).

**THE 6 YEAR FOLLOW-UP**

The 6 year follow-up was primarily aimed to evaluate neurocognitive outcomes through a battery of neuropsychological tests – [administered by the PhD candidate, with some assistance (5%) from a trained psychologist] and questionnaires completed by parents and teachers. The battery of tests included: the Child Clinical Evaluation of Language Fundamentals 4th Ed. (CELF-4), Wechsler Abbreviated Scale of Intelligence (WASI), Renfrew Bus Story, Self-Ordered Pointing test (SOPT) and the ‘Fruit Stroop’. They were delivered in the same order for each participant, and frequent short breaks were allowed. The total amount of time required to administer these tests was approximately two hours.

Information from both teachers and parents was requested to gather data about behaviour both at school and at home. Questionnaires were posted to parents prior to the assessment and included three parent questionnaires (the Autism Spectrum Quotient: Children’s Version (AQ-Child), Children’s Communication Checklist- 2nd Ed.
(CCC-2), Child Behavior Checklist (CBCL) and two teacher questionnaires (the Teachers Report Form (TRF) and the Gifted Rating Scale (GRS).

**LANGUAGE AND COMMUNICATION ASSESSMENTS AND QUESTIONNAIRE**

**CELF-4 CORE LANGUAGE COMPOSITE SCORE**

A Core Language Composite score was derived from performance on the CELF-4. This is a standardised test for the assessment of a child’s communication skills and other aspects of language-related neurocognitive development, as evident through spoken language (Semel et al., 2003). Four subtests were conducted: Concepts and Following Directions, Word Structure, Recalling Sentences and Formulating Sentences. Scores from these subtests were age adjusted and compiled to form a Core Language Composite score (mean = 100; SD = 15).

**RENFREW BUS STORY NARRATIVE TEST**

The Renfrew Bus Story test assessed the participant’s ability to re-tell a narrative after hearing it read once by the examiner. Scoring was conducted according to sentence length, sentence complexity and total information. The Renfrew bus story has been shown to be predictive of later language development and academic performance (Pankratz et al., 2007; Stothard et al., 1998). At 6 years of age, an information score of > 22 and sentence length of > 9 is considered average.

**CHILDREN’S COMMUNICATION CHECKLIST (CCC-2)**

The CCC-2 quantified 10 key areas of communication: speech, syntax, semantics, coherence, inappropriate initiation, stereotyped language, context, non-verbal communication, social relations and interests. These sub-areas were combined to provide a General Communication Composite (GCC) score that was standardised according to age and gender. GCC scores >59 are considered to be within the normal range.
Chapter 4

**BEHAVIOURAL QUESTIONNAIRES**

**THE CHILD BEHAVIOR CHECKLIST (CBCL)**

The CBCL is a standardised questionnaire completed by parents in order to assess the competencies and problems of the child. It derives an Internalising, Externalising and Total Behavioural score. 8 syndrome scores and 6 behavioural profile scores pertaining to the Diagnostic & Statistical Manual of Mental Disorders (DSM) are also ascertained: Affective, Anxiety, Somatic, Attention Deficit/Hyperactivity, Oppositional Defiant and Conduct Problems. Additionally, several scores pertaining to the child’s behavioural competencies can be calculated (Social, School, Activities and Total Competence). All raw scores are converted into standardised, age adjusted T-scores. A T-score of 50 indicates average functioning in reference to other children of the same age and gender and every 10 points represents one standard deviation (scores < 65 considered to be within the normal range).

**TEACHERS REPORT FORM (TRF) 6-18**

School teachers were invited to complete the Teachers Report Form (TRF), an adapted version of the CBCL. The TRF derives five scores indicative of the child’s Adaptive Functioning, i.e. Academic Performance, Working Hard, Behaving Appropriately, Learning and Happy. The TRF provides the same 8 syndrome scores as the CBCL and 5 DSM-orientated profile scores. The TRF offers insight into the child’s functioning at school as evaluated by their primary teacher. Used in conjunction with the CBCL, the TRF helps to provide a comprehensive assessment of the child’s behavioural functioning and may highlight areas of strength and or weakness. All raw scores are converted into standardised, age adjusted T-scores (scores < 65 considered to be within the normal range).

**AUTISM SPECTRUM QUOTIENT: CHILDREN’S VERSION (AQ-CHILD)**

Parents completed the AQ-Child questionnaire that quantitatively measures autistic-like traits (Auyeung et al., 2008). The questionnaire concentrates on areas such as social interaction, communication, behavioural flexibility, attention-switching, attention-to-detail, communication and imagination. Scores < 70 were considered within the normal range.
**WORKING MEMORY AND EXECUTIVE-FUNCTIONS**

**WORKING MEMORY**
An age adjusted Working Memory Composite Score (mean: 100; SD: 15) was derived from the CELF-4 subtests: Familiar Sequences and Number Repetition Total (comprised of a forwards and a backwards number sequence).

**CELF-4 SUBTESTS OF EXECUTIVE FUNCTION**
Rapid Autonomic Naming (RAN) was evaluated via a subtest from the CELF-4. The RAN required the participant to quickly identify the names of familiar colours and shapes. Scores were based on speed and accuracy. The Word Association test score was also derived from the CELF-4. It required participants to name as many types of animals as they could think of, followed by types of food and then types of jobs/occupations.

**THE FRUIT STROOP TEST**
To assess executive functioning and working memory, we created the Fruit Stroop test based on previously established protocol (Archibald & Kerns, 1999). The test took the form of a 4-page booklet containing shapes and pictures in 4 colours (Appendix C). Working as quickly as possible (from left to right), participants were required to identify as many colours as they could on each page before the examiner told them to stop (after 45 seconds). When participants reached the end of the page before the timer, they were instructed to continue from the beginning of the page. The first page contained rows of coloured rectangles in a pseudorandom order. The same order of colours populated the second page, however this time as correctly coloured fruit. The third page was the same except in black and white, so participants needed to recall the previously presented colour sequence from working memory. The final page contained the same sequence of fruits; however, this time they were incorrectly coloured (e.g. purple banana) so that participants needed to inhibit their automatic response in order to name the correct colour (i.e. yellow). The final score was based on the number of correctly identified colours per page: \( \frac{(Page\ 1 \times \ Page\ 3)}{(Page\ 1 \ + \ Page\ 3)} - Page\ 4 \). The total number of errors were also recorded.
THE SELF ORDERED POINTING TEST (SOPT)

To assess non-spatial, complex working memory, the SOPT was developed by the current investigators based on previously established protocol (Cragg & Nation, 2007). Participants were shown a sequence of different pictures and were required to point to a different picture on each page so that each picture was selected once (Appendix D). There were 4 items of increasing complexity – proportionate to the number of pictures per item (6, 8, 10 and 12, pictures respectively). The order of the pictures changed pseudorandomly on each page so that spatial location was not informative. Participants achieved a score quantifying any errors i.e. repeated selection of any picture more than once. There were no time restrictions, however, in our study, all participants completed the task in approximately 10 minutes.

GLOBAL INTELLIGENCE MEASURES

WECHSLER ABBREVIATED SCALE OF INTELLIGENCE (WASI)

The WASI is one of the gold standard tests used to evaluate general intelligence in those aged 6 to 90 years of age (Axelrod, 2002). The test informs verbal IQ (VIQ), performance IQ (PIQ) and full scale IQ (FSIQ) scores (Wechsler, 1999) each with a mean: 100; SD: 15.

GIFTED RATING SCALE (GRS)

The child’s school teacher was invited to complete the GRS by providing information on 6 scales surrounding current theories of giftedness, i.e. intellectual ability, academic ability, creativity, artistic talents, leadership ability and motivation. These 6 norm-referenced scores provided a standardized method for identifying gifted and talented students based on teacher observations (mean: 50; SD: 10).

ETHICAL APPROVAL AND PARTICIPANT CONSENT

The 6 year follow-up study was approved by the University of Western Australia and the Princess Margaret Hospital Ethics Committee. Informed consent was obtained from the children’s parents. The trial is registered under the grant ID: 458502, funded by the National Health and Medical Research Council (NHMRC) of Australia. Informed consent was obtained from participating families.
STATISTICS

Statistical analysis was performed using the IBM statistical software, Statistical Package for the Social Sciences (SPSS) Version 21 for PC. Statistical significance was assessed at the two-tailed P < 0.05. All children were included in the analysis, irrespective of compliance with the intervention.

Any difference in demographic or neonatal characteristics between the groups were determined by independent T-tests where data was normally distributed (normality having been checked visually through histograms and confirmed using Q-Q plots). Where variables were not normally distributed, logarithm and square root transformations were performed. However untransformed data are referred to in the descriptive statistics for ease of interpretation, as transformation did not alter the final results. When normality was not achieved, non-parametric Mann-Whitney U tests were performed and/or Friedman two-way analysis of variance of ranks applied. Pearsons Chi square ($\chi^2$) tests were used for nominal/categorical data.

Many of the scores from the neurodevelopmental tests/questionnaires were age-standardized according to the test-manufacturers norm-references. Subsequently, unadjusted analyses (independent t-tests) were performed when comparing the fish oil and control groups composite t-scores from the CELF-4, CCC-2, CBCL, TRF, WASI and the GRS between the fish oil and control groups. For the remaining test data that were not age-standardised (including the Renfrew Bus Story, SOPT, Fruit Stroop, AQ-Child, RAN & Word Association test), multivariate linear regression analyses were used, controlling for age (in months) at the time of assessment. Regression models were also employed to analyse the raw scores of the WASI individual subtests (vocabulary, similarities, block design and matrix reasoning) while adjusting for age. This was necessary since the standardised WASI IQ scores were only accurate from age 6 years, yet not all participants had reached 6 years of age by the time of their assessment.

RESULTS

PARTICIPANTS

At the 6 year visit, n = 335 children participated in the neurocognitive assessment (80% of initial enrolment); n = 156 from the control group and n = 179 from the fish oil
treatment group. Attrition rates were similar for the two groups. In total, n = 85 participants did not attend the 6 year follow-up due to the reasons outlined in Figure 4.1. The percentage of participants retained within the study was higher than other large-scale long-term longitudinal studies in this field (Cheatham et al., 2011; Jensen et al., 2010). Overall, the mean age ± SD of children at the time of the assessment was 72 ± 7 months, and the range was between 61 and 97 months.

Of the participants who attended the 6 year follow-up, 100% of the control group and 97.5% of the treatment group reported breastfeeding after birth; and 45.8% of the control group and 54.1% of the treatment group continued to be breastfed beyond 6 months of age (Table 4.1.)

**Figure 4.1.** Flow of the neurocognitive developmental arm of the study and number of participants in the trial as for June 2014.
BASELINE CHARACTERISTICS OF ATTENDEES COMPARED TO NON-ATTENDEES AT THE 6 YEAR FOLLOW-UP

The population characteristics of the children who participated in the follow-up at 6 years were compared to those who did not participate (data not shown). It was observed that participants who attended the 6 year follow-up had significantly higher maternal education levels ($t (334) = 3.77, P = 0.001$), maternal age ($t (112) = 3.12, P = 0.002$) and paternal age ($t (408) = 3.32, P = 0.004$) than those who did not participate. No other baseline characteristics were different including: ethnicity, birth order, playgroup/childcare within the first 12 months or allergic diagnosis by 12 months. Furthermore, there was no difference in the fatty acid levels (sampled at birth, 6 months or 5 years) between the participants who participated at 6 years and those who did not.

BASELINE CHARACTERISTICS OF RANDOMISED GROUPS

There were few significant differences between the baseline characteristics of the fish oil treatment group versus the control group at the 6 year follow-up (Table 4.1.) There were no differences in the exact age of participants at the 6 year follow-up between the two groups (treatment group: $72 \pm 7$; control group: $73 \pm 7$; $P = 0.339$). Nor were there differences in pregnancy, neonatal or early childhood characteristics with the exception of gestation (mean group difference was 2.3 days; 95% CI: 0.07 – 0.59, $P = 0.010$), whereby the fish oil group was born approximately 2 ½ days earlier on average. However, the clinical significance of this values is likely negligible so was not adjusted for in subsequent analyses.

Comparison of the number of capsules consumed between the treatment and control groups (Figure 4.2.) indicated that the treatment group took a mean of 62% (SE: 3%) of capsules, rather than the prescribed 100% capsules, and the control group took a mean of 70% (SE: 2.4%) capsules. However, Mann Whitney U test ascertained that this difference in compliance between the two groups was not statistically significant ($P = 0.089$). Considering the fish oil treatment intended to supply DHA ~265 mg per day, yet the compliance of this group was only 62%, it could be argued that fish oil supplementation delivered approximately 164.3 mg per day.
**Table 4.1.** Baseline population characteristics of those within the treatment group and the control group – Including only those who attended the 6 year follow-up

<table>
<thead>
<tr>
<th>Neatnalt Anthropometrics</th>
<th>Control Group</th>
<th>Treatment Group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (grams)</td>
<td>3516±486</td>
<td>3465±432</td>
<td>.361</td>
</tr>
<tr>
<td>Gestation (weeks)</td>
<td>39.59±1.27</td>
<td>39.29±1.17</td>
<td>.010*</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>34.64±1.33</td>
<td>34.92±1.48</td>
<td>.121</td>
</tr>
<tr>
<td>Ethnicity (% Caucasian)</td>
<td>94.1%</td>
<td>90.7%</td>
<td>.432</td>
</tr>
<tr>
<td>Birth order (% first born)</td>
<td>47.1%</td>
<td>48.7%</td>
<td>.809</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>46.4%</td>
<td>52.8%</td>
<td>.329</td>
</tr>
<tr>
<td>Environmental / Family Characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>32.45±5.37</td>
<td>33.23±5.21</td>
<td>.063</td>
</tr>
<tr>
<td>Partner age (years)</td>
<td>34.70±5.41</td>
<td>35.81±6.12</td>
<td>.079</td>
</tr>
<tr>
<td>Maternal education (years)</td>
<td>13.50±2.83</td>
<td>14.90±3.59</td>
<td>.110</td>
</tr>
<tr>
<td>Partner smokes (% yes)</td>
<td>12.9%</td>
<td>10.5%</td>
<td>.560</td>
</tr>
<tr>
<td>Alcohol units/wk - Pre-pregnancy</td>
<td>4.03±4.56</td>
<td>3.65±5.40</td>
<td>.578</td>
</tr>
<tr>
<td>Alcohol units/wk - Pregnancy</td>
<td>0.38±8.40</td>
<td>0.35±6.64</td>
<td>.699</td>
</tr>
<tr>
<td>Infant Characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. months breast fed - 12m</td>
<td>6.32±3.03</td>
<td>6.53±3.65</td>
<td>.790</td>
</tr>
<tr>
<td>Ever breastfed (% yes)</td>
<td>100%</td>
<td>97.5%</td>
<td>.600</td>
</tr>
<tr>
<td>Breastfed beyond 6m (% yes)</td>
<td>45.8%</td>
<td>54.1%</td>
<td>.668</td>
</tr>
<tr>
<td>Ear problems - 12m (% yes)</td>
<td>25.6%</td>
<td>25.3%</td>
<td>1.00</td>
</tr>
<tr>
<td>Allergic disease - 12m (% yes)</td>
<td>51.3%</td>
<td>37.0%</td>
<td>.114</td>
</tr>
<tr>
<td>Child takes fish oil - 12m (% yes)</td>
<td>15.4%</td>
<td>11.1%</td>
<td>.425</td>
</tr>
<tr>
<td>Childcare/Playgroup 12m (% yes)</td>
<td>70.3%</td>
<td>83.5%</td>
<td>.067</td>
</tr>
<tr>
<td>Age at 6y assessment (months)</td>
<td>73±7</td>
<td>72±7</td>
<td>.339</td>
</tr>
</tbody>
</table>

*Results provided as either mean ± SD or percentage of the group*
Table 4.2. Comparison of DHA levels of control vs treatment groups at birth and at 6 months

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Treatment</th>
<th>P -value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHA (erythrocytes) - Birth</td>
<td>(n = 57) 7.44 ± 1.44</td>
<td>(n = 52) 7.36 ± 1.15</td>
<td>.745</td>
</tr>
<tr>
<td>DHA (erythrocytes) - 6m</td>
<td>(n = 71) 6.22 ± 1.59</td>
<td>(n = 68) 6.83 ± 1.74</td>
<td>.033*</td>
</tr>
<tr>
<td>DHA (plasma) - 6m</td>
<td>(n = 80) 4.49 ± 1.33</td>
<td>(n = 65) 5.35 ± 1.60</td>
<td>.001**</td>
</tr>
</tbody>
</table>

A Results provided as mean ± SD; DHA levels given as a percentage of total fatty acids
Significance * P <0.05; ** P<0.001

Figure 4.2. Range of mean capsule compliance for the two groups, calculated based on a composite of parent reports and pharmacy count.

**FATTY ACID CONCENTRATIONS**

There was no significant difference between the DHA levels of the treatment and control groups at birth. After the 6 month supplementation period, infants in the fish oil treatment group had significantly higher levels of DHA within plasma phospholipids ($P = 0.001$) and erythrocytes ($P = 0.033$).

**NEUROCOGNITIVE DEVELOPMENTAL OUTCOMES AT 6 YEARS**

**LANGUAGE AND COMMUNICATION**

Core language scores did not differ between treatment and control groups, nor were there any group differences for any of the core language subtests (Table 4.3.) There
were no significant differences between the groups for the sentence length, information scores or mean number or subordinate clauses according to the Renfrew bus story, either before or after adjusting for age. Results from the CCC-2 identified no significant differences between the two groups in terms of parents’ perceptions of their child’s communicative skills (Table 4.3).

Table 4.3. Language scores of the control placebo group compared to the fish oil treatment group

<table>
<thead>
<tr>
<th>LANGUAGE</th>
<th>Control Group</th>
<th>Treatment Group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical Evaluation of Language Fundamentals (CEL-F-4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core Language Composite</td>
<td>104.01 (13.15)</td>
<td>102.92 (11.83)</td>
<td>.361</td>
</tr>
<tr>
<td>Concepts/Follows Directions</td>
<td>10.53 (3.03)</td>
<td>10.73 (2.62)</td>
<td>.100</td>
</tr>
<tr>
<td>Word Structure</td>
<td>10.42 (2.55)</td>
<td>10.38 (2.73)</td>
<td>.121</td>
</tr>
<tr>
<td>Recalling Sentences</td>
<td>9.97 (2.80)</td>
<td>9.57 (2.62)</td>
<td>.432</td>
</tr>
<tr>
<td>Formulating Sentences</td>
<td>11.42 (2.53)</td>
<td>11.24 (2.42)</td>
<td>.809</td>
</tr>
<tr>
<td>The Renfrew Bus Story Test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Information Score</td>
<td>34.85 (8.13)</td>
<td>33.56 (8.40)</td>
<td>.184</td>
</tr>
<tr>
<td>Sentence Length</td>
<td>11.68 (2.61)</td>
<td>11.45 (2.29)</td>
<td>.427</td>
</tr>
<tr>
<td>Subordinate Clause</td>
<td>1.89 (1.60)</td>
<td>1.80 (1.58)</td>
<td>.778</td>
</tr>
<tr>
<td>Children’s Communication Checklist - 2nd Ed. (CCC-2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCC (Composite)</td>
<td>79.01 (17.94)</td>
<td>78.68 (17.33)</td>
<td>.870</td>
</tr>
</tbody>
</table>

A Results provided as mean ± SD

**BEHAVIOURAL OUTCOMES**

As illustrated in Table 4.4, children in the treatment group exhibited significantly more externalising behaviours compared to controls, with the mean externalising T score of the fish oil group 2.30 points higher than controls (95% CI: 4.39 – 0.21; \( P = 0.035 \)). Further analysis revealed that the mean T scores of oppositional defiant problems (as defined from the DSM-orientated scales; see Figure 4.4. was 1.60 points higher in the treatment group (95% CI: 0.40 – 2.75 \( P = 0.006 \)) compared to controls.

