Enteral Docosahexaenoic Acid Supplementation To Attenuate Inflammation In The Preterm Infant

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A thesis submitted for the degree of Doctor of Philosophy

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February 2017
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ABSTRACT

Preterm infants have an underdeveloped immune system and as such they are predisposed to developing unregulated inflammatory responses that are associated with disease in the postnatal period. Docosahexaenoic acid (DHA) is an omega-3 long-chain polyunsaturated fatty acid (LCPUFA) with known immunomodulatory properties, however the effect of dietary DHA on the regulation of immune responses in preterm infants is largely unknown. This thesis employs a multi-system approach to address questions related to the efficacy of omega-3 DHA to regulate inflammation in preterm infants and in human type II alveolar epithelial cells (AEC).

The N3RO randomised controlled trial (RCT) provided the opportunity to carry out a single-centre nested study to examine the effect of supplemental DHA in preterm infants on pro-inflammatory and regulatory biomarkers in blood and levels of a common bacterial pathogen in the gastrointestinal tract. The aim of the N3RO RCT was to assess the efficacy of an enteral DHA emulsion to reduce bronchopulmonary dysplasia (BPD) in preterm infants < 29 weeks gestation compared to a standard soy emulsion without DHA.

Prior to analysis of biological samples from preterm infants, the immune response to enteral DHA and soy emulsions in human type II AECs, one of the primary cell types affected in respiratory disorders, was assessed in vitro. The enteral emulsions assessed in the N3RO RCT were tested in conjunction with other commercially available parenteral lipid emulsions. Omega-3 DHA in both enteral and parenteral emulsions significantly reduced pro-inflammatory cytokines (IL-1β, IL-8 and IFNγ) when compared to soy-based emulsions.

There are very few studies that have assessed what, if any, targets DHA interacts with to exert an immunomodulatory effect in preterm infants. Inflammatory cytokines are known to play a crucial role in the progression of airway inflammation, epithelial and vascular damage and subsequent development of BPD. Such inflammatory mediators are also involved in the
development of other neonatal inflammatory disorders such as sepsis, necrotising enterocolitis and retinopathy of prematurity. A total of 144 blood samples were collected from 51 preterm infants enrolled in the nested study. Supplemental DHA did not reduce pro-inflammatory cytokine levels in plasma or whole blood culture supernatants (after a 24 hour incubation with *E. coli* lipopolysaccharide).

Inflammatory mediators in the gut environment can influence initial colonisation and resulting abundance of both commensal and pathogenic bacteria. *Staphylococcus* is among the first colonisers of the respiratory and gastrointestinal tracts and it is one of the most important pathogens in the neonatal intensive care unit. Colonisation by methicillin-resistant bacteria including *Staphylococcus* in preterm infants also causes significant morbidity and mortality in the neonatal intensive care unit. In the neonatal period, diet has a significant effect on microbial colonisation of the gut, however the effect of supplemental omega-3 LCPUFA on *Staphylococcus* colonisation in preterm infants is unknown. A total of 220 stool samples were collected from 41 preterm infants enrolled in the nested study. Levels of *Staphylococcus* and bacteria carrying the gene coding for methicillin-resistance (*mecA*) decreased significantly over time in both groups, but DHA did not have an effect on abundance.

The original contribution this thesis makes to the knowledge base is that supplementing preterm infants < 29 weeks gestation enterally with 60 mg/kg/day of DHA does not affect circulating levels of pro-inflammatory or regulatory cytokines, the immune response to an infectious stimuli nor does it influence *Staphylococcus* and *mecA*+ bacteria in the gut. This thesis contributes important information regarding the use of DHA at supplemental levels in nutrition regimens for preterm infants.
DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Signed:

Naomi H. Fink

Date: 17 February, 2017
ACKNOWLEDGEMENTS

My deepest gratitude goes to my supervisors A/Prof Irmeli Penttila, Prof Maria Makrides and Prof Robert Gibson, who expertly guided me through all stages of my postgraduate education and provided invaluable feedback. I am extremely grateful to these leaders for investing in their students. A special thanks to Irmeli Penttila who was a tremendous mentor and exemplary role model. Your scholarly advice, meticulous scrutiny and scientific approach inspired me to strive for greatness during my research pursuit. Thank you for opening my eyes to new stages of opportunity and strength; your contribution towards my success today is highly acknowledged.

My appreciation also extends to my laboratory colleagues Colleen Bindloss, Irene Kanter, and Dr. Adaweyah Donato for your expert tutelage and unwavering patience as I grasped new techniques in the lab. Thank you to A/Prof Geraint Rogers and Dr. Lex Leong for guiding me through the unfamiliar territory of qPCR and for providing a welcoming work environment.

I would like to extend my gratitude to the team at CNRC, particularly Dr Jacquie Gould, Dr Carmel Collins, Dr Merryn Netting, Dr Lisa Yelland and Dr Edna Bates for your advice and academic support. To colleagues both past and present, Dr Lenka Malek, Dr Karen Best, Ashlee Davies and Chloe Douglas, for fostering such a positive atmosphere. Thank you also to Dr Jennie Louise and Suzanne Edwards at the Data Analysis and Management Centre for your statistical support.

I am extremely grateful for the financial support provided by Centre for Research Excellence “Foods for Future Australians” scholarship and the Healthy Development Adelaide and Channel 7 Children’s Research Foundation top-up scholarship. This support allowed me to focus my time solely on my research. Thank you also to my supervisors for providing opportunities to present at domestic and international conferences and engage with other experts in the field.
This study would not have been possible without the families and infants participating in the N3RO trial; your willingness to partake in research is much appreciated. Thank you also to the clinical staff in the neonatal intensive care unit at the Women’s and Children’s Hospital, in particular Ros Lontis, Louise Goodchild and Dr. Andrew McPhee for your support and guidance.

I would like to thank my parents and siblings for always being by my side, even during my time overseas. Witnessing the exceptional work ethic and perseverance demonstrated by my parents over the years has developed in me a strong sense of personal responsibility and taught me that hard work is its own reward. Thank you to my fiancé Adam for your unconditional love and support every step of the way from undergraduate, to masters and throughout doctoral studies.
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<thead>
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<th>Abbreviation</th>
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<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACTRN</td>
<td>Australian Clinical Trials Registry Number</td>
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<tr>
<td>AEC</td>
<td>Alveolar epithelial cell</td>
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<td>ALA</td>
<td>Alpha linolenic acid</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<td>BPD</td>
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<td>CA</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
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<td>Dimethylsulfoxide</td>
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<td>DPPC</td>
<td>Dipalmitoylphosphatidylcholine</td>
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<td>EFA</td>
<td>Essential fatty acid</td>
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<td>EN</td>
<td>Enteral nutrition</td>
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<td>EPA</td>
<td>Eicosapentaenoic acid</td>
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<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>GA</td>
<td>Gestational age</td>
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<td>GC</td>
<td>Gas chromatography</td>
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<tr>
<td>GPR</td>
<td>G-protein coupled receptor</td>
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LIST OF ABBREVIATIONS (CONTINUED)

GSH-PX Glutathione peroxidase
LCPUFA Long-chain polyunsaturated fatty acids
HCl Hydrochloric acid
H₂SO₄ Sulfuric acid
KCl Potassium chloride
KH₂PO₄ Potassium phosphate
IL Interleukin
IVH Interventricular haemorrhage
ITT Intention to treat
LA Linoleic acid
LPS Lipopolysaccharide
LxA4 Lipoxin A4
MIP Macrophage inflammatory protein
MinDC Minimum detectable concentration
MUFA Monounsaturated fatty acid
NaCl Sodium chloride
NaOH Sodium hydroxide
Na₂HPO₄ Sodium phosphate
NICU Neonatal intensive care unit
NEC Necrotising enterocolitis
PBS Phosphate buffered saline
PN Parenteral nutrition
PMA Postmenstrual age
LIST OF ABBREVIATIONS (CONTINUED)

PP Per protocol
PPAR Peroxisome proliferator-activated receptor
PUFA Polyunsaturated fatty acid
RBC Red blood cell
RCT Randomised controlled trial
ROP Retinopathy of prematurity
RvD1 Resolvin D1
SCBU Special care baby unit
SFA Saturated fatty acid
SOD Superoxide dismutase
SOP Standard operating procedure
SP Surfactant protein
TAE Tris base, acetic acid and EDTA buffer
TAP Total antioxidant potential
T-AOC Total antioxidant capacity
TBL Total bacterial load
TGF Transforming growth factor
Th T helper
TLR Toll-like receptor
TNF Tumor necrosis factor
TPN Total parenteral nutrition
T-reg T regulatory
VLBW Very low birth weight
LIST OF ABBREVIATIONS (CONTINUED)

WCH  Women’s and Children’s Hospital

<  Less than

>  Greater than
CONTEXTUAL STATEMENT

Each year, approximately 12% of all babies are born preterm (before 37 completed weeks gestation). Complications due to preterm birth are the leading cause of death for children under five years of age. As a consequence of in utero growth disruption, preterm infants are born at a time when most organs and the immune system are not yet fully developed. Fundamental differences exist between the immune system of a preterm infant and that of an adult or even a term infant, resulting in an increased risk for inappropriate immune activation and chronic inflammation. A vicious cycle can emerge wherein chronic inflammation inflicts damage on immature organs and may result in widespread systemic effects and subsequent development of inflammatory diseases. Infants that suffer from inflammatory diseases such as bronchopulmonary dysplasia (BPD) in the early postnatal period experience an increased hospital stays and multiple re-admissions throughout childhood.

In the context of the preterm infant, this thesis addresses research questions from the themes of immunonutrition, inflammation and the effect of a dietary intervention on microbial colonisation of the gut. These questions are addressed through an examination of the effect of dietary omega-3 docosahexaenoic acid (DHA), a known anti-inflammatory agent, on markers of inflammation in preterm infants enrolled in a single-centre nested study in the multi-centre N3RO randomised controlled trial (RCT) (n-3 fatty acids for Improvement in Respiratory Outcomes: ACTRN 12612000503820). The N3RO RCT provided supplemental DHA to 1273 infants born < 29 weeks gestational age (GA) with the aim of reducing the incidence of BPD. The N3RO RCT was conducted at 13 perinatal centres in Australia, New Zealand and Singapore and the single-centre nested study forms the basis of this PhD.
The capacity for omega-3 long-chain polyunsaturated fatty acids (LCPUFA) to regulate the immune response is well known and has been extensively reviewed in adult as well as animal models. However, it is not appropriate to extrapolate immunological results obtained from adults to preterm infants, given the fundamental differences in their immune systems. Limited studies have assessed the relationship between omega-3 LCPUFA and inflammatory biomarkers as a primary outcome in preterm infants and the available data is not conclusive. Several high-quality trials investigating the effect of omega-3 LCPUFA supplementation in preterm infants on outcomes such as neurodevelopment, visual acuity and growth in preterm infants have been conducted worldwide. Some of these trials have reported on the relationship between omega-3 LCPUFA supplementation and neonatal inflammatory outcomes when included as part of their safety measures, but were not powered to detect differences in outcomes such as BPD, sepsis, necrotising enterocolitis (NEC) and retinopathy of prematurity (ROP). Therefore, the mechanisms by which omega-3 LCPUFA could act to prevent or attenuate inflammation in a preterm infant are not clear. These trials and the few that have assessed inflammatory biomarkers in omega-3 LCPUFA supplementation trials are reviewed in the context of neonatal inflammatory outcomes (BPD, sepsis, NEC, ROP) in Chapter 1, the literature review component of the thesis which is not presented in manuscript format. The literature review was later adapted into a manuscript and accepted for publication in the peer-reviewed Journal of Nutrition and Intermediary Metabolism. This article has been included in Appendix 2.

Triggers of inflammation in the newborn lung include oxygen exposure, respiratory support effects and foreign particles and organisms, both commensal and pathogenic. This can result in lung inflammation if responses to stimuli are not appropriately regulated, and in preterm infants can result in BPD. In the lung, type II AECs produce pulmonary surfactant and are progenitors for type I AECs that conduct gas exchange. Type II AECs cells also secrete inflammatory
cytokines and are targets in many lung diseases and acute respiratory distress syndrome. The immunomodulatory capability of omega-3 and omega-6 lipid emulsions provided to infants in the N3RO RCT were assessed in vitro in a type II AEC model, A549 cells, which have been used extensively as an type II AEC experimental model. This is the first in vitro investigation of these emulsions on inflammatory biomarkers. Alongside a series of commercially available parenteral lipid emulsions used in nutrition regimens for preterm infants, the efficacy of the enteral emulsion containing omega-3 DHA to attenuate the pro-inflammatory lung cytokine milieu was assessed using A549 cells. The aim of the first manuscript presented in this thesis (Chapter 2) is to describe the effect of omega-3 and omega-6 LCPUFA in parenteral and enteral lipid emulsions on secretion of immune markers by A549 cells after an inflammatory stimulus. Detailed methodologies for this and the following chapters, which are broader than those presented in manuscript format, are described in Appendix 9.

The in vivo and ex vivo data presented in Chapters 3 and 4 was obtained from 51 preterm infants enrolled in a nested study in the N3RO RCT at the Women’s and Children’s Hospital in Adelaide, South Australia. The growing body of evidence supports a role for omega-3 LCPUFA to attenuate inflammation and promote immune regulation. An assessment of markers of inflammation in preterm infants alongside the collection of clinical data will provide a broader understanding of how omega-3 LCPUFA affects the developing immune system and the mechanisms by which it may confer a protective effect against neonatal inflammatory disorders. Immune markers in biological samples collected from preterm infants were assessed at three time points from the first postnatal days of life until 36 weeks postmenstrual age (PMA) or discharge/transfer from hospital (whichever came first). Samples were analysed for inflammatory biomarkers relating to common diseases in the neonatal period, such as sepsis and BPD. The aim of the manuscript presented in Chapter 3 was to determine if supplemental omega-3 DHA could reduce the burden of inflammatory mediators in the preterm infant
compared to the control emulsion containing omega-6 linoleic acid (LA) and no DHA. The study protocol and supporting documentation for this nested study are included in Appendices 3–8.

A diverse microbial colonisation is imperative for proper gastrointestinal functioning and appropriate immune development in a preterm infant. *Staphylococcus* is one of the most common opportunistic pathogens in the preterm infant and has been associated with inflammatory diseases such as BPD. It is one of the first species to colonise the respiratory tract, and in parallel via the common mucosal system, one of the first colonisers of the gut. Almost half of the total energy consumed by infants is in the form of lipids, therefore it is plausible that type and amount of certain fatty acids may influence the initial colonisation of microbes in the gut directly or indirectly through other signalling mechanisms. LCPUFA are part of a preterm infant’s nutritional regimen and constituted the supplementation regimen in the N3RO RCT. However, it is not known if LCPUFA have the ability to affect the colonisation patterns of *Staphylococcus* bacteria in the gut. The aim of the manuscript presented in Chapter 4 was to describe the colonisation patterns of *Staphylococcus* in the preterm infant and assess the effect of omega-3 LCPUFA supplementation on the prevalence and abundance of this species over time. Methicillin-resistant staphylococci infections also cause significant morbidity and mortality in the neonatal intensive care unit (NICU) and therefore the abundance of bacteria carrying the gene for methicillin resistance was also examined. This data was compared with clinical data to gain a deeper understanding of how parameters such as mode of birth (vaginal/Caesarean), degree of prematurity and how exposure to antibiotic, probiotic and antifungal medications may affect *Staphylococcus* colonisation patterns. The relationship between omega-3 LCPUFA and other bacterial species was also assessed by a collaborating group but this work was considered outside of the context of this thesis.
The *in vitro* data generated from type II AECs combined with *in vivo* and *ex vivo* data from preterm infants < 29 weeks contributes important information on the use of omega-3 LCPUFA as an immunomodulatory agent. Omega-3 LCPUFA are supplied to preterm infants in low amounts in breast milk, some human milk fortifiers and infant formula. Omega-3 LCPUFA are also becoming more prevalent in parenteral nutrition regimens for preterm infants worldwide with the introduction of a mixed-oil lipid emulsion containing fish oil. The data generated from this thesis adds to the understanding of the immunological effects of introducing omega-3 LCPUFA into feeding regimens in the early postnatal period.
CHAPTER 1

OMEGA-3 LONG-CHAIN POLYUNSATURATED FATTY ACIDS AND INFLAMMATION IN THE PRETERM INFANT: A REVIEW OF THE LITERATURE

PREFACE

This chapter comprises the literature review portion of the thesis and is not presented in manuscript format. Concepts within this review were adapted into a manuscript and accepted for publication in the peer reviewed *Journal of Nutrition and Intermediary Metabolism* in April 2016 (Appendix 2).

Fink NH, Collins CT, Gibson RA, Makrides M, Penttila IA. Targeting inflammation in the preterm infant: The role of the omega-3 fatty acid docosahexaenoic acid. JNIM. 2016:5;55-60.
1.1. INTRODUCTION
Preterm birth, defined as birth at less than 37 completed weeks gestation (GA), occurs in around 12% of deliveries worldwide with major implications for the long term health of the child.\textsuperscript{1,2} Mortality rates for preterm infants have decreased substantially over the last few decades due to advancements in medical care.\textsuperscript{2} However, morbidity rates, particularly in the extremely preterm infant (born less than 28 weeks GA) have continued to rise because more infants survive preterm birth.\textsuperscript{2} As a consequence of in utero growth disruption, preterm infants are born at a time when most organ systems are not fully developed.\textsuperscript{3} Degree of prematurity is subclassified as extremely preterm (<28 weeks GA), very preterm (28 to <32 weeks GA) and moderate to late preterm (32 to <37 weeks GA).\textsuperscript{1} Preterm birth is usually divided into spontaneous and physician-initiated births and can result from a variety of causes such as infection, diabetes and high blood pressure.\textsuperscript{27}

Preterm infants have a lower birth weight and as a result, increased susceptibility to infection and other clinical conditions to which older, larger birth weight infants do not usually succumb.\textsuperscript{28} Functionally and immunologically immature, the very preterm infant requires intensive support, and the medical interventions necessary for their survival can trigger a local or systemic inflammatory response.\textsuperscript{3} Because preterm infants have an under-developed immunoregulatory system, there is the potential for chronic inflammation to develop.\textsuperscript{29} Dysregulation of inflammatory responses is thought to play a central role in the aetiology of many life-threatening neonatal disorders, including BPD, NEC and sepsis.\textsuperscript{3,29-32} and presents a continuing challenge to the clinical team involved in their care.

Interest is intensifying in dietary compounds such as omega-3 LCPUFA that may promote the resolution of inflammation and confer a protective effect against development of neonatal inflammatory disorders.\textsuperscript{33,34} There is some controversy as to whether or not preterm infants
can synthesise sufficient LCPUFA such as DHA and arachidonic acid (AA) from essential fatty acid (EFA) precursors because genetic variants in the fatty acid desaturase genes may affect synthesis rates of LCPUFA. However, all infants receive an exogenous source of EFA and/or LCPUFA, via breast milk, lipid emulsions, formula or a combination of these sources. Interest in DHA supplementation and its effect on clinical outcomes in preterm infants has escalated because these sources may not provide sufficient levels of DHA for these infants.

1.2. EARLY IMMUNE DEVELOPMENT IN THE PRETERM INFANT

The development of a complex and dynamic immune response to protect the host from infection and chronic inflammation requires careful interplay and precise regulation between both the innate and adaptive immune system. The innate immune system operates non-specifically as a front line of immune defence against infection, unlike the adaptive immune system which requires interaction with a pathogen prior to activation, in order to mount an antigen specific response. The adaptive immune system develops an immunological memory after exposure to a pathogen, leading to a fast and effective response upon the next encounter. These two systems have very important individual and collaborative roles for immune protection and maintenance of homeostasis.

1.2.1. Transition from innate to adaptive immune response in the preterm infant

Preterm infants have a fundamentally different immune system to that of an adult or even a term infant, making them especially susceptible to exaggerated immune activation. A preterm infants’ surroundings change from a low bacterial load in the intrauterine environment to the microbial-rich (commensal and pathogenic) NICU environment where they are exposed to many life-saving but invasive interventions.
Until the immune system develops, the preterm infant relies heavily on the non-specific innate immune response for defence. This reliance can result in inappropriate immune activation and chronic inflammation until the homeostatic mechanisms are developed. The antigen-specific adaptive immune system of a preterm infant is underdeveloped at birth, particularly with regard to T cells mediating inflammatory responses (T helper 1: Th1) and the important T cells involved in regulating the immune response (T-regulatory: T-reg). Ineffective T-reg function after birth, when the infant is exposed to a massive environmental antigenic onslaught during the birth process and in the NICU, can result in excess inflammation and an impaired ability to down-regulate immune responses once initiated. Because a preterm infant’s early immunological development takes place in the protective, largely sterile environment of the womb, they lack the antigenic exposure necessary to trigger maturation of the adaptive response. Infants born preterm also do not benefit from the trans-placental transfer of antibodies during the third trimester.

1.2.2. Polarisation of the immune system
To avoid compromising either the fetus or the mother, a delicate balance of immune tolerance and suppression is required. A mechanism to counteract the excessive pro-inflammatory responses is required otherwise uncontrolled tissue damage can result. To reduce the risk of miscarriage, a strong Th2 response is necessary to modify the Th1 cellular response in utero. Hence, a newborn infant’s immune response is polarised towards a Th2 profile and a progressive maturation towards a Th1-like response develops with age. If, after birth, the neonate is not successful in down-regulating the pre-existing Th2 dominance, an allergenic phenotype may develop.

1.2.3. Characterising the immune response in a preterm infant
In the literature, there is some controversy whether or not a preterm infant is capable of mounting a sufficient immune response. With regard to preterm infants, it has been reported
that production of some pro-inflammatory cytokines fall below adult levels, particularly interleukin (IL) 12/IL-23. Cultured monocytes from preterm infants exhibit reduced pro-inflammatory IL-6 and Tumor necrosis factor (TNF) α production in response to bacterial antigens. Given results of this nature, it has been suggested that a preterm infant’s reduced ability to defend against infection is primarily due to suppression of the pro-inflammatory response mediated by IL-12/IL-23. A reduced cytotoxic response by natural killer T cells, deficiency in antibody production as a result of T- and B-cell immaturity and reduced ability to clear intracellular pathogens have also been cited as reasons for increased susceptibility to infection in a preterm infant.

In contrast to the above findings, it has also been proposed that pro-inflammatory pathways are upregulated in preterm infants. Cells involved in initiating immune responses, such as antigen-presenting cells (APC) and monocytes, have been found to produce as much or even more IL-23 than adult cells in vitro, particularly in response to bacterial stimulation. IL-23 is important as it can promote the activation of IL-17-secreting T-cells, which mediate inflammation and tissue damage. Although existing evidence supports the claim that preterm infants’ immune systems are polarised towards a pro-inflammatory profile, a failure of regulatory mechanisms in the preterm infant could also explain the high levels of pro-inflammatory cytokines. Systemic inflammatory responses have been shown to be most evident in the first 72 hours of life in the preterm infant and sustained inflammatory responses are still common in the first few weeks of life. Chronic inflammation may result from a breakdown in homeostasis and lack of immune regulation leading to inflammation in multiple organ sites. This has the potential to inflict irreversible long-term damage to organ systems.
While controversy exists in the characterisation of the preterm infant’s immune system, the inflammatory process is central to maintaining tissue homeostasis. Therefore exaggerated, prolonged or a lack of inflammatory response can all be pathogenic. Omega-3 LCPUFA in the diet may play a role in the resolution of inflammation while the regulatory immune system is maturing.

1.3. FATTY ACIDS IN THE DIET
1.3.1. Lipid metabolism
Forty percent of the total energy ingested by preterm infants is derived from lipids and a complex orchestration of events is required to digest and solubilise dietary fat and deliver the metabolites. Dietary fatty acids can exist in the body as part of complex lipids such as triglycerides or cholesterol esters, as part of a lipoprotein complex or in a free form (ie. unbound to any protein or other moiety). Fatty acids are usually stored and carried within the body as triglycerides and serve as a concentrated energy source. Triglycerides consist of three fatty acid molecules attached to the carbon atoms of a glycerol molecule. Gastric lipase hydrolys approximately 20% of triglycerides and the remainder are hydrolysed in the intestine into free fatty acids and glycerol molecules and transported into the enterocyte. The free fatty acids are reassembled back into triglycerides and packaged into chylomicrons in order to remain stable in aqueous phase during transport via the bloodstream. The triglycerides in the chylomicron core are surrounded by polar phospholipids and apolipoproteins. Lipoprotein lipase, released from the capillary endothelium, recognises the apolipoproteins, uptakes the triglycerides in the chylomicron core and delivers the dietary lipid to targeted tissues and the liver. Adipocytes are the primary storage tissue for fatty acids, and is the main site of turnover between absorption and release.
1.3.2. Brief overview of fatty acids

Generally, fatty acids are composed of a hydrocarbon chain of varied length with a methyl group at one end and a carboxyl group at the other. They are systematically named according to carbon chain length, degree of unsaturation, position of the first double bond and orientation \((cis/trans)\) around that bond \(^{64,65}\). For example, \(cis\)-C18:1n9 (or \(cis\)-C18:1ω9) is an 18-carbon chain, one degree of unsaturation occurring 9 positions from the terminal methyl group and displays a \(cis\) orientation around the double bond. A \(cis\) orientation indicates that the hydrogen atoms attached to the double bond are oriented on the same side and \(trans\) refers to opposite side configuration \(^{65}\).

Preformed fatty acids can be obtained from the diet or synthesised in the body from other fatty acids \(^{37,63}\). Fatty acids are classified as short (< 8 carbon atoms), medium (8-12 carbon atoms) or long chain (> 12 carbon atoms) and range in chain length from 4-30 carbon atoms \(^{63}\). They are also classified based on the number of double bonds. Fatty acids without any double bonds are termed ‘saturated’ fatty acids (SFA), ‘monounsaturated’ fatty acids (MUFA) if they contain one double bond, and ‘polyunsaturated’ (PUFA) if they contain more than one double bond \(^{63}\).

Short-chain SFA such as butyric (C4:0) and caproic acid (C6:0) are commonly found in dairy fat, MUFA such as oleic acid (C18:1n9) are found in olive oil and LCPUFA such as DHA (C22:2n6) are found in fish and some nuts and seeds. SFA are the least reactive and are stable for long periods of time, while fatty acids containing double bonds are less stable due to the potential for oxidation \(^{63}\). Breast milk contains high proportions of SFA such as C16:0 and C18:0 and also supplies PUFA such as DHA and AA.
1.3.3. **Endogenous synthesis of LCPUFA**

Humans can synthesise LCPUFA from two PUFA precursors, α-linolenic acid (ALA) and LA. ALA and LA are EFA, so named because they cannot be synthesised by humans and therefore must be obtained from the diet. LCPUFA are synthesised from EFA via the action of desaturase and elongase enzymes (Figure 1). As the desaturase and elongase enzymes are common to both the omega-3 and omega-6 pathways, the precursors present in the highest amounts will shift the reaction towards the production of its downstream products. A preterm infant has the necessary enzymes for conversion of EFA to downstream LCPUFA (ie. DHA), but synthesis rates are low.
Figure 1. Simplified schematic of the anabolic pathway of essential fatty acids
1.3.4. Lipid derivatives and their role in inflammation
Omega-3 and omega-6 PUFA are the biosynthetic precursors of eicosanoids (prostaglandins, leukotrienes and thromboxanes) and docosanoids (E- and D-series resolvins, protectins and maresins). Omega-3 and omega-6 PUFA such as eicosapentaenoic acid (EPA) and AA are eicosanoid precursors, while the omega-3 DHA serves as the docosanoid precursor. These biologically active lipid mediators can amplify or reduce inflammation, influence tissue repair processes, cell growth and proliferation and contraction/dilation of smooth muscle. Although eicosanoids are synthesised from both omega-3 and omega-6 precursors, the most common substrate for eicosanoid synthesis is AA. Therefore, omega-3 LCPUFA exert an anti-inflammatory effect by antagonising AA metabolism to reduce the synthesis of pro-inflammatory prostaglandins and by synthesising docosanoids to resolve inflammation. Dietary levels of omega-3 and omega-6 PUFA influence levels of eicosanoid and docosanoid production in the body. Furthermore, cytokines, eicosanoids and docosanoids interact together as part of signalling pathways to influence inflammation.

1.4. NUTRITION FOR PRETERM INFANTS
Preterm infants receive macronutrients (carbohydrates, protein, lipids) and micronutrients (vitamins and minerals) enterally from breast milk and formula and/or parenterally from parenteral nutrition regimens. Often, preterm infants receive parenteral lipid emulsions in the first days or weeks of life alongside the introduction of small volumes of enteral breast milk and/or formula until the infant is able to tolerate full enteral feeding. Target levels of nutrients and daily feeding volumes are based on feeding guidelines and also on the infant’s tolerance, as measured by gastric residuals. Early introduction of enteral feeding is recommended because in addition to promoting development of oral tolerance, which will be discussed later in section 1.4.2, it reduces the risk of sepsis as catheters required for parenteral nutrition (PN) are removed earlier.
Growth stages in a preterm infant are more energy and nutrient-intensive than in a term infant and in some countries, human milk fortifier is often added to the breast milk from mothers of preterm infants. Human milk fortifiers can be derived from both bovine and human milk and also provide the preterm infant with additional LCPUFA, including DHA. Table 1 summarises the comprehensive guidelines for feeding preterm infants obtained from a recent systematic review.

Table 1. Summary of current enteral feeding guidelines for preterm infants

<table>
<thead>
<tr>
<th>Enteral feeding parameter</th>
<th>Guideline</th>
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</table>
| Type                      | Order of preference:  
|                           | 1. Breast milk (fresh if available, frozen if not)  
|                           | 2. Donor milk  
|                           | 3. Formula  
| When                      | Should be initiated as early as the infant tolerates enteral feeds. Aim to achieve full enteral feeding in 1 week (>1000g at birth) to 2 weeks (1000-1500g at birth)  
| Amount                    | < 1kg at birth: start at 15-20 mL/kg/day  
|                           | >1kg at birth: start at 30 mL/kg/day  
| Fortification             | Milk should be fortified when enteral intake reaches 100 mL/kg/day at a concentration of 1:50 and then increased to 1:25 if tolerated for 48 hours  

1.4.1. Fatty acids in nutritional regimens

At birth, the preterm infant becomes disconnected from the nutrient supply of the placenta. In order for proper growth and development to take place outside of the womb, an exogenous source of nutrition that can sustain the high accretion rate of LCPUFA is required. While breast milk is preferred, other nutrition products are supplied in some circumstances, such as infant formula if breast milk supply is not sufficient or intravenous lipid emulsions if the infant
cannot tolerate enteral feeds \(^{80}\). Breast milk contains between 0.06-1.4\% (wt/wt) DHA and formula content ranges from 0.2-0.3\% (wt/wt) \(^{81}\).

Current feeding recommendations centre on the concept of adjusting nutrition regimens so that the postnatal growth of preterm infants matches the growth of an age-matched healthy fetus\(^{80,82}\). Fetal accretion rates of LCPUFA have been used to inform guidelines for target levels of breast milk and formula supplementation in preterm infants \(^{80}\). The assumption is that the infant’s nutrition source (breast milk/formula/PN) should contain similar levels of LCPUFA to that supplied by the placenta, had the infant remained *in utero* until term.

Guidelines for recommended levels of fatty acids do not exist beyond those set for LCPUFA \(^{77}\) and total lipids are supplied at a rate of approximately 2-3g/kg/day. Data on fetal accretion rates of DHA are obtained mainly from fetal autopsy results, which are used as an estimate of DHA accumulation in the central nervous system \(^{80}\). Even with these levels as a guideline for appropriate DHA levels in nutrition sources, Lapillonne et al. (2013) stress that current nutritional practices in the NICU do not provide adequate levels of DHA during periods of PN and enteral nutrition (EN) in very preterm/very low birth weight infants \(^{43,80}\).

### 1.4.2. Docosahexaenoic acid and its role in early immune development

Breast milk has long been considered the gold standard for infant nutrition and is essential for promoting appropriate immune development in newborns \(^{83}\). Breast milk contains DHA, albeit the concentration is highly variable and dependent on dietary intake (≈0.3 - 1\%) \(^{84,85}\). In addition to LCPUFA, breast milk also contains a complex mixture of immunologically active components such as growth factors, lactoferrin, prostaglandins, immunoglobulins, cytokines and immune cells \(^{53,86}\). Together with LCPUFA, the immunoregulatory bioactives in breast milk such as IL-10, transforming growth factor (TGF) \(\beta\) and DHA serve as mediators to promote oral tolerance and they also modulate developing immune responses while the infant
develops their own immunoregulatory ability \(^3, 28, 44, 46, 50, 87, 88\). This immune maturation is crucial in order for a complex and dynamic relationship to develop between the innate and adaptive immune system \(^29, 46, 49, 89\), allowing infants to respond effectively and appropriately to self and pathogenic environmental stimuli \(^44, 45\). Without appropriate regulation, an unchecked inflammatory pathophysiology can result, leading to many neonatal morbidities \(^3, 29, 48\).

1.5. OMEGA-3 LCPUFA AND NEONATAL INFLAMMATORY DISORDERS

A heightened immune response leading to an exaggerated release of inflammatory mediators is a hallmark of BPD and other inflammatory disorders in the neonatal period, including sepsis, NEC and ROP \(^3\). These disorders have a multi-factorial pathogenesis for which a single medication or comprehensive treatment is not available. Data from both preterm infant \(^29, 90\) and animal studies \(^91, 92\) support the potential for DHA to serve as a general preventative agent against inflammation without inhibiting development or function of underdeveloped organs.

1.5.1. Evidence for an effect of omega-3 LCPUFA supplementation on neonatal inflammatory outcomes

A literature review was performed to evaluate the clinical outcomes associated with omega-3 LCPUFA supplementation in preterm infants ((preterm infant*[ALL] OR premature infant*[ALL] OR infant, premature[MH])) AND (Docosahexaenoic acid*[ALL] OR Fatty acids, omega-3[MH] OR n-3 PUFA*[ALL] OR n-3 Fatty Acid*[ALL] OR n-3 Polyunsaturated Fatty Acid*[ALL] OR Omega-3 Fatty Acid*[ALL] OR alpha-Linolenic Acid*[ALL] OR Eicosapentaenoic Acid*[ALL] OR DHA[ALL] OR EPA[ALL] OR ALA[ALL])). The key studies reviewing the effect of direct supplementation of omega-3 LCPUFA to preterm infants on inflammatory clinical outcomes relevant to this thesis are summarised in Table 2. Studies providing maternal omega-3 supplementation during pregnancy were excluded because the final dose of omega-3 LCPUFA received by the infant is highly variable and maternal
compliance cannot be confirmed. Only studies in which infants <36 weeks GA were directly supplemented with omega-3 LCPUFA were included.
Table 2. Characteristics of key studies reporting effect of omega-3 long-chain polyunsaturated fatty acids on inflammatory clinical outcomes in preterm infants

<table>
<thead>
<tr>
<th>STUDY (AUTHOR, YEAR, LOCATION)</th>
<th>DESIGN</th>
<th>POPULATION</th>
<th>a. INTERVENTION</th>
<th>b. CONTROL</th>
<th>c. TIME/DURATION OF SUPPLEMENTATION</th>
<th>d. PARENTERAL OR ENTERAL SUPPLEMENTATION</th>
<th>TRIAL OUTCOMES*</th>
<th>SIGNIFICANT RESULTS RELATING TO NEONATAL INFLAMMATORY OUTCOMES</th>
<th>COMMENT</th>
</tr>
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<tbody>
<tr>
<td>RANDOMISED CONTROLLED TRIALS</td>
<td></td>
<td></td>
<td>a. SMOFlipid (n=17)</td>
<td>b. ClinOleic (n=17)</td>
<td>c. From &lt; 7 days postnatal for at least 7 days</td>
<td>d. Parenteral</td>
<td>Primary: LCPUFA levels, lipid peroxidation</td>
<td>No difference in the incidence of chronic lung disease, sepsis</td>
<td>1. ITT analysis 2. Power calculations not conducted for inflammatory clinical outcomes 3. No definition for “chronic lung disease” provided</td>
</tr>
<tr>
<td>Deshpande G et al., Australia 2014</td>
<td>RCT</td>
<td>n=30 (additional 4 neonates recruited after randomisation to account for incomplete intervention) Infants &lt; 30 weeks GA</td>
<td></td>
<td></td>
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<tr>
<td>Yang Q et al., USA 2014</td>
<td>RCT</td>
<td>n=37 Infants born &lt; 37 weeks GA and &lt; 2 months at randomisation</td>
<td>a. Standard nutrition + enteral fish oil supplement (n=18)</td>
<td>b. Standard nutrition (n=18)</td>
<td>c. Once EN rate is 20 mL/kg/d until hospital discharge</td>
<td>d. Enteral</td>
<td>Primary: Amount of days on PN after an ostomy and EN intake</td>
<td>↓ evaluations of sepsis</td>
<td>1. ITT analysis 2. Power calculations not conducted for inflammatory clinical outcomes 3. Sepsis defined as “positive culture from both central line and peripheral vein”</td>
</tr>
</tbody>
</table>
Table 2. continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>n</th>
<th>GA Group</th>
<th>Intervention 1</th>
<th>Intervention 2</th>
<th>Outcome 1</th>
<th>Outcome 2</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beken S et al., Turkey 2014</td>
<td>RCT</td>
<td>80</td>
<td>Infants &lt; 32 weeks GA</td>
<td>SMOFlipid (n=40)</td>
<td>Intralipid (n=40)</td>
<td>ROP</td>
<td>No difference in the incidence of chronic lung disease, sepsis</td>
<td>↓ ROP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>a.</td>
<td>b.</td>
<td></td>
<td></td>
<td>1. Power calculations conducted for ROP 2. ROP definition was “diagnosis by paediatric ophthalmologist and need for laser photoagulation”. 3. Chronic lung disease defined as “oxygen dependency beyond 36 weeks of CA”. NEC defined as “grade ≥ 2”</td>
</tr>
<tr>
<td>Manley B et al., Australia 2011</td>
<td>Sub-study of RCT</td>
<td>657</td>
<td>Infants &lt; 33 weeks GA</td>
<td>Breast milk from mothers supplemented with DHA (tuna oil)/High-DHA formula (n=322)</td>
<td>BM from mothers supplemented with placebo (soy oil)/standard formula (n=335)</td>
<td>Overall no difference in risk of BPD between groups.</td>
<td></td>
<td>1. ITT analysis 2. Power calculations not conducted for inflammatory clinical outcomes 3. BPD defined as “oxygen requirement at a corrected age of 36 weeks’ GA”</td>
</tr>
<tr>
<td>Tomsits et al., Hungary 2010</td>
<td>RCT</td>
<td>60</td>
<td>Infants &lt; 34 weeks GA</td>
<td>SMOFlipid (n=30)</td>
<td>Intralipid (n=30)</td>
<td>Safety (serum triglycerides and laboratory safety parameters) and efficacy (weight, CRP levels, sepsis, respiratory support) parameters</td>
<td>No difference in incidence of sepsis or need for respiratory support between groups</td>
<td>1. ITT and PP analysis (results were similar- final ITT data presented) 2. Power calculations not conducted for inflammatory clinical outcomes 3. Respiratory support defined as “days with oxygen therapy”</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>n</td>
<td>Duration</td>
<td>Intervention(s)</td>
<td>Primary: Cognitive development at 6 months of age</td>
<td>No difference in incidence of NEC, ROP or need for respiratory support between groups</td>
<td>1. Power calculations not conducted for inflammatory clinical outcomes</td>
<td>2. NEC defined as “suspected/proven”</td>
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<tr>
<td><strong>Henriksen C et al., Norway 2008</strong></td>
<td>RCT</td>
<td>141</td>
<td>Infants &lt; 31 weeks GA</td>
<td>a. Human milk + DHA/AA (n=68)</td>
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<td></td>
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<td>b. Human milk with no added DHA/AA (n=73)</td>
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<td>c. Enteral feeding til hospital discharge</td>
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<td>d. Enteral</td>
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<tr>
<td><strong>Clandinin M et al., Canada 2005</strong></td>
<td>RCT</td>
<td>361</td>
<td>Phase I: n=361, Phase II: n=245 Infants &lt; 35 weeks GA</td>
<td>a. Formula with added DHA from algal (Phase I, n=112, Phase II, n=52) or fish oil (Phase I, n=130, Phase II, n=65)</td>
<td>Primary: Weight at 57 and 92 weeks PMA</td>
<td>Secondary: Intake, tolerance, general development, morbidity, adverse outcomes</td>
<td>Phase 1: No difference in incidence of BPD, sepsis, NEC or ROP between groups</td>
<td>1. Power calculations not conducted for inflammatory clinical outcomes</td>
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<td>b. Formula with no added DHA or AA (Phase I, n=119, Phase II, n=62)</td>
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<td>c. Phase I: 40 weeks PMA, Phase II: 92 weeks PMA</td>
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<td>d. Enteral</td>
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<tr>
<td><strong>Groh-Wargo S et al., USA 2005</strong></td>
<td>RCT</td>
<td>60</td>
<td>Infants &lt; 33 weeks GA</td>
<td>a. Formula with added DHA/ARA from fish/fungal oil (n=18) or egg/fish oil (n=18)</td>
<td>Growth and body composition, bone mineral density, plasma and erythrocyte fatty acid levels</td>
<td>No difference in incidence of BPD between groups.</td>
<td>1. Power calculations not conducted for inflammatory clinical outcomes</td>
<td>2. BPD defined as “supplemental oxygen beyond 1 month postnatal or 36 weeks CA”</td>
</tr>
</tbody>
</table>
### Table 2. continued