Table 4.4. Behaviour scores of the control group compared to the fish oil treatment group

<table>
<thead>
<tr>
<th>BEHAVIOUR</th>
<th>Control Group</th>
<th>Treatment Group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Child Behaviour Checklist - Parent Report Form (CBCL)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 4

Internalising T-Score  49.28 (9.29)  48.03 (10.43)  .265
Externalising T-Score  48.49 (9.49)  50.74 (9.11)  .035*
Total Behaviours T-Score  48.29 (9.14)  48.51 (10.13)  .842
Total Competence T-Score  46.61 (9.63)  46.56 (8.90)  .965

Autism Spectrum Questionnaire - Children's Version (AQ-Child)

AQ-Child  46.06 (15.44)  45.51 (15.64)  .757

*A Results provided as mean ± SD

** Behavioural Problems at 6 Year Follow-up – Parent Reports

Figure 4.3. Behavioural problems reported by parents via the Child Behaviour Checklist- Parent Report Form DSM: Diagnostic & Statistical Manual of Mental Disorders. Box’s denote mean T-scores ± 1 SE in the control and treatment groups.

Post hoc analyses revealed both of these effects were stronger in boys only (n = 165). Specifically, in boys the mean difference in oppositional defiance T scores were 2 points higher for boys in the fish oil group compared to boys in the control group (95% CI: 0.49 – 3.52, \( P = 0.01 \)), while the mean externalising behaviour T scores were 4 points higher for boys in the fish oil group compared to boys in the control group (95%
CI: 1.24 – 6.58, \( P = 0.004 \)). However, this was not a pre-planned analysis and thus statistical power was reduced accordingly.

No other significant differences were observed for the CBCL questionnaire including Internalising or Total Behavioural scores. The Teacher Report Form identified no significant differences between the two groups, as was the case for the AQ-Child questionnaire.

**Working Memory and Specialised Executive-Functions Assessments**

Working Memory was not significantly different between the two groups (Table 4.5). However, participants in the treatment group scored higher on the familiar sequences subtest compared to controls (95% CI: -1.30, 0.062; \( P = 0.031 \)). Conversely, the ability to repeat a sequence of numbers (forwards) was lower in the treatment group, versus controls; (95% CI: 0.03 – 1.29; \( P = 0.040 \)).

There were no group differences for the secondary outcomes of the CELF-4, i.e. the two supplementary tests, namely Word Associations and Rapid Autonomic Naming (RAN), after adjusting for age by way of linear regression. Similarly, there were no differences between the treatment versus the control group for the Fruit Stroop test or the SOPT (Table 4.5.).

**Table 4.5. Working memory scores of the control group compared to the fish oil treatment group**

<table>
<thead>
<tr>
<th>WORKING MEMORY &amp; INHIBITORY CONTROL</th>
<th>Control Group</th>
<th>Treatment Group</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Memory Composite</td>
<td>103.21 (13.86)</td>
<td>104.40 (13.16)</td>
<td>.448</td>
</tr>
<tr>
<td>No(^{-}) Repetition Forwards</td>
<td>10.85 (2.79)</td>
<td>10.19 (2.75)</td>
<td>.040*</td>
</tr>
<tr>
<td>No(^{-}) Repetition Backwards</td>
<td>10.07 (2.58)</td>
<td>10.11 (2.54)</td>
<td>.876</td>
</tr>
<tr>
<td>Familiar Sequences</td>
<td>10.59 (2.77)</td>
<td>11.27 (2.70)</td>
<td>.031*</td>
</tr>
<tr>
<td>Rapid Autonomic Naming</td>
<td>121.17 (38.88)</td>
<td>118 (39.34)</td>
<td>.563</td>
</tr>
<tr>
<td>Word Association</td>
<td>24.98 (7.51)</td>
<td>24.31 (7.32)</td>
<td>.459</td>
</tr>
</tbody>
</table>

**Additional Tests: Executive Functioning, Memory and Inhibition**

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Treatment Group</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOPT</td>
<td>2.66 (2.46)</td>
<td>2.32 (2.06)</td>
<td>.226</td>
</tr>
<tr>
<td>Fruit Stroop</td>
<td>2.87 (4.41)</td>
<td>2.53 (4.74)</td>
<td>.551</td>
</tr>
<tr>
<td>Fruit Stroop Errors</td>
<td>4.79 (3.98)</td>
<td>4.14 (3.61)</td>
<td>.188</td>
</tr>
</tbody>
</table>
GLOBAL INTELLIGENCE MEASURES

There were no significant group differences between the treatment group and control group for the global measures of IQ (VIQ, PIQ or FSIQ) derived from the WASI (Table 4.6). Given that the WASI IQ scores were age standardised from 6 years of age, we also analysed the group differences for each of the WASI subtest raw scores while adjusting for age. There were no significant differences between the groups for any of the WASI raw scores before or after adjusting for age. Furthermore, results from the Gifted Rating Scale (GRS) identified no significant differences between the two groups for intelligence or any other measures including academic, creativity, artistic, leadership or motivation.

Table 4.6. Global intelligence scores of the control group compared to the fish oil treatment group

<table>
<thead>
<tr>
<th>GLOBAL INTELLIGENCE</th>
<th>Control Group</th>
<th>Treatment Group</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verbal IQ</td>
<td>111.17 (13.41)</td>
<td>111.33 (13.08)</td>
<td>.915</td>
</tr>
<tr>
<td>Performance IQ</td>
<td>103.28 (11.41)</td>
<td>103.85 (12.01)</td>
<td>.667</td>
</tr>
<tr>
<td>Full Scale IQ</td>
<td>108.03 (12.03)</td>
<td>108.52 (11.33)</td>
<td>.717</td>
</tr>
<tr>
<td>Gifted Rating Scale Score</td>
<td>49.20 (9.35)</td>
<td>48.62 (7.76)</td>
<td>.801</td>
</tr>
</tbody>
</table>

DISCUSSION

We predicted that fish oil supplementation during infancy would have a positive neurocognitive effect, particularly on language development at 6 years of age – based on our earlier findings at 12 and 18 months (Meldrum et al., 2012). We hypothesised that any long-term effects would be more pronounced at this age due to the emergence of further language and cognitive capacities subsequent to on going brain maturation, together with the commencement of formal schooling. However, this was not evident despite the use of several neurocognitive tests that were specifically chosen to investigate language and communication. Rather, our findings suggest that the advantage of fish oil supplementation on language and communication had no lasting effect at the 6 year follow-up.
Externalising behaviour problems and oppositional defiant behaviours were found to be significantly higher in the treatment group. This is in line with our previous findings in this sample at 18 months, which reported higher anxiety/depression in the treatment group. However, the average externalising scores observed at 6 years were well within the normal range (i.e. T-score < 65), so unlikely to be clinically significant. It is not clear why fish oil supplementation in early life would have negative behavioural effects in childhood. It is possible that the fish oil preparation contained an unknown negative component (although, the components known to be deleterious e.g. methyl-mercury were monitored and complied with Australian and international standards).

It is curious that these adverse behavioural findings are supported by recent work from a randomised control trial that reported more behavioural problems in 4 year old children born to mothers who were supplemented with DHA during pregnancy (Makrides et al., 2014). Similarly, in the 7 year follow-up by randomised control trial by Cheatham et al., (2011) and colleagues, children whose mothers were supplemented with fish oil during breastfeeding were found to have lower pro-social scores compared to the control group. Neither of these studies have offered plausible explanations for these findings, instead citing reasons such as chance outcomes (i.e. Type I statistical errors), which is possible. But the consistency of this unexpected result adds weight, particularly considering that to the best of our knowledge there is no evidence to the contrary from similar follow-ups of infant DHA supplementation.

There was no significant difference between the treatment group and the control group in terms of the composite measures of working memory. Despite this, there were some effects within the subtests of working memory. Since the directions of these effects were inconsistent (i.e. the fish oil group performed significantly better in the familiar sequences subtest yet significantly worse recalling numbers) these may be random effects. Therefore, it is premature to conclude that fish oil supplementation provides any benefit to working memory in children. The null effect with respect to overall working memory performance was echoed in the additional tests of executive functioning and inhibitory control (the Fruit Stoop and SOPT) which we included to overcome some of the limitations of global tests of neurocognitive outcomes and investigate more specific cognitive domains. These tests are believed to be more
specialised and sensitive measures with greater potential to detect more modest effects on cognitive performance (Bellisle et al., 1998). The utility and merit of these tests has been evaluated (Willoughby, Wirth, & Blair, 2011).

The baseline fatty acid levels of participants was similar for both groups at birth, indicating that randomised groups started with comparable lipid profiles. We expected the DHA levels in erythrocytes and plasma phospholipids would be substantially elevated in the treatment group compared to the control group after the 6 month supplementation period. We ascertained that DHA in plasma and erythrocytes were statistically significantly higher in the fish oil treatment group at 6 months, however, the difference was only modest, considering the relatively high dose of ω-3 LC-PUFA used in our study. It is known that erythrocyte fatty acid levels are a better biomarker of long-term fatty acid intake over the preceding months compared to plasma levels (Olsen et al., 1995). We acknowledge that direct postnatal fish oil supplementation was not exceptionally effective in raising the DHA status of infants. Reasons as to why the intervention did not have a greater effect on fatty acid levels include low compliance, possible issues with bioavailability and absorption of the ethyl ester supplements (in a large bolus delivery), genetic differences in fatty acid metabolism or DHA sufficient population.

The overall compliance to taking the fish oil and placebo capsules was less than ideal (<80% compliance). This may be attributable to the inconvenience and/or difficulty in administering the oil directly into the infants’ mouth each day. Also, it is conceivable that some participants were deterred by the capsules odour and/or taste, particularly those within the fish oil treatment group. Despite our best efforts to mask the natural aroma of fish oil (with peppermint oil), the odour may have been unpleasant, especially in conjunction with infant reflux.

It is also possible that the formulation of ω-3 fatty acids (as ethyl esters) affected the bioavailability. There have been suggestions that ethyl esters are more slowly digested and absorbed by the gastrointestinal tract when compared to triglyceride formulations (Mu & Mullertz, 2015). However, this remains speculative since there have been no previous studies comparing the bioavailability of ethyl esters to other formulations in infants which support (or reject) this theory.
Furthermore, it is possible that individual response to fish oil supplementation varied, due to genetic differences in fatty acid metabolism. Genetic polymorphisms in the FADS gene cluster are known to have a direct impact upon the amount of LC-PUFAs available to the developing foetus/infant (Lattka, 2011). It could be speculated that heterogeneity in fatty acid metabolism may explain the relatively modest increase in DHA despite high dose fish oil supplementation.

The IFOS sample population were well-educated, high-income and in turn, demonstrated a high rate of breastfeeding compared to the general Australian population (Australian Institute of Health and Welfare, 2011). It is well known that breastfeeding is associated with social advantage. This is because well-educated, high-income parents are generally more informed about the benefits of breastfeeding and/or may be in a better position (e.g. employment wise) to continue breastfeeding for the first 6 months. Yet we believe that the social characteristics of our sample population enhance the real-world utility of this study. Parents within this demographic are more likely to possess the financial means to purchase commodities such as infant fish oil products. Subsequently, this demographic subgroup may potentially be misled into purchasing unnecessary products which they believe may confer a neurocognitive advantage for their child.

The results of this study must be considered within the context of the IFOS sample population. We do not expect that many children in our cohort were DHA or EPA deficient, owing to the aforementioned population characteristics, specifically the high rate of breastfeeding. Therefore, the lack of significant benefit from ω-3 LC-PUFA supplementation could be because participants in this study were already receiving enough dietary DHA for optimal neurocognitive development. This line of thought is shared by (B. Carlson & Kingston, 2007), who state: “the lowest observed concentrations of DHA within human breast milk should prove sufficient for normal brain and retinal development”. Indeed, Gibson et al., (1997) has established that DHA incorporation into infant plasma and erythrocytes is saturable. While a dose response to increased dietary DHA occurs initially, the curve reaches a plateau once the DHA level in breast milk exceeds approximately 260 mg per day.
CONCLUSION

On the basis of these findings, direct fish oil supplementation of healthy West Australian infants cannot be recommended for the purposes of enhancing neurocognitive outcomes in early childhood. Our results using ~265 mg DHA supplementation indicate no positive behavioural effects among 6 year-old children (especially in boys); thus appearing to run counter to the hypothesis that fish oil supplementation during infancy incites long-term improvement in brain development. These findings indicate that the negative effects of fish oil supplementation on externalising and oppositional/defiant behaviours in childhood deserves more consideration.

Candidate contribution:

This randomised control trial commenced in 2005, before the initiation of this PhD. The intervention, collection of blood samples/ fatty acid analysis also occurred prior to candidature. Alexandra Heaton re-enrolled participants at 6 years, performed neuropsychometric testing, was responsible for data entry, statistical analysis, in addition to the writing, drafting and editing of this manuscript.
CHAPTER 5

The correlation between infant long-chain polyunsaturated fatty acid status, neurocognitive performance and behaviour at 6 years of age

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To be submitted to Maternal and Child Nutrition
ABSTRACT

Some previous studies have reported positive correlations between early long-chain polyunsaturated omega-3 fatty acid (ω-3 LC-PUFA) docosahexaenoic acid (DHA, 22:6ω-3) levels and neurocognitive outcomes while others have not. Overall the results are mixed with little clarity demonstrated particularly in healthy, term-born child populations. The study examines ω-3 and ω-6 LC-PUFA status over several time points (umbilical cord blood, 6 months and 5 years of age) and assesses the relationship between LC-PUFA status at these ages and neurocognitive development at 6 years. Of the 420 neonates who were enrolled in the IFOS study, 258 participants were available for inclusion in the present correlational analyses. Neurocognitive development and behaviour were evaluated via a battery of sensitive neurocognitive assessments and questionnaires. DHA status at any age point was not correlated with neurocognitive development including language, working memory or IQ. However, from a subgroup of teacher reports (n = 30), we found a significant negative relationship between cord blood DHA status and the number of externalising problem behaviours ($B = -2.943; P = 0.001$), particularly hyperactivity ($B = -0.347; 95\% CI: -0.538, -0.126; P = 0.004$). We also revealed significant negative correlations between cord blood arachidonic acid (AA, 20:4ω-6) status and language outcomes, and AA status at 5 years with academic ($B = -2.12; P = 0.017$) and intellectual ability ($B = -1.50; P = 0.011$) at 6 years. The present study is the first to show high cord blood levels of AA and total ω-6 LC-PUFA were negatively associated with language outcomes and scholastic ability which may indicate that ω-6 fatty acid status during infancy should be monitored.

INTRODUCTION

Omega-3 (ω-3) and ω-6 fatty acid bioavailability during infancy and early childhood may have lasting effects on neurocognitive and behavioural outcomes. The ω-3 and ω-6 long-chain polyunsaturated fatty acids (LC-PUFAs), docosahexaenoic acid (DHA) and arachidonic acid (AA), rapidly accumulate within the cerebral cortex from the last trimester of gestation to approximately 2 years after birth (Martinez, 1992). Coinciding within this window of development, synapses form and neuronal cells grow and differentiate (Clandinin, 1999). DHA is incorporated into neural cell membranes, improving neuronal flexibility and signalling (Sidhu, Huang, & Kim, 2011). The negative
consequences of DHA deficiency during critical periods of brain development are widespread, and include alterations to serotonergic and dopaminergic function (Tanaka, Farooqui, Siddiqi, Alhomida, & Ong, 2012a).

The most efficient means of raising DHA concentrations requires dietary consumption of DHA-containing foods (Brenna & Diau, 2007). An alternate way of obtaining DHA is via the endogenous desaturation (i.e. addition of a double bond) and elongation (i.e. addition of 2-carbon) of ω-3 fatty acid precursors originating from α-Linolenic acid (α-LA); (Figure 5.1). However, human capacity to metabolise DHA is variable (Plourde & Cunnane, 2007). Indeed, infants raised on formula milk devoid of DHA display lower concentrations of DHA within plasma, erythrocytes and post-mortem brain tissue than those receiving dietary DHA (Farquharson et al., 1995; Makrides et al., 1994). Such differences in brain concentrations of DHA imply that dietary DHA is needed to meet early human requirements.

![Figure 5.1](image.png)

**Figure 5.1.** The metabolic pathways involved in synthesising ω-6 and ω-3 long chain polyunsaturated fatty acids from their respective shorter chain precursors.
<table>
<thead>
<tr>
<th>Country/ Reference</th>
<th>Age</th>
<th>DHA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western Australia -IFOS</td>
<td>6 month</td>
<td>6.2% Total FAs RBCs</td>
<td></td>
</tr>
<tr>
<td>South Australia (Makrides et al., 1993)A</td>
<td>22 wksA</td>
<td>6.16% Total FAs RBCsA</td>
<td>N/AA</td>
</tr>
<tr>
<td></td>
<td>16 wksB</td>
<td>4.3% Total FAs RBCsB</td>
<td>15.2% RBCsB</td>
</tr>
<tr>
<td>Gibson et al., (1997)</td>
<td>12 weeks</td>
<td>7.9% RBCs</td>
<td>14.1% RBCs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.7% Plasma</td>
<td>11.2% Plasma</td>
</tr>
<tr>
<td>USA {Miller, 2010 #1079}</td>
<td>6 months</td>
<td>2.8% Total FAs RBCs</td>
<td>11.2% Total FAs RBCs</td>
</tr>
<tr>
<td>USA Birch et al.,(2001)</td>
<td>17 weeks</td>
<td>3.8% Total FAs RBCs</td>
<td>3.9% RBCs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.6% Total FAs Plasma</td>
<td>1.5% Plasma</td>
</tr>
<tr>
<td>USA McNamara et al., 2010</td>
<td>8 years</td>
<td>3% Total FAs RBCs</td>
<td>21% Total FAs RBCs</td>
</tr>
<tr>
<td>Europe {Wolters, 2014 #1080}</td>
<td>3 years</td>
<td>1.2% Total FAs in Whole Blood</td>
<td>7.6% Total FA in Whole Blood</td>
</tr>
<tr>
<td>The Netherlands (Ghys et al., 2002)</td>
<td>47 month</td>
<td>4.7% Total FAs RBCs</td>
<td>14.1% Total FAs RBCs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.8% Total FAs Plasma</td>
<td>16.5% Total FAs Plasma</td>
</tr>
<tr>
<td>South Africa Dalton et al., (2009)</td>
<td>7-9 years</td>
<td>2.8% Total FAs PC in RBCs</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.8% Total FAs PE in RBCs</td>
<td></td>
</tr>
<tr>
<td>South Africa (Baumgartner et al., 2012)</td>
<td>6-11 years</td>
<td>3.1% Total FAs Phospholipid Fraction in RBCs</td>
<td>N/A</td>
</tr>
<tr>
<td>India (Muthayya et al., 2009)</td>
<td>6-10 years</td>
<td>3.3% Total FAs Phospholipid Fraction in RBCs</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Epidemiological studies have reported a positive relationship between ω-3 LC-PUFA intake and child neurocognitive performance (Boucher et al., 2011; Hibbeln et al., 2007; Mendez et al., 2009; Oken et al., 2008; Oken et al., 2005). Boucher et al., (2011) reported that child DHA intake was positively associated with brain activity during...
performance of a specific task requiring familiarity processing - in children from Arctic Quebec, aged 10 to 13. Benefits of DHA-rich foods have also been reported for child intelligence; from ages 6 months to 9 years, particularly for verbal IQ (Hibbeln et al., 2007, Oken et al., 2008; Gale et al., 2008). However, prospective observational studies reliant on food frequency questionnaires are subject to recall bias as participants may under- or overestimate their true intake.