<table>
<thead>
<tr>
<th>Study Authors, Year, Location</th>
<th>Study Design</th>
<th>n</th>
<th>Treatment Groups</th>
<th>Primary Outcomes</th>
<th>Secondary Outcomes</th>
<th>Inclusion Criteria</th>
<th>Additional Notes</th>
</tr>
</thead>
</table>
| Fewtrell M et al., United Kingdom 2004 | RCT | 238 | a. Formula with added DHA from fish oil (n=122)  
  b. No-LCPUFA formula (n=116)  
  c. From first days of enteral feeding to 9 months of age  
  d. Enteral | Primary: Developmental scores  
  Secondary: growth | No difference in incidence of respiratory disease, NEC, or infection between groups | Infants < 35 weeks GA | 1. Power calculations not conducted for inflammatory clinical outcomes  
  2. Infection was defined as “microbiologically confirmed skin sepsis, systemic infection with clinical and hematologic evidence only (high or low white cell count and/or low platelet count), and bacteriologically proven systemic infection”  
  3. NEC was defined as “confirmed either at surgery, post-mortem examination, or by the presence of portal or intramural gas on abdominal radiography”  
  4. Respiratory diseases defined as “requirement for respiratory support (number of days requiring >30% oxygen, number of days of mechanical ventilation)” |
| Innis S et al., Canada 2002 | RCT | 194 | a. Formula with added DHA and DHA/ARA (n=66)  
  b. No-LCPUFA formula (n=62)  
  c. Enteral feeding until at least 28 days postnatal up to 4 months of age  
  d. Enteral | Growth, visual acuity, adverse events, blood fatty acid analysis | No difference in incidence of NEC, ROP or sepsis between groups | Infants < 32 weeks GA | 1. Power calculations not conducted for inflammatory clinical outcomes  
  2. No definition for NEC, sepsis, ROP |
Table 2. continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>n</th>
<th>Age</th>
<th>Formula Details</th>
<th>Outcomes</th>
<th>Notes</th>
</tr>
</thead>
</table>
| O'Connor D et al., USA 2001   | RCT    | 470| Infants < 33 weeks GA | a. Formula with added DHA/AA from egg/fish or fish/algal oils  
  b. No-LCPUFA formula  
  c. First enteral feeding until 12 months  
  d. Enteral | Growth, visual acuity and development | No difference in the incidence of NEC, chronic lung disease or systemic infections between groups  
1. ITT analysis  
2. Power calculations not conducted for inflammatory clinical outcomes  
3. Confirmed NEC was defined as “roentgenographic, surgical or post-mortem evidence of pneumatosis, intra-abdominal free air or gas in the portal tract, or perforation”  
4. Systemic infection was defined as “positive blood culture”  
5. Chronic lung disease was defined as “supplemental oxygen beyond one month postpartum” |
| Carlson S et al., USA 1996     | RCT    | 94 | Infants < 30 weeks GA | a. Fish oil enriched formula (n=26)  
  b. No-LCPUFA formula (n=33)  
  c. ≤ postnatal day 5 to discharge  
  d. Enteral | Visual acuity and growth | No difference in incidence of BPD between groups  
1. 100 subjects/group would have been required to determine an effect of diet on incidence of sepsis, NEC  
2. High loss to follow-up  
3. Disproportionate control/intervention groups  
4. BPD was defined as “requirement for supplemental oxygen for 28 d and had lung changes on X-rays characteristic of BPD” |
<table>
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<tr>
<th>Foreman-van Drongelen et al., Netherlands 1996</th>
<th>RCT</th>
<th>n=43 Infants &lt; 37 weeks GA</th>
<th>a. Formula with added LCPUFA from algal or fungal oils (n=15) or breast milk (n=12)</th>
<th>Plasma and RBC phospholipid fatty acid levels</th>
<th>No difference in incidence of BPD or sepsis between groups</th>
<th>1. Power calculations not conducted for inflammatory clinical outcomes 2. No definition for BPD provided</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marc I et al., Canada 2011</td>
<td>Longitudinal intervention trial with reference group</td>
<td>n=36 Infants &lt; 29 weeks GA</td>
<td>a. Breast milk from mothers supplemented with fish oil capsules</td>
<td>Primary: DHA concentration in infant plasma on day 49 postnatal</td>
<td>No difference in the incidence of respiratory distress syndrome, respiratory support or NEC, between groups</td>
<td>1. Power calculations not conducted for inflammatory clinical outcomes 2. No definition of respiratory distress syndrome or NEC. Newborn outcomes were collected from the hospital charts</td>
</tr>
</tbody>
</table>
Table 2. continued

<table>
<thead>
<tr>
<th>STUDY (AUTHOR, YEAR, LOCATION)</th>
<th>DESIGN</th>
<th>POPULATION</th>
<th>a. DESCRIPTION OF DIET/SUPPLEMENTATION</th>
<th>b. TIME/DURATION OF DIET/SUPPLEMENTATION</th>
<th>c. PARENTERAL OR ENTERAL DIET/SUPPLEMENTATION</th>
<th>STUDY OUTCOMES*</th>
<th>SIGNIFICANT RESULTS RELATING TO NEONATAL INFLAMMATORY OUTCOMES</th>
<th>COMMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skouroliakou M et al., Greece 2012</td>
<td>Prospective observational</td>
<td>n=282 Infants &lt; 36 weeks GA VLBW group (&lt; 1500g): n=129 LBW group (&lt; 1500-2500g): n=153</td>
<td>a. SMOFlipid (VLBW: n=54, LBW: n=83) AND Intralipid (VLBW: n=75, LBW: n=70)</td>
<td>b. postnatal day 1 or 2, for at least 7 days</td>
<td>c. Parenteral</td>
<td>Cholestasis and BPD</td>
<td>VLBW group: ↓ BPD In the VLBW group, type of lipid emulsion was independently associated with BPD No difference in NEC/sepsis (treated as single outcome) between groups</td>
<td>1. Nutrition was selected according to the attending neonatologist’s preference and product availability 2. Power calculations to detect differences in BPD not conducted 3. BPD was defined as “the need for supplementary oxygen at day 28 of life and classified as mild, moderate, and severe according to the needs for oxygen at 36 weeks postconceptional age for infants with GA &lt;32 weeks or at 56 days postnatally for infants with GA ≥32 weeks.” 4. NEC/sepsis definition not provided</td>
</tr>
<tr>
<td>Martin C et al., USA 2011</td>
<td>Retrospective cohort study</td>
<td>n=88 Infants &lt; 30 weeks GA</td>
<td>a. Standard nutrition regimen</td>
<td>Fatty acid profiles in infant plasma, nutrition intake and growth, clinical outcomes (chronic lung disease, sepsis, IVH, NEC, ROP)</td>
<td>↓ DHA levels are associated with ↑ in chronic lung disease and late-onset sepsis. No association between DHA and NEC or ROP</td>
<td>1. Power calculations not conducted for inflammatory clinical outcomes 2. Chronic lung disease was defined as “requiring supplemental oxygen at 36 weeks’ PMA” 3. ROP was determined by an examining ophthalmologist 4. Early and late onset sepsis were defined as “positive blood culture within first 3 postnatal days and after 3 postnatal days, respectively” 5. NEC was defined by “the presence of pneumatosis on abdominal radiograph or by the clinical spectrum of bloody spools, abnormal abdominal exams and change in clinical status resulting in withholding of enteral feedings and provision of antibiotics for ≥10 days”</td>
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Table 2. continued

| Pawlik D et al., Poland 2011 | Observational study with historical reference group | n=84 Infants < 32 weeks GA (< 1250g) | a. 50/50 mixture of Omegaven+ClinOleic (n=40) compared to ClinOleic (n=44, historical reference group) | b. postnatal day 1 until full enteral feeding | c. Parenteral | Primary: ROP and cholestasis Secondary: infections, IVH, BPD, patent ductus arteriosus, total PN and length of hospitalisation | No difference in the incidence of BPD, sepsis, ROP *Requirement for laser therapy to treat ROP was reduced in the intervention group | 1. Power calculations not conducted for inflammatory clinical outcomes 2. ROP was defined as “ROP and the need for laser photocoagulation” 3. BPD was defined as “as an oxygen requirement and/or respiratory support at 36 weeks corrected gestational age” |

Legend to Table 2. ‘*’= Information on outcome hierarchy (ie. primary/secondary/etc) is included if specified by the authors. Abbreviations: Corrected age (CA), C-reactive protein (CRP), gestational age (GA), intraventricular haemorrhage (IVH), postmenstrual age (PMA), randomised controlled trial (RCT), red blood cell (RBC), parenteral nutrition (PN), total parenteral nutrition (TPN), very low birth weight (VLBW).
1.5.2. Characterising the evidence

Thirteen of the 14 interventional trials summarised in Table 2 were RCTs, the gold standard for evaluating the effect of an intervention versus control with limited bias. Of these, infants were supplemented via the enteral route in 11 of the interventional trials, two of the observational studies investigated the effect of supplementation via the parenteral route and one observational study did not provide supplementation but measured omega-3 and omega-6 status to assess associations with clinical outcomes. Five RCTs reported that analyses were performed in intention to treat (ITT), others did not define the analysis as either ITT or per protocol (PP). Overall, the majority of the RCTs were designed to assess visual acuity, neurodevelopment, growth, and/or fatty acid levels. Inflammatory clinical outcomes were mainly reported because they were included as a measure of safety or as an adverse outcome of the trial. At least one neonatal inflammatory outcome (BPD, sepsis, NEC, ROP) was included as either a primary or secondary outcome in six of the intervention trials and three of the observational studies.

The definition of inflammatory outcomes (BPD, sepsis, NEC, ROP) varied substantially between studies. For example, BPD was defined in several studies as “supplemental oxygen at 36 weeks PMA”\(^{95, 101, 104, 106}\), Carlson et al. (1996) defined it as “supplemented oxygen for > 28 days and/or pulmonary changes on radiologic examination”\(^{102}\) whereas Foreman-Van Drongelen et al. (1996) did not provide any description of how BPD was defined\(^{103}\). If a definition was provided for sepsis/infections, these tended to be consistent and required a positive blood culture in most studies\(^ {34, 93, 97, 99, 101}\). When incidence of NEC was reported, several studies did not provide a definition\(^ {100, 104, 105}\) and other studies’ definitions contained a range of detail from “suspected/proven”\(^ {96}\) to “modified Bell staging criteria”\(^ {97}\) to “roentgenographic, surgical or postmortem evidence of pneumatosis, intra-abdominal free air or gas in the portal tract, or perforation”\(^ {101}\). Similarly for ROP, definitions were either not provided\(^ {100}\), vague\(^ {96}\) or detailed\(^ {34, 94, 106}\).
More recent trials that have included inflammatory disorders in the primary and secondary outcomes have reported mixed effects of omega-3 LCPUFA supplementation; a decrease in ROP \textsuperscript{94, 108}, a decrease in sepsis evaluations \textsuperscript{93}, a decrease in BPD in very low birth weight (VLBW) infants \textsuperscript{105} and no effect on chronic lung disease or sepsis \textsuperscript{8}. It is important to note that other than Beken et al. (2014) \textsuperscript{94} and Carlson et al. (1996) \textsuperscript{102}, power calculations were not conducted for any inflammatory outcomes (BPD, sepsis, NEC, ROP). Therefore, it is not known if these studies were sufficiently powered to detect differences in outcomes based on diet. Furthermore, while Carlson et al. (1996) \textsuperscript{102} determined that 100 infants per group would be needed to detect diet-induced differences in sepsis or NEC, they did not reach this sample size and therefore lacked sufficient power.

Contrasting results in inflammatory outcomes from trials summarised in Table 2 are likely due to variations in study design, definition of outcomes/safety measures, dose of omega-3, timing of administration, duration of intervention, and/or delivery (ie. PN vs. EN, formula vs. breast milk). For example, Manley et al. (2011) \textsuperscript{95} and Henriksen et al. (2008)\textsuperscript{96} employed a similar delivery method (breast milk), but in the former, the breast milk omega-3 LCPUFA content was increased via maternal supplementation while in the latter the omega-3 LCPUFA was added directly to mother/donor milk. Many of the studies that followed a similar study design included different GA, such as < 32 weeks GA in the study by Innis et al. (2002) \textsuperscript{100} and < 35 weeks GA in the study by Clandinin et al. (2005) \textsuperscript{97}. Some inflammatory conditions such as BPD are highly dependent on GA, therefore consideration must be given when comparing outcomes of omega-3 supplementation at different GA. Timing of introduction and duration of intervention also varied between the above studies, for example in the first days of life until day 14 \textsuperscript{11}, first days of life until term \textsuperscript{101, 104} and first days of life until nine months of age \textsuperscript{99}. Lastly, the dose of omega-3 LCPUFA is perhaps the most difficult parameter to compare between the
studies presented in table 2. Some authors report the weight of the lipid dose itself (i.e. 100 mg of pure fish oil), some report relative percentage of omega-3 LCPUFA (i.e. % of total fatty acids) and some report volume (mL) of parenteral lipid emulsion that was administered per kilogram of body weight. Standardising these values for DHA and other omega-3 LCPUFA in order to compare the absolute dose in each study is a major challenge.

In summary, most of the RCTs were not designed with inflammatory outcomes as primary or even secondary outcomes and therefore may not be sufficiently powered to detect differences between groups. Results from these trials regarding the effect of omega-3 LCPUFA on neonatal inflammatory disorders may only be suggestive. The trials described in Table 2 are discussed below in the context of BPD, sepsis, NEC and ROP outcomes.

1.5.3. Bronchopulmonary dysplasia
BPD is a disorder of prematurity characterised by the need for assisted ventilation or supplemental oxygen at 36 weeks PMA and signs of impaired alveolarisation and vasculogenesis in the lungs \(^{29,109}\). BPD occurs in approximately 45% of infants born less than 29 weeks GA \(^{29,110}\). Ongoing lung damage may be caused by the preterm infant’s inability to down-regulate and maintain control of the inflammatory immune response, leading to a chronic inflammatory state \(^{52,59,60,110}\). Perinatal infection, surfactant deficiency, barotrauma, volutrauma and oxygen toxicity also elicit inflammatory responses in the lung \(^{109}\). As the pathogenesis of BPD is multi-factorial, a single medication or comprehensive treatment to prevent or treat this disorder is not currently available \(^{59}\). The current practise to combat neonatal lung inflammation is systemic corticosteroid administration \(^{32,59}\). Systemic corticosteroids can interfere with proper lung development via inhibition of alveolar septation \(^{32}\). While corticosteroids effectively reduce acute inflammation, the long-term effects while the infant’s immune system is still developing remain to be assessed.
1.5.3.1. Intervention studies

Twelve intervention studies reported on the effect of omega-3 LCPUFA supplementation on incidence of either BPD, chronic lung disease, respiratory disease or respiratory support. Eleven of the intervention trials were RCTs with seven administering omega-3 LCPUFA via the enteral route and four via the parenteral route. Duration of supplementation ranged from seven days \(^8\) to one year of age \(^9\). Eleven intervention trials reported no difference in the incidence of respiratory outcomes between groups. Evidence of the ability for DHA to improve respiratory outcomes in preterm infants comes from the “Docosahexaenoic acid for Improvement in Neurodevelopmental Outcomes (DINO) trial”. In the subgroup of infants born weighing less than 1250 g, those who received higher-DHA breast milk or formula had a reduced rate of BPD \(^9\). BPD was assessed as part of the secondary outcomes in this sub-study of the DINO RCT \(^9\).

1.5.3.2. Observational studies

Three observational studies investigated the relationship between physiological levels of or supplementation with omega-3 LCPUFA. One supplementation study reported neutral effects and two studies found a significant association between omega-3 supplementation and the incidence of BPD in samples sizes of 282 neonates \(^10\) and 88 neonates \(^3\) respectively. BPD was reduced in the VLBW group following parenteral supplementation with a lipid emulsion containing fish oil when compared to soy \(^10\) and type of lipid emulsion was independently associated with BPD in this group. Martin et al. (2011) reported that decreased DHA levels in infant plasma (n=88) were significantly associated with an increase in BPD \(^3\).

1.5.4. Necrotising enterocolitis

NEC is predominantly a disease of prematurity, it is the most common gastrointestinal illness in newborns and has a high mortality rate \(^1\). As the disease progresses, inflammation in the intestine worsens causing breakdown of the mucosal barrier and an escalating immune cascade
leading to sepsis, shock and even death \cite{31,111,114}. The risk for developing NEC is strongly influenced by commensal bacteria which exert metabolic, nutritional and immunological effects on the host \cite{115}. A preterm infant has very low bacterial diversity and the establishment of a more complex microbiome is easily disrupted by events related to premature birth, for example, early antibiotic administration and Caesarean sections \cite{23,116,117}. This process, termed dysbiosis, is implicated in the development of both sepsis and NEC \cite{115}.

The protective effect of omega-3 supplementation against development of NEC \cite{118} and mucosal inflammation \cite{119} has been reported in animal models. Further large-scale studies in humans are required to first determine if DHA can reduce the incidence of NEC, and second, if it is through a direct anti-inflammatory action such as those reported by Ohtsuka et al. (2011) \cite{119} and Lu et al. (2007) \cite{118} or if DHA influences microbial communities directly in the gut as described Andersen et al. (2011) \cite{91}.

1.5.4.1. Intervention studies

Seven interventional trials reported on the effect of omega-3 LCPUFA supplementation and incidence of NEC; six of these were RCTs. In six of the intervention trials, supplementation was via the enteral route. Results of all intervention trials were neutral; with the incidence of NEC unchanged between intervention and control groups.

1.5.4.2. Observational studies

Two observational studies found that NEC was not significantly associated with parenteral omega-3 LCPUFA supplementation \cite{105} or with plasma DHA levels \cite{34}. 
1.5.5. Sepsis
Sepsis is a systemic inflammation caused by infection. Globally, sepsis is responsible for approximately 15% of neonatal deaths \(^{120}\), with rates of infection dependent on the geographic region \(^{30,121,122}\). In preterm infants, sepsis is classified as either early-onset (< 72 hours of life) or late-onset (> 72 hours of life), with the latter being a common complication associated with prolonged admission to NICU \(^{121,122}\). The distinction between the two is of clinical importance, as early-onset sepsis usually results from exposure to bacteria in utero or during delivery and late-onset sepsis is acquired from bacteria in the environment (ie. nosocomial infections) \(^{121}\). Different microorganisms are responsible for the pathogenesis of sepsis; staphylococci infections are most common, but fungal, viral and parasitic infections occur as well \(^{20,121}\). An exaggerated immune response leading to an over-exuberant release of inflammatory mediators in the pathogenesis of sepsis makes immunomodulatory therapy with DHA an attractive option in clinical management \(^{31,111}\).

1.5.5.1. Intervention studies
The effect of omega-3 LCPUFA supplementation in preterm infants on the development of sepsis or infection was reported in nine RCTs; five with enteral and four with parenteral administration. In a sample of 37 infants with an enterostomy, a decrease in sepsis was reported in neonates receiving fish oil supplementation compared to standard nutrition \(^{93}\). Deshpande et al. (2014) assessed the incidence of sepsis as part of their secondary outcomes but did not find any significant differences between intervention and control groups (n=30) \(^{8}\). All other RCTs reported neutral results for the association between omega-3 LCPUFA and incidence of sepsis and/or infection in their sample.
1.5.5.2. Observational studies

Three observational trials investigated the effect of omega-3 LCPUFA supplementation on the incidence of sepsis. Two of the three trials reported neutral results \(^{105, 106}\) and one trial demonstrated that increased plasma DHA was associated with decreased risk of late-onset sepsis in preterm infants \(^{123}\). The authors state that an appropriate balance of omega-3 and omega-6 LCPUFA has an impact on disease risk and alterations in these LCPUFA could be responsible for immune dysregulation and subsequent increase in the risk of developing sepsis \(^{123}\).

1.5.6. Retinopathy of prematurity

ROP is the second leading cause of childhood blindness worldwide \(^{124, 125}\). Impaired vasculogenesis and improper retinal neuronal development are responsible for the pathogenesis of ROP \(^{126}\). As DHA is an integral part of cell membrane phospholipids, it may protect against processes that impair vascular formation in ROP and thus may help to ameliorate vascular abnormalities and disease development \(^{127-130}\). A recent meta-analysis has shown no clear trend in risk for ROP between groups supplemented with omega-3 LCPUFA and without \(^{29}\). However, individual studies, as discussed below, have reported that omega-3 LCPUFA supplementation was associated with a reduction in the incidence of ROP in preterm infants.

1.5.6.1. Interventional studies

Four RCTs reported on the incidence of ROP in omega-3 LCPUFA versus control groups; two administered omega-3 LCPUFA enterally and two parenterally. Two of the trials noted a neutral effect. One study in 130 infants found a significant reduction in the incidence of the primary outcome, ROP, in the group of infants receiving a parenteral lipid emulsion containing omega-3 compared to a soy/olive oil based emulsion \(^{108}\). In a recent study by Beken et al. (2014) in 80 infants, a significant reduction in the incidence of ROP was reported in infants receiving a parenteral lipid emulsion containing omega-3 compared to a soy-based emulsion during periods
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of total PN. ROP was a primary outcome of this trial which had a 72.9% power ($\alpha=0.05$) to detect a 20% reduction in ROP.

1.5.6.2. Observational studies

One observational study in 88 neonates indicated increased DHA levels were not associated with a reduction in the incidence of ROP. A second reported that although omega-3 LCPUFA supplementation had no effect on ROP, infants in the omega-3 supplementation group had a reduced requirement for laser therapy to treat ROP.

The data from these intervention and observational studies support the concept that there may be an early window of opportunity for effective immunomodulation with DHA depending on dose, duration of supplementation and timing and route of introduction. The critical period is most likely when the immune system is still developing and before clinical phenotypes have been established in the infant. Results from clinical studies in preterm infants and animal studies support the need for further large-scale RCTs to determine the clinical efficacy of DHA supplementation to prevent or attenuate inflammatory conditions in preterm infants.

1.6. OMEGA-3 LCPUFA AND IMMUNE REGULATION

1.6.1. Known targets for immunoregulation by omega-3 LCPUFA

The therapeutic potential of LCPUFA was recognised in the early 1980s, as epidemiologic data described a lower incidence of inflammatory disorders in populations regularly consuming large quantities of fish. Since then, evidence obtained from adult, animal and in vitro models indicate that omega-3 LCPUFA such as DHA influence a wide variety of mechanisms. These include alterations in cell signalling pathways, modifications to receptor-mediated pathways to inhibit or attenuate inflammation, decreases in oxidative stress and increases in anti-inflammatory prostaglandin synthesis. DHA may also influence the gut
microbiome to promote immune regulation. These targets have been extensively reviewed in adults and animals.

1.6.2. Evidence for an effect of omega-3 LCPUFA supplementation on immune responses
A growing body of evidence suggests dietary intake of LCPUFA early in life could influence immune development and other health outcomes. While there is extensive literature on the mechanisms of action of DHA and inflammation in adult disease, the targets that DHA acts on during initial immune development and resulting clinical conditions in preterm infants remains unclear.

A literature search was performed to review the reported inflammatory outcomes associated with omega-3 LCPUFA supplementation in preterm infants using the following search terms: ((preterm infant* [ALL] OR premature infant* [ALL] OR infant, premature [MH])) AND (Docosahexaenoic acid* [ALL] OR Fatty acids, omega-3 [MH] OR n-3 PUFA* [ALL] OR n-3 Fatty Acid* [ALL] OR n-3 Polyunsaturated Fatty Acid* [ALL] OR Omega-3 Fatty Acid* [ALL] OR alpha-Linolenic Acid* [ALL] OR Eicosapentaenoic Acid* [ALL] OR DHA [ALL] OR EPA [ALL] OR ALA [ALL]) AND ((inflamm* [ALL] OR biomarker* [ALL] OR immun* [ALL] OR cytokine* [ALL])). The key studies reviewing the effect of direct supplementation of omega-3 LCPUFA to preterm infants on physiological outcomes are summarised in Table 3. Only the trials reporting in vivo effects of omega-3 LCPUFA are included in Table 3.
Table 3. Characteristics of key studies reporting effect of omega-3 long-chain polyunsaturated fatty acids on functional outcomes in preterm infants **

<table>
<thead>
<tr>
<th>STUDY (AUTHOR, YEAR, LOCATION)</th>
<th>DESIGN</th>
<th>POPULATION</th>
<th>a. INTERVENTION b. CONTROL c. TIME/DURATION OF SUPPLEMENTATION d. PARENTERAL OR ENTERAL SUPPLEMENTATION</th>
<th>TRIAL OUTCOMES*</th>
<th>SIGNIFICANT RESULTS OF OMEGA-3 LCPUFA SUPPLEMENTATION ON INFLAMMATION</th>
<th>COMMENT</th>
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<tbody>
<tr>
<td>RANDOMISED CONTROLLED TRIALS</td>
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<tr>
<td>Skouroliakou M et al., Greece 2015 10</td>
<td>RCT</td>
<td>n=51 Infants &lt; 32 weeks GA</td>
<td>a. SMOFlipid (n=25) b. Intralipid (n=26) c. First days of life until postnatal day 30 or when EN reached 80% of total caloric intake (whichever was earlier) d. Parenteral</td>
<td>Cytokine and fatty acid profiles</td>
<td>SMOFlipid was associated with ↓ final IL-6 and IL-8 levels compared to Intralipid</td>
<td>1. Statistical analysis was PP based and not ITT (no cases lost to follow-up) 2. A priori sample size calculation conducted (80% power (α=0.05) to detect a clinically meaningful difference in IL-8)</td>
</tr>
<tr>
<td>Wang Y et al., China 2015 9</td>
<td>RCT</td>
<td>n=150 Infants &lt; 37 weeks GA</td>
<td>a. 3 groups: Intralipid (soy oil, n=53), Lipofundin (medium chain/long chain triglyceride, n=51) and ClinOleic (olive oil, n=52) c. Within 72 of birth for at least 14 days (mixed with EN) d. Parenteral</td>
<td>Lipid profiles, fatty acid composition and antioxidant capacity</td>
<td>No difference between groups in antioxidant capacity</td>
<td>1. Power calculation not conducted for outcomes 2. Antioxidant capacity defined as “SOD, MDA, GSH-Px, and T-AOC”</td>
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<tr>
<td>Study</td>
<td>Type</td>
<td>n</td>
<td>Inclusion Criteria</td>
<td>Interventions</td>
<td>Outcomes</td>
<td>Analysis and Calculations</td>
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</table>
| Deshpande G et al., Australia | RCT  | 30 | (2 additional neonates recruited in each group to account for incomplete intervention) Infants < 30 weeks GA | a. SMOFlipid (n=17)  
  b. ClinOleic (n=17)  
  c. From < 7 days postnatal for at least 7 days  
  d. Parenteral | Primary: LCPUFA levels, lipid peroxidation  
  Secondary: growth, sepsis, IVH, duration of hospital stay and PN, mechanical ventilation, mortality | 1. ITT analysis  
  2. A priori sample size calculations conducted for primary outcomes (90% power (α=0.05) to detect a difference of 1 SD in primary outcomes)  
  3. Lipid peroxidation was measured via F2-isoprostane levels |
| Tomsits et al., Hungary       | RCT  | 60 | Infants < 34 weeks GA                                                             | a. SMOFlipid (n=30)  
  b. Intralipid (n=30)  
  c. First feeding until at least postnatal day 7, up to postnatal day 14  
  d. Parenteral | Safety (serum triglycerides and laboratory safety parameters) and efficacy (weight, CRP levels, sepsis, respiratory support) parameters | 1. ITT and PP analysis (results were similar-final ITT data presented)  
  2. A priori sample size calculations not conducted for primary outcomes  
  3. Lipid peroxidation was measured via MDA |
| Skouroliakou M et al., Greece | RCT  | 38 | Infants < 32 weeks GA                                                             | a. SMOFlipid (n=14)  
  b. Intralipid (n=18)  
  c. Postnatal day 0 until at least 14 days  
  d. Parenteral | Primary: Oxidative stress  
  Secondary: growth, blood count, clinical condition, length of hospitalisation | 1. A priori sample size calculations conducted for primary outcome Oxidative stress was defined as “Serum vitamin A, E and TAP”  
  2. TAP = all antioxidants present in the sample |
### Table 3. continued

<table>
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<tr>
<th>INTERVENTION TRIALS</th>
<th>n=73 **Term and preterm infants after sepsis diagnosis</th>
<th>n=44 Infants &lt; 36 weeks GA</th>
<th>a. 100 mg DHA (n=29)</th>
<th>b. 100 mg olive oil (n=34)</th>
<th>c. 14 days</th>
<th>d. Enteral</th>
<th>Clinical course of sepsis, plasma IL-1β, IL-6, and TNFα, leukocyte fatty acid composition</th>
<th>DHA supplementation during sepsis results in attenuated IL-1β response and a less severe clinical course of the disease</th>
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<tbody>
<tr>
<td>Field C et al., Canada 2000 [13]</td>
<td></td>
<td></td>
<td>a. 3 groups: a) Formula + LCPUFA (n=15), b) Standard formula (n=12), c) breast milk (n=17)</td>
<td></td>
<td></td>
<td></td>
<td>Isolated peripheral blood lymphocytes and lipid composition</td>
<td>↑ IL-10 in the formula+LCPUFA group compared to standard formula (levels were not different from breast milk fed infants) ↑ % of CD3+CD4+ T cells and CD20+ cells in the BM group and formula + LCPUFA group vs. standard formula ↑ proportion of total CD4+CD45RO+ (antigen mature) T cells in the breast milk and formula + LCPUFA group vs. standard formula</td>
</tr>
</tbody>
</table>

Legend to Table 3. ‘*’= Information on outcome hierarchy (ie. primary/secondary/etc) is included if specified by the authors. ‘**’= Term and preterm infants were included together in this study and were not analysed separately. Abbreviations: cluster of differentiation (CD), glutathione peroxidase (GSH-PX), intention-to-treat (ITT), malonyldiadehyde (MDA), parenteral nutrition (PN), per protocol (PP), superoxide dismutase (SOD), total antioxidant potential (TAP), total anti-oxidising capacity (T-AOC).
1.6.3. Characterising the evidence

The seven studies outlined in Table 3 were intervention trials; five of them were RCTs, one was quasi-experimental and one was an intervention trial with a secondary randomisation step. As with Table 2, the studies described in Table 3 exhibit widespread heterogeneity with respect to study design and definition of outcome measures. Most of the studies were randomised and both researchers/clinical team and parents/participants were blinded to treatment group. One intervention study employed a semi-randomisation strategy, in which mothers initially chose whether their infant would be enrolled in the breast milk or formula arm of the trial, but the type of formula (standard vs. standard+LCPUFA) was randomised. GA of the infants in the trial included in Table 3 ranged from < 30 weeks to < 37 weeks to term infants. While the focus is on preterm infants, the one study with a mixed term/preterm population was included in Table 3 because these groups were not analysed separately.

Definitions of outcome measures varied widely between studies, for example lipid peroxidation/antioxidant capacity was defined as “SOD, MDA, GSH-Px, and T-AOC” "as a combined action of all antioxidants present in the sample” or “F2-isoprostanes” or “MDA”. Of the seven trials in Table 3, only three conducted an *a priori* power calculation to determine the sample size necessary to detect omega-3 LCPUFA-induced differences in outcomes. Two RCTs were powered to detect differences in antioxidant capacity and lipid peroxidation and one RCT was powered to detect differences in cytokine levels. Three of the seven studies included in Table 3 were analysed according to ITT and one study that had no loss to follow-up performed their analysis according to PP.

None of the above studies reported long-term *in vivo* effects of early postnatal omega-3 LCPUFA supplementation on immune status. Of the seven studies summarised above, five had supplementation and follow-up periods of only one to two weeks. Overall, the literature
on the effects of omega-3 fatty acids on inflammatory biomarkers contains mainly small sample sizes and report mixed effects. Therefore, there is a considerable gap in the literature relating to which aspects of inflammation are responsive to DHA in a neonate, and more specifically, in a preterm infant. In the following sections, evidence for the immunoregulatory efficacy of DHA, effect of downstream metabolites of omega-3 LCPUFA and oxidative stress in preterm infants is reviewed. Given the paucity of in vivo data from preterm infants, preterm models (animal and in vitro studies) are also presented. Important trends in the data from studies utilising LCPUFA supplementation in term infants or children is also included when relevant or when data in preterm infants are unavailable.

1.6.4. Cytokine synthesis and release

Omega-3 LCPUFA in cellular membranes can inhibit the production of omega-6 derived pro-inflammatory eicosanoids and can also form potent anti-inflammatory lipid mediators such as resolvins and protectins. Together, these actions directly and indirectly influence the production of pro-inflammatory cytokines via transcription factors and receptor-mediated signalling.

1.6.4.1. Studies in preterm infants

In preterm infants, LCPUFA supplementation has been reported to modulate cytokine synthesis and immune cell phenotypes. In a double-blind, randomised-controlled clinical trial in preterm infants, a medium-chain triglyceride/omega-3 LCPUFA emulsion was found to attenuate the inflammatory response when compared to a soy (omega-6) emulsion. Pro-inflammatory cytokines (IL-6 and IL-8) were assessed as part of the primary outcome and were significantly reduced at study-end in the omega-3 LCPUFA group, highlighting the potential for omega-3 LCPUFA to dampen the heightened immune response seen in preterm infants after birth. The study had 80% power (α=0.05) to detect clinically meaningful differences in IL-8 and < 50% power to detect differences in IL-6 and TNFα. In a study by Field et al. (2000),
the addition of DHA and AA to standard infant formula has been shown to increase the production of immunoregulatory IL-10 and the proportion of antigen-mature memory (CD45RO+CD4+) cells (important in adaptive immunity) in preterm infants to levels comparable to those seen in the breast milk-fed group, indicating an effect of DHA on immune maturation. In an analysis of 73 preterm and term infants with confirmed sepsis, enteral DHA supplementation for 14 days resulted in an attenuation in IL-1β levels and a less severe course of sepsis. Cytokine response and sepsis were assessed as part of the primary outcome and no difference was observed in the levels of other inflammatory cytokines (IL-6 and TNFα) with DHA supplementation. Although cytokine response was the primary outcome of this study, the calculated power to detect differences in cytokine levels was 6%-18% at postnatal day 7 and 11%-58% at postnatal day 14.

1.6.4.2. Studies in term infants

While immune response data from preterm infants are limited, data from term infants are supportive of an immunoregulatory role for DHA. Field et al. (2008) compared cellular immune responses in term neonates who received either standard formula, formula supplemented with DHA/AA or breast milk from two to six weeks of life. Although results were not significantly different, infants that received the formula supplemented with DHA/AA had similar cytokine and T cell responses to those who received breast milk. Cultured lymphocytes from both formula groups produced more pro-inflammatory cytokines than the human milk fed group. However, at six weeks, an increased pro-inflammatory TNFα response was seen in the formula group but not for the formula + DHA/AA group. In addition, the group without LCPUFA supplementation exhibited delayed maturation of the adaptive immune response (fewer CD4+CD28+ cells) and had significantly fewer peripheral blood effector memory T cells (CD3+CD44+ cells). This suggests that these fatty acids influence the degree of T cell maturation and development of effector memory T cells. A decrease in pro-
inflammatory cytokines in response to *in vitro* stimulation by allergens (ovalbumin, house dust mite, cat hair, phytohaemagglutinin) was also reported for immune cells from term infants, whose mothers were supplemented with fish oil (3.7g/day omega-3 LCPUFA) during pregnancy. The data supports a role for early dietary LCPUFA in immune development and regulation.

1.6.4.3. *Animal and in vitro studies*

Animal studies also reinforce the anti-inflammatory role of omega-3 LCPUFA seen in preterm and term infants. A reduction in systemic inflammation would be reflected by a reduction in pro-inflammatory mediators and/or an increase in regulatory cytokines. In a neonatal piglet model, increased dietary DHA and decreased omega-6 LCPUFA were associated with a reduction in systemic inflammation (as measured by CRP). Mice receiving an omega-3 LCPUFA supplemented diet produced offspring with significantly increased immunoregulatory IL-10 levels in the colon and spleen. Importantly, variations in the local tissue cytokine milieu also have the potential to influence other immune cells, such as APC that are critical for initiating immune responses. This is important because DHA-induced changes in the cytokine environment may have downstream effects on priming of APC and other immune cells for the promotion of an anti-inflammatory response during initial antigen encounter. Omega-3 LCPUFA have been associated with a reduction in TNFα in a mouse model of hyperoxia-induced retinopathy through an increase in omega-3 bioactive metabolites. Increased tissue levels decreased the avascular area of the retina through an increase in vessel regrowth following an injury. Connor et al. (2007) concluded that their results supported the idea that omega-3 LCPUFA supplementation may prevent against retinopathy through a reduction in pathological angiogenesis.
Gold et al. (2006) observed that omega-3 EPA and AA reduced antigen- and mitogen-stimulated IFN\(\gamma\) production in vitro in cord blood samples from a US birth cohort study (30-42 weeks GA)\(^\text{157}\). Additionally, lymphocyte proliferation was reduced by EPA and AA, indicating an attenuation of the immune response by both omega-3 and omega-6 LCPUFA\(^\text{157}\).

### 1.6.5. Lipid mediators and the resolution of inflammation

Downstream metabolites of LCPUFA, such as resolvins and protectins, are also important in mediating an anti-inflammatory response. These metabolites promote the resolution of inflammation and tissue repair\(^\text{67}\). Importantly for the preterm infant, this occurs without compromising host defence\(^\text{67}\). Much of the work that examines the interaction between LCPUFA, resolvins and inflammation has been conducted in vitro in samples obtained from adult and animal models, leaving a gap in knowledge for the efficacy in neonates.

#### 1.6.5.1. Animal and in vitro studies

Results from neonatal animal models serve as the best indicator for effectiveness of LCPUFA’s ability to trigger resolution of inflammation. Martin et al. (2014) recently examined the effect of supplementation with a downstream metabolite of DHA, resolvin D1 (RvD1), along with a downstream metabolite of AA, lipoxin A4 (LxA4), in a neonatal mouse model of BPD\(^\text{158}\). The results demonstrate that these metabolites reversed the histologic and biochemical changes associated with lung injury in the mouse model. Whilst RvD1 and LxA4 were shown to reduce alveolar damage when administered on their own, the most significant reduction in alveolar damage was seen when the two were combined\(^\text{158}\). Similarly, Velten et al. (2014) also reported that prenatal DHA supplementation reduced neonatal lung inflammation in a mouse model\(^\text{159}\). These recent findings suggest biological plausibility for a mechanistic role of terminal metabolites of DHA in combatting inflammatory lung damage.
1.6.6. Mediation of oxidative stress

Supplemental oxygen therapy is vital to the survival of infants with BPD but it places a large oxidative stress burden on the infant. The antioxidant capacity of a preterm infant is reduced and lipids in breast milk, infant formula and PN are thus subject to an increased risk for peroxidation. DHA and its downstream metabolites have the potential to reduce oxidative stress.

1.6.6.1. Studies in preterm infants

A double-blind randomised controlled trial in preterm infants (n=38) reports that a parenteral lipid emulsion containing omega-3 LCPUFA reduced oxidative stress compared to a standard soy product. Oxidative stress levels were determined by Vitamin A, E and total antioxidant potential and assessed as a primary outcome with 80% power (α=0.05) to detect differences in antioxidant markers. Positive results with respect to omega-3 LCPUFA and oxidative stress were also obtained when very preterm infants (n=30) were randomised to either a lipid emulsion containing soy and olive oils (ClinOleic) or one containing soy, medium-chain triglycerides, olive oil and fish oil (SMOFlipid). Vitamin E levels and plasma F2-isoprostanes (lipid peroxidation marker) were used to quantify levels of oxidative stress in the infants and the study had a 90% power (α=0.05) to detect a difference of 1 SD in primary outcomes. Compared to ClinOleic, SMOFlipid reduced oxidative stress in this high risk infant population and was determined to be safe and well-tolerated. Neutral effects on oxidative stress have also been reported on the effect of LCPUFA in 150 preterm infants on oxidative stress compared to olive- and soy-based lipids. Neutral effects of omega-3 LCPUFA on lipid peroxidation, as an indicator of anti-oxidant capacity, were reported by Tomsits et al. (2010).
1.6.6.2. Studies in children

Results from research conducted in children and adolescents with acute lymphoblastic lymphoma are relevant as they add strength to the use of DHA as an adjunct therapy to combat oxidative stress in preterm infants. In a randomised, double-blind, placebo-controlled trial, participants (n=70) received either 1000 mg/day fish or placebo oil in combination with their methotrexate regimen. Methotrexate is a necessary treatment in patients with acute lymphoblastic lymphoma but is known to induce hepatotoxicity, the pathogenicity of which is mainly due to oxidative stress. Omega-3 LCPUFA as an adjunct therapy in these patients ameliorated the hepatotoxicity compared to placebo and no adverse reactions to the fish oil were observed. These results are promising but more in-depth studies are required in larger cohorts to establish the robustness of these findings and the mechanisms underlying the action of DHA against oxidative stress.

1.6.6.3. Animal studies

In a neonatal guinea pig model, indicators of oxidative stress (peroxide and aldehyde concentrations) were reduced when guinea pigs were fed an omega-3 LCPUFA emulsion compared to an omega-6 LCPUFA emulsion. Further research in humans to determine appropriate dose and timing regimens is required to reveal whether DHA would serve as an effective adjunct therapy to combat oxidative stress in infants in the NICU.