There is prevailing concern that sub-optimal $\omega$-3 LC-PUFA levels during critical periods of early neurological development may put children at a neurodevelopmental disadvantage. Experimental studies in animal models have shown that $\omega$-3 LC-PUFA deficiencies cause detrimental effects on brain functioning, learning and behaviour (Reisbick, Neuringer, & Connor, 1996)(Grayson, Kroenke, Neuringer, & Fair, 2014)(Catalan et al., 2002; Wainwright, 2002)(Takeuchi, Iwanaga, & Harada, 2003)(DeMar et al., 2006). Such animal studies artificially induce total $\omega$-3 fatty acid deficiency by supplying diets that are very high in LA and very low in $\alpha$-LA. This experimental paradigm results in reduced brain levels of DHA and elevated levels of 22:5 $\omega$-6 which is a chain elongation-desaturated product of AA. It is not known whether the range of dietary LA and $\alpha$-LA intakes in humans, (which in most common circumstances do not reach the extremes used in the animal studies), lead to changes in DHA and 22:5 $\omega$-6 levels in the brain.

Positive correlations between early $\omega$-3 LC-PUFA levels and neurocognitive outcomes have been cited in the academic literature (Agostoni, Riva, et al., 1995; Dijck-Brouwer et al., 2005; Jacobson et al., 2008; Makrides et al., 2000). There is evidence that higher $\omega$-3 LC-PUFA may be favourably associated with child behavioural outcomes (Kirby et al., 2010; Krabbendam et al., 2007) and attention (Colombo et al., 2004). Pertaining to the present (IFOS) cohort, Meldrum et al., (2012), found that DHA status at 6 months was positively correlated with communication skills at 18 months. However, statistically significant correlations are not always identifiable (Bakker et al., 2003; Ghys, Bakker, Hornstra, & van den Hout, 2002; Kirby, Woodward, Jackson, Wang, & Crawford, 2010). Overall, the results are mixed with little clarity demonstrated particularly in healthy, term-born child populations.
A frequently proposed reason for the inconsistencies of previous research is that the consequence of ω-3 LC-PUFA deficiency/sufficiency depends on the developmental stage of the child. As noted above, pregnancy and the first two years of life is expected to be the most critical time for DHA bioavailability since this period coincides with maximal DHA absorption by the brain (Makrides et al., 1994). It is also plausible that DHA bioavailability may be increasingly important during childhood as the brain continues to develop. The chronological age at which the brain is most receptive to LC-PUFA supply will be investigated in the present study. Furthermore, it is speculated that ω-3 LC-PUFA supplementation may induce benefits which become easier to detect as a child ages, and undergoes more sophisticated neuro-psychometric testing.

The present study is a longitudinal follow-up of participants who were enrolled at birth. The study was initially designed to assess the relationship between child allergy outcomes and ω-3 LC-PUFA. Leading on from this, a wealth of information and data has been collected on these participants, at intervals from birth. During the 5 year follow-up (primarily for the purpose of another research project exploring cardiovascular outcomes), blood samples were collected and fatty acid levels profiled. The fact that 5 year fatty acid data was available, provided us with the opportunity to explore the associations between neurocognitive outcomes at 6 years and ω-3 and ω-6 LC-PUFA levels at a similar point in time. We also took advantage of the fatty acid data collected from cord blood and at 6 months of age.

Here we report the correlations between ω-3 and ω-6 LC-PUFA in erythrocytes and neurocognitive performance utilising the randomised control trial cohort described in Chapter 3. The study collected data on LC-PUFA levels at three time points (umbilical cord blood, 6 months and 5 years of age). As aforementioned, blood sampling at these time points was prompted by adjunct research projects sharing the IFOS cohort. This study is opportunistic since these developmental time points are also linked to periods of rapid regional brain growth and differentiation (Dobbing & Sands, 1979). Specifically: i) LC-PUFA status at birth represents prenatal availability which may be important for later cognitive function; ii) 6 months after birth represents another critical point of neurodevelopment, as neural myelination within the frontal lobes begins at this age and continues throughout childhood; iii) erythrocyte LC-PUFA at 5
years represents status during early childhood when important cognitive capacities such as literacy and numeracy are being acquired.

**METHODS**

The present paper describes the correlations between $\omega-3$ and $\omega-6$ LC-PUFA status from one or more samples (either cord blood, venous blood at 6 months and/or venous blood at 5 years) and 6 year neurodevelopmental outcome periods; (Figure 5.2.). As mentioned, this study is part of a longitudinal investigation of the neurocognitive, immunological and cardiovascular effects of fish oil supplementation during infancy (Meldrum, D’Vaz, et al., 2011). This double-blind randomised control trial was carried out through the University of Western Australia. It was deemed appropriate to combine the treatment and control groups for a pooled analysis of $\omega-3$ and $\omega-6$ LC-PUFA status since we observed that fish oil supplementation did not significantly affect neurocognitive outcomes at 6 years (Chapter 4).

![Figure 5.2. Stages of the trial from pregnancy to 6 years with the three blood sample collections.](image)

Four hundred and twenty pregnant women in their third trimester with confirmed allergic disease were enrolled in the current study between June 2005 and October 2008. Maternal allergic disease was an inclusion criteria because allergy prevention in the offspring was one aim of the randomised control trial. Participants were not eligible if they took fish oil during pregnancy (>1000 mg), typically ate fish more than three times per week or smoked during pregnancy. All children were enrolled if they delivered at term (>36 weeks) with an uncomplicated perinatal period and no congenital abnormalities. Parents completed questionnaires regarding infant anthropometric details (i.e. gender, birth weight, and birth order within their family) and provided information relating to socio-demographic characteristics (e.g. number of years of maternal education). Clinical follow-up over the course of the trial included
the assessment of allergic disease (3 months, 6 months, 1 year, 2½ years and 5 years),
neurodevelopmental status (1 year, 1½ years and 6 years) and language capacity (1½
years and 6 years).

**ASSESSMENTS AND QUESTIONNAIRES OF 6 YEAR NEUROCOGNITIVE OUTCOMES**

At the 6 year follow-up, a battery of five neuropsychological tests was used to assess
language and cognition (including behaviour and memory). The tests included the
Clinical Evaluation of Language Fundamentals 4th Ed. (CELF-4), the Wechsler
Abbreviated Scale of Intelligence (WASI), the Renfrew Bus Story, the Fruit Stroop Test
and the Self Ordered Pointing Test (SOPT). The assessment took approximately two
hours to administer in full. Three questionnaires were completed by parents including
the Children’s Communication Checklist 2nd Ed. (CCC-2), the Child Behaviour Checklist 6
– 18 (CBCL) and the Autism Spectrum Quotient – Child Version (AQ-Child). Two
additional questionnaires were completed by the children’s school teachers, which
included the Teachers Report Form 6 – 18 (TRF) of the CBCL and the Gifted Rating Scale
(GRS).

**LANGUAGE (ASSESSMENTS AND QUESTIONNAIRES)**

The CELF-4 assessed the child’s ability to grasp concepts, follow directions, understand
word structure, formulate sentences, and other aspects of communication via spoken
language (Semel et al., 2003). Four language subsets were combined to give a Core
Language score. The Renfrew Bus Story test assesses the child’s ability to complete a
story retelling with the aid of picture prompts (Pankratz et al., 2007). The Renfrew Bus
Story provided three scores including sentence length, sentence complexity and the
quality of information corresponding to the original story line. Parents completed the
Children’s Communicative Competence Scale (CCC-2), which provided a General
Communication Composite (GCC) (W. Helland, Biringer, Helland, & Heimann, 2009).
GCC scores >59 are considered to be within the normal range.

**BEHAVIOUR (QUESTIONNAIRES)**

Parents completed the CBCL, which provided insight into the child’s behavioural
competencies and problems. The CBCL derived an Internalising behaviours score and
an Externalising behaviours score and a Total Score for all problems reported. T-scores below 65 were classified as within the normal range for this age group (Berglund, Westrup, Hagglof, Hernell, & Domellof, 2013; Schmeck et al., 2001). The predictive validity of the CBCL- parent version is strengthened by the separate evaluation from the child’s school teacher (Smith, 2007). CBCL teachers’ ratings have been shown in previous research to be a highly reliable indicator of child behavioural problems (Verhulst, Koot, & Van der Ende, 1994). Parents also completed the AQ-Child questionnaire, which measures autism-spectrum-like traits in children 4 – 11 years (Auyeung et al., 2008).

**WORKING MEMORY (ASSESSMENTS)**

A working memory composite score was derived from the CELF-4. The working memory composite score was comprised of two subtests including number recall and familiar sequences.

The Self Ordered Pointing Test (SOPT) assessed non-spatial, working memory. Participants were presented with a booklet containing familiar images on each page. They were required to point to a different picture on each successive page, taking care not to point to the same picture more than once. The number of errors was calculated, (i.e. pointing to a picture which they had selected already). The SOPT was developed by the current investigators and adapted from a previously established protocol (Cragg & Nation, 2007).

The Fruit Stroop Test was designed to assess executive function, inhibition of response and working memory. The test was developed by the current investigators and again adapted from a previously established protocol (Archibald & Kerns, 1999). Participants were presented with pictures of common fruits and were required to quickly name fruit colours while ignoring interference (i.e. when fruits were wrongly coloured). An interference score denoted how well the child was able to ignore interference and answer correctly and negative values indicated greater interference.
GLOBAL INTELLIGENCE (ASSESSMENTS AND QUESTIONNAIRES)

The Wechsler Abbreviated Scale of Intelligence (WASI) was administered to evaluate overall intellectual development, as well as, verbal and non-verbal performance (Wechsler, 1999). The test consisted of the four subtests Vocabulary, Similarities, Block Design and Matrix Reasoning. Three IQ scores were derived: Performance IQ (PIQ), Verbal IQ (VIQ) and Full Scale IQ; possible scores ranged from 40 – 160, however scores of 100 were considered average.

The Gifted Rating Scale (GRS) questionnaire was completed by the child’s schoolteacher and included questions about the child’s intelligence, academic ability, creativity, artistic ability, motivation and leadership abilities (Y. Liu, Lien, Kafka, & Stein, 2010). The GRS mean score is 50.

FATTY ACID COMPOSITIONAL ANALYSIS

Umbilical cord blood was collected at birth and processed by research staff within 12 hours of delivery or sample collection. At 6 months and 5 years, up to 10 mL of blood was collected from infants/children where possible (via venipuncture of the cubital fossa vein). Fatty acid analysis was conducted using previously published methods (Mori et al., 2000). The phospholipid fraction of erythrocyte lipids was extracted by thin-layer chromatography. Fatty acid methyl esters were analysed via gas–liquid chromatography (Agilent Technologies Australia Private Limited) using Supelco SP-2560 column’s (Sigma-Aldrich Private Limited) and hydrogen carrier gas. Peaks were identified by comparing them with a known standard mixture. Individual fatty acids were calculated as a relative percentage with the evaluated fatty acids set at 100%. For the present report, fatty acid levels expressed as weight percentage (wt %) of total fatty acids.

COVARIATES

Covariates were established a priori, having been identified as important determinants of early neurocognitive development in the previous literature. The relative influences of maternal education (Bacharach & Baumeister, 1998) and the child’s gender (Andersson, Sonnander, & Sommerfelt, 1998) were analysed. Participant data were retrieved from the original study questionnaires. The number of years engaged in
education was used as a proxy for maternal intelligence. Since fish oil supplementation is known to increase DHA and EPA status and reduce AA concentrations (Simopoulos, 2002), we also adjusted in the analyses for randomised controlled trial group allocation (fish oil treatment vs control).

STATISTICAL ANALYSIS

Descriptive analyses of ω-3 and ω-6 fatty acid levels and maternal and infant characteristics were conducted. Correlations (r) were performed between neurocognitive developmental outcomes at 6 years, and ω-3 and ω-6 LC-PUFA data from cord blood and venous blood at 6 months and 5 years. We tested for associations between neurocognitive and behavioural outcomes and the following fatty acids: DHA, AA, EPA, total ω-3 LC-PUFA, total ω-6 LC-PUFA and the ω-3 LC-PUFA/ ω-6 LC-PUFA ratio. These models were decided a priori. We hypothesised that higher DHA and total ω-3 LC-PUFA would be positively associated with neurocognitive outcomes. All models were run twice, first as a simple (un-adjusted) linear regression (bivariate Pearson product-moment correlations); and then again, adjusting for covariates.

As aforementioned, covariates had already been established before statistical analyses commenced (i.e. maternal education, gender, birth weight, parity and randomised control trial treatment group). These covariates were decided upon based on their reported associations in the literature with neurologic, neurocognitive, developmental/behavioural outcomes.

Because multiple tests were undertaken, correlation coefficients were considered statistically significant at the two-tailed $P = 0.01$ level. Missing cases were excluded pairwise. Assumptions of linearity, independence of errors, homoscedasticity and normality of residuals were met. There was no multi-colinearity of independent variables in the final regression models. All statistical analyses were performed using SPSS version 21 for Windows.
RESULTS

**Population Characteristics**

Two hundred and fifty eight participants were available for inclusion in the correlational analyses - as they had both participated in the 6 year neurodevelopmental follow-up and had agreed to blood sample collection at one or more previous appointment(s); see Figure 5.3. Fluctuations in attendance and consent to blood sampling affected the number of participants per correlation (cord blood, 6 months and/or 5 years; see Figure 5.4., Figure 5.5. and Figure 5.6. respectively) varied. Furthermore, the sample sizes of the main correlations are shown in Table 5.1.

The baseline health and demographic characteristics of the 258 participants included in correlation analyses were not significantly different to the 335 participants who attended the 6-year follow-up (Table 5.2.). For example, the gender distribution of those who attended the 6-year follow-up was not significantly different to those who attended at 6 years and provided blood sample(s); (see Table 5.2). Furthermore, amongst the participants with data on neurocognitive outcomes at 6 years in addition to fatty acid data, (from cord, 6 months or 5 years) the gender distribution was equivalent with the percentage of males at each time point; 53%, 51% and 52%.

The mean ± SD of erythrocyte fatty acid levels of participants included in the present correlation analyses are shown in Table 5.3. and are in line with other Australian studies that have looked at infant/chid LC-PUFA levels (see Appendix E) In the present study, we report mean DHA in erythrocyte membranes as a percentage of total fatty acids (6.21%). This is higher than previous reports from Miller et al., (2010) in the USA who found DHA in erythrocytes was 2.81% total fatty acids. The DHA levels reported in our study echo those previously reported by another Australian research group (Makrides et al., 1993); at 6.2% of total fatty acids, as measured in erythrocytes.
**Figure 5.3.** Venn diagram showing the number of IFOS participants with data available for correlation analyses: as they have blood samples collected at birth and/or 6 months, and/or 5 years in addition to their attendance at the 6 year neurodevelopment follow-up (n = 258).

**Figure 5.4.** Venn diagrams showing the number of IFOS participants with data available for correlations between: a) cord blood LC-PUFA levels and 6 year neurodevelopment (n = 97); b) 6 month LC-PUFA levels and 6 year neurodevelopment (n = 126); c) 5 year LC-PUFA levels and 6 year neurodevelopment (n = 232).

**Table 5.1.** Number of participants with fatty acid data at birth, 6 months and 5 years and neurocognitive outcomes at 6 years: Sample sizes of the main correlations

<table>
<thead>
<tr>
<th>Blood Samples Collected at Each Time Point</th>
<th>6 year Neurocognitive Data</th>
<th>Participants with Both Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth (n = 97)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core Language - CELF-4</td>
<td>n = 92</td>
<td></td>
</tr>
<tr>
<td>Behaviour - CBCL (parent; teacher)</td>
<td>n = 95; n = 30</td>
<td></td>
</tr>
<tr>
<td>Working Memory - CELF-4</td>
<td>n = 91</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 5

Full Scale IQ - WASI \( n = 91 \)

6 Months \( n = 139 \)

Core Language - CELF-4 \( n = 118 \)

Behaviour - CBCL (parent; teacher) \( n = 121; n = 31 \)

Working Memory - CELF-4 \( n = 116 \)

Full Scale IQ - WASI \( n = 117 \)

5 Years \( n = 239 \)

Core Language - CELF-4 \( n = 219 \)

Behaviour - CBCL (parent; teacher) \( n = 213; n = 54 \)

Working Memory - CELF-4 \( n = 214 \)

Full Scale IQ - WASI \( n = 219 \)


Table 5.2. The key outcomes and characteristics of the sub-group of participants included in the present correlational analyses compared to the total IFOS cohort.

<table>
<thead>
<tr>
<th>Sub-group ( (n = 258) )</th>
<th>All 6 Yr Data ( (n = 335) )</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Outcomes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core Language</td>
<td>104.32 ± 12.39(^a)</td>
<td>103.43 ± 12.50</td>
</tr>
<tr>
<td>Total Behaviours</td>
<td>48.38 ± 9.98</td>
<td>48.41 ± 9.63</td>
</tr>
<tr>
<td>Working Memory</td>
<td>104.20 ± 13.13</td>
<td>103.83 ± 13.49</td>
</tr>
<tr>
<td>Full Scale IQ</td>
<td>108.26 ± 11.06</td>
<td>108.37 ± 11.58</td>
</tr>
<tr>
<td><strong>Covariates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group - % Fish oil</td>
<td>131 (51%)</td>
<td>216 (52%)</td>
</tr>
<tr>
<td>Gender - %Male</td>
<td>141 (54%)</td>
<td>216 (52%)</td>
</tr>
<tr>
<td>Maternal Education (years)</td>
<td>15.13 ± 2.62</td>
<td>14.66 ± 2.69</td>
</tr>
<tr>
<td>Age at 6 year visit</td>
<td>72.60 ± 7.02</td>
<td>72.99 ± 7.00</td>
</tr>
<tr>
<td><strong>Other Factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head Size at Birth (cm)</td>
<td>34.92 ± 1.39</td>
<td>34.87 ± 1.45</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3469.83 ± 424.09</td>
<td>3475.40 ± 443.16</td>
</tr>
<tr>
<td>Birth Order - Eldest Child %yes</td>
<td>121 (47%)(^c)</td>
<td>202 (48%)</td>
</tr>
<tr>
<td>Allergic Disease at 1 year - %no</td>
<td>150 (65%)</td>
<td>196 (61%)</td>
</tr>
<tr>
<td>Breastfed Ever - %yes</td>
<td>225 (97%)</td>
<td>321 (98%)</td>
</tr>
<tr>
<td>Breastfed (No. months)</td>
<td>6.75 ± 3.69</td>
<td>6.51 ± 3.57</td>
</tr>
</tbody>
</table>
Breastfed still at 1 year - %no 123 (69%) 188 (67%) .472
Childcare 1 year - %no 34 (15%) 57 (18%) .236

\(^a\) Scores of the sub-group compared to the total cohort using one-sample t-tests.
\(^b\) Mean ± SD.
\(^c\) All Such Values (Percentage).

Table 5.3. Fatty acids in erythrocyte phospholipids at birth, 6 months and 5 years (fatty acids expressed as a % of total fatty acids ± SD) of those participants who attended the 6 year follow-up.

<table>
<thead>
<tr>
<th></th>
<th>Cord Blood (n =97)</th>
<th>6 months (n = 126)</th>
<th>5 years (n = 232)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Omega-3 PUFAs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPA</td>
<td>0.32 ± 0.11</td>
<td>0.84 ± 0.43</td>
<td>0.94 ± 0.50</td>
</tr>
<tr>
<td>DHA</td>
<td>7.36 ± 1.30</td>
<td>6.56 ± 1.67</td>
<td>4.90 ± 1.34</td>
</tr>
<tr>
<td>Total ω-3 LC-PUFA</td>
<td>8.36 ± 1.46</td>
<td>9.27 ± 2.12</td>
<td>8.57 ± 1.79</td>
</tr>
<tr>
<td><strong>Omega-6 PUFAs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>15.60 ± 1.95</td>
<td>14.12± 2.08</td>
<td>16.31 ± 1.58</td>
</tr>
<tr>
<td>Total ω-6 LC-PUFA</td>
<td>26.96 ± 3.31</td>
<td>21.38 ± 3.01</td>
<td>22.27 ± 2.05</td>
</tr>
<tr>
<td><strong>Ratios</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio DHA/AA</td>
<td>0.47 ± 0.08</td>
<td>0.47 ± 0.13</td>
<td>0.31 ± 0.10</td>
</tr>
<tr>
<td>Ratio ω-3/ ω-6 LC-PUFA</td>
<td>0.31 ± 0.05</td>
<td>0.44 ± 0.12</td>
<td>0.39 ± 0.12</td>
</tr>
</tbody>
</table>

Fatty acids included in total ω-3 LC-PUFA and total ω-6 LC-PUFA;
CB total ω-3 LC-PUFA = 20:5n3, 22:5n3, 22:6n3;
6m total ω-3 LC-PUFA = 20:5n3, 22:5n3, 22:6n3;
5y total ω-3 LC-PUFA = 20:5n3, 22:5n3, 22:6n3;
CB total ω-6 LC-PUFA = 20:3n6, 20:4n6, 22:3n6, 22:4n6;
6m total ω-6 LC-PUFA = 20:3n6, 20:4n6, 22:4n6, 22:3n6;
5y total ω-6 LC-PUFA = 20:2n6, 20:3n6, 20:4n6, 22:4n6, 22:5n6;

**COVARIATES**

The pre-chosen covariates were maternal education, gender, birth weight, parity and randomised control trial treatment group. We examined the influence these covariates and confirmed that maternal education was a strong predictor of Core Language (r = 0.16; P < 0.01), Total Behaviours (r = -0.03; P < 0.01) and Full Scale IQ (r = 0.18; P < 0.01). Core Language was also significantly influenced by the child’s gender whereby females achieved scores 5 points higher than their male counterparts on average (r = -
3.25; \( P < 0.01 \)). We therefore concluded that the choice of covariates was appropriate. Since the WASI standardisation guidelines are contracted to \( \geq 6 \) years of age, we also chose to include exact age at the time of the 6 year follow-up, when analysing the WASI IQ data. We found exact age at the 6 year follow-up was significantly correlated with Full Scale IQ \( (r = 0.18; P < 0.01) \).

**LANGUAGE RESULTS**

The Core Language composite score derived from the CELF-4 was not significantly correlated with erythrocyte LC-PUFA levels at birth, 6 months or 5 years of age. However, analysis of the CELF-4 language subtests found a significant negative correlation between Recalling Sentences and cord blood \( \omega-6 \) LC-PUFA \( (r = 0.260, P = 0.014) \); which remained significant after adjustment for covariates \( (B = -0.246; P = 0.010) \).

Negative correlations were identified between \( \omega-6 \) LC-PUFA status and several secondary language outcomes. The number of subordinate clauses in the Renfrew Bus Story was negatively correlated with cord blood \( \omega-6 \) LC-PUFA status \( (B = -0.182; P = 0.001) \). This trend continued at 6 months whereby \( \omega-6 \) LC-PUFA negatively predicted subordinate clauses at 6 years \( (B = -0.145; P = 0.006) \).

**BEHAVIOUR RESULTS**

Parent reports of behavioural problems via the CBCL showed no significant correlations between Internalising or Externalising behaviours and \( \omega-3 \) or \( \omega-6 \) LC-PUFAs at birth, 6 months or 5 years. However, in a smaller subset of participants who provided teacher reports for the CBCL \( (n = 30; \textbf{Table 5.1.}) \) we found a significant negative relationship between the number of Externalising Problem Behaviours at 6 years and cord blood DHA \( (B = -2.943; P = 0.001; \textbf{Table 5.4.}) \). Similarly, this negative relationship was found for cord blood \( \omega-3 \) LC-PUFA status \( (B = -2.337; P = 0.023) \). Subtest analysis revealed a strong negative relationship between cord blood DHA and teacher reports of Hyperactivity \( (B = -0.347; 95\% CI: -0.538, -0.126; P = 0.004) \). Teacher and parent reports of Attention Problems and ADHD characteristics showed several non-significant negative trends with DHA levels at birth and 6 months (\textbf{Table 5.4.}).
**Table 5.4.** Correlations between externalising behaviours as reported by parents and teachers and DHA in erythrocytes at birth, 6 months and 5 years.

<table>
<thead>
<tr>
<th>Externalising Behaviour Scores</th>
<th>Erythrocyte DHA</th>
<th>B</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Externalising Behaviours</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Externalising - Parent</td>
<td>Cord Blood</td>
<td>-0.824</td>
<td>-2.378 - 0.730</td>
<td>0.295</td>
</tr>
<tr>
<td>Externalising - Teacher</td>
<td>Cord Blood</td>
<td>-2.935</td>
<td>-5.117 - -0.752</td>
<td>0.001</td>
</tr>
<tr>
<td>Externalising - Parent</td>
<td>6 Months</td>
<td>-0.037</td>
<td>-1.178 - 1.104</td>
<td>0.948</td>
</tr>
<tr>
<td>Externalising - Teacher</td>
<td>6 Months</td>
<td>-0.312</td>
<td>-2.345 - 1.721</td>
<td>0.754</td>
</tr>
<tr>
<td>Externalising - Parent</td>
<td>5 Years</td>
<td>0.497</td>
<td>-0.478 - 1.472</td>
<td>0.316</td>
</tr>
<tr>
<td>Externalising - Teacher</td>
<td>5 Years</td>
<td>0.023</td>
<td>-1.643 - 1.690</td>
<td>0.978</td>
</tr>
<tr>
<td><strong>ADHD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADHD - Parent</td>
<td>Cord Blood</td>
<td>-0.240</td>
<td>-0.938 - 0.458</td>
<td>0.496</td>
</tr>
<tr>
<td>ADHD - Teacher</td>
<td>Cord Blood</td>
<td>-0.470</td>
<td>-1.469 - 0.528</td>
<td>0.339</td>
</tr>
<tr>
<td>ADHD - Parent</td>
<td>6 Months</td>
<td>-0.164</td>
<td>-0.673 - 0.346</td>
<td>0.525</td>
</tr>
<tr>
<td>ADHD - Teacher</td>
<td>6 Months</td>
<td>0.001</td>
<td>-0.822 - 0.823</td>
<td>0.999</td>
</tr>
<tr>
<td>ADHD - Parent</td>
<td>5 Years</td>
<td>0.249</td>
<td>-0.187 - 0.685</td>
<td>0.262</td>
</tr>
<tr>
<td>ADHD - Teacher</td>
<td>5 Years</td>
<td>-0.085</td>
<td>-0.749 - 0.579</td>
<td>0.798</td>
</tr>
<tr>
<td><strong>Attention Problems</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attention - Parent</td>
<td>Cord Blood</td>
<td>-0.214</td>
<td>-0.829 - 0.401</td>
<td>0.490</td>
</tr>
<tr>
<td>Attention - Teacher</td>
<td>Cord Blood</td>
<td>0.047</td>
<td>-0.446 - 0.540</td>
<td>0.846</td>
</tr>
<tr>
<td>Attention - Parent</td>
<td>6 Months</td>
<td>-0.019</td>
<td>-0.469 - 0.431</td>
<td>0.932</td>
</tr>
<tr>
<td>Attention - Teacher</td>
<td>6 Months</td>
<td>-0.114</td>
<td>-0.509 - 0.282</td>
<td>0.558</td>
</tr>
<tr>
<td>Attention - Parent</td>
<td>5 Years</td>
<td>0.159</td>
<td>-0.226 - 0.543</td>
<td>0.417</td>
</tr>
<tr>
<td>Attention - Teacher</td>
<td>5 Years</td>
<td>-0.142</td>
<td>-0.473 -0.188</td>
<td>0.391</td>
</tr>
<tr>
<td><strong>Hyperactivity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperactivity - Teacher</td>
<td>Cord Blood</td>
<td>-0.347</td>
<td>-0.568 - -0.126</td>
<td>0.004</td>
</tr>
<tr>
<td>Hyperactivity - Teacher</td>
<td>6 Months</td>
<td>-0.019</td>
<td>-0.235 - 0.196</td>
<td>0.855</td>
</tr>
<tr>
<td>Hyperactivity - Teacher</td>
<td>5 Years</td>
<td>-0.081</td>
<td>-0.258 - 0.095</td>
<td>0.360</td>
</tr>
</tbody>
</table>

*Correlation data was available for cord blood DHA and CBCL parent and teacher questionnaires (n = 95 and n – 30 respectively); 6 month erythrocyte DHA and CBCL parent and teacher questionnaires (n = 121 and n = 31 respectively); 5 year erythrocyte DHA and CBCL parent and teacher questionnaires (n = 213 and n = 54 respectively).

*Note that parent report forms of the CBCL did not yield a separate score for hyperactivity.*
**WORKING MEMORY COMPOSITE SCORE**

The primary outcome for Working Memory (Working Memory Composite Score derived from the CELF-4) was not significantly correlated with erythrocyte LC-PUFA levels at birth, 6 months or 5 years of age.

Subtest analysis revealed a positive correlation between cord blood ω-6 LC-PUFA status and scores on familiar sequences ($r = 0.290$, $P = 0.002$). The strength of this association remained significant after adjustment with respect to the four aforementioned covariates ($B = 0.274; P = 0.009$).

**GENERALISED INTELLIGENCE MEASURES**

Un-adjusted correlations found significant negative relationships between ω-6 LC-PUFA concentrations at birth, 6 months and 5 years and measures of IQ at 6 years. However, after adjustment with respect to the aforementioned five covariates, these relationships were no longer significant (Table 5.5).

Academic ability, as reported by teachers through the Gifted Rating Scale (GRS), was found to be significantly negatively correlated with AA at 5 years ($B = -1.97; P = 0.011$). Similarly, this was found with 5 years ω-6 LC-PUFA status and Academic score ($B = -1.63; P = 0.006$). Scores from the GRS are not shown in a table. All correlations were adjusted for covariates.

Intellectual score from the GRS was found to be significantly negatively correlated with AA at 5 years ($B = -2.12; P = 0.017$). Likewise this was found with 5 year ω-6 LC-PUFA status and Intellectual score at 6 years ($B = -1.50; P = 0.011$).

<table>
<thead>
<tr>
<th>Table 5.5. Correlations between ω-6 LC-PUFA at birth, 6 months and 5 years and measures of IQ at 6 yearsA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cord Blood ω-6 LC-PUFA</strong></td>
</tr>
<tr>
<td>VIQ</td>
</tr>
<tr>
<td>PIQ</td>
</tr>
<tr>
<td>FSIQ</td>
</tr>
</tbody>
</table>
Adjusted B (95% CI), P-value

Correlation data was available for cord blood DHA and IQ (n = 91); 6 month erythrocyte DHA and IQ (n = 117); 5 year erythrocyte DHA IQ (n = 219).

**POST-HOC ANALYSIS**

As an exploratory post-hoc stratified analyses separated participants into quartiles according to DHA index. The neurodevelopmental outcomes of participants in the lowest DHA quartile were compared to those of the three upper DHA quartiles. We also compared the neurodevelopmental outcomes of participants in the highest DHA quartile to those in the lower three quartiles. Neither approach produced statistically significant findings (Appendix F).

**DISCUSSION**

The primary objective of this study was to explore the relationship between erythrocyte DHA status at birth, 6 months and 5 years and measures of neurocognitive/behavioural development in healthy 6 year-old children. In general, the results from the present study show little clear relationship between DHA and/or total ω-3 LC-PUFA on neurodevelopment within this healthy cohort. There was some support for a role of ω-3 LC-PUFAs in child behaviours, where children born with higher DHA status at birth had less externalising behavioural traits, specifically hyperactivity, as reported by their schoolteachers’ on the CBCL. Yet no significant correlations were observed between DHA levels at any time point and language, working memory or global intelligence outcomes. Therefore the earlier finding reported by Meldrum et al., (2012) was no longer evident in the current study of 6 year olds.

**DHA STATUS AND EXTERNALISING BEHAVIOUR**

We observed that the children born with higher DHA status at birth had less externalising behavioural traits, as reported by their schoolteachers’ on the CBCL. Further analysis of the CBCL externalising behaviour subtests revealed a significant negative relationship between teacher reports of hyperactivity and cord blood DHA. Several non-significant trends were also observed with respect to the relationships between subtests of externalising behaviours and early DHA status. Specifically, both
parent and teacher reports of ADHD and attention problems displayed non-significant negative trends in terms of their association with DHA status at all ages (particularly from cord blood and at 6 months).

The consideration of externalising behaviours, particularly attention problems is important, given that numerous epidemiologic and clinical studies have indicated low levels of DHA are associated with ADHD symptomology and could contribute to its aetiology (Gow & Hibbeln, 2014; Gow, Hibbeln, & Parletta, 2015). Our findings are in line with studies of children already diagnosed with ADHD (Gow et al., 2013; Milte et al., 2012) or other mild learning difficulties (Montgomery, Burton, Sewell, Spreckelsen, & Richardson, 2013). For example, in a UK cohort of healthy children with below average reading ability, aged 7 – 9 years, Montgomery et al., (2013) found a significant correlation between higher DHA status and fewer ADHD-type symptoms.

The present results indicate that DHA status at birth may be important for child behaviour. We previously reported that postnatal ω-3 LC-PUFA supplementation from birth to 6 months provided no behavioural benefit (Chapter 4). Clearly, cord blood DHA status is not dependent upon the trial intervention, rather on maternal dietary habits and other factors during gestation. Together, these findings highlight the complexity of the relationship between DHA and behaviour and the fact that there are probably several other important factors at play. One such factor may be due to genetic polymorphisms that modulate endogenous LC-PUFA metabolism (Brookes, Chen, Xu, Taylor, & Asherson, 2006).

**Effects of High Omega-6 LC-PUFA During Early Development**

In addition to the discussed findings for DHA, high levels of ω-6 LC-PUFA during early life were associated with poorer recalling sentences and some other language outcomes at 6 years. Despite the fact that AA does play important roles within the brain, (Schmitz & Ecker, 2008);(Sidhu et al., 2011), high ω-6 LC-PUFA can be detrimental (Katsuki & Okuda, 1995) and can stimulate apoptotic neural cell death via lipid peroxidation and oxidative damage to membrane proteins, ion channels, and receptors occurs (Zaleska & Wilson, 1989). These effects could explain why children with high ω-6 LC-PUFA had lower language scores.
Another plausible explanation as to the negative relationship between ω-6 LC-PUFA levels and neurocognitive development is the fact that the ω-6 and ω-3 fatty acid families compete for the same enzymes and transport systems (Brenna, Salem, Sinclair, & Cunnane, 2009). Thus, higher levels of ω-6 LC-PUFA inhibit endogenous DHA synthesis (Gibson, Neumann, Lien, Boyd, & Tu, 2013). Therefore while no relationship between ω-3 LC-PUFA and language was observed, the significant negative association with ω-6 LC-PUFA could suggest the involvement of ω-3 LC-PUFA.

Whether the observed negative effect on language can be attributed to excess ω-6 LC-PUFA, insufficient ω-3 LC-PUFA or some other factor is unclear. Regardless of the mechanisms involved, our study highlights the fact that heightened levels of AA during times of perinatal and infant brain development can induce negative effects at 6 years of age. This should be followed-up to see if this effect persists into later childhood.

**Strengths and Limitations of the IFOS 6 Year Follow-up**

A strength of the present study was the use of a variety of neurocognitive and behavioural assessments developed to evaluate specific cognitive domains in addition to global outcomes. Specifically, the test battery included several non-standardised assessments capable of detecting subtle differences in healthy children. Other strengths of the present study include questionnaire data collected from both parents and teachers, providing insight into the child’s academic ability, behavioural competencies and problems from two separate sources.

CBCL teachers' ratings are a reliable indicator of child behavioural problems (Verhulst et al., 1994), with especially high predictive validity diagnosing externalising behavioural problems (Smith, 2007). We recognise that we have a small sample size due to the absence of teacher reports returned to the study and so these results should be treated with caution.