1.6.7. The role of surfactant proteins in the innate immune system

Surfactant demonstrates a wide variety of anti-inflammatory properties and because it is comprised primarily of lipids, it is another promising target for immunoregulation via DHA. Low concentrations of LCPUFA in pulmonary surfactant system are thought to contribute to the increased vulnerability of the preterm lung to oxidative lung injury. In addition to reducing the surface tension in the lung, surfactant demonstrates a variety of anti-inflammatory
and immunomodulatory properties. The surfactant protein (SP)-A and SP-D network play a role in neonatal inflammatory diseases such as BPD. Although surfactant is composed predominantly of phospholipid, it has a small protein component (10%) and four SP have been defined: SP-A, SP-B, SP-C, SP-D. The protein component lends surfactant an immunological role; SP-A and SP-D are known constituents of the innate immune system and mediate host defence functions.

1.6.7.1. Animal studies
Decreased levels of SP-D have been associated with a need for supplemental oxygen and implicated as a possible risk factor for BPD development. Data on the relationship between omega-3 LCPUFA and the immunomodulatory properties of surfactant are lacking in preterm infants, but data from an animal model during pregnancy is promising. Following maternal DHA supplementation, preterm fetuses had increased amounts of surfactant in amniotic fluid in the lungs compared to control. Increased levels of DHA have also been reported to increase the production of dipalmitoylphosphatidylcholine (DPPC) in baboon neonates. DPPC is the major surfactant lipid in the fetal and neonatal lung. While neither of these studies measured levels of surfactant proteins, results suggest a link between DHA and surfactant production and therefore it is biologically plausible to expect that increase lipid composition of surfactant may influence the incorporation of surfactant proteins.

1.6.8. Interaction between gut bacteria and host immune system
Dietary LCPUFA have the potential to influence the cross-talk between the host immune system and bacterial species in the gut. Incorporation of omega-3 LCPUFA into gut mucosal tissue can influence the environment in the gut via an attenuation of the inflammatory response. Inflammation in the gut can cause the epithelial barrier integrity to become compromised and thereby weaken host defence mechanisms. This results in an increased potential for adherence
of pathogenic bacteria to mucosal surfaces and/or an increase in gut permeability which allows pathogenic bacteria to cross the epithelial barrier into the peripheral border and access internal tissues $^{176-178}$.

*Staphylococcus* is one of the first species to colonise the respiratory tract, and in parallel, the gut $^{22}$. *Staphylococcus* has been associated with the development of neonatal inflammatory disorders $^{19-21}$ and methicillin-resistant staphylococci are known to cause significant morbidity and mortality in preterm infant in the NICU $^{24, 25}$. There is a well-established link between infection and inflammation in the preterm infant and bacterial colonisation of the gut plays a role in this interaction. It has been shown that dietary lipids can affect specific populations of bacteria in the gastrointestinal tract and their metabolic end products $^{176, 179}$ but the effect of omega-3 LCPUFA in the diet on *Staphylococcus* is not known.

Intervention studies in preterm infants on the effect of omega-3 supplementation on diversity of microbial species as a whole in the gastrointestinal tract are also lacking $^{23, 180}$. However, it is known that breast milk promotes bacterial colonisation of the gut, which in turn, is a major promoter of the development of immunoregulatory pathways $^{181}$. Enteral feeding regimens for preterm infants consist of breast milk, preterm formula, or more commonly in the first few weeks, a combination of the two $^{74}$. Both are sources of omega-3 LCPUFA which has been shown to influence bacterial species in the gut, albeit with controversial efficacy $^{91, 144, 145, 182}$. Nutrition in early life has the potential to induce lasting changes in the cross-talk between the gut bacteria and immune system $^{180, 183, 184}$.

Appropriate colonisation of the gut is also required for the maintenance of intestinal barrier function and to program both the innate and adaptive arms of the immune system $^{185}$. Colonisation of mucosal surfaces influence mucosal effector tissues in the lamina propria and
the epithelium of the intestinal and respiratory tracts. Disruption in the stepwise process of bacterial colonisation can lead to inappropriate stimulation, a lack of homeostasis in the host-microbial relationship and adverse clinical outcomes. The postnatal period represents a critical window to influence specific microbial species because the balance is especially sensitive and responsive to external influences.

1.6.8.1. Studies in infants

It has been proposed that fat intake and type of fat (saturated vs. unsaturated) influences the distribution of beneficial and protective bacteria in preterm infants but very little data exists in preterm infants and is mainly observational. In infants, omega-3 supplementation has been reported to affect microbial diversity when administered along with cow’s milk and to affect the abundance of large bacterial groups in infants that have stopped breastfeeding before nine months of age.

1.6.8.2. Animal studies

Data obtained from animal studies have reported neutral effects of omega-3 LCPUFA as well as significant changes in colonisation patterns in response to supplementation. Animal and in vitro studies also highlight a potential role of omega-3 LCPUFA in the maintenance of gut integrity and epithelial barrier function.

1.7. STUDY RATIONALE

The abundant evidence from adults and animal models provides a convincing rationale to study how DHA supplementation might improve clinical outcomes in preterm infants by targeting the critical window of time when immunomodulation has the greatest potential for benefit in at-risk infants. Further large-scale interventions are required in order to determine firstly, the clinical efficacy of DHA during the neonatal period and secondly, the targets which the
fatty acid and its downstream mediators may interact with to exert a clinical benefit. This knowledge would allow clinicians to determine whether DHA would be best used as a preventative or treatment option in preterm infants.

A major criticism of the inconsistent findings in previous studies with respect to the clinical efficacy of omega-3 LCPUFA is the variation of study design. A variation in route of administration (maternal versus direct infant supplementation, parenteral versus enteral) can result in an inconsistent delivery to the infant because of biological variation, delay in the infant reaching full enteral feeds and issues with maternal compliance. Other variations in study design such as timing of introduction (pre- vs. postnatal), dose and duration also make it difficult to summarise the effect of omega-3 LCPUFA supplementation on clinical outcomes. Therefore, the ability to delve into the mechanisms that underpin inflammation and the sites of action of omega-3 LCPUFA is complicated by such variation in study design.

The N3RO RCT directly supplemented preterm infants < 29 weeks GA with 60 mg/kg/day of enteral DHA to examine the effect of supplemental DHA on incidence of BPD. This is an advantage because direct DHA supplementation to the infant ensures that the target dose is received and does not require the mother to adhere to a supplementation regimen. The N3RO RCT was conducted at 13 perinatal centres across Australia, New Zealand and Singapore. Clinician, researcher and participant/family were blinded to treatment group. The design and implementation of the trial is of high quality and ensures that results will reflect the impact of supplementation. The RCT offers a unique opportunity to nest a study with the purpose of examining a subset of the infants in order to explore some of the targets with which DHA may be interacting in a preterm infant to exert an anti-inflammatory effect. The targets of interest described in the aims below were chosen based on their implication in neonatal inflammatory disorders. The RCT provides clinical data to which the functional outcome data from the nested

...
study can be compared. Omega-3 LCPUFA have been incorporated into many nutritional regimens in the NICU over the past decades as part of infant formula, breast milk fortifier and next-generation lipid emulsions. The results of this study are expected to contribute to the knowledge regarding the effect of dietary omega-3 LCPUFA on immune markers in a preterm infant < 29 weeks GA.

1.8. AIMS OF THE THESIS

Taking a multi-system approach through in vitro, in vivo and ex vivo investigation allows for an in-depth examination of research questions related to inflammation and nutrition in the preterm infant. This thesis aims to provide evidence on mechanisms of action underpinning the potential protective effect of DHA against inflammatory outcomes in the neonatal period (ie. BPD, sepsis, NEC). In the context of a cell culture experiment, this thesis first aims to determine:

The effect of the DHA and LA enteral lipid emulsions used in the N3RO trial on secretion of cytokines associated with BPD from a lung epithelial cell model in vitro.

In the context of a nested study in an RCT, this thesis aims to determine:

1) If supplemental DHA has an immuno-regulatory effect by assessing the levels of pro-inflammatory and regulatory cytokines in plasma and supernatants from whole blood culture following stimulation with E. coli lipopolysaccharide (LPS).

2) If supplemental DHA has an effect on the levels of SP-D in plasma as an indicator of pathological lung changes.
3) If supplemental DHA has an influence on *Staphylococcus* bacteria and bacteria carrying the gene for methicillin resistance (*mecA*) in the gut from a subset of infants by assessing stool samples.
CHAPTER 2

THE EFFECT OF OMEGA-3 AND OMEGA-6 FATTY ACIDS IN LIPID EMULSIONS ON ALVEOLAR CYTOKINE RELEASE

PREFACE

The manuscript in chapter 2 describes the effect of omega-3 DHA- and omega-6 LA-rich enteral emulsions on pro-inflammatory and regulatory cytokine release from a human type II AEC model \textit{in vitro}. Commercially available parenteral emulsions containing omega-3 DHA and omega-6 LA that are currently used in neonatal intensive care units worldwide were also assessed.

\textbf{Manuscript: Omega-3 long-chain polyunsaturated fatty acids in lipid emulsions and the impact on cytokine release from human alveolar cells}
Statement of Authorship

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**Principal Author**

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TITLE: Omega-3 long-chain polyunsaturated fatty acids in lipid emulsions and the impact on cytokine release from human alveolar cells

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ABSTRACT

BACKGROUND: Lipid emulsions are the only source of long-chain polyunsaturated fatty acids (LCPUFA) while a preterm infant is receiving parenteral nutrition. Inflammatory cytokines are key elements in the pathogenesis of neonatal inflammatory disorders such as bronchopulmonary dysplasia and omega-3 LCPUFA may help regulate inflammatory responses. We assessed the efficacy of omega-3 and omega-6 fatty acids in enteral and parenteral lipid emulsions to modulate the release of pro-inflammatory cytokines from a type II human alveolar cell (AEC) model.

METHODS: We examined cytokine release from human type II AEC (A549 cells) stimulated with TNFα in the presence of the omega-3 LCPUFA docosahexaenoic acid (DHA) and omega-6 PUFA linoleic acid (LA) in commercially available parenteral lipid emulsions and the enteral emulsions used in the N3RO randomised controlled trial (RCT) (ACTRN 12612000503820). Cytokines were assessed by flow cytometry and ELISA, surfactant protein D by ELISA and fatty acids by gas chromatography.

RESULTS: Relative to LA-predominant emulsions, incubation of cells with DHA-predominant parenteral and enteral lipid emulsions resulted in a significant decrease in pro-inflammatory IL-1β (P<0.05), IL-8 (P<0.001) and IFNγ (P<0.01) secretion after TNFα stimulation. DHA was completely metabolised from the post-incubation parenteral and enteral media preparations while a large proportion of LA remained.

CONCLUSION: This study highlights the immunomodulatory potential of omega-3 LCPUFA in both enteral and parenteral lipid emulsions. It is also the first in vitro assessment of the immunoregulatory efficacy of enteral emulsions used in the N3RO RCT.
INTRODUCTION

Omega-3 long chain polyunsaturated fatty acids (LCPUFA) such as docosahexaenoic acid (DHA) are known to reduce inflammation, whereas the general consensus is that omega-6 polyunsaturated fatty acids (PUFA) such as linoleic acid (LA) can elicit a pro-inflammatory response \(^{66, 87}\). The preterm infant is highly susceptible to unregulated local and systemic inflammatory responses. Inflammation has been shown to be most evident in the first 72 hours of life and sustained inflammatory responses are still common for the first few weeks of life \(^{58}\). This coincides with the time period that parenteral (PN) and enteral nutrition (EN) regimens are usually established for a preterm infant \(^{194}\). While the overarching goal of nutrition regimens for preterm infants is to reach full enteral feeding as soon as possible, PN is essential when nutritional requirements cannot be met via the enteral route. The lipid emulsion component is an important source of calories and the only source of LCPUFA while the infant is on PN \(^{195}\).

The conventional lipid emulsions used worldwide in standard PN regimens for preterm infants have been soy-based and therefore comprised mainly of LA \(^{196}\). Excess omega-6 PUFA and LCPUFA have been associated with release of pro-inflammatory mediators, which has led to the recent development of next-generation lipid emulsions derived from other lipid sources (coconut, olive, and fish) \(^{197, 198}\). Before the introduction of the next-generation lipid emulsions, omega-3 LCPUFA did not form part of standard PN regimens in the neonatal intensive care unit (NICU) \(^{196}\). Once preterm infants are able to tolerate enteral feeds, they receive low levels of omega-3 LCPUFA in breast milk or infant formula \(^{83, 199}\). The balance of omega-3 and omega-6 fatty acids in an infant’s EN and/or PN regimen has the potential to exacerbate or attenuate inflammation \(^{200}\).

Preterm infants have an underdeveloped immune system and therefore the homeostatic mechanisms that normally regulate an inflammatory response is impaired \(^{3, 48}\). Chronic inflammation plays a central role in the aetiology of many life-threatening neonatal disorders.
including bronchopulmonary dysplasia (BPD) \(^\text{32}\), necrotising enterocolitis (NEC) \(^\text{201}\) and sepsis \(^\text{58}\). BPD is a lung disorder of prematurity affecting approximately 45% of infants < 29 weeks gestation and is a constant risk to the survival of preterm infants while in the NICU \(^\text{29, 202}\). An intense immune response that triggers an exaggerated release of pro-inflammatory cytokines is a hallmark of BPD \(^\text{203, 204}\). There are few therapies known to effectively prevent or treat BPD and although postnatal corticosteroid administration is common, their use is not without side effects such as immunosuppression \(^\text{52, 204, 205}\).

A large multicentre randomised controlled trial (\(n\)-3 fatty acids for Improvement in Respiratory Outcomes (the N3RO trial) ACTRN 12612000503820) \(^\text{6}\) assessed the efficacy of an omega-3 (DHA) emulsion to reduce the incidence of BPD in preterm infants < 29 weeks gestational age. Using the enteral emulsions assessed in the N3RO trial and a series of commercially available parenteral lipid emulsions as a comparison, we aimed to assess the ability of the omega-3 DHA and omega-6 LA in lipid emulsions to modulate alveolar cytokine release. The lung is constantly exposed to commensal and pathogenic foreign particles and organisms, which can elicit inflammatory responses. Type II AECs play an important role in lung epithelial injury and repair, secrete pulmonary surfactant and surfactant proteins (SP), and can give rise to type I AEC that conduct gas exchange at the blood-air barrier \(^\text{16}\). The early pro-inflammatory state of the lung and subsequent development of BPD make type II AEC the targets in many lung diseases and acute respiratory distress syndrome \(^\text{16-18}\). A549 cells have been used extensively as an experimental model of alveolar type II AEC and they exhibit an appropriate biological response to pro-inflammatory stimulation by upregulating cytokines and chemokines \(^\text{17}\). In this current study we used A549 cells to examine the hypothesis that DHA-predominant parenteral and enteral emulsions could reduce the pro-inflammatory response in alveolar cells exposed to an inflammatory stimulus (TNF\(\alpha\)) compared to their LA-based counterparts. We also aimed to determine the effect of DHA on one of the SPs, SP-D, secreted by type II AEC. SP-D has been proposed to exert an immunoregulatory role in the lung \(^\text{170}\).
**METHODS**

*A549 cells and cell culture*

Human adenocarcinoma AEC (A549) cells (CCL-185- American Type Cell Culture, USA) were used throughout these experiments. The cells were maintained in a continuous culture prior to each experiment at 37°C and 5% CO₂. The standard culture medium contained Ham's F-12K (Kaighn's) Medium²⁰⁶ (Thermo Fisher Scientific, Sydney, Australia), supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich, Sydney, Australia), penicillin (50 U/mL) and streptomycin (50 µg/mL) (Thermo Fisher Scientific, Sydney, Australia) unless otherwise indicated. All media were purchased endotoxin free.

**Cellular proliferation assay**

A range of molarities (0-400 µM) of DHA and LA in the enteral emulsions (Table 1) were assessed to determine the threshold concentration at which cellular proliferation of A549 cells was inhibited using the WST-1 cellular proliferation reagent (WST1- Takara Bio Inc, Japan). Consistent with the literature²⁰⁷, results of the cellular proliferation assay determined that 50 µM of both DHA and LA from the enteral lipid emulsions did not inhibit growth. This molarity for DHA and LA was used throughout these experiments.
Table 1. Oil sources of enteral and parenteral lipid emulsions used in A549 cell culture

<table>
<thead>
<tr>
<th>Emulsion name (oil source)</th>
<th>DHA- or LA-predominant</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parenteral lipid emulsions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omegaven (fish oil)</td>
<td>DHA</td>
<td>Fresenius Kabi, Australia</td>
</tr>
<tr>
<td>Intralipid (soy oil)</td>
<td>LA</td>
<td>Fresenius Kabi, Australia</td>
</tr>
<tr>
<td>ClinOleic (olive oil/soy oil)</td>
<td>LA</td>
<td>Baxter Healthcare Pty Ltd, Australia</td>
</tr>
<tr>
<td>SMOFlipid (soy/medium chain triglycerides/olive/fish oil)</td>
<td>LA</td>
<td>Fresenius Kabi, Australia</td>
</tr>
<tr>
<td><strong>Enteral lipid emulsions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-DHA fish oil emulsion (fish oil)</td>
<td>DHA</td>
<td>Clover Corporation Ltd., Australia</td>
</tr>
<tr>
<td>Soy oil emulsion (soy oil)</td>
<td>LA</td>
<td>Clover Corporation Ltd., Australia</td>
</tr>
</tbody>
</table>

Legend to Table 1. Abbreviations: docosahexaenoic acid (DHA), linoleic acid (LA).

Cell culture preparation

Attempts were made to normalise treatment oil preparations to 50 µM DHA (or 50 µM LA when the emulsion did not contain DHA). Product inserts detailing the world standard composition of each emulsion were used to calculate the approximate molarity of DHA and LA, while certificates of analyses for the enteral DHA and soy emulsions were used to calculate exact molarity. Solutions of 50 µM DHA or 50 µM LA of pure emulsion in Ham’s F-12K media were prepared prior to each experiment. Ham’s F-12K media served as the no-LCPUFA control.

A549 cells were cultured in 24-well plates at a seeding density of 2.5 x 10^5 cells/well with the standard media preparation. For all experiments (n=3-4), cells were between passage 16 and 20 and each emulsion preparation was assayed in triplicate. Plates were incubated at 37°C and 5% CO₂ for a 24 hour incubation period. After this incubation period, cells were cultured with/without 2 ng/mL TNFα (R&D Systems, Minneapolis, USA) and media was replaced with serum (FBS)-free enteral and parenteral media preparations (Table 1). After a 24 hour incubation period with/without TNFα and respective media preparation, aliquots of supernatant
were immediately frozen at -80°C until cytokine analysis. Culture plates containing the remaining A549 cells were then frozen at -80°C for a single freeze-thaw cycle to lyse cells.

For lipid analysis, cells were incubated in Ham’s F-12K media for 24 hours, followed by a 24 hour incubation with each serum-free emulsion preparation and Ham’s F-12K media alone. After 24 hours, the post-incubation media was removed and frozen at -80°C. Each well was rinsed with 2 mL warm PBS to remove lipids and media and 1 mL warm 1x trypsin/EDTA (Sigma-Aldrich, Sydney, Australia) was added to each well. Plates were incubated for 5-10 mins or until cells detached and 4 mL of media was added to each well. The contents of each set of triplicate wells were centrifuged at 400 g to pellet cells. After washing, the pellet was resuspended in 50 µL PBS and frozen at -80°C. Samples of each pre-incubation media preparation were frozen at -80°C for later fatty acid analysis.

Analysis of inflammatory mediators: cytometric bead array, flow cytometry

Cell culture supernatants were assayed for inflammatory cytokines using an enhanced sensitivity human cytokine bead array (BD Biosciences, San Diego, USA) and according to the manufacturer’s protocol supplied with the kit. Cell culture supernatants incubated in the presence of lipid emulsions with TNFα assayed between 1:10 and 1:100. Cell culture supernatants incubated without TNFα were assayed undilated. Cytokines analysed included IL-1β, IL-6, IL-8, IL-10 and IFN γ (BD CBA Human Enhanced Sensitivity Flex Sets- BD Biosciences, San Diego, USA). Detection limits for each cytokine were 0.64 pg/mL (IL-1β), 6.21 pg/mL (IL-6), 0.89 pg/mL (IL-8), 0.93 pg/mL (IL-10) and 0.81 pg/mL (IFNγ). Cytokines in supernatant were analysed on a BD Biosciences FACS Canto flow cytometer (BD Biosciences, San Diego, USA). Mean fluorescence intensity was generated by BD FacsDiva™ software version 6.1.3 (BD Biosciences, San Diego, USA, 2009). Standard curves were generated to model the protein concentration as a function of mean fluorescence intensity.

Analysis of surfactant protein D (SP-D): ELISA
Cell culture supernatants were analysed for SP-D using a Surfactant Protein D Human Quantikine ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer’s protocol supplied with the kit. The SP-D ELISA is a solid phase sandwich ELISA with an assay range of 0.6 - 40 ng/mL. Cell lysate samples were assayed undiluted and standards were prepared as outlined in the protocol. Absorbance was measured by a multi-well spectrophotometer (Victor™ x4 Multilabel reader, Perkin Elmer, Singapore) at 450 nm with a reference wavelength of 595 nm. Standard curves were generated to model the protein concentration as a function of mean absorbance.