**Conclusion**

In summary, we found no evidence of any significant positive relationships between neurocognitive skills and ω-3 LC-PUFA status at birth, 6 months or 5 years. The present study is the first to show high cord blood levels of ω-6 LC-PUFA, particularly AA, were
negatively associated with language outcomes and scholastic ability which may indicate that ω-6 LC-PUFA status during infancy should be closely monitored. This study found that higher concentrations of DHA at birth was associated with less externalising behaviours namely hyperactivity, although, this behavioural data was limited by its sample size and may not have clinical relevance.

Candidate contribution:

Alexandra Heaton re-enrolled participants at 6 years, wrote the ethics application that was submitted to the education department, performed neuropsychometric testing, was responsible for data entry, statistical analysis, writing, and editing of this manuscript. Alexandra also presented this research at national and international meetings.
CHAPTER 6

FADS genetic factors modulating individual dietary requirements for omega-3 LC-PUFA

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To be submitted to Prostaglandins, Leukotrienes and Essential Fatty Acids
ABSTRACT

Neural supply of long-chain polyunsaturated fatty acids (LC-PUFA) is important for neurocognitive and behavioural development. Circulating concentrations of LC-PUFA are determined by diet and genetics. Research conducted in our laboratory and elsewhere have consistently found strong associations between the fatty acid desaturase (FADS) genes and circulating LC-PUFAs. Using regression analyses, we evaluated whether FADS gene variants or haplotypes modulate neurocognitive and behaviour development either directly or via an interaction with fish oil supplementation. A total of 277 participants from the Infant Fish Oil Study (IFOS) of Western Australia were included in the analysis, encompassing 21 FADS gene cluster SNPs. We found FADS genotypes and haplotypes were associated with behaviour at 6 years. Specifically, FADS1-MaA haplotype, consisting of the 5 major FADS1 alleles: rs174545, rs174546, rs174548, rs174553, rs174556, i.e. CCCAC respectively, significantly predicted more internalising behavioural problems. However, there was no significant interaction with fish oil treatment. Major allele carriers of several SNPs within FADS1, FADS2 and FADS3 (rs174548, rs99780, rs174570, rs2727271 and rs174455) were found to independently predict significantly higher internalising behaviour. Major alleles of these SNPs have been shown to be associated with better endogenous metabolism of arachidonic acid (AA) and other LC-PUFAs. These results highlight the idea that genetic variants of FADS1, FADS2 and FADS3 may modulate behavioural traits via their effect on LC-PUFA metabolism.

INTRODUCTION

Fish oil and breast milk both contain omega-3 (ω-3) polyunsaturated fatty acids (PUFAs), including the long-chain polyunsaturated fatty acid (LC-PUFA) docosahexaenoic acid (DHA; 22:6 ω-3). Modern Western diets are overall low in DHA (Meyer, 2011). This is concerning because the alternative way of obtaining DHA is via the desaturation and elongation of dietary α-Linolenic acid (α-LA); (Figure 6.1.). Yet, this reaction cascade is thought to be inefficient and can be complicated by genetic polymorphisms in the fatty-acid desaturase (FADS) gene cluster. This same reaction cascade, involving desaturases and elongases is also responsible for the metabolism of dietary linoleic acid (LA) into arachidonic acid (AA, 20:4 ω-6).
Figure 6.1. The metabolic pathways involved in synthesising ω-6 and ω-3 long chain polyunsaturated fatty acids from their respective shorter chain precursors.

**DHA within the Brain**

DHA is the most abundant ω-3 LC-PUFA found within the human brain. The amount of DHA taken up by the brain depends on bioavailability – particularly during critical times of early brain growth (Novak, Dyer, & Innis, 2008). Low bioavailability of ω-3 LC-PUFA and/or comparably high levels of ω-6 LC-PUFA within the circulation forces the brain to compensate by accreting AA and other ω-6 LC-PUFAs in place of DHA (Guesnet & Alessandri, 2011). Though, due to their structural and functional dissimilarities, this substitution is supposed to have disadvantageous consequences.

AA gives rise to inflammatory mediators whereas the ω-3 LC-PUFAs [eicosapentaenoic acid (EPA, 20:5 ω-3) and DHA] are relatively anti-inflammatory (Chapkin, Kim, Lupton, & McMurray, 2009). Yet the beneficial neural effects of DHA are complex and go beyond the physical displacement of AA within cell membranes. DHA also increases
neuroprotectin-D1 and resolvins, which prevent neural apoptosis (Joffre, Nadjar, Lebbadi, Calon, & Laye, 2014). Furthermore, DHA influences cell signalling, neurotransmitters, and receptor expression and function, in addition to gene regulation and expression (Bazinet & Layé, 2014). Based on animal literature with extreme deprivation of ω-3 PUFA, it is possible that in some fairly extreme situations there could be effects on behaviour and learning in children (McNamara & Carlson, 2006).

Low dietary consumption of ω-3 LC-PUFAs and abnormal lipid metabolism have been implicated in several neuropsychiatric diseases, as recently reviewed by L. Liu et al. (2014). Despite the putative benefits of enhancing dietary DHA, the findings from a number of randomised controlled trials evaluating the neurocognitive and behavioural effects of ω-3 LC-PUFA supplementation during infancy have indicated no developmental benefit to children (Beyerlein et al., 2010; Gould et al., 2013; Qawasmi et al., 2012; Simmer, 2001; Simmer et al., 2008, 2011). In line with previous randomised control trials, we recently reported that fish oil supplementation during infancy was not associated with neurocognitive outcomes at 6 years, albeit positively associated with the development of more externalising behavioural problems (Chapter 4).

**GENETIC VARIABLES MODULATING LC-PUFA STATUS**

The lack of consistent evidence is suggested to be at least partially caused by differing abilities to endogenously synthesise ω-3 and ω-6 LC-PUFA due to individual genetic polymorphisms (Chilton et al., 2014). In mammals, members of ω-3 and ω-6 fatty acid families compete with each other for enzymes involved in the elongation-desaturation of applicable ω-3 and ω-6 LC-PUFAs. The precise enzymes at the heart of this are delta-5 and delta-6 desaturases (Δ5D and Δ6D, respectively). The availability and activity of Δ5D and Δ6D form the rate-limiting step of the reaction. The genes coding for Δ5D and Δ6D are FADS1 and FADS2, respectively, which are located on human chromosome 11 (Mathias et al., 2014). Less is understood about FADS3, however, it is clustered with family members FADS1 and FADS2 at 11q12-q13.1 (Park, Kothapalli, Reardon, & Brenna, 2009).
The proposition that FADS polymorphisms play a key role in neurocognitive development is strengthened by previous reports that major allele carriers of the FADS genotypes are associated with up-regulation of desaturase activity – display decreased levels of desaturase substrates (i.e. α-LA and LA) and increased levels of desaturase products (i.e. DHA and AA) (Hester et al., 2014). With few exceptions, the majority of studies conclude that minor FADS allele carriers display lower levels of LC-PUFA products and higher proportions of substrate fatty acids. Indeed, such relationships have been confirmed by recent (unpublished) work conducted in our laboratory; whereby major allele single nucleotide polymorphisms (SNPs) within the FADS gene cluster predict significantly higher LC-PUFA levels (particularly AA) in the circulation compared to minor allele carriers.

Genetic studies have underscored the importance of FADS genotypes by linking SNPs in these genes with numerous fatty acid related phenotypes including coronary artery disease (Martinelli et al., 2008) allergy (Standl et al., 2011) and atopic eczema (Rzehak et al., 2009), as well as cognitive outcomes such as attention-deficit hyperactivity disorder (ADHD) symptomology, neurocognitive development (Brookes et al., 2006; Steer et al., 2010), schizophrenia (Y. Liu, Jandacek, Rider, Tso, & McNamara, 2009) and bipolar disorder (Y. Liu & McNamara, 2011).

A study conducted in two large birth cohorts by Caspi et al., (2007) found breastfed children carrying one or two copies of the major allele for rs174575 (FADS2) had significantly higher IQ (5.8 points) compared to carriers of the same allele who were not breastfed. Therefore, minor allele carriers benefited most from receiving breast milk in terms of their later IQ. Steer et al., (2010) performed a similar analysis on the Avon Longitudinal Study of Parents and Children (ALSPAC); when children were 8 years of age (n = 5934). They reported significant results in the opposite direction to those found by Caspi et al., (2007), showing that the IQ of breastfed children was similar, irrespective of FADS2 genotype.

There have been no previous randomised controlled trials investigating the interactional effect between FADS polymorphisms and ω-3 LC-PUFA supplementation, on neurocognitive and behavioural outcomes during early childhood. Since the aforementioned randomised control trial supplementation studies could have been
inadvertently confounded by inextricable factors relating to breastfeeding, it is important for the present study to elucidate on this interesting question – within the framework of a methodologically robust randomised control trial.

Growing understanding about the FADS region as a key locus for LC-PUFA biosynthesis has caused us to reconsider the belief that raising dietary DHA and EPA via supplements (i.e. fish oil) during the infant brain growth spurt will improve neurocognitive/behavioural outcomes amongst a population of genetically heterogeneous, healthy, term-born children. Therefore research has turned to the investigation of specific genetic subgroups which may differentially benefit from supplementation.

To the best of our knowledge, this is the first randomised controlled trial to investigate whether FADS polymorphisms positively predict neurocognitive/behavioural outcomes in children. The novel objective in the present report, was to determine whether fish oil supplementation interacts with SNPs/haplotypes in the FADS1-FADS2-FADS3 gene cluster to influence neurocognitive/behavioural outcomes in children.

Thus providing an explanation as to why previous research into the benefits from LC-PUFA supplementation yielded somewhat diluted results amongst genetically heterogeneous sample populations. If indeed the FADS genes moderate the beneficial effect of dietary ω-3 LC-PUFA, this may have significant implications for identifying which children are more likely to derive benefits from fish oil supplementation.

**METHODS**

This study is part of the Infant Fish Oil Study (IFOS), the methodology of which has been described elsewhere (Meldrum, D'Vaz, et al., 2011). Briefly, 420 pregnant mothers were recruited during the third trimester. Prior to birth, infants were randomized to receive either fish oil (containing 250 - 280 mg DHA and 60 – 110 mg EPA per day) or placebo (olive oil) once daily from birth to 6 months. Blood samples were collected at birth (via umbilical cord blood), 6 months and at 5 years of age (via venipuncture). Neurocognitive development was assessed at the 6 year follow-up.
SIX YEAR NEUROCOGNITIVE/ BEHAVIOURAL ASSESSMENT

At 6 years, all participants were invited to take part in the neurocognitive/behavioural follow-up and 80% of participants attended (n = 335). The 6 year assessment consisted of a 2 hour appointment at the Children’s Research Facility at Princess Margaret Hospital in Perth where they were assessed via the Clinical Evaluation of Language Fundamentals 4th Edition (CELF-4) which gave rise to a core language composite score and a working memory composite score and the Wechsler Abbreviated Scale of Intelligence (WASI) which yielded the Performance IQ, Verbal IQ and Full Scale IQ scores. These tests provided standardised scores that were age adjusted and normally distributed. Parents and teachers completed Child Behavioural Checklist (CBCL) to provide information on internalising and externalising behavioural traits.

As described in Chapter 4, in this same randomised controlled trial, while overall we found no benefits from supplementation, we found that the group of children who were received fish oil supplementation from birth to 6 months reported adverse behavioural effects, particularly in the domain of oppositional/defiance, compared to the control group. However, when we looked at the concentrations of fatty acids in erythrocytes at birth, 6 months and 5 years (as described in Chapter 5), DHA appeared to be having a protective effect on behavioural problems, namely externalising behaviours particularly hyperactivity. On the other hand, we found AA levels in cord blood and 6 months were negatively correlated with language development at 6 years.

DNA EXTRACTION AND PROCESSING

As aforementioned, blood samples were collected from IFOS study participants at three time points: at birth, 6 months and at 5 years of age. Blood cell pellets (0.3 – 2ml) were stored at -80°C until processed using QIAamp® DNA Midi Kits (Qiagen) according to manufacturer’s instructions. Where participants had multiple blood samples in storage, samples collected at 5 years, or at 6 months were preferentially chosen for DNA purification. However, if blood was not collected at either of these time points, cord blood samples were utilized instead (n = 14). A sub-group (n = 8) of participants who consented to DNA collection but did not desire venipuncture,
provided saliva samples as an alternative means of DNA attainment, utilizing Oragene® oral DNA collection kits. DNA was extracted from 277 participants in total and sample purity was evaluated on a NanoDrop spectrophotometer and was confirmed to be of good quality (260/280 nm >1.80); (Figure 6.2.).

**Figure 6.2.** Events Preceding FADS Genotyping. Blood samples collected at three timepoints (i) birth (via umbilical cord); (ii) at 6 months of age; (iii) at 5 years of age. Samples were stored at -80⁰C until DNA extraction was performed via a QIAamp® DNA Blood Midi kit (Qiagen).

**Genotyping**

Genotyping was performed for 21 SNPs within the FADS gene cluster. SNP selection was conditional on their inclusion within previous research pertaining to essential fatty acid metabolism or LC-PUFA concentrations in humans. Secondly, the frequency of the minor alleles was ≥20%, as this would ensure that an adequate number of participants homozygous for the minor (less common) allele would be probable within our sample population.

International collaboration with researchers (Koletzko et al.) in Munich, Germany was engaged due to their extensive experience genotyping FADS. SNPs were genotyped according to manufacturer recommended protocols on the AB7900 TaqMan platform. Genotyping was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to detect allele-specific primer extension products (Mass Array, Sequenom, San Diego, CA) according to predefined protocol developed by Sequenom http://www.sequenom.com/Sites/Genetic-Analysis/Applications/SNP-Genotyping. PCR primers were designed by Sequenom’s MassArrayAssayDesign program, and then aligned to the gene cluster in order to check for accuracy and to eliminate false results from genotyping repeat regions.
Standard quality control was assured through the inclusion of (10%) duplicate and negative samples (Figure 6.3.).

Figure 6.3. Isolated DNA samples from 277 participants were then transported to Germany where FADS genotyping was performed and then genotype data sent back to Perth Laboratory via email communication.

Characteristics of Selected SNPs

Genotyping was performed for 21 SNPs within the FADS gene cluster. The characteristics of these SNPs, including their positions on chromosome 11, their possible functions and their genotype and allele frequencies are summarised in Table 6.1. The genotype distribution for each SNP did not deviate from Hardy-Weinberg equilibrium (Hardy-Weinberg equilibrium $P$-values <0.05). The average minor allele frequency (MAF) of those genotyped was 31%. Only one SNP was excluded from the analysis (rs968567) as it had a minor allele frequency (MAF) below 5% and most SNPs ranged from between 20 and 42%, (Table 6.1).

Table 6.1. Characteristics of Single Nucleotide Polymorphisms within the FADS gene cluster

<table>
<thead>
<tr>
<th>SNP</th>
<th>Possible functional region</th>
<th>Position</th>
<th>Alleles Major/Minor</th>
<th>MAF</th>
<th>Subjects with genotype MM; Mm; mm</th>
<th>HW Sig</th>
<th>Genotyping Success Rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs174545</td>
<td>FADS1</td>
<td>61569306</td>
<td>C/G</td>
<td>.29</td>
<td>108 129 36</td>
<td>.749</td>
<td>98.9</td>
</tr>
<tr>
<td>rs174546</td>
<td>FADS1</td>
<td>6159830</td>
<td>C/T</td>
<td>.29</td>
<td>108 130 36</td>
<td>.705</td>
<td>99.3</td>
</tr>
<tr>
<td>rs174548</td>
<td>FADS1</td>
<td>61571348</td>
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<td>.33</td>
<td>111 131 30</td>
<td>.669</td>
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</tr>
<tr>
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<td>FADS1</td>
<td>61575158</td>
<td>A/G</td>
<td>.29</td>
<td>108 131 37</td>
<td>.797</td>
<td>99.6</td>
</tr>
<tr>
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<td>C/T</td>
<td>.28</td>
<td>109 129 31</td>
<td>.405</td>
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</tr>
<tr>
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<td>61639573</td>
<td>T/C</td>
<td>.40</td>
<td>107 126 41</td>
<td>.695</td>
<td>99.9</td>
</tr>
<tr>
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<td>61640379</td>
<td>A/G</td>
<td>.48</td>
<td>107 125 43</td>
<td>.519</td>
<td>99.3</td>
</tr>
<tr>
<td>rs99780</td>
<td>FADS2</td>
<td>61596633</td>
<td>C/T</td>
<td>.39</td>
<td>103 127 41</td>
<td>.80</td>
<td>98.6</td>
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<tr>
<td>rs174570</td>
<td>FADS2</td>
<td>61597212</td>
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<td>195 73 4</td>
<td>.47</td>
<td>98.6</td>
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<tr>
<td>rs174574</td>
<td>FADS2</td>
<td>61600342</td>
<td>C/A</td>
<td>.47</td>
<td>106 129 40</td>
<td>.90</td>
<td>99.6</td>
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<tr>
<td>rs174575</td>
<td>FADS2</td>
<td>61602003</td>
<td>C/G</td>
<td>.21</td>
<td>149 99 27</td>
<td>.08</td>
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<tr>
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<td>108 127 38</td>
<td>.898</td>
<td>98.9</td>
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<td>104 129 38</td>
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<tr>
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<td>FADS2</td>
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<td>169 88 16</td>
<td>.384</td>
<td>98.6</td>
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<tr>
<td>rs174583</td>
<td>FADS2</td>
<td>61609750</td>
<td>C/T</td>
<td>.37</td>
<td>110 123 40</td>
<td>.522</td>
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<tr>
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<td>FADS2</td>
<td>61624414</td>
<td>A/G</td>
<td>.42</td>
<td>169 85 19</td>
<td>.574</td>
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<td>FADS2</td>
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<td>G/A</td>
<td>.31</td>
<td>116 120 35</td>
<td>.595</td>
<td>98.2</td>
</tr>
</tbody>
</table>
The genewise regions of FADS1 and FADS2 appropriate for haplotype estimation were identified through calculating linkage disequilibrium (LD) blocks. Haplotype blocks were statistically reconstructed via PHASE (version 2.1.1) using SNPs with a minor allele frequency >0.1. Haplotype blocks were further defined as regions with Lewontin’s D’ >0.8 between consecutive SNPs. One LD block was observed in the FADS1 gene region (Figure 6.4A), from rs174545 to rs174556 and another LD block was observed in FADS2, from rs968567 to rs526126 (Figure 6.4B). Haplotype blocks were identified using Haploview version 4.2 (Broad Institute). Data were restricted to the haplotype for each individual with a conditional probability exceeding 0.9 for haplotypes generated from all seventeen SNP.

**Figure 6.4.** The SNP linkage disequilibrium patterns of **A)** FADS1 and **B)** FADS2 were assessed and visually presented as a heat map (generated by Haploview software). The numbers in the squares are pairwise D’ values (%), with D’ = 100 if no number is given.
STATISTICAL ANALYSIS

Statistical analysis was performed using the IBM statistical software, Statistical Package for the Social Sciences (SPSS) Version 21 for PC. The outcome measures (neurocognitive/behavioural results), obtained at 6 years, were tested for normality and if necessary, log transformations were applied prior to further analysis.