*Fatty acid extraction, methylation and analysis by gas chromatography*

Fatty acids were extracted, methylated and analysed by gas chromatography and flame ionisation detection according to a previously published procedure. Fatty acids were reported as percent of total fatty acids.

*Statistical analysis*

Data are presented as mean (pg/mL) ± SEM. Differences in cytokine secretion after exposure to each lipid emulsion were examined using a linear mixed effects model. A mixed model was used to adjust for clustering on media preparation (up to four replicates were taken for each media preparation). A mixed model was chosen because effects within the replicates would be more similar than effects between media preparations, thus correlation would occur and result in erroneous standard errors and P values without adjustment. Logarithmic transformation was applied to IL-8 and IFNγ values in order to uphold assumptions of a linear model. Post-hoc tests were conducted with the soy oil emulsion as the reference group for enteral emulsion comparisons and Intralipid for parenteral emulsion comparisons. All analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, USA).
RESULTS

Cytokine secretion from unstimulated cells at baseline

Prior to exposure of A549 cells to the lipid emulsion preparations, cells at baseline constitutively produced a mean of 43.40 pg/mL Interleukin (IL) 8 and 0.35 pg/mL IL-1β, while IL-6, IL-10 and IFNγ were below the detection limit of the assay.

Cytokine secretion from unstimulated cells in the presence of lipid emulsions

In the supernatants of unstimulated A549 cells exposed to each lipid emulsion preparation, mean IL-6 and IL-10 levels were below the detection limit of the assay. There was no significant association between the type of lipid emulsion used in cell culture and either IFNγ or IL-1β release from unstimulated cells (data not shown). Relative to Intralipid, treatment with Omegaven resulted in 80% less IL-8 from A549 cells (P <0.01, Figure 1), while SMOFlipid, ClinOleic, and the no-LCPUFA control were without effect (Figure 1). Incubation of A549 cells with the high-DHA emulsion resulted in 72% less IL-8 compared to IL-8 secretion from cells in the presence of the LA emulsion (P<0.05, Figure 1).
Figure 1. Effect of incubation of unstimulated A549 cells with parenteral and enteral lipid emulsions on secretion of IL-8.
Legend to Figure 1. Results following incubation with parenteral (n=3) and enteral (n=4) emulsions are expressed as pg/mL ± SEM. Points with different superscripts indicate significant differences (* = P <0.05, ** = P <0.01). Parenteral and enteral categories are separated by the dotted line and compared to the soy-based emulsion in each category, Intralipid and soy oil emulsion, respectively. Abbreviations: docosahexaenoic acid (DHA).

Cytokine secretion in the presence of lipid emulsions in response to TNFα stimulation

Incubation of A549 cells with TNFα increased IL-6, IL-8 and IFNγ when compared to baseline levels of unstimulated cells in Ham’s F-12K media alone. IL-10 in supernatants from TNFα-stimulated A549 cells was below the detection limit of the assay (data not shown). The no-LCPUFA control and the mixed-oil emulsion, SMOFlipid, did not have a significant effect on cytokine release from A549 cells after TNFα stimulation compared to cells in the presence of Intralipid (Figure 2).

Compared to incubation with Intralipid, treatment of A549 cells with Omegaven resulted in 98% less IL-8 secretion (110.42 pg/mL vs. 3478.23 pg/mL, P <0.0001, Figure 2C) and 60% less IFNγ secretion (4.45 pg/mL vs. 10.29 pg/mL, P <0.01, Figure 2D) after TNFα stimulation.
IL-6 release from A549 cells after TNFα stimulation was 49% higher in the presence of Omegaven compared to IL-6 release from cells incubated with Intralipid (10.88 pg/mL vs. 5.57 pg/mL, P <0.05, Figure 2B). IFNγ secretion from cells stimulated with TNFα was 46% higher in the presence of ClinOleic compared to Intralipid (20.05 pg/mL vs. 10.29 pg/mL, P <0.05, Figure 2D).

Relative to the soy oil emulsion, treatment of A549 cells with the high-DHA fish oil emulsion resulted in 92% less IL-8 (514.19 pg/mL vs. 5626.32 pg/mL, P <0.0001, Figure 2C), 52% less IFNγ (9.44 pg/mL vs. 21.65 pg/mL, P <0.01, Figure 2D) and 37% less IL-1β secretion (0.50 pg/mL vs. 0.79 pg/mL, P <0.05, Figure 2A) after TNFα stimulation. The high-DHA fish oil emulsion did not significantly affect IL-6 release from stimulated cells when compared to the soy oil emulsion (Figure 2B).
Figure 2. Effect of incubation with parenteral and enteral lipid emulsions on secretion of IL-1β, IL-6, IL-8 and IFNγ from A549 cells following TNFα stimulation

Legend to Figure 2. (A) IL-1β (B) IL-6 (C) IL-8 and (D) IFNγ. Results following incubation with parenteral (n=3) and enteral (n=4) emulsions are expressed as pg/mL ± SEM. Points with different superscripts indicate significant differences (* = P <0.05, ** = P <0.01, † = P <0.0001). Parenteral and enteral categories are separated by the dotted line and compared to the soy-based emulsion in each category, Intralipid and soy oil emulsion, respectively. Abbreviations: docosahexaenoic acid (DHA).
Fatty acid analysis of pre- and post-incubation media

In the pre-incubation media, the proportion of DHA was 18.31% of total fatty acids in the Omegaven preparation and 54.53% in the high-DHA fish oil emulsion media preparation (Table 2). DHA was not detected in either the Omegaven or high-DHA fish oil emulsion post-incubation media samples. A decrease in the relative percentage was observed for DHA in SMOFlipid; 2.57% DHA in the pre-incubation media vs. 2.03% post-incubation media. Proportional changes in LA in the pre- and post-incubation media were observed for ClinOleic (21.33% vs. 18.74%), SMOFlipid (24.44% vs. 21.07%) and Intralipid (31.58% vs. 24.18%). The percentage of LA in the soy oil emulsion was higher in the post-incubation media than in the pre-incubation media (36.03% vs. 37.17%). The no-LCPUFA control did not contain DHA, LA, EPA or AA in detectable amounts.

Table 2. Fatty acid analysis of pre- and post-incubation media preparations for parenteral and enteral lipid emulsions

<table>
<thead>
<tr>
<th>Media preparation</th>
<th>DHA (%)</th>
<th>LA (%)</th>
<th>EPA (%)</th>
<th>AA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td><strong>Parenteral lipid emulsions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omegaven</td>
<td>18.31</td>
<td>ND</td>
<td>2.84</td>
<td>3.04</td>
</tr>
<tr>
<td>Intralipid</td>
<td>ND</td>
<td>ND</td>
<td>31.58</td>
<td>24.18</td>
</tr>
<tr>
<td>SMOFlipid</td>
<td>2.57</td>
<td>2.03</td>
<td>24.44</td>
<td>21.07</td>
</tr>
<tr>
<td>ClinOleic</td>
<td>ND</td>
<td>ND</td>
<td>21.33</td>
<td>18.74</td>
</tr>
<tr>
<td>no-LCPUFA control</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Enteral lipid emulsions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-DHA fish oil emulsion</td>
<td>54.53</td>
<td>ND</td>
<td>ND</td>
<td>3.50</td>
</tr>
<tr>
<td>Soy oil emulsion</td>
<td>ND</td>
<td>ND</td>
<td>36.03</td>
<td>37.17</td>
</tr>
</tbody>
</table>

Legend to Table 2. Results are expressed as percent of total fatty acids. Abbreviations: arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA), not detected (ND).
Fatty acid analysis of the cell membrane

The fatty acids in the cell membrane did not exhibit changes in LA or DHA proportional to the levels contained in the emulsion, when compared with control (Table 3); all values are within 2% of the no-LCPUFA control cell membrane fatty acid values. The same trend exists for the EPA and AA percentages after incubation with each lipid emulsion.

Table 3. Omega-3 and omega-6 fatty acids in unstimulated cell membranes of A549 cells incubated with parenteral and enteral emulsions

<table>
<thead>
<tr>
<th>Media preparation</th>
<th>DHA (%)</th>
<th>LA (%)</th>
<th>EPA (%)</th>
<th>AA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parenteral lipid emulsions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Omegaven</td>
<td>1.97</td>
<td>1.02</td>
<td>0.86</td>
<td>2.78</td>
</tr>
<tr>
<td>Intralipid</td>
<td>2.27</td>
<td>1.59</td>
<td>0.74</td>
<td>4.07</td>
</tr>
<tr>
<td>SMOFlipid</td>
<td>2.58</td>
<td>1.92</td>
<td>0.90</td>
<td>4.18</td>
</tr>
<tr>
<td>ClinOleic</td>
<td>2.37</td>
<td>2.68</td>
<td>0.61</td>
<td>4.34</td>
</tr>
<tr>
<td>no-LCPUFA control</td>
<td>2.31</td>
<td>0.88</td>
<td>0.65</td>
<td>3.39</td>
</tr>
<tr>
<td><strong>Enteral lipid emulsions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-DHA fish oil emulsion</td>
<td>1.95</td>
<td>0.86</td>
<td>0.53</td>
<td>2.99</td>
</tr>
<tr>
<td>Soy oil emulsion</td>
<td>2.33</td>
<td>0.96</td>
<td>0.58</td>
<td>3.44</td>
</tr>
</tbody>
</table>

Legend to Table 3. Results are expressed as percent of total fatty acids. Abbreviations: arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA), not detected (ND).

Surfactant protein D

Surfactant protein D (SP-D) was not detected in cell culture lysates.

DISCUSSION

This study aimed to assess cytokine release from AECs in the presence of omega-3 DHA and omega-6 LA in the enteral emulsions and in four commercially available parenteral emulsions. Overall, incubating type II AECs with DHA-predominant parenteral and enteral emulsions
resulted in a significant reduction in the release of pro-inflammatory cytokines IL-8, IFNγ and IL-1β when compared to the LA-predominant emulsions after exposure to pro-inflammatory TNFα stimulation. TNFα is released in response to lipopolysaccharide and other bacterial products and as a mediator of the innate immune system, it significantly contributes to chronic inflammatory disease 209.

Fatty acid analysis of cell culture media following incubation revealed a preference for omega-3 DHA to be taken up from the media by the A549 cells compared to omega-6 LA. Both the parenteral and enteral emulsion preparations with a high pre-incubation concentration of DHA (Omegaven and high-DHA fish oil emulsion, respectively) showed negligible post-incubation DHA levels. In contrast, the predominantly LA-based parenteral and enteral emulsions (Intralipid and soy oil emulsion, respectively) resulted in similar pre- and post-incubation percentages, indicating low levels of uptake and/or metabolism. Interestingly, even though the data suggest that only a small proportion of LA in the emulsions was metabolised, it was enough to result in a significant difference in pro-inflammatory IL-8, IFNγ and IL-1β release compared to the emulsions containing DHA. Differences in cytokine release were also not likely due to the immunomodulatory effects of other omega-3 and omega-6 LCPUFA such as EPA and AA because levels were low-to-none in pre-incubation media preparations. Because of its immunoregulatory potential, we aimed to assess SP-D secretion from A549 cells as an indicator of pathological lung changes 168 associated with omega-3 and omega-6 LCPUFA, but levels did not reach threshold of detection. While SP-D was not detected from A549 cells, the literature provided strong support for the rationale to investigate SP-D in light of the relationship to neonatal respiratory disorders 168, 210, immune response 211, and its ability to modulate the inflammatory response in A549 cells 212. Literature suggests that SP-A 170, and more recently SP-B and SP-C 213 may also influence the immune response.

Many of the effects of LCPUFA are dependent on their incorporation into the cellular membrane 33, 150. DHA and LA percentages in the membrane fatty acids did not change in
proportion to supplementation during this study, suggesting that the DHA and LA that was taken up from the media was most likely used as an energy substrate instead of incorporated into membranes. Lack of DHA and LA in tissue after supplementation may reflect differences in transport mechanisms or indicate that fatty acids were used as part of the inflammatory process itself, likely as a substrate for eicosanoid synthesis. Our results suggest that DHA and LA in lipid emulsions are capable of exerting an immunomodulatory effect independent of their incorporation into the cell membrane. This finding is promising in situations where cell membrane composition of DHA cannot be increased pre-emptively via supplementation, for example infants at risk of BPD or patients diagnosed with acute lung injury. Both BPD and acute lung injury have been associated with lower than normal levels of omega-3 LCPUFA.

Elevations in inflammatory cytokines IL-1β, 6, 8, 10 and IFNγ have been shown to be predictive of BPD development in extremely low birthweight infants. Chemotactic factors such as IL-8 produced by the respiratory airways play a pivotal role in the progression of airway inflammation and recruitment, and the subsequent activation of granulocytes and macrophages lead to damage of the vascular endothelium and alveolar epithelium. Our results demonstrate that omega-3 LCPUFA reduces secretion of some pro-inflammatory cytokines predictive of BPD. Furthermore, DHA reduced release of IL-8 from unstimulated cells, indicating an attenuation effect separate from infectious or inflammatory stimuli. Given the established role of inflammation in the pathogenesis of BPD, modulation of the inflammatory cascade using omega-3 LCPUFA is an attractive option.

In conclusion, results of this study show that parenteral and enteral emulsions containing omega-3 LCPUFA can reduce alveolar secretion of important pro-inflammatory cytokines compared to omega-6 LCPUFA emulsions, highlighting the potential immunomodulatory effect of dietary LCPUFA in the clinical setting. Very little published data exists from preterm infants examining if omega-3 LCPUFA in lipid emulsions exhibit the same attenuation of pro-inflammatory cytokines in vivo, emphasising the need for further large-scale trials. We are
currently aiming to address this gap in knowledge in preterm infants by conducting an \textit{in vivo} assessment of the systemic immune response to a high-dose omega-3 DHA in enteral lipid emulsion as part of a nested study in the N3RO RCT.
CHAPTER 3

DOCOSAHEXAENOIC ACID AS AN IMMUNOMODULATORY AGENT IN PRETERM INFANTS

Preface

The manuscript in chapter 3 details the results of the nested side study in the N3RO RCT in preterm infants < 29 weeks gestation. The aim of the nested study was to assess the effect of enteral supplementation with 60 mg/kg/day of omega-3 DHA on pro-inflammatory and regulatory cytokine levels in the preterm infant. Levels of SP-D were also assessed as an indicator of pathological lung changes. The detailed study protocol describing the framework of the nested study is included in Appendix 3. Other supporting documents relating to the nested study and N3RO RCT are included in Appendices 4-8.

Manuscript: Effect of omega-3 LCPUFA on the immune response of preterm infants < 29 weeks gestation: Results from a single-centre nested study in the N3RO randomised controlled trial
Statement of Authorship

| Title of Paper | Effect of omega-3 LCPUFA on the immune response of preterm infants <29 weeks gestation: Results from a single centre nested study in the N3RO randomised controlled trial |
| Publication Status | Published | Accepted for Publication |
| | Submitted for Publication | Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | N/A |

Principal Author

| Name of Principal Author (Candidate) | Naomi H Fink |
| Contribution to the Paper | Contributed to the conception and design of the research. Contributed to the acquisition, analysis and interpretation of the data. Drafted the first version of the manuscript and subsequent versions after revision. |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the sole author of this manuscript. |
| Signature | Date | 10/10/2015 |
TITLE: Effect of omega-3 LCPUFA on the immune response in preterm infants < 29 weeks gestation: Results from a single-centre nested study in the N3RO randomised controlled trial

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Statistical support and review: Dr. Jennie Louise; School of Public Health, The University of Adelaide; Adelaide, SA, Australia 5005
ABSTRACT

INTRODUCTION: Preterm infants are predisposed to developing inflammatory disorders during the neonatal period. Omega-3 long-chain polyunsaturated fatty acids are known to exert an immunoregulatory effect in adults and animals and preliminary data in preterm infants suggests a benefit. This study aimed to assess the effect of supplemental enteral docosahexaenoic acid (DHA) on levels of inflammatory biomarkers in preterm infants.

METHODS: This single-centre nested study in the N3RO randomised controlled trial (ACTRN 12612000503820) included a total of 51 preterm infants (27 intervention, 24 control) < 29 weeks gestational age with no major congenital malformations. Infants in the intervention group received an enteral emulsion providing 60 mg/kg/day DHA from <3 days after first enteral feed to study end (36 weeks postmenstrual age) and the control emulsion was soy-based (no DHA). A blood sample was collected at baseline, approximately two weeks postnatal and at study end. Pro-inflammatory (IL-1β, IL-6, IL-8, IL-12p70, IL-17A, IL-23, MIP-1α, TNFα and IFNγ) and regulatory cytokines (IL-10) (MILLIPLEX® MAP high sensitivity magnetic bead panel), surfactant protein D (SP-D) (ELISA) and fatty acid levels (gas chromatography) were assessed in blood samples. Remaining blood was incubated with/without E. coli lipopolysaccharide and pro-inflammatory and regulatory cytokines were assessed in supernatants (flow cytometry).

RESULTS: No differences in pro-inflammatory or regulatory cytokine release were observed between groups. IL-6, IL-8, IL-10, TNFα, TGFβ1 and SP-D levels changed significantly over time but were not significantly different at any time point between groups. SP-D levels were significantly reduced in the intervention group at study end.

CONCLUSION: Supplemental DHA at a dose of 60 mg/kg/day does not attenuate the release of pro-inflammatory mediators nor does it up-regulate regulatory mediators in preterm infants.
< 29 weeks gestation. SP-D was reduced at 36 weeks postmenstrual age in the intervention group. DHA at this dose cannot be recommended to attenuate inflammation in preterm infants.
INTRODUCTION

Preterm infants are born with an underdeveloped regulatory immune system and thus are predisposed to developing diseases associated with an inflammatory response, such as bronchopulmonary dysplasia (BPD), necrotising enterocolitis (NEC), sepsis and retinopathy of prematurity (ROP)\(^3\)\(^4\). Minimising the injury resulting from unregulated inflammation is a priority for clinicians while infants are in the neonatal intensive care unit (NICU). An anti-inflammatory nutritional intervention in the early postnatal period is an attractive option. Preterm infants receive omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFA) via breast milk, formula, and/or parenteral lipid emulsions. Omega-6 LCPUFA are known precursors of inflammatory eicosanoids with experimental studies in adults, animal and \textit{in vitro} models indicating that omega-6 LCPUFA can induce pro-inflammatory cytokine production\(^{221}\). An opposite, anti-inflammatory effect has been reported for omega-3 LCPUFA\(^{221}\).

An inability to downregulate immune responses and the resulting pro-inflammatory cytokine cascade initiated by pre- and postnatal stimuli is central to the pathogenesis of inflammatory disorders in preterm infants. In the case of BPD, a lung disorder of prematurity, pro-inflammatory cytokines have been shown to disrupt alveolar growth and vasculogenesis through periodic cycles of damage and repair\(^{109,222}\). Elevations in inflammatory cytokines IL-1\(\beta\), 6, 8 and IFN\(\gamma\) have been reported to be effective predictors of BPD development in extremely low birthweight infants\(^{216-219}\).

BPD disrupts the saccular stage of development in the lung, which coincides with the period during which surfactant is produced\(^{223}\). Surfactant reduces surface tension in the lung and while composed predominantly of phospholipids, it also contains a small protein component \(^{167}\) including two surfactant proteins (SP), SP-A and SP-D. These are part of the innate immune system and can exert a regulatory influence on other innate immune cells, such as macrophages\(^{167,168}\). Decreased levels of surfactant and specifically SP-D in preterm infants have been
associated with a need for supplemental oxygen and implicated as a possible risk factor for the development of BPD\textsuperscript{173, 224, 225}. Furthermore, omega-3 LCPUFA have been reported to attenuate inflammation\textsuperscript{221} and influence the composition of surfactant lipids in adults and in animal models\textsuperscript{174, 175}.

There are very few studies assessing the effect of parenteral\textsuperscript{10} or enteral\textsuperscript{13, 14} omega-3 LCPUFA supplementation on cytokine production or surfactant in preterm infants. A single-centre nested study in the N3RO randomised controlled trial (RCT) (n-3 fatty acids for Improvement in Respiratory Outcomes ACTRN 12612000503820) was conducted to assess the role of omega-3 LCPUFA in attenuating inflammation in the preterm infant. Preliminary evidence in preterm infants\textsuperscript{34, 95, 102} as well as a strong supporting evidence from \textit{in vivo} and \textit{in vitro} studies\textsuperscript{221} underpinned the rationale for the N3RO RCT and the choice of 60 mg/kg/day as the dose\textsuperscript{6}. The N3RO RCT assessed the efficacy of enteral omega-3 docosahexaenoic acid (DHA) supplementation to reduce the incidence of BPD in preterm infants < 29 weeks gestational age (GA). The aim of this study was to test the hypothesis that enteral DHA supplementation at a dose of 60 mg/kg/day could attenuate the inflammatory response. To our knowledge, the effect of enteral supplementation with this dose on inflammatory biomarkers has not been assessed previously in preterm infants.