HAPLOTYPE STATISTICAL ANALYSES

For haplotype analysis, additive inheritance models were assumed, based on the number of copies of the major alleles, i.e. FADS haplotypes were encoded as a 3-category, ordinal variables; 0 = negative haplotype; 1 = heterozygous (1 Allele); and 2 = homozygous (2 Alleles). Similarly, FADS SNPs were encoded as a 3-category, ordinal variables: mm = minor allele homozygotes; Mm = heterozygotes; and MM = major allele homozygotes.

Generalized linear regression models were used to analyse the associations between haplotypes and SNPs with neurocognitive/behavioural outcomes. The neurocognitive/behavioural variable was entered as the dependent variable and SNP or haplotype was entered separately as the (primary) independent variable.

In the first model, the crude bivariate, linear relationship between these variables was investigated, with no variables controlled for (unadjusted). The second model, (a multivariable regression analysis) had neurocognitive/behavioural variable entered as the dependent variable, the SNP or haplotype entered as the independent variable with gender and supplementation group (fish oil vs placebo: from birth to 6 months) controlled for in the model. This was deemed appropriate since previous research has indicated that females tend to metabolise ω-3 and ω-6 fatty acids more efficiently than males (Decsi & Kennedy, 2011). Moreover, some randomised control trials of infant LC-PUFA supplementation have found sex-specific differences in some neurocognitive/behavioural outcomes (Meldrum, Smith, et al., 2011).

In separate models, the supplementation-by-genotype interaction effect was investigated with the inclusion of an interaction term (genotype x intervention).
SNP Statistical Analyses

Importantly, to reduce the number of comparisons, the statistical analyses were conducted in a systematic manner. Haplotypes were used as the primary outcome. SNPs were only examined if indicated by a significant haplotype association. We additionally analysed the 2 SNPs within the intergenic region of FADS1/FADS2 (rs174448 and rs174449) and the single FADS3 SNP, (rs174455) despite exclusion from haplotypes.

RESULTS

Haplotype Association with Neurocognitive and Behavioural Outcomes

Only 6 haplotypes had a frequency >5%: FADS1-MaA, FADS1-MiA, FADS2-MaA, FADS2-Hapl.1, FADS2-Hapl.2 and FADS2-Hapl.3 (Table 6.2 and Table 6.3). Subsequently, these 6 haplotypes were investigated further for associations with neurocognitive and/or behavioural outcomes (Table 6.4).

No significant relationships were identified for the key neurocognitive outcomes including working memory, core language or full scale IQ. However, three significant associations were found between behavioural outcomes (internalising and externalising behavioural traits) and haplotypes.

Table 6.2. Haplotype characteristics for FADS1

<table>
<thead>
<tr>
<th></th>
<th>rs174545</th>
<th>rs174546</th>
<th>rs174548</th>
<th>rs174553</th>
<th>rs174556</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADS1-MaA</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>60.50%</td>
</tr>
<tr>
<td>FADS1-MiA</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>G</td>
<td>T</td>
<td>35.50%</td>
</tr>
<tr>
<td>FADS1-Hapl.1</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>2.50%</td>
</tr>
<tr>
<td>FADS1-Hapl.2</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>1.30%</td>
</tr>
<tr>
<td>FADS1-Hapl.3</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>0.90%</td>
</tr>
<tr>
<td>FADS1.Hapl.Rare</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>T</td>
<td>3%</td>
</tr>
</tbody>
</table>

MaA, major allele, haplotype carrying only common alleles; MiA minor allele, haplotype carrying only rare alleles.
### Table 6.3. Haplotype characteristics for FADS2

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADS2-MaA</td>
<td>26.70%</td>
</tr>
<tr>
<td>FADS2-Haplo.1</td>
<td>27%</td>
</tr>
<tr>
<td>FADS2-Haplo.2</td>
<td>6.70%</td>
</tr>
<tr>
<td>FADS2-Haplo.3</td>
<td>5.90%</td>
</tr>
<tr>
<td>FADS2-Haplo.4</td>
<td>3.10%</td>
</tr>
<tr>
<td>FADS2-Haplo.5</td>
<td>3%</td>
</tr>
<tr>
<td>FADS2-Haplo.6</td>
<td>2.50%</td>
</tr>
<tr>
<td>FADS2-Haplo.7</td>
<td>2.30%</td>
</tr>
<tr>
<td>FADS2-Haplo.8</td>
<td>2.20%</td>
</tr>
<tr>
<td>FADS2-Haplo.9</td>
<td>2.20%</td>
</tr>
<tr>
<td>FADS2-Haplo.</td>
<td>3%</td>
</tr>
<tr>
<td>Rare</td>
<td></td>
</tr>
</tbody>
</table>

*MaA, major allele, haplotype carrying only common alleles.*
**FADS1 Major Allele Variants and Behaviour**

FADS1-MaA haplotype, consisting of the 5 major FADS1 alleles: rs174545, rs174546, rs174548, rs174553, rs174556, i.e. CCCAC respectively, significantly predicted more internalising behavioural problems (Figure 6.5A). This association remained significant after controlling for the effects of gender and treatment group. FADS1-MiA haplotype consisting of all the minor alleles (i.e. GTGGT), displayed a significant negative relationship with internalising behaviours (Figure 6.5B), which also retained significance after adjustment for gender and treatment group.

The interaction effect between FADS1-MaA haplotype and capsule treatment group on internalising behaviour was not significant F = 2.01(df=2); P = 0.14, nor was the interaction between FADS1-MiA and capsule treatment group significant F = 2.1(df=2); P = 0.126.

**FADS2 Major Allele Variants and Behaviour**

FADS2-MaA haplotype, consisting of the 13 major FADS2 alleles rs968567, rs99780, rs174570, rs174574, rs174575, rs2727271, rs174576, rs174578, rs174579, rs174583, rs174602, rs498793, rs526126, i.e. GCCCACTCCAGC significantly predicted more externalising behavioural problems (Figure 6.5C). However, this was not significant after adjustment for gender and treatment group.

Capsule treatment group was not found to significantly interact with FADS2-MaA haplotype to affect externalising behaviour F = 1.7(df=2); P = 0.846.

No significant relationships for the other neurocognitive or behavioural outcomes investigated and FADS haplotypes were identified.
Figure 6.5. Internalising Behaviour Scores and FADS1 and FADS2 Haplotype – Box-Whisker plot showing the distributions of internalising scores among participants of A) FADS1-MaA major allele haplotype, B) FADS1-MiA minor allele haplotype and C) FADS2- major allele haplotype.

Significance indicated above each plot. Boxes indicate 25 to 75% percentiles, horizontal lines indicate mean values and vertical lines indicate ranges.

FADS Gene Cluster SNP Associations with Behaviours

Closer investigation of the relationship between internalising behaviours and SNPs revealed significant associations for rs174548, rs174553, rs174448, rs174449, rs99780, rs174570, rs174578, rs2727271 and rs174455 (Figure 6.6). The results showed that major allele carriers had higher internalising behavioural scores. The associations for rs174548, rs99780, rs174570, rs2727271 and rs174455 were significant after controlling for the effects of capsule treatment group and gender (Figure 6.6). Other SNPs were not found to be statistically significantly associated with internalising behaviour.

Analysis of the relationship between externalising behaviour and FADS SNPs revealed one significant interaction between the FADS3 SNP rs174455, ($B = -0.08$ (SE = 0.90); $P = 0.030$) however, this did not retain significance after adjusting for gender and
Chapter 6

treatment group ($B = -1.17$ (SE = 0.9); $P = 0.196$). No significant relationships were identified for FADS1 or FADS2 SNPs and externalising behaviour.

**Figure 6.6.** Box-Whisker plots showing the distributions of internalising behaviour scores and the 5 FADS1 SNPs, those FADS2 SNPs where significance was met in addition to the FADS3 SNP.

Significance indicated above each plot pertains to the results for all three groups, and the $P$-values above specific lines only for the 2 groups specifically. Genotypes are listed at the bottom of each plot. Boxes indicate 25 to 75% percentiles, horizontal lines indicate mean values and vertical lines indicate ranges. For rs174570 and rs2727271, the heterozygotes and minor allele homozygotes were combined since the number of participants homozygous for the minor allele was below $n = 4$. 

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### Table 6.4: Associations Between FADS1 and FADS2 Haplotypes and Neurodevelopmental/Behavioural Outcomes

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Neuro/Behavior</th>
<th>Not carriers</th>
<th>One allele</th>
<th>Two alleles</th>
<th>Unadjusted Sig.</th>
<th>Adjusted Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FADS1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FADS1 - MajorA</strong></td>
<td>N = 35</td>
<td>N = 112</td>
<td>N = 83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11111</td>
<td>Working Memory</td>
<td>102.97 (14.7)</td>
<td>104.84 (13.51)</td>
<td>103.54 (13.13)</td>
<td>P = 0.704</td>
<td>P = 0.892</td>
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<tr>
<td></td>
<td>Core Language</td>
<td>104.45 (13.36)</td>
<td>102.75 (12.77)</td>
<td>104.17 (12.31)</td>
<td>P = 0.664</td>
<td>P = 0.989</td>
</tr>
<tr>
<td></td>
<td>Full-Scale IQ</td>
<td>12.19 (2.43)</td>
<td>11.18 (2.39)</td>
<td>11.61 (2.54)</td>
<td>P = 0.104</td>
<td>P = 0.438</td>
</tr>
<tr>
<td></td>
<td>Autism Q-Child</td>
<td>42.52 (13.36)</td>
<td>46.25 (14.04)</td>
<td>44.62 (17.39)</td>
<td>P = 0.432</td>
<td>P = 0.755</td>
</tr>
<tr>
<td>22222</td>
<td>Internalising</td>
<td>47.6 (11.47)</td>
<td>46.79 (9.48)</td>
<td>50.48 (10.07)</td>
<td>( \text{P} = 0.037^* )</td>
<td>( \text{P} = 0.045^* )</td>
</tr>
<tr>
<td></td>
<td>Externalising</td>
<td>50 (8.19)</td>
<td>49.05 (9.19)</td>
<td>51.16 (10.29)</td>
<td>P = 0.310</td>
<td>P = 0.328</td>
</tr>
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<td><strong>FADS1 - MinorA</strong></td>
<td>N = 94</td>
<td>N = 112</td>
<td>N = 24</td>
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<tr>
<td>22222</td>
<td>Working Memory</td>
<td>104.41 (13.3)</td>
<td>104.42 (13.21)</td>
<td>101.3 (15.89)</td>
<td>P = 0.579</td>
<td>P = 0.541</td>
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<td></td>
<td>Core Language</td>
<td>104.64 (12.6)</td>
<td>102.58 (12.57)</td>
<td>103.61 (13.53)</td>
<td>P = 0.511</td>
<td>P = 0.542</td>
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<tr>
<td></td>
<td>Full-Scale IQ</td>
<td>11.5 (2.51)</td>
<td>11.3 (2.43)</td>
<td>12.26 (2.36)</td>
<td>P = 0.245</td>
<td>P = 0.328</td>
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<tr>
<td></td>
<td>Autism Q-Child</td>
<td>45.5 (17.45)</td>
<td>45.25 (13.64)</td>
<td>42.75 (13.16)</td>
<td>P = 0.735</td>
<td>P = 0.495</td>
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<td>Internalising</td>
<td>50.13 (9.96)</td>
<td>46.56 (9.81)</td>
<td>48.71 (11.18)</td>
<td>( \text{P} = 0.040^* )</td>
<td>( \text{P} = 0.077 )</td>
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<td>Externalising</td>
<td>50.98 (10.16)</td>
<td>48.75 (9.18)</td>
<td>51.58 (7.45)</td>
<td>P = 0.164</td>
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<td><strong>FADS2</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FADS2 - MajorA</strong></td>
<td>N = 126</td>
<td>N = 87</td>
<td>N = 17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1111111111111</td>
<td>Working Memory</td>
<td>104.91 (14.22)</td>
<td>103.4 (12.46)</td>
<td>102.26 (13.86)</td>
<td>P = 0.602</td>
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<td>Core Language</td>
<td>102.82 (12.67)</td>
<td>103.67 (12.44)</td>
<td>107.16 (13.64)</td>
<td>P = 0.376</td>
<td>P = 0.293</td>
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<td></td>
<td>Full-Scale IQ</td>
<td>11.46 (2.37)</td>
<td>11.32 (2.43)</td>
<td>12.26 (3.12)</td>
<td>P = 0.327</td>
<td>P = 0.629</td>
</tr>
<tr>
<td></td>
<td>Autism Q-Child</td>
<td>46.92 (14.96)</td>
<td>42.54 (15.37)</td>
<td>45.18 (15.62)</td>
<td>P = 0.120</td>
<td>P = 0.132</td>
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<tr>
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<td>Internalising</td>
<td>47.55 (10.53)</td>
<td>48.48 (9.59)</td>
<td>52.18 (9.13)</td>
<td>P = 0.201</td>
<td>P = 0.969</td>
</tr>
<tr>
<td></td>
<td>Externalising</td>
<td>50.32 (9)</td>
<td>48.43 (10.15)</td>
<td>55.12 (7.53)</td>
<td>( \text{P} = 0.023^* )</td>
<td>( \text{P} = 0.589 )</td>
</tr>
<tr>
<td>Haplotype</td>
<td>Neuro/Behavior</td>
<td>Not carriers</td>
<td>One allele</td>
<td>Two alleles</td>
<td>Unadjusted Sig.</td>
<td>Adjusted Sig.</td>
</tr>
<tr>
<td>-----------</td>
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<td>--------------</td>
<td>------------</td>
<td>-------------</td>
<td>----------------</td>
<td>--------------</td>
</tr>
<tr>
<td><strong>FADS2 - HAP#1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11111111111121</td>
<td>Working Memory</td>
<td>103.68 (13.23)</td>
<td>104.29 (13.93)</td>
<td>106.2 (13.63)</td>
<td>( P = 0.783 )</td>
<td>( P = 0.485 )</td>
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<tr>
<td></td>
<td>Core Language</td>
<td>104.64 (12.88)</td>
<td>102.29 (13.01)</td>
<td>101.93 (7.28)</td>
<td>( P = 0.357 )</td>
<td>( P = 0.208 )</td>
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<td></td>
<td>Full-Scale IQ</td>
<td>11.73 (2.6)</td>
<td>11.27 (2.35)</td>
<td>10.66 (1.71)</td>
<td>( P = 0.193 )</td>
<td>( P = 0.084 )</td>
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<tr>
<td></td>
<td>Autism Q-Child</td>
<td>43.61 (13.46)</td>
<td>46.83 (16.35)</td>
<td>45.55 (19.99)</td>
<td>( P = 0.310 )</td>
<td>( P = 0.229 )</td>
</tr>
<tr>
<td></td>
<td>Internalising</td>
<td>48.27 (10.53)</td>
<td>48.13 (9.33)</td>
<td>48.69 (12.18)</td>
<td>( P = 0.979 )</td>
<td>( P = 0.069 )</td>
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<td>Externalising</td>
<td>49.58 (9.21)</td>
<td>50.21 (9.62)</td>
<td>51.19 (10.88)</td>
<td>( P = 0.772 )</td>
<td>( P = 0.553 )</td>
</tr>
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<td>2212212222222222</td>
<td>Working Memory</td>
<td>104.47 (13.21)</td>
<td>101.88 (15.24)</td>
<td></td>
<td>( P = 0.315 )</td>
<td>( P = 0.298 )</td>
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<tr>
<td></td>
<td>Core Language</td>
<td>103.48 (12.26)</td>
<td>103.59 (15.13)</td>
<td></td>
<td>( P = 0.962 )</td>
<td>( P = 0.94 )</td>
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<td></td>
<td>Full-Scale IQ</td>
<td>11.38 (2.44)</td>
<td>12.08 (2.56)</td>
<td></td>
<td>( P = 0.151 )</td>
<td>( P = 0.167 )</td>
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<td>Autism Q-Child</td>
<td>45.65 (15.77)</td>
<td>41.75 (11.14)</td>
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<td>( P = 0.180 )</td>
<td>( P = 0.181 )</td>
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<tr>
<td></td>
<td>Internalising</td>
<td>47.97 (10.12)</td>
<td>49.85 (10.06)</td>
<td></td>
<td>( P = 0.326 )</td>
<td>( P = 0.306 )</td>
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<td></td>
<td>Externalising</td>
<td>49.99 (9.61)</td>
<td>49.76 (8.77)</td>
<td></td>
<td>( P = 0.897 )</td>
<td>( P = 0.968 )</td>
</tr>
<tr>
<td><strong>FADS2 - HAP#3</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1222122212212121</td>
<td>Working Memory</td>
<td>104.07 (13.56)</td>
<td>103.84 (13.37)</td>
<td></td>
<td>( P = 0.588 )</td>
<td>( P = 0.888 )</td>
</tr>
<tr>
<td></td>
<td>Core Language</td>
<td>103.61 (12.75)</td>
<td>101.48 (10.95)</td>
<td></td>
<td>( P = 0.068 )</td>
<td>( P = 0.774 )</td>
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<tr>
<td></td>
<td>Full-Scale IQ</td>
<td>11.47 (2.51)</td>
<td>11.25 (1.8)</td>
<td></td>
<td>( P = 0.440 )</td>
<td>( P = 0.886 )</td>
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<td>Autism Q-Child</td>
<td>45.29 (15.36)</td>
<td>43.8 (14.74)</td>
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<td>( P = 0.788 )</td>
<td>( P = 0.556 )</td>
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<td></td>
<td>Internalising</td>
<td>48.67 (10.18)</td>
<td>45 (9.31)</td>
<td></td>
<td>( P = 0.203 )</td>
<td>( P = 0.459 )</td>
</tr>
<tr>
<td></td>
<td>Externalising</td>
<td>49.81 (9.61)</td>
<td>51.48 (8.31)</td>
<td></td>
<td>( P = 0.454 )</td>
<td>( P = 0.580 )</td>
</tr>
</tbody>
</table>

A 1: major allele, 2: minor allele
B Autism Q-Child: Autism Questionnaire-Child version
C Where minor allele homozygotes count (n) was <10, combined with heterozygous group.
DISCUSSION

To our knowledge, this is the first report of a direct assessment of FADS genetic variants and neurocognitive and behavioural outcomes in 6 year olds from a ω-3 LC-PUFA treatment, randomised controlled trial. No SNP or haplotype × treatment group interaction was evident for core language, working memory, full scale IQ, autistic-like traits or behavioural outcomes for those who were supplemented with DHA+EPA-rich fish oil during the first 6 months of life. Thus, our data suggests that FADS genotypes have no interactional effect with early fish oil supplementation on 6 year neurocognitive development. This lack of effect may be due to the number of years since supplementation occurred relative to neurocognitive/behavioural assessments at 6 years. However, it remains undetermined as to whether an interaction may indeed occur at an earlier time point (i.e. sooner after the supplementation period).

HAPLOTYPE - BEHAVIOURAL ASSOCIATION

We found evidence that FADS haplotypes and SNPs may have direct effects on behavioural traits at 6 years. Specifically, the FADS1 and FADS2 haplotypes representing all major alleles (FADS1- and FADS2- MaA Haplo) showed the strongest positive associations with internalising and externalising behavioural outcomes (respectively). In the current study, the FADS3 SNP rs174455 also showed a significant association with internalising behaviour both before and after correction for gender and treatment group. In keeping with the (presently unpublished) findings in our laboratory – identifying major allele carriers of SNPs and haplotypes with higher arachidonic acid (AA) concentrations, our findings help corroborate a functional role of FADS alleles in the fatty acid desaturation pathway and effect on child behaviour.

These results highlight the idea that genetic variants of FADS1, FADS2 and FADS3 are likely to modulate behavioural traits via their effect on LC-PUFA metabolism/desaturation. The direction of these effects were consistent with the major allele carriers (associated with better efficiency/capacity for LC-PUFA synthesis) achieving higher behavioural scores (i.e. having more behavioural problems). Subsequently, it can be inferred that FADS polymorphisms associated with better metabolism of AA are also associated with more behaviour problems. The exact mechanism of this is
unknown however it appears to be related to the expression of major alleles associated with a high efficiency/capacity for improved LC-PUFA synthesis.

**Major Allele FADS Variants**

Preliminary data in our cohort and other research suggests, FADS haplotypes and associated major allele SNPs (favouring high desaturase activity) give rise to more gene expression and ω-6 LC-PUFA. Therefore, it could be reasoned that higher concentrations of ω-6 LC-PUFA incite more problem behaviour, namely internalising traits, in children 6 years of age. This is in line with our previous work (Chapter 5) that demonstrated a significant negative correlation between AA status at birth and language development at 6 years as well as AA status at 6 months and 5 years associated with poorer intellectual and academic reports.

It is not clear from these data why FADS polymorphisms associated with higher ω-6 LC-PUFA would be predictive of more internalising behavioural problems at 6 years. However, two important caveats must be noted. First, the internalising behavioural scores of participants in the present study were well within the normal range according to the scoring criteria, and would not typically warrant clinical intervention. Second, it is possible that slightly higher internalising behavioural scores (as long as they remain within the normal range) could have positive, not adverse implications for child development: predicting greater emotional intelligence and fostering personality traits important in learning and future success (Almlund, Duckworth, Heckman, & Kautz, 2011). This finding is also akin to the study by Brookes et al., (2006), who found a significant association between rs498793 and ADHD, suggesting that mental health and behaviour may be particularly affected by FADS genotypes.

**Additional Considerations**

The present study has many strengths including novelty, randomisation of fish oil supplementation and the investigation of both SNPs and haplotypes. Our findings are also strengthened by the fact that we chose to adjust for gender. This was deemed appropriate since previous research has indicated that females tend to metabolise ω-3 and ω-6 fatty acids more efficiently than males (Decsi & Kennedy, 2011). Moreover, some randomised controlled trials of infant LC-PUFA supplementation have found sex-
specific differences in some neurocognitive/behavioural outcomes. However, there are a couple of potential weaknesses that need be addressed. First, we acknowledge that access to a larger sample size would have provided greater certainty about these findings. However, this study was considered opportunistic and exploratory. Furthermore, the sample population was in line with similar studies conducted. Second, we are conscious that correlations between multiple variables may give rise to non-specific random significant results. We chose not to statistically adjust for multiple comparisons as this study was considered investigative. Moreover, we deliberately attempted to reduce the number of comparisons overall by employing a systematic approach which reduced the large number of (neurocognitive/behavioural and polymorphism) variables into a smaller set of variables.

CONCLUSION

There was no significant interaction between FADS genetic variants and fish oil supplementation (from birth to 6 months) on neurocognitive and behavioural outcomes at 6 years. However, a consistent relationship was observed between the major allele variants of SNPs in the FADS1, FADS2 and FADS3 genes and higher internalising behaviour scores: both when SNPs were evaluated one by one and when considered as the FADS1 haplotype containing all the major allele variants. Continuity in outcome results affords confidence, however the present study was not without limitations. Greater sample sizes are recommended for the design of similar studies in the future. The results presented here provide valuable information about the effect of FADS genetic variants on behavioural outcomes at 6 years and offer the basis for future studies to elucidate these long term effects from which we will be able to draw stronger conclusions.

Candidate contribution:

This randomised control trial commenced in 2005, before the initiation of this PhD. The intervention, collection of blood samples/fatty acid analysis also occurred prior to candidature. Alexandra Heaton re-enrolled participants at 6 years, performed
neuropsychometric testing, was responsible for data entry, all statistical analysis including linkage disequilibrium, Hardy-weinberg calculations, SNP and haplotype analyses, in addition to the writing, drafting and editing of this manuscript.
CHAPTER 7

GENERAL DISCUSSION
General discussions arising from this thesis

One of the most controversial and topical issues is whether or not current dietary DHA intakes of Western nations are adequate for the developing human brain. Differential findings reported by numerous studies have led to confusion in everyday clinical practice regarding the efficacy of fish oil supplementation for neurocognitive/behavioural optimization. While numerous epidemiological/observational and randomised controlled trials have reported positive findings, others have not (Heaton, Meldrum, Foster, Prescott, & Simmer, 2013). The results of randomised controlled trials in this field have been encumbered by various limitations notwithstanding methodological differences and inconsistencies which make them difficult to compare.

The 6 year follow-up of the IFOS randomised controlled trial demonstrated that across-the-board, ω-3 LC-PUFA supplementation during early infancy provides little to no benefit to the neurocognitive or behavioural development of healthy children at 6 years.

In this chapter, the key findings of the 6 year follow-up are summarised. The theoretical basis behind these results and the possible mechanisms involved as well as interpretation in the context of the wider literature are discussed. Potential limitations and considerations of the study are detailed in addition to recommendations for future research before final remarks and study conclusions are made.

SUMMARY OF THE RESULTS OF THE 6 YEAR FOLLOW-UP

In the current 6 year follow-up study of neurocognitive and behavioural development, we addressed three overarching hypotheses and found:

1. The randomised controlled trial revealed that children who received ω-3 LC-PUFA supplementation during infancy did not display enhanced neurocognitive or behavioural development at 6 years compared to children within the control group. Those who received the fish oil treatment scored higher only on the familiar sequences sub-test of the CELF-4. In another subtest of the CELF-4,
Number Repetition Forwards, the control group scored higher than the treatment group. Children who received the fish oil treatment also displayed higher externalising behaviours, specifically in the realm of oppositional defiance, compared to controls.

2. Circulating ω-3 LC-PUFA (specifically, DHA) status at birth, 6 months and 5 years were generally not positively correlated with child neurocognitive development at 6 years of age. Circulating DHA status at birth was associated with adverse behavioural outcomes at 6 years, specifically externalising behaviours, including child hyperactivity. However, DHA status at other ages (6 months or 5 years) were not associated with behavioural outcomes. Circulating ω-6 LC-PUFA, specifically AA status at birth and at 6 months was significantly negatively correlated with language scores (as assessed via the Renfrew Bus Story Test). Furthermore, AA status at 5 years was significantly negatively correlated with Academic and Intellectual ability (as identified via the Gifted Rating Scale, completed by school teachers).

3. Major FADS allele carriers (associated with high endogenous LC-PUFA synthesis) did not achieve better scores on neurocognitive and/or behavioural outcomes at 6 years compared to minor allele carriers. Indeed, major FADS allele carriers displayed more internalising behaviour compared to minor allele carriers. Any neurocognitive or behavioural effect of infant fish oil supplementation did not appear to be modulated by individuals FADS genotype. Thus, at 6 years we found no evidence that a gene-diet interaction had occurred as a result of early ω-3 LC-PUFA supplementation.

**DISCUSSION OF THE RESULTS OF THE IFOS 6 YEAR FOLLOW-UP**

**NEUROCOGNITIVE OUTCOMES**

The 6 year follow-up of the IFOS randomised controlled trial indicates that fish oil supplementation from birth to 6 months provides little to no benefit for later neurocognitive development at 6 years.
Based on our understanding of how DHA interacts within neural tissue (i.e. affecting physicochemical processes that underlie hippocampal and frontal-cortex based cognition), we hypothesised that the treatment group would display enhanced working memory compared to controls. However, our results did not fully support this (Chapter 4). The results described in Chapter 4 were not consistent for working memory. While the fish oil group performed significantly better than controls in the test of Familiar Sequences, in contrast, the control group performed significantly better in the Number Repetition Forwards subtest. Admittedly, the significant difference in the Familiar Sequences subtest could be considered more meaningful than the finding in Number Repetition Forwards; since the latter is essentially a subtest within a subtest (Number Repetition Forwards and Number Repetition Backwards are combined to form a Total Number Repetition subtest score, which is then combined with another subtest to derive the Working Memory Composite score). However, the fact that we identified statistically significant group-differences, in paradoxical directions, flags the inconsistency of effects for working memory, lessening the results’ credibility.

The beneficial effect of fish oil supplementation on the Familiar Sequences subtest is supported by physicochemical evidence and previous observational research in humans. DHA has been shown to promote various cellular mechanisms that underlie memory and learning (Cao et al., 2009); enhancing activity in hippocampal neurons (Calderon & Kim, 2004) – networks central for memory formation (Berger et al., 2012; Rolls, 2008). Evidence in favour of DHA supplementation for enhanced working memory comes from Boucher et al., (2011), who found a positive correlation between DHA status at birth and working memory at 11 years, based on two measures – including a Digit Span Forwards subtest (Boucher et al., 2011). Furthermore, a very large observational study revealed a significant association between LC-PUFA intake of children aged 6 – 16 and performance on the digit span test of working memory (Zhang, Hebert, & Muldoon, 2005).

Yet other studies, such as the trial by Kirby et al., (2010) found no relationship between working memory and DHA status at 8 – 10 years (Kirby et al., 2010). In contrast,
(Cheatham et al., 2011) found higher erythrocyte DHA at 4 months was negatively associated with working memory at 7 years.

The reports of null effects of DHA supplementation on measures of working memory are consistent with other findings in the literature utilising randomised control trials (Heaton et al., 2013; Simmer et al., 2011). Albeit, these previous trials are not without their methodological limitations; as reviewed by (Meldrum, Smith, et al., 2011), and do not rule out the possibility that DHA can enhance working memory. Based on our results, however, it can not be deduced that infant fish oil supplementation enhances working memory at 6 years of age. Despite the implementation of both global and specialised assessments designed to detect more modest effects on cognition (Bellisle et al., 1998), we found no correlation between DHA status and any tests of working memory (Chapter 5). To this point, we conclude that DHA supplementation during infancy yields no obvious benefits at 6 years in terms of working memory or other neurocognitive processes in well-nourished healthy term-born children.

DHA supplementation is associated with lowered AA levels in erythrocytes. By this reasoning, higher AA status may allude to the control group of the present randomised controlled trial, reflecting the typical Western diet of the control participants. Participants in the control group after 6 months of supplementation had significantly higher AA in erythrocytes. We report an association between high AA status during infancy and worse neurocognitive development in early childhood. We found that AA status at birth, 6 months and 5 years was associated with various neurocognitive and behavioural adversities. For example, we found high cord blood levels of AA and total ω-6 LC-PUFA were negatively associated with language outcomes, academic and intellectual abilities. The implications of this are unclear and while it appears that fish oil supplementation incurred no effect, it might be inferred that children who did not receive fish oil during infancy were worse off than those in the treatment group.

**Behavioural Outcomes**

In terms of behavioural outcomes, the 6 year follow-up of the IFOS randomised controlled trial (Chapter 4) indicated that fish oil supplementation from birth to 6 months did not protect against behavioural problems. Rather, the randomised control
trial results suggest that fish oil supplementation may induce more externalising behavioural problems at 6 years. This is supported by similar findings in the IFOS cohort at the 18 month follow-up, as it was noted that toddlers in the fish oil treatment group had higher anxiety scores than control group participants (Meldrum et al., 2012). Furthermore, children within the FADS genetic subgroup – synonymous with higher LC-PUFA concentrations (major allele carriers) – demonstrated significantly more internalising behaviour problems at 6 years. IFOS is not the first randomised control trial to report adverse associations between early ω-3 LC-PUFA supplementation and child problem behaviour (Cheatham et al., 2011; Makrides et al., 2014).

In direct contrast, higher DHA status in erythrocytes at birth had a protective effect on externalising behaviour at 6 years (Chapter 5). Furthermore, children with higher DHA status at birth showed significantly less ADHD-type traits at 6 years of age. These finding were akin to several previous reports of child behaviour and DHA status (Gow et al., 2013; Milte et al., 2012; Montgomery et al., 2013). Specifically, low ω-3 LC-PUFA status during early development has been linked to an increased risk of depression/anxiety, ADHD and autism (Ramakrishnan, Imhoff-Kunsch, & DiGirolamo, 2009).

It is reasonable to speculate that the associations between early DHA status and child behaviour are mediated by changes in dopaminergic and serotonergic systems (i.e. neurotransmitters involved in the regulation of behaviours, emotions and other neurocognitive processes) (de la Presa Owens & Innis, 1999). Research has found that severe DHA deficiency during development impedes monoamine neurotransmission (Tanaka, Farooqui, Siddiqi, Alhomida, & Ong, 2012b) while also reducing synaptogenesis and overall synaptic function (Cao et al., 2009).

A plausible explanation as to the negative behavioural consequences associated with the modification of ω-3 LC-PUFA intake (via fish oil supplementation) from birth to 6 months is the fact that it creates an environmental “mismatch” once supplementation ceases (Wong-Goodrich et al., 2008). It could be speculated that metabolic imprinting by early ω-3 LC-PUFA availability may cause the brain to become differentially sensitive to later ω-3 LC-PUFA supply, by setting the range of ω-3 LC-PUFA intake that is optimal
for behavioural development. This supposition was also shared by Cheatham et al., (2011); who similarly reported higher behavioural problems at 7 years in the participants who received higher ω-3 LC-PUFA (indirectly, via maternal supplementation) during infancy.

A small number of other randomised controlled trials of enhanced dietary ω-3 LC-PUFA during infancy also reported adverse neurocognitive / developmental outcomes; including reduced head circumference (Bergmann et al., 2007; Jensen et al., 1997) and poorer verbal skills (Lauritzen, Jørgensen, Olsen, Straarup, & Michaelsen, 2005). One explanation for the growth and developmental deficit associated with ω-3 LC-PUFA supplementation during infancy is due to oxidative stress caused by increased lipid peroxidation (Church, Jen, Jackson, Adams, & Hotra, 2009). All PUFA is highly vulnerable to oxidation-induced lipid peroxidation; thought to be capable of inciting cytotoxic and genotoxic effects (Michalski, Calzada, Makino, Michaud, & Guichardant, 2008). DHA is particularly susceptible to non-enzymatic peroxidation due to its reactive hydrogen molecules positioned adjacent to Carbon=Carbon double bonds which invite electron-stealing reactive oxygen species from the atmosphere (Xu, Davis, & Porter, 2009). The long term consequences of ingesting oxidised LC-PUFA during infancy has not been elucidated. However, it could have particularly negative consequences, considering capacity for hydroperoxide-detoxification may be less proficient at this stage of development (Awada et al., 2012). Heightened oxidative stress has been implicated in the pathogenesis of various disorders and psychiatric conditions, such as autism (Meguid, Dardir, Abdel-Raouf, & Hashish, 2011), thus, could have potentially contributed to the higher externalising behavioural problems amongst the fish oil group.

Unlike Al-Hilal and colleagues in 2013, who found evidence of a significant interaction between dietary ω-3 LC-PUFA and FADS genotype on Δ5D activity, we did not find evidence of a gene-diet interaction for any of our functional outcomes of neurocognition / behaviour. This result was contrary to our hypothesis; that SNP and haplotypes within the FADS gene cluster would have interacted with the fish oil treatment in determination of enhanced development.
CONSIDERATIONS AND IMPLICATIONS FOR FUTURE RESEARCH

**LONGITUDINAL FOLLOW-UP**

As aforementioned, the results from the randomised controlled trial in Chapter 5 found a significant adverse effect of fish oil supplementation on externalising behaviour and ADHD at 6 years; which was corroborated by FADS genetic subgroups accompanying higher LC-PUFA. Together, these findings from the 6 year follow-up add weight to the earlier report from the 18 months follow-up revealing an association between fish oil supplementation and anxiety. While these reoccurring findings may be due to chance, further investigation is warranted. Since the brain continues to develop well beyond the age of six and into early adulthood, it is recommended that behavioural follow-up studies are performed so to enable longitudinal exploration.

One of the strengths of the present study is the fact that all of the assessments employed in the 6 year follow-up are also applicable in older children. It is particularly helpful that the behavioural questionnaire used (the CBCL) is age-standardised up until 18 years. Thus, longitudinal monitoring of child behaviour can be implemented with great ease. Moreover, the behavioural questionnaire used at the 18 month follow-up was also a CBCL derivative appropriate for younger children. Such continuity of assessments facilitates the most robust means of mapping human development, and the potential effects of ω-3 LC-PUFA supplementation, across time.

**SUBJECTIVITY OF PARENT REPORTS**

While the CBCL has been shown to have excellent reliability and validity, evaluations of child behaviour are derived from parent and teacher reports and may be considered somewhat subjective (Berglund et al., 2013; Schmeck et al., 2001). It is worth noting, if the behaviours are not reported to be within the clinical cut-off range, they are not necessarily problem behaviours – but may be reflections of a child’s personality (i.e. the behaviour of a strong-willed or emotional child may be considered oppositional/defiant by some). It is important to note that mean scores of oppositional defiance were not within the clinical range, rather, most participants from both the treatment and control groups scored well within the normal range for all behavioural outcomes.
The validity of results derived from parent questionnaires at 6 years may have been compromised by the fact that parents were informed (at 2 ½ years) about which treatment group their child was allocated to. This may have biased their rating of their child’s behaviour. It is possible that parents within the treatment group placed higher expectations on the behaviour of their children as a result of their group allocation and therefore judged externalising behaviours more harshly. Therefore, while the present results suggest a link between DHA and child behavioural development, more scientifically rigorous research is required to confirm this observation.

**Possible Chance Effect**

Considering the large number of outcomes measured and statistical comparisons performed, our findings should be interpreted with caution. No statistical adjustments were made to compensate for the large number of comparisons as we wanted to reduce the risk of committing Type-II error (not identifying a significant effect despite its existence).

The one significant positive finding associated with fish oil supplementation from birth to 6 months in terms of the familiar sequence subtest of working memory from the CELF-4. However, this is likely a chance finding, given the contradictory outcome for the Number Repetition Forwards subtest of working memory. Consequently, it may be premature to make unequivocal recommendations about working memory benefits of DHA supplementation in healthy term infants based on the currently available research findings.