\textbf{METHODS}

\textit{Trial framework}

This single-centre nested study in the N3RO RCT was conducted at one participating centre, the Women’s and Children’s Hospital (WCH) in Adelaide, South Australia. This study was approved by the WCH Human Research Ethics Committee. All trial procedures for the nested study were conducted according to the N3RO trial framework\textsuperscript{6}. Briefly, preterm infants randomly allocated to the intervention (DHA) group were supplemented with 60 mg/kg/day DHA via the enteral route between baseline (approximately three days of age) and study end.
(36 weeks postmenstrual age (PMA)). Infants in the control group received an enteral soy oil emulsion, without any DHA, between baseline and at study end. Baseline characteristics and clinical outcomes were recorded in a separate case report form (CRF) for each infant.

**Participants and sample collection**

The study sample included preterm infants born < 29 weeks GA with no major congenital malformations. For the nested study, a blood sample was collected in conjunction with a venous puncture or capillary collection for diagnostic purposes at T0 (baseline: approximately three days postnatal), T1 (mid-point: approximately 14 days postnatal) and T2 (study end: 36 weeks PMA/discharge/transfer to other hospital, which ever occurred first). Blood (0.5 mL) was collected into lithium heparin coated tubes according to standard clinical practice by clinical staff. If the infant had an arterial line, blood was preferentially collected from this route over venous puncture or capillary collection. Blood samples were used for analysis of biomarkers in whole blood after *E. coli* lipopolysaccharide (LPS) stimulation, or in plasma without stimulation, as described below.

**Stimulation of whole blood with *E. coli* LPS**

LPS stimulation of whole blood was carried out according to a previously described method. Blood was incubated for 21-24 hours with and without LPS (Sigma-Aldrich, Sydney Australia; final concentration 1 µg/mL), after which time supernatants were collected and stored in 60 µL aliquots at -80°C for later batch analysis of cytokines by flow cytometry. The remaining blood sample was centrifuged at 2000 g for 10 minutes to isolate plasma. Plasma was stored at -80°C until batch analysis of immune markers.

**Analysis of inflammatory mediators in supernatants from whole blood culture**

Supernatants from whole blood which had been incubated with or without *E. coli* LPS were assayed for inflammatory cytokines using an enhanced sensitivity human cytokine bead array
(BD Biosciences, San Diego, USA) according to the manufacturer’s protocol supplied with the kit. Supernatants incubated with/without LPS were assayed undiluted. Cytokines analysed included IL-6, IL-8, IL-10, IL-12p70, TNFα and IFNγ (BD CBA Human Enhanced Sensitivity Flex Sets- BD Biosciences, San Diego, USA). Minimum concentration detected and ranges of standards for each cytokine are presented in Table S1. Cytokines in supernatant were analysed on a BD Biosciences FACS Canto flow cytometer (BD Biosciences, San Diego, USA). Mean fluorescence intensity was generated by BD FacsDiva™ software version 6.1.3 (BD Biosciences, San Diego, USA, 2009).

Analysis of inflammatory mediators in plasma

A MILLIPLEX® MAP panel (Merck Millipore, Australia) was used to measure plasma cytokines/chemokines according to the manufacturer’s protocol supplied with the kit. Multiple cytokines (IL-1β, IL-10, IL-12p70, IL-17A, IL-23, TNF-α, IFN-γ, MIP-1α, IL-6 and IL-8) were assessed using a MILLIPLEX® MAP Human High Sensitivity T Cell Magnetic Bead Panel on Luminex 200 and MAGPIX® instruments. Limits of detection and range of standards for each cytokine are presented in Table S2. Seven standard samples and two quality controls were added to wells in duplicate and samples were assayed undiluted in a single detection. When volume was sufficient, samples were assayed in duplicate.

Analysis of SP-D in plasma

Plasma samples were analysed for SP-D using an SP-D Human Quantikine ELISA (R&D Systems, Minneapolis, USA), according to the manufacturer’s instructions. Plasma samples were assayed at 1:5 to 1:10 dilution and standards were prepared as outlined in the protocol. Absorbance was measured by a multi-well spectrophotometer (Victor™ x4 Multilabel reader, Perkin Elmer, Singapore) at 450 nm with a reference wavelength of 595 nm.

Fatty acid extraction, methylation and analysis by gas chromatography
Blood samples (30 µL) were collected from infants at baseline (T0) and study end (T2). Fatty acids were extracted, methylated and analysed by gas chromatography and flame ionisation detection according to a previously published procedure. Results were reported as percent of total fatty acids.

Statistical analysis

All participants were analysed according to the group to which they were randomised (intention-to-treat principle). Continuous variables were presented as mean (SE) or median (IQR)/(SD) depending on value distribution. Between-group differences in baseline and clinical outcomes were assessed using a student’s t-test or ANOVA, as appropriate, using SPSS statistical software (IBM SPSS Statistics v24, Chicago, IL). For cytokines, SP-D and fatty acids, longitudinal Tobit regression models were used to assess between group differences and changes over time. A generalised estimating equation was used to account for repeated measures and a time-by-treatment interaction was included to test for differences between groups in rate of change over time. Both unadjusted and adjusted analyses were conducted with adjusted analyses including GA (< 27 weeks) and infant sex as covariates.

While sample size was determined by the recruitment rate at WCH, recent studies have detected clinically meaningful differences in cytokine expression in similar sample sizes in preterm infants with group sizes of n=12, n=15 and n=25.

RESULTS

A total of 51 neonates were recruited for the nested study between March 2014 and October 2015. All 51 infants recruited received either the intervention (DHA) or control (soy, no DHA) emulsion (Figure 1). At least one blood sample was collected from all neonates and the majority (n=43) had samples collected from all three time points.
Figure 1. Flow diagram for the N3RO nested study according to the CONSORT statement.
Legend to Figure 1. Abbreviations: docosahexaenoic acid (DHA), postmenstrual age (PMA).
Baseline and clinical characteristics of participants

The intervention (DHA) and control (no DHA) groups did not differ significantly with regard to sex distribution, GA at birth, singleton/multiple birth, mode of delivery or maternal steroid administration (Table 1). Clinical characteristics such as postnatal steroid administration, surgery, inflammatory outcomes (ie. BPD, sepsis, NEC) or feeding parameters were not significantly different between groups at study end (Table 2). All infants received ClinOleic lipid emulsion (Baxter Healthcare) during periods of parenteral nutrition and three infants (two intervention, one control) received both ClinOleic and Omegaven (Fresenius Kabi) during the study period.

Table 1. Baseline characteristics of participants by group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Intervention (DHA) (n=27)</th>
<th>Control (no DHA) (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>14/13</td>
<td>13/11</td>
</tr>
<tr>
<td>Mean gestational age</td>
<td>26 $^{37/}$</td>
<td>26 $^{37/}$</td>
</tr>
<tr>
<td>&lt;27 weeks gestational age</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Singleton/multiple birth</td>
<td>18/9</td>
<td>18/6</td>
</tr>
<tr>
<td>Mode of delivery (vaginal/C-section)</td>
<td>19/8</td>
<td>14/10</td>
</tr>
<tr>
<td>Maternal steroids</td>
<td>26</td>
<td>21</td>
</tr>
</tbody>
</table>

Legend to Table 1. There were no differences in baseline characteristics between groups. Abbreviations: docosahexaenoic acid (DHA).
Table 2. Clinical characteristics of participants at study end by group

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Intervention (DHA) (n=27)</th>
<th>Control (no DHA) (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% compliance</td>
<td>90%</td>
<td>89%</td>
</tr>
<tr>
<td>(total doses/total possible doses)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postnatal steroids</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Surgery</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Bronchopulmonary dysplasia</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sepsis</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Necrotising enterocolitis</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Days parenteral nutrition</td>
<td>26.7</td>
<td>25.1</td>
</tr>
<tr>
<td>Days intravenous lipids</td>
<td>19.8</td>
<td>19.4</td>
</tr>
<tr>
<td>Days to reach full enteral feeds</td>
<td>20.69</td>
<td>17.63</td>
</tr>
<tr>
<td>Type of enteral feed at discharge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. breast milk</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>b. formula</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>c. both</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

Legend to Table 2. There were no differences in clinical characteristics between groups. Abbreviations: docosahexaenoic acid (DHA).

*Fatty acid levels*

Fatty acids in whole blood expressed as percent of total fatty acids and the effect of time and treatment group are presented in Table 3. Overall, omega-3 DHA and eicosapentaenoic acid (EPA) levels were significantly higher in the intervention group compared with control (P<0.001) and the rate of change over time was significantly different between groups. Omega-6 arachidonic acid (AA) levels were significantly higher in the control group compared to intervention (P<0.01). Within groups, DHA increased over time in the intervention group (P<0.001) and decreased over time in the control group (P<0.01). Both EPA and linoleic acid (LA) significantly increased over time in both groups (P<0.001) and AA decreased over time in both groups (P<0.001).
Table 3. Fatty acid levels at baseline and study end in blood samples

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Intervention (n=27)</th>
<th>Control (n=24)</th>
<th>Main Effect Between Groups (Intervention vs. Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td></td>
</tr>
<tr>
<td>OMEGA-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHA</td>
<td>T0 3.10 (0.84) T2 4.05 (0.81)</td>
<td>↑ P&lt;0.001</td>
<td>T0 3.18 (0.87) T2 2.61 (0.48) ↓ P&lt;0.01</td>
</tr>
<tr>
<td>EPA*</td>
<td>T0 0.27 (0.21, 0.39) T2 0.82 (0.72, 1.03)</td>
<td>↑ P&lt;0.001</td>
<td>T0 0.28 (0.18, 0.44) T2 0.45 (0.32, 0.43)</td>
</tr>
<tr>
<td>OMEGA-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA</td>
<td>T0 8.06 (2.29) T2 14.51 (1.38)</td>
<td>↑ P&lt;0.001</td>
<td>T0 7.85 (3.11) T2 14.68 (2.00)</td>
</tr>
<tr>
<td>AA</td>
<td>T0 12.03 (2.01) T2 8.57 (0.94)</td>
<td>↓ P&lt;0.001</td>
<td>T0 12.15 (2.94) T2 9.49 (1.06)</td>
</tr>
</tbody>
</table>

Legend to Table 3. Fatty acids are reported as percent of total fatty acids in blood samples collected from intervention (DHA) and control (no DHA) groups. “*” Descriptives are median (IQR). “†” denotes a significant overall treatment by time effect within the statistical model (ie. there is a different pattern of change over time for intervention vs. control). Abbreviations: Arachidonic acid (AA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA), not significant (NS), baseline (T0), study end: 36 weeks PMA/discharge/transfer (T2).

Outcomes

For all outcomes, unadjusted and adjusted analyses were conducted with infant sex and GA (<27 weeks) as covariates. Adjusted significance values are reported unless otherwise stated.

Plasma cytokine levels: Values for outcome parameters and the effect of time and treatment group are presented in Table 4. A selection of pro-inflammatory and regulatory immune markers in plasma over time are represented graphically in Figure 2. The rate of change over time for all pro-inflammatory cytokines assessed was not significantly different between groups. Plasma levels of IL-6 and IL-8 significantly decreased between baseline and mid-point (P<0.05) and between mid-point and study end (P<0.001) in the control group and between mid-point and study end in the intervention group (P<0.001), but plasma IL-6 and IL-8 levels were not significantly different between groups at any time point. Plasma levels of IFNγ, IL-
12p70, IL-17A and IL-23 were below the limit of detection in the majority of infants (Table S2).

The regulatory cytokine, IL-10, significantly increased in plasma between mid and end points of the study in the intervention group (P<0.05) but was not significantly different at any time point from levels in the control group. Rate of change over time in IL-10 levels was also not significantly different between groups.

SP-D: Levels of SP-D in the plasma decreased significantly from mid- and end points of the study in both groups (P<0.001) and was significantly lower in the intervention group (P<0.05) at study end compared to control group (Table 4 and Figure 2). The rate of change over time in SP-D was not significantly different between groups.
### Table 4. Pro-inflammatory and regulatory cytokines and SP-D in plasma

<table>
<thead>
<tr>
<th>Immune marker</th>
<th>Intervention (n=27) Median (IQR)</th>
<th>Control (n=24) Median (IQR)</th>
<th>Change over time (P value)</th>
<th>Change over time (P value)</th>
<th>Main Effect (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro-inflammatory cytokines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP1α (pg/mL)</td>
<td>8.16 (3.70, 13.79)</td>
<td>8.87 (4.73, 15.78)</td>
<td>10.57 (4.18, 19.60)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>13.01 (10.7, 14.64)</td>
<td>12.59 (10.79, 16.27)</td>
<td>14.21 (10.27, 16.2)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IFNγ* (pg/mL)</td>
<td>0.59 (0.59, 10.01)</td>
<td>0.59, 0.59, 8.86)</td>
<td>NS</td>
<td>NS</td>
<td>0.59 (0.59, 14.01)</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>0.17 (0.17, 0.21)</td>
<td>0.17 (0.17, 1.39)</td>
<td>0.17 (0.17, 0.17)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Regulatory factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>6.83 (2.21, 12.53)</td>
<td>4.19 (1.33, 7.81)</td>
<td>0.79 (0.12, 1.92)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>SP-D (ng/mL)</td>
<td>20.30 (6.08, 45.28)</td>
<td>21.43 (9.65, 65.72)</td>
<td>4.15 (2.39, 11.61)</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Legend to Table 4. “*” result is presented as median (minimum, maximum). “-” too few observations (≤25 observations) within detectable range for statistical analysis. Abbreviations: docosahexaenoic acid (DHA), not significant (NS), surfactant protein (SP), baseline (T0), postnatal day 14 (T1), study end: 36 weeks PMA/discharge/transfer (T2).
Figure 2. Pro-inflammatory cytokines, regulatory cytokines and surfactant protein D in plasma.

Legend to Figure 2. Pro-inflammatory A) IL-6, B) IL-8, C) MIP1α, D) TNFα and regulatory E) IL-10 and F) SP-D results from intervention (DHA) and control (no DHA) groups are expressed as median (IQR). Abbreviations: docosahexaenoic acid (DHA), baseline (T0), postnatal day 14 (T1), study end: 36 weeks PMA/discharge/transfer (T2).
Response to *E. coli* LPS: Values for outcome parameters and the effect of time and treatment group are presented in Table 5. Cytokine release from unstimulated and LPS-stimulated whole blood culture supernatants after a 24 hour period were similar between the groups. IL-8 levels (unstimulated) significantly increased between baseline and mid-point in the control group (P<0.05) and between mid-point and study end in the intervention group (P<0.01) but were not significantly different at any time point between groups. Similarly, TNFα and TGFβ1 levels (unstimulated) significantly increased between baseline and mid-points in both control and intervention groups and TGFβ1 also decreased significantly between mid-point and study end in the control group. Levels of TGFβ1 were not significantly different at any time point between groups, but there was a significant time-by-treatment interaction indicating a different pattern of change over time for control versus intervention. IL-12p70 and IFNγ levels (unstimulated) were below the limit of detection at all time points (Table S1).

In supernatants from whole blood culture following LPS stimulation, there was an increase in IL-10 and TNFα levels between mid-point and study end in both control and intervention groups but levels were not significantly different at any time point between groups. Likewise for TGFβ1, levels increased between mid-point and baseline but were not significantly different between groups. IL-12p70 and IFNγ levels (LPS-stimulated) were below the detection limit at all time points (Table S1) and the majority of IL-6 and IL-8 levels (LPS-stimulated) were greater than the detection limit of 200 pg/mL.
Table 5. Cytokine levels at in supernatants from unstimulated and LPS (*E. coli*) stimulated whole blood culture

<table>
<thead>
<tr>
<th>Immune marker</th>
<th>Intervention (n=27)</th>
<th>Change over time (P value)</th>
<th>Control (n=24)</th>
<th>Change over time (P value)</th>
<th>Main Effect (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Time point (postnatal day ± SE)</td>
<td>Time point (postnatal day ± SE)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ (ng/mL)</td>
<td>0.44 (1.52, 39.36)</td>
<td>3.74 ± 0.20</td>
<td>NS</td>
<td>0.63 (0.38, 0.93)</td>
<td>↑ P&lt;0.01 NS NS NS</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>1.38 (0.44, 3.26)</td>
<td>3.31 (2.14, 5.88)</td>
<td>↑ P&lt;0.01 NS</td>
<td>1.01 (0.47, 1.75)</td>
<td>↑ P&lt;0.01 ↓ P&lt;0.001 NS</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>0.43 (0.43, 1.90)</td>
<td>1.17 (0.44, 1.85)</td>
<td>NS</td>
<td>1.01 (0.47, 1.75)</td>
<td>↑ P&lt;0.01 ↓ P&lt;0.001 NS</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>1.06 (0.43, 2.44)</td>
<td>1.15 (0.38, 0.74)</td>
<td>↑ P&lt;0.001 NS</td>
<td>3.59 (1.52, 95.07)</td>
<td>NS NS NS NS</td>
</tr>
<tr>
<td>IL-8 (pg/mL)</td>
<td>4.84 (1.52, 72.32)</td>
<td>T0 vs. T1 12.51 (4.70, 28.40)</td>
<td>NS</td>
<td>6.13 (1.52, 20.23)</td>
<td>NS NS NS NS</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.03 (0.33, 0.78)</td>
<td>T2 vs. T1 193.50 (193.50)</td>
<td>NS</td>
<td>93.50 (193.50)</td>
<td>NS NS NS NS</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>3.31 (2.14, 5.88)</td>
<td>T1 vs. T0 5.59 (1.52, 95.07)</td>
<td>NS</td>
<td>193.50 (193.50)</td>
<td>NS NS NS NS</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>1.06 (0.43, 2.44)</td>
<td>T2 vs. T0 193.50 (193.50)</td>
<td>NS</td>
<td>193.50 (193.50)</td>
<td>NS NS NS NS</td>
</tr>
</tbody>
</table>

Legend to Table 5. Blood samples were obtained from infants in intervention (DHA) and control (no DHA) groups and results are expressed as median (IQR). “↑” denotes a significant overall treatment by time effect within the statistical model (i.e. there is a different pattern of change over time for intervention vs. control). Abbreviations: docosahexaenoic acid (DHA), not significant (NS), surfactant protein (SP), baseline (T0), postnatal day 14 (T1), study end: 36 weeks PMA/discharge/transfer (T2).
Figure 3. Pro-inflammatory and regulatory cytokines in supernatants from unstimulated and *E. coli* LPS-stimulated whole blood.

**A**

TNFα

**B**

TGFβ1
Legend to Figure 3. Pro-inflammatory A) TNFα, and regulatory B) TGFβ1 and C) IL-10 cytokine levels are expressed as median (IQR). Blood samples were collected from infants in the intervention (DHA) and control (no DHA) groups. Abbreviations: docosahexaenoic acid (DHA) baseline (T0), postnatal day 14 (T1), study end: 36 weeks PMA/discharge/transfer (T2).

DISCUSSION

To our knowledge, this is the first assessment of inflammatory biomarkers in response to an enteral supplementation regimen of 60 mg/kg/day DHA in preterm infants < 29 weeks GA. In this single-centre nested study in an RCT, we report that enteral omega-3 LCPUFA supplementation did not attenuate the inflammatory cytokine response in plasma or in peripheral blood mononuclear whole blood culture supernatants (after a 24 hour incubation with E. coli LPS). The intervention emulsion (DHA) was associated with a significant reduction in plasma SP-D levels between groups at study end. While neutral\textsuperscript{230} and negative\textsuperscript{231} results have been reported for fish oil supplementation in clinical care, a wealth of evidence from infant\textsuperscript{232}, adult\textsuperscript{214,234}, animal\textsuperscript{235-237} and \textit{in vitro}\textsuperscript{238} models suggests omega-3 LCPUFA can attenuate inflammation either via a reduction in pro-inflammatory cytokines or an increase in regulatory...
cytokines. Our results do not support a regulatory effect for omega-3 DHA at the dose of 60 mg/kg/day in preterm infants.

Very few studies have assessed the effect of LCPUFA on cytokine responses in preterm infants\textsuperscript{10, 13, 14} and results are difficult to compare directly with those in the current study due to variations in study design. Firstly, the GA assessed in these trials ranges from 26-32 weeks GA\textsuperscript{10}, <36 weeks GA\textsuperscript{13} to include both preterm and term infants\textsuperscript{14}. Secondly, the route of supplementation was either parenteral\textsuperscript{10} or enteral\textsuperscript{13, 14} and neither the dose nor preparation were comparable between studies (emulsion, pure oil, breast milk/formula). Lastly, the control groups in two of the enteral LCPUFA supplementation studies\textsuperscript{13, 14} are not comparable to the control group in this current study. Previous studies have reported omega-3 LCPUFA-induced effects on cytokine production in preterm infants. A reduction in IL-6 and IL-8 by Skouroliakou et al. (2015) was reported following parenteral administration of omega-3 LCPUFA (SMOFlipid). Enteral supplementation with 100 mg pure fish oil resulted in a reduction in IL-1β and IL-6 by Lopez-Alarcon et al. (2012) and an increase in IL-10 was described by Field et al. (2000) in LCPUFA-enriched formula group when compared with the standard no-LCPUFA formula group. The neutral effect of omega-3 LCPUFA supplementation from our nested study in the N3RO RCT on cytokines in comparison with the above results suggests that DHA has the potential to elicit a varied effect on cytokine production in preterm infants depending on dose, duration, GA and route of administration.