**Effectiveness of Direct Infant Supplementation**

We recognise that direct fish oil supplementation of infants was associated with some difficulty. Direct fish oil supplementation is a method regularly used in adult studies. However, the IFOS trial is the only randomised control trial to directly provide infants with high dose fish oil from birth to 6 months. The main advantage with this study design was the fact that it did not affect the parents decision to breastfeed and/or formula feed. However, we resolve that direct fish oil supplementation may not be ideally pragmatic in an infant population.
While fatty acid status observed at 6 months demonstrated that direct infant fish oil supplementation raised the circulating plasma and erythrocyte DHA of those in the fish oil group, this effect was somewhat modest. The fish oil group’s mean erythrocyte DHA at 6 months was approximately 6.83% of total fatty acids (Appendix D). Comparatively, Gibson et al., (1997) was able to raise infant erythrocyte DHA to 9.8% total fatty acids by supplying DHA enriched breast milk (1.13% DHA). Assuming the infants daily milk intake is ~780 mL per day and contains 4% fatty acids (DHA 1.13%, of total fatty acids); this equates to around 406 mg of DHA per day. Based upon a similar calculation, we can calculate that the majority of IFOS participants received at least 100 mg DHA as part of their normal breast milk diet (since average DHA content of breast milk was 0.32% of total fatty acids at 6 months). Those in the treatment group received an additional ~265 mg of DHA per day, resulting in a total of ~365 mg of DHA per day. The amount of DHA supplied in Gibson et al., is not much higher than IFOS, so it is therefore surprising that the level of DHA in erythrocytes was not more similar.

There are several possibilities that may help explain why (high dose) fish oil supplementation did not raise DHA levels as much as expected. First, it may be attributable to the inconvenience in administering the oil directly into the infants mouth. Second, it is conceivable that some participants in the fish oil treatment group were deterred by the smell of the oil, particularly in conjunction with infant reflux. Third, there may have been issues with the fatty acid formulation (provided as ethyl esters) that could have impacted bioavailability and/or absorption of the supplements. Fourth, it is possible that issues of bioavailability and/or absorption arose from the single daily dose (large bolus delivery). Regardless of the mechanism at play, we note that direct postnatal fish oil supplementation was not exceptionally effective in raising the DHA status of infants.

**Genetic Polymorphisms**

An important consideration as to why DHA levels in erythrocytes and plasma did not respond more significantly to high dose supplementation is due to genetic differences in fatty acid metabolism. Genetic polymorphisms in the FADS gene cluster are known to have a direct impact upon the amount of LC-PUFAs available to the developing infant (Schaeffer et al., 2006). Concurrent research conducted in our laboratory
(beyond the scope of this PhD thesis) has explored the whether FADS polymorphisms modulate the effectiveness of fish oil supplementation. Briefly, we have found that FADS genotypes influence the effect of supplementation, with the minor allele homozygous carriers receiving the greatest benefit. The full paper discussing this finding (Meldrum et al., 2015) is currently under review, however, is important to acknowledge here as it helps to elucidate on the relatively modest increase in DHA despite high dose fish oil supplementation.

Breastfeeding

The IFOS sample population were well-educated, high-income and, in turn, demonstrated a high rate of breastfeeding compared to the general Australian population (Australian Institute of Health and Welfare, 2011). It is well known that breastfeeding is associated with social advantage. This is because well-educated, high-income parents are generally more informed about the benefits of breastfeeding and/or may be in a better position (e.g. employment wise) to continue breastfeeding for the first 6 months. Owing to the aforementioned population characteristics, we do not expect that many children in our cohort were DHA or EPA deficient. Therefore, the lack of significant benefit from ω-3 LC-PUFA supplementation could be because participants in this study were already receiving adequate dietary DHA for optimal brain development.

The majority of the IFOS cohort were already receiving ~111 mg of DHA per day from birth to 6 months via breast-milk (assuming 780 mL/day with 4.2% total fat, together with the mean breast-milk DHA 0.32% total fatty acids in this cohort at 6 months). This may help to explain why the IFOS treatment group displayed only a modest increase in DHA after 6 months supplementation with ~265 mg DHA. Indeed, Gibson et al., (1997) established that DHA incorporation into infant plasma and erythrocytes reaches a point of saturation once dietary DHA levels exceed approximately 260 mg per day i.e. breast-milk DHA 0.8% total fatty acids (assuming 780 mL of breast-milk per day containing 4.2% total fat).
**DHA Sufficiency Across the IFOS Cohort**

The results from the 6 year follow-up indicate that the IFOS participants already have adequate DHA for optimal neurocognitive development. Due to the aforementioned characteristics of the IFOS cohort (i.e. high-income, high-education), it is likely that the study participants were well-informed about good nutrition and followed dietary recommendations to increase DHA to improve their health outcomes as well as those of their children. Such recommendations are increasingly being adopted with a recent Australian study noting that 64% of women screened for the DOMInO trial were excluded because they were already taking a prenatal supplement that contained DHA (Makrides et al., 2010). Similarly in IFOS, fish oil supplementation during pregnancy was an exclusion criterion, however, there were no restrictions on maternal fish oil supplementation during breastfeeding. While it is expected that a balanced proportion of mothers from each group took dietary supplements containing ω-3 fatty acids during lactation, it remains possible that this could have diluted the observable difference between the two groups (Forsyth, 2012).

Furthermore, the IFOS participants who attended the 6 year follow-up were largely breastfed (i.e. 90% of mothers reported breastfeeding primarily until 6 months), as in line with other populations utilising well-educated, affluent parents. This is tantamount to the fact that such populations are generally more informed about the benefits of breastfeeding and/or may be in a better position (i.e. employment wise) to continue breastfeeding for the first 6 months. Considering the present findings within the context of the high rate of breastfeeding leads us to believe that breast milk provides sufficient quantities of DHA for normal brain development.

Overall, the pre-existing nutritional sufficiency within the IFOS cohort may have created a ‘ceiling effect’ with respect to functional outcomes. However, studies incorporating such population subsets are most relevant as it is these population subsets that are most likely to purchase fish oil supplements.

Our results from the 6 year follow-up pertain to a niche Australian population and may not be transferable to alternate populations. More studies are required in order to ascertain whether ω-3 LC-PUFA supplementation would offer benefit to infants from
disadvantaged families, those exposed to environmental stressors, born prematurely or with very low birth weight. Future randomised control trials that specifically target at-risk groups are needed to offer insight into the effect of supplementation in these sub-groups.

**CONFOUNDING EFFECTS AND BACKGROUND NOISE**

It is possible that by 6 years of age other factors were more important in contributing to cognitive performance than dietary intake of ω-3 LC-PUFA during the first 6 months. Such factors include upbringing, parenting style and classroom teaching methods. These highly subjective confounders are difficult to quantify and to the best of our knowledge previous randomised control trials within this area do not always measure such confounders. It should also be acknowledged that fatty acid intake over the subsequent years are not accounted for. The process of randomisation generally ensures that the characteristics of participants are equally distributed between the groups. However, detecting subtle effects amongst all the background noise may require much greater sample sizes or the development of more sensitive tools to measure neurocognitive/behavioural outcomes.

Also, despite using the best neuropsychometric tools available to us at this current time, our ability to measure the relative influence of ω-3 LC-PUFA supplementation on human neurocognitive and behavioural development may be too imprecise.

**PARTICIPANT RETENTION**

It is important that new tools and technologies are embraced and used to our advantage such as utilising Facebook and other forms of modern communication to contact participants when conventional methods fail. The internet and online forums could be used as a way of keeping participants up-to-date with the latest findings, rewarding their contribution and encouraging further participation. This may have a positive effect and subsequently mean higher participation retention. Follow-up is very important in research and it needs to be pragmatic. The best way to retain participants should be re-evaluated so that cohort studies can be more easily observed into adulthood. Future research studies need to include these new technologies into ethics applications and protocols.
GENERALISABILITY OF FINDINGS TO OTHER POPULATIONS

The results of this study must be considered within the context of the IFOS sample population. Despite recruiting pregnant women from both private and public metropolitan antenatal clinics, most participants tended to be Caucasian, high-income earning, with high levels of education. Subsequently, the generalisability and external validity of these findings may be somewhat constrained since the study population displayed limited socioeconomic and cultural diversity. The fact that people from cultural minorities or those with low-income/ low-educational status are particularly under-represented in the IFOS cohort may be attributable to the fact that voluntary (and non-remunerated) participation is more achievable for those with the means to engage various supports and services (e.g. hired domestic assistance, child care providers and conducive employment arrangements). Subsequently, there is a potential risk that participant enrolment could have been biased - as with any study utilising human volunteers.

Final Remarks and Study Conclusions

In conclusion, trial found that direct fish oil supplementation of healthy, predominantly breastfed, full-term infants with parents of high-education and high-income, was not effective in enhancing cognitive development, nor was it successful in preventing child behavioural problems.

The small number of significant findings may have been due to chance as we performed several analyses and the scores that were detected as being different between the groups were sub-scores of the main tests of neurocognition. However further studies with long-term follow up are required to ascertain whether these findings were likely to be due to chance.

The lack of observable neurocognitive effect of fish oil supplementation noted in this randomised controlled trial may be confounded by factors that naturally arise due to differing parenting styles, child diet (i.e. once solid foods are introduced), experiences and events that have occurred during the course of the child’s 6 years of life. There is an innate difficulty conducting research aimed at evaluating the neurocognitive impact of an isolated variable such as fish oil supplementation since; after birth, myriad...
environmental factors contribute to the brain’s development and structural reorganization - in addition to nutrition. It may be difficult for randomisation to ensure balance in these factors after a long-term follow up.

The mothers and infants in the present study were well endowed with DHA, in line with their higher education and financial standing. It is therefore likely that the present cohort exceeded the typical intake of ω-3 LC-PUFA. Due to the probability that DHA intake is sufficient in this unique population it may be concluded that additional ω-3 LC-PUFA supplementation does not confer measurable improvements in cognition during childhood. Furthermore, a universal approach of supplementing healthy individuals with fish oil during infancy does not protect against the development of externalising behavioural problems at 6 years.

We do acknowledge that a limitation the trial was the somewhat modest (albeit statistically significant) increase in the DHA content of infant erythrocytes and plasma phospholipids from direct fish oil supplementation. It remains possible that ω-3 LC-PUFA supplementation via a different method may have been more successful in increasing DHA levels and potentially positive neurocognitive effects.

The conclusion that normal human breast-milk should provide sufficient DHA for healthy neurocognitive and behavioural development is reassuring. However, the IFOS cohort is not necessarily an accurate representation of all infant-mother pairs in Australia it remains possible that some subgroups may indeed benefit from additional dietary ω-3 LC-PUFA. Albeit, we believe that the social characteristics of our sample population enhance the real-world utility of this study. This is because parents within this demographic are more likely to possess the financial means to purchase commodities such as infant fish oil products. Subsequently, this demographic subgroup may potentially be misled into purchasing unnecessary products which they believe may confer a neurocognitive advantage for their child.
Our findings therefore concur with the conclusion of others; that in our cohort, postnatal ω-3 LC-PUFA supplementation does not enhance neurocognitive nor behavioural development of healthy term-born infants.

While ω-3 LC-PUFA supplementation randomised control trials of have reported conflicting results, it is thought that deficiencies in ω-3 LC-PUFA status cause neurodevelopmental harm. It is possible that DHA deficiency during early periods of brain growth and structural organization may render the brain vulnerable to neurological or neurodegenerative diseases later in life (Farquharson et al., 1995). This is because, during early development the neural architecture paves the foundation for brain connectivity. By the same token, it should be considered that ω-3 LC-PUFA intake excess to requirements may also cause deficit.

On the whole, our randomised controlled trial demonstrated no benefit from supplementing healthy, term-born, predominantly breast-fed infants with dietary ω-3 LC-PUFA from birth to 6 months. Our results largely support the view of several Cochrane reviews and meta-analyses that have concluded there is currently no definitive evidence that additional dietary ω-3 LC-PUFA is beneficial to neurocognitive development of primarily breastfed, term infants.
APPENDICES

APPENDIX A: PREVIOUS FOLLOW-UP ON THE IFOS COHORT

Antenatal/ Recruitment Visit
An antenatal-clinic visit was scheduled when participating women were in their 36th week (or beyond) of pregnancy. They completed a semi-quantitative food frequency questionnaire that explored maternal diet and the use of any multivitamins and/or fish oil supplements during pregnancy. Information was also collected on maternal characteristics including; age, parity, education, stress levels, ethnicity, socio-demographic and income. A blood sample was taken and skin prick test performed (to assess their immune responses to a panel of common allergens; cat, rye, cow’s milk, egg, house dust mite, grasses, moulds, peanut, histamine, dog, cockroach, feathers).

Information Collected at Birth
Umbilical cord blood was collected by participating hospital staff via a 19-gauge needle and syringe. Cord blood was maintained within a solution of 20mL Roswell Park Memorial Institute (RPMI) (Invitrogen, CA) and 500μl of preservative-free heparin and processed by research staff within 12 hours of delivery. Following birth, parents completed a questionnaire regarding factors including: delivery mode, medical complications, drugs used during labour and infant anthropometric details including gender, birth weight, length, Apgar score and head circumference.

Three Month Follow-up
The 3 month follow-up involved a parent questionnaire documenting any symptoms, treatments and clinical diagnosis of allergic disease. Information on breastfeeding and/or formula feeding was recorded and samples of breast milk were collected where possible.

Six Month Follow-up
The 6 month follow-up was identical to the previous visit at 3 months, with the additional collection of a peripheral blood sample from infants where possible. Up to 10 mL of blood collected via venipuncture of the cubital fossa vein.
Appendices

Twelve Month Follow-up

The 12 month follow-up was identical to the previous visit, with the addition of an eczema evaluation using the SCORAD index and a skin prick test to common allergen extracts (egg, milk, peanut, house dust mite, cat, rye grass and southern grass mix; Hollister-Stier Laboratories). Those displaying signs of food sensitization were referred to a paediatric immunologist for assessment. Further, the MacArthur Bates Communicative Development Inventory- Words and Gestures, a developmental questionnaire of language development for children 8 to 16 months old, was mailed to parents prior to their 12 month appointment.

Eighteen Month Follow-up

Neurocognitive and language development was assessed at the 18 month follow-up using the Bayley Scales of Infant Toddler Development (3rd Edition) (BSID-III) along with two parent questionnaires. The BSID-III is a neuro-psychometric assessment, designed to diagnose developmental delay and assess developmental outcomes in very young children (1 to 42 months). The parent questionnaires included the Achenbach Childhood Behaviour Checklist (CBCL), that assesses cognitive-behavioural development in children (18 months to 5 years), and the Macarthur Communicative Development Inventory Words and Gestures, as described above.

Two and a Half year Follow-up

The clinical follow-up at two and a half years was identical to the follow up at 12 months, with the exception of the Macarthur Communicative Development Inventory Words and Sentences form (designed for children aged 17 to 30 months), that replaced with Words and Gestures form. Further, asthmatic symptoms were assessed.

5 year Follow-up

At 5 years, the clinical follow-up was identical to the previous visit, with several additional assessments including: body composition, blood pressure, skin folds and heart disease risk factors. Urine samples were also collected where possible. Further, saliva samples were collected from children where venous blood samples had not been collected (at any visit) yet parents consented to DNA extraction.

IFOS BIOLOGICAL SAMPLES
Blood Processing

Both cord and peripheral blood were processed by the same technique that enabled separation into plasma, mononuclear cell and erythrocyte fractions by centrifugation at 1500 revolutions per minute (RPM) for 30 minutes. The process was enhanced by the addition of an endotoxin solution, Lymphoprep™ (Nycomed Pharmacia, Norway). The neutrophils were isolated and stored at -80°C for DNA extraction. The red blood cells or erythrocytes were analysed for fatty acid composition as was the plasma phospholipid component of the blood (cord blood and 6 months). Mononuclear cells were primarily used for immunology research. They could be isolated from the layer above the concentration gradient and washed, once with RPMI only and then twice with a solution containing RPMI plus 2% heat-inactivated foetal calf serum (FCS) (Australian Biosearch, Australia).

Fatty Acid Compositional Analysis

The fatty acid analysis was conducted using a previously published methods (Mori et al., 2000). The phospholipid fraction of the blood plasma and the erythrocyte lipids were extracted from whole blood using methanol–chloroform (2:1). Lipid fractions were analysed using thin layer chromatography. Fatty acid methyl esters were analysed by gas-liquid chromatography (Agilent Technologies Australia Private Limited) using Supelco SP-2560 column’s (Sigma-Aldrich Private Limited) and hydrogen as the carrier gas.

Breast Milk Samples

Breast milk samples were analysed for fatty acid composition by a technique that has been described elsewhere (Dunstan et al., 2004; Meldrum, D’Vaz, et al., 2011). Briefly, fatty acid methyl esters were separated from whole milk samples by gas chromatography and flame ionization (Shimadzu GC-14A, Shimadzu Corporation, Kyoto, Japan). Individual FAs were identified by comparing their retention time with those of known lipid standards (Nu-Chek-Prep Inc., Elysian, MN, USA). Those with a carbon chain length from C14 to C22 were analysed. Breast milk fatty acid levels are expressed as weight percentage (wt%) of total FAs measured.
APPENDIX B: FATTY ACID LEVELS: CONTROL VS TREATMENT GROUPS

Comparison of fatty acid levels of control and treatment groups at birth, 6 months and 5 years.

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*DHA levels given as a % of total fatty acids
Appendices

APPENDIX B: FATTY ACID LEVELS: CONTROL VS TREATMENT GROUPS

[Box plots showing fatty acid levels for control vs treatment groups.]

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143 | Page
APPENDIX C: THE FRUIT STROOP TEST

Page 1
Fruit Stroop Test

Page 2
Fruit Stroop Test
APPENDIX D - THE SELF ORDERED POINTING TEST

SOPT Pictures Included in Item 1
   – Consists of Six Pages

SOPT Pictures Included in Item 2
   – Consists of Eight Pages
SOPT Pictures Included in Item 3
- Consists of ten pages

SOPT Pictures Included in Item 4
Consists of twelve pages
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<td>4.7% Plasma</td>
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## APPENDIX F: DHA QUARTILE ANALYSIS

### Percentiles of DHA

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DHA as a % of total FA

Neurodevelopmental outcomes of participants in the bottom DHA quartile were compared to those of the three upper DHA quartiles.

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Top Quartiles       106.75  10.80

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Neurodevelopmental outcomes of participants in the top DHA quartile were compared to those of the bottom three DHA quartiles.
### Appendices

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#### 5y RBC

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Appendices

REFERENCES


Appendices


Forsyth, S. (2012). Why are we undertaking DHA supplementation studies in infants who are not DHA-deficient? The British Journal of Nutrition, 108(05), 948-948.


Appendices


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Neuringer, M., Connor, W., Lin, D., Barstad, L., & Luck, S. (1986). Biochemical and functional effects of prenatal and postnatal omega 3 fatty acid deficiency on
retina and brain in rhesus monkeys. *Proceedings of the National Academy of Sciences (USA)*, 83(11), 4021-4025.


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