In addition to assessing the effect of LCPUFA on plasma levels of cytokines, the effect of omega-3 on the pro-inflammatory response to stimulation with bacterial endotoxin was investigated. \textit{E. coli} is a standard model stimulant and also a common pathogen encountered by preterm infants in the NICU\textsuperscript{239, 240}, thus \textit{E. coli} endotoxin (LPS) was chosen to mimic an infectious stimuli \textit{ex vivo}. An attenuated pro-inflammatory response or increased regulatory response by peripheral blood mononuclear cells after incubation with \textit{E. coli} LPS has been reported in other studies following omega-3 LCPUFA supplementation in infants\textsuperscript{241}, adults\textsuperscript{242}. 
and animals. Omega-3 LCPUFA did not have an effect on cytokine levels after LPS stimulation in blood from infants enrolled in this study. The only difference observed between groups was the rate of change of TGFβ1 over time in unstimulated samples after a 24 hour incubation period. TGFβ is a regulatory cytokine which is also essential for appropriate lung growth and development. The TGFβ1 isoform is increased in various forms of lung pathology, including chronic lung disease of prematurity. However, levels were not significantly different between groups at any of the three time points.

The results of the N3RO RCT indicate that DHA does not provide any protection against BPD development, and the cytokine results presented in this nested study support this conclusion. A sample size of 51 infants has previously detected clinically meaningful differences in IL-8, which is one of the critical chemokines involved in the progression of BPD. While DHA supplementation resulted in a significant increase in BPD in the N3RO RCT in a sample size of 1273 infants (unpublished data), we observed no difference in plasma IL-8 concentration between control and the intervention group at any time point assessed. The results of this nested study are important in the context of the N3RO RCT results because they suggest that the increase in BPD in the intervention group was not likely due to an increase in pro-inflammatory cytokines and that DHA was acting via alternate mechanisms.

An increase in SP-D levels have been implicated in the most severe pulmonary outcomes and conversely, decreased SP-D levels have been associated with BPD and respiratory distress, and also with an increased requirement for supplemental oxygen and surfactant administration. The majority of the literature suggests a positive benefit for increased SP-D levels in preterm infants. In light of the increased incidence of BPD observed in the N3RO RCT, the decrease in SP-D levels in the intervention group compared to control group warrants further investigation. SP degradation or inactivation are associated with an increased susceptibility to inflammation and lung infection and may also contribute to an increased incidence of BPD. No studies have reported a relationship between omega-3...
LCPUFA supplementation and SP levels in surfactant. While there is no supporting data, we suggest that in a similar manner to the way in which changes to the lipid composition of cell membranes and lipid rafts can influence how membrane proteins associate with the cell, altering the lipid composition of surfactant via supplementation may also influence the type and number of SPs. This is only a suggested mechanism and robust data is needed to test this hypothesis.

Preterm infants have a low blood volume and therefore only a small blood sample (0.5 mL) could be ethically and safely obtained for this study. While multiplex assays were the most efficient way to obtain information on several cytokines from a small sample volume (25 ul), a limitation of this study was there was not sufficient volume to assay all samples in duplicate or triplicate. Furthermore, while this serves as excellent pilot data for understanding the relationship between LCPUFA and inflammation and is comparable to sample sizes for other LCPUFA/biomarker studies in the literature, there may not be sufficient power to detect differences in some cytokines (ie. IL-6 and TNFα). A strength of both the nested study and the N3RO RCT is the ability to confirm the success of the intervention; DHA and EPA levels in the blood of preterm infants increased in response to omega-3 LCPUFA supplementation. Relative proportions of omega-3 and omega-6 fatty acids in the blood are comparable to other results reported in the literature for preterm infants in omega-3 LCPUFA supplementation and observational studies. The major strengths of this nested study are the high-quality randomised controlled design and longitudinal assessment of numerous inflammatory mediators in both resting and stimulated conditions. To our knowledge, no other studies have performed such an extensive longitudinal evaluation of cytokines in preterm infants following omega-3 LCPUFA supplementation.

In conclusion, 60 mg/kg/day omega-3 DHA did not affect circulating levels of pro-inflammatory or regulatory cytokines in preterm infants < 29 weeks GA, nor did it attenuate the response to an infectious stimuli (E. coli LPS). DHA at this dose was associated with a
reduction in SP-D which may contribute to an impairment in the immunoregulatory capability of the lung. While the levels of DHA in current infant formulas appear to be safe and well-tolerated, further consideration should be given to new products if the dose of DHA is to exceed what is currently supplied in standard clinical care. Determining the dose-related effects of DHA on the immune system and specifically on the lung and BPD development in preterm infants are important next steps.
## SUPPLEMENTARY TABLES

Supplementary Table 1 (S1). Minimum concentration detected and range of standards for each cytokine assessed with the BD Biosciences enhanced sensitivity human cytometric bead array

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Minimum concentration detected (pg/ml)</th>
<th>Range of standards (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>1.52</td>
<td>0-200</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.42</td>
<td>0-200</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.44</td>
<td>0-200</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.77</td>
<td>0-200</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.44</td>
<td>0-200</td>
</tr>
<tr>
<td>IFNγ</td>
<td>4.04</td>
<td>0-200</td>
</tr>
</tbody>
</table>

Supplementary Table 2 (S2). Limit of detection and range of standards for each cytokine assessed with the MILLIPLEX® MAP high sensitivity T cell magnetic bead panel

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Minimum detectable concentration (pg/ml)</th>
<th>Range of standards (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>0.19</td>
<td>0.49-2000</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.12</td>
<td>0.18-750</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.14</td>
<td>0.31-1250</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.64</td>
<td>1.46-6000</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.19</td>
<td>0.49-2000</td>
</tr>
<tr>
<td>IL-17A</td>
<td>0.75</td>
<td>0.73-3000</td>
</tr>
<tr>
<td>IL-23</td>
<td>12.94</td>
<td>7.93-32.50</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.15</td>
<td>0.43-1750</td>
</tr>
<tr>
<td>MIP1α</td>
<td>2.00</td>
<td>0.31-1250</td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.59</td>
<td>0.61-2500</td>
</tr>
</tbody>
</table>

Legend to Table S2. Each respective software calculated the true limits of detection (minimum Detectable Concentration-MinDC) by mathematically determining what the empirical MinDC would be if an infinite number of standards were run under the same conditions for that assay.
CHAPTER 4

OMEGA-3 LCPUFA AND GASTROINTESTINAL COLONISATION BY STAPHYLOCOCCUS AND METHICILLIN-RESISTANT BACTERIA

PREFACE

The manuscript in chapter 4 describes the results of the quantitative PCR analysis of total bacteria, *Staphylococcus* and bacterial carriage of the gene coding for methicillin-resistance (*mecA*) by bacteria in stool samples collected from a subset of infants in the nested side study in the N3RO RCT. The effect of enteral omega-3 LCPUFA supplementation on colonisation by *Staphylococcus* and *mecA*+ bacteria was investigated. The detailed study protocol describing the framework of the nested study is included in Appendix 3. Other supporting documents relating to the nested study and N3RO RCT are included in Appendices 4-8.

**Manuscript: Assessment of Staphylococcus and methicillin-resistant bacteria in preterm infants and the influence of omega-3 long-chain polyunsaturated fatty acids**
# Statement of Authorship

<table>
<thead>
<tr>
<th>Title of Paper</th>
<th>Assessment of Staphylococcus and methicillin-resistant bacteria in preterm infants and the influence of omega-3 long-chain polyunsaturated fatty acids</th>
</tr>
</thead>
</table>
| Publication Status | - Published  
- Submitted for Publication  
- Accepted for Publication  
- Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | N/A |

## Principal Author

| Name of Principal Author (Candidate) | Naomi H Fink |
| Contribution to the Paper | Contributed to the conception and design of the research.  
Contributed to the acquisition, analysis and interpretation of the data.  
Drafted the first version of the manuscript and subsequent versions after revision. |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the sole author of this manuscript. |
| Signature | Date | 10/10/2016 |
TITLE: Assessment of *Staphylococcus* and methicillin-resistant bacteria in preterm infants and the influence of omega-3 long-chain polyunsaturated fatty acids

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BACKGROUND AND AIM: Mucosal inflammation in preterm infants has the potential not only influence gut permeability but also adherence of pathogenic bacteria to epithelial cells. *Staphylococcus* and other methicillin-resistant bacteria are known pathogens causing significant morbidity and mortality in preterm infants. Evidence suggests that omega-3 docosahexaenoic acid (DHA) can attenuate inflammation and may influence colonisation by pathogenic bacterial species. Using a nested study within the N3RO randomised controlled trial (RCT) (ACTRN12612000503820), we assessed the effect of supplemental oral DHA (60 mg/kg/d) on stool *Staphylococcus* spp. bacteria and species carrying the gene coding for methicillin resistance (*mecA*) in infants < 29 weeks gestation.

METHODS: Stool samples were collected weekly from infants at baseline to study end (36 weeks postmenstrual age (PMA)/discharge/transfer; whichever occurred first) for infants enrolled in the nested study. Total DNA was extracted from stool and quantification of total bacteria, *Staphylococcus* spp. and *mecA*+ bacteria was assessed by real time quantitative polymerase chain reaction (qPCR).

RESULTS: A total of 221 stool samples were collected from 41 neonates. There was a significant reduction over time in relative abundance (% of total bacteria detected) of both *Staphylococcus* spp. and *mecA*+ bacteria in stools but levels were not significantly different between groups. Antibiotic and probiotic exposure was also not different between groups.

CONCLUSION: Our data show that staphylococci and *mecA*+ bacteria levels in the gut decreased significantly between birth and 36 weeks PMA but that they are not altered by enteral DHA supplementation. Further work is warranted to assess the effect of dietary modulations on bacterial species in the gut to prevent adverse outcomes in preterm infants.
INTRODUCTION

Microbial colonisation in newborn infants occurs with exposure to the mother’s body, other human contacts, and from inanimate objects present in the infant’s environment. Appropriate microbial colonisation of the gut is imperative for proper gastrointestinal functioning and immune development in preterm infants with disruptions to this correlating with adverse clinical outcomes. Many factors associated with preterm birth such as tube feeding, exposure to antimicrobial agents and invasive medical interventions are known to disrupt the stepwise establishment of the intestinal microbiota. Nutritional, microbiological and immunological disturbances in preterm infants all contribute to disease progression but the relationship among these determinants is not fully understood. There is a well-established link between infection and inflammation in the preterm infant and the mucosal environment is likely to play a role in this interaction.

One of the most important human bacterial pathogens associated with nosocomial and community infections is *Staphylococcus*. *Staphylococcus* is a gram-positive pathogen and one of the first species to colonise the respiratory tract, and in parallel, the gut. *Staphylococcus* has been associated with the development of neonatal inflammatory disorders and methicillin-resistant staphylococci are known to cause significant morbidity and mortality in preterm infants in Australian neonatal intensive care units (NICU). Prolonged antibiotic exposure is very common in preterm infants in the NICU and is also associated with an increased risk for the development of methicillin-resistant bacterial infections. Unless infection is detected, levels of staphylococci and methicillin-resistant bacteria in the preterm infant gut are not routinely monitored. The few studies performed so far show that staphylococci are one of the most predominant constituents of the gut microbiome in the early postnatal period.
Nutrition in early life can induce lasting changes in microbial colonisation and subsequent cross-talk between the gut microbiota and the immune system\textsuperscript{176, 180, 183, 184}. The composition of the bacterial species in the gut can influence the nature of the immune response (pro-inflammatory versus regulatory) and, importantly, inflammation in the gut can influence the colonisation pattern of both commensal and pathogenic bacteria in the gastrointestinal tract\textsuperscript{177, 186}. An inflammatory environment promotes adherence of pathogenic bacteria to the mucosal surface and can also compromise epithelial barrier integrity allowing access of bacteria to deeper tissues\textsuperscript{23, 177, 178, 144}. Diet can influence bacteria directly, as is the case with probiotics, or indirectly via an influence on the inflammatory response in the gastrointestinal environment\textsuperscript{177, 180}.

Preterm infants receive dietary omega-3 long chain polyunsaturated fatty acids (LCPUFA) via breast milk, human milk fortification products, infant formula and next-generation lipid emulsions. LCPUFA are known to modulate the inflammatory response in human, animal and \textit{in vitro} models\textsuperscript{221} with the general consensus being that omega-3 LCPUFA are anti-inflammatory and omega-6 LCPUFA are pro-inflammatory\textsuperscript{195}. While there is evidence to suggest that amount and type of fat may influence bacteria in the gut\textsuperscript{176, 258}, there is a paucity of mechanistic evidence from preterm infants on the relationship between modulation of inflammation by dietary LCPUFA and resulting bacterial colonisation during the postnatal period\textsuperscript{23, 180}. As part of a single-centre nested study in the N3RO RCT, we aimed to test the hypothesis that enteral DHA could influence the inflammatory environment in the gut to alter levels of common opportunistic pathogens, \textit{Staphylococcus} and bacteria carrying the gene coding for methicillin-resistance (\textit{mecA}). The N3RO RCT was conducted in preterm infants < 29 weeks gestation to assess the efficacy of omega-3 DHA to reduce the incidence of bronchopulmonary dysplasia (BPD), an inflammatory lung disorder of prematurity\textsuperscript{6}. We quantified \textit{Staphylococcus} spp. and \textit{mecA}+ bacteria in stool samples collected from infants.
METHODS

Trial framework

In the N3RO RCT \textit{(n-3 fatty acids for the improvement in Respiratory Outcomes; ACTRN 12612000503820)} preterm infants were supplemented with 60 mg/kg/day of an omega-3 DHA emulsion from randomisation (approximately day 3 postnatal) to 36 weeks PMA/transfer/discharge (whichever occurred first). Infants in the control group received an enteral soy oil emulsion, without any DHA, between baseline and study end. The stool analysis sub-study reported here was a nested study conducted at one participating trial centre, the Women’s and Children’s Hospital (WCH) in Adelaide, Australia between March 2014 and October 2015. Ethical approval for the study was granted by the Human Research Ethics Committee at the WCH. Baseline characteristics and clinical outcomes were recorded in a separate case report form (CRF) for each infant.

Sample collection

Stool samples were collected at baseline (approximately postnatal day 3-5), once weekly thereafter and at study completion. Nappies containing stool were collected from the NICU, and Special Care Baby Unit (SCBU) at the WCH. Faecal matter was aseptically transferred from the nappy to cryotubes and frozen immediately at -80°C for later DNA extraction.

DNA extraction and quantification

Total DNA was extracted from the faecal samples using MoBio PowerSoil Powerlyzer DNA isolation kits according to the manufacturer’s instructions with slight modifications (Mo Bio Laboratories, Inc., Carlsbad, USA). Approximately 0.15g of stool sample was added to the bead solution and samples were mechanically lysed with a MP Biomedicals FastPrep-24 (Jomar Life Research, Welland, Australia) with two pulses of maximum speed for 60s. Eluted DNA
concentration was quantified fluorometrically with a Qubit dsDNA HS Assay kit (Life Technologies, Carlsbad, USA).

**PCR-based quantification of total bacteria, staphylococci, and mecA+ bacteria**

Determination of total bacterial load was performed by qPCR using primers (Sigma-Aldrich, Sydney, Australia) specific to conserved regions of the 16S ribosomal RNA gene. Enumeration of staphylococci was achieved using primers specific to the *tuf* gene, and *mecA+* bacteria were quantified using a separate qPCR assay specific to the *mecA* gene (Table 1). Specificity of these primers was assessed against pure reference bacteria by PCR.

qPCR reactions were performed in a total volume of 10 µL, and contained 1 µL DNA (1-100 ng DNA/µL), 0.8 µL forward and reverse 16S universal primer (0.23 µM) or 0.91 µL forward and reverse *tuf* or *mecA* primers (0.26 µM) and 5 µL SYBR Green (Thermo Fisher Scientific, Sydney, Australia). PCR reactions were performed using a QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Sydney, Australia) with the following parameters: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C (*tuf*) or 58°C (*mecA*) or 60°C (16S) for 60 seconds. The final melt curve program was 95°C for 15 seconds, 55°C (*Tstag*) or 58°C (*mecA*) or 60°C (16S) for 60 seconds and 95°C for 15 seconds. Samples and standards were assayed in triplicate. Final outcome was reported as DNA yield (amount of DNA (ng)/amount of faecal material (ng)).
Table 1. PCR primers for qPCR assay to quantify total bacteria, *Staphylococcus* spp. and meca+ bacteria in DNA extracts from stool samples

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S: Universal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amplification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qB1114</td>
<td>5'-CGG CAA CGA GCG CAA CCC-3'</td>
<td>130</td>
<td>Denman, 2006</td>
</tr>
<tr>
<td>qB1275</td>
<td>5'-CCA TTG TAG CAC GTG TGT AG CC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tuf:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>specific</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TStaG422</td>
<td>5'-CGT GTT GAA GTG CGT CAA ATC A-3'</td>
<td>370</td>
<td>Martineau, 2001</td>
</tr>
<tr>
<td>TStaG765</td>
<td>5'-CAT TTC AGT TCC TTC TGG TAA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>meca: Methicillin resistance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>meca1</td>
<td>5'-TGG CTA TCG TGT CAC AAT CG-3'</td>
<td>310</td>
<td>Vannuffel, 1995</td>
</tr>
<tr>
<td>meca2</td>
<td>5'-CTG GAA CTT GTT GAG CAG AG-3'</td>
<td></td>
<td>and Ryffel 1990</td>
</tr>
</tbody>
</table>

Antibiotic, probiotic and antifungal medication exposure

Information on antibiotic, antifungal and probiotic exposure was recorded from the WCH case note files for each participant. This information was not available for research purposes for one participant. Date, type of medication, dose in mg/kg/day, final dose and time(s) of administration were recorded for each participant between baseline and study end. The type of probiotic used in the NICU and SCBU at the WCH was standard for all infants. If parents consented, the infant received Infloran (supplying *Bifidobacterium bifidum* and *Lactobacillus acidophilus*) from approximately first enteral feed to 36 weeks PMA. Classification of antibiotic and antifungal medications for the purposes of this study are outlined in Table 2.
Table 2. Classification of antibiotic and antifungal medications administered to preterm infants enrolled in the N3RO nested study

<table>
<thead>
<tr>
<th>Group</th>
<th>Class</th>
<th>Type (generic names)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic</td>
<td>β-lactam</td>
<td>penicillin (penicillin, amoxicillin, flucloxacillin, piperacillin-tazobactam, ampicillin), carbanapenem (meropenem), cephalosporin (cefotaxime, cephalexin, ceftazidime, cefazolin)</td>
</tr>
<tr>
<td></td>
<td>Aminoglycoside</td>
<td>gentamycin</td>
</tr>
<tr>
<td></td>
<td>Glycopeptide</td>
<td>vancomycin</td>
</tr>
<tr>
<td></td>
<td>Macrolide</td>
<td>azithromycin</td>
</tr>
<tr>
<td></td>
<td>Sulfonamide</td>
<td>trimethoprim</td>
</tr>
<tr>
<td></td>
<td>Miscellaneous</td>
<td>metronidazole, mupirocin</td>
</tr>
<tr>
<td>Antifungal</td>
<td>Oral/IV</td>
<td>fluconazole, nystatin, voriconazole, amphotericin</td>
</tr>
<tr>
<td></td>
<td>Topical</td>
<td>clotrimazole</td>
</tr>
</tbody>
</table>

Statistical analysis

Differences in baseline patient characteristics and clinical outcomes were assessed using an independent samples t-test or ANOVA, as appropriate, in SPSS v24. Gene expression outcomes were expressed as percent of the total bacteria detected (TBD) by the universal primer (log-transformed). Staphylococcus spp. and mecA+ bacteria over time were analysed using mixed-effects linear regression models. Treatment group and time (postnatal days) were modelled as fixed effects. Repeated measures over time were accounted for by including a random intercept and slope for each infant. An additional random effect was included to account for clustering due to multiple births. Time was modelled as a continuous variable. A treatment-by-time interaction term was included in the model to test for differences between groups in pattern of change over time. Rate of change in Staphylococcus spp. and mecA+ bacteria in increments of seven days postnatal was assessed within groups and between groups.

In order to investigate differences between birth mode and change over time in Staphylococcus spp. and mecA+ bacteria (expressed as percent of TBD, log-transformed), linear mixed models were fitted with a random intercept and slope. Full enteral feeds was assessed as a predictor for
the change over time in *Staphylococcus* spp. and *mecA+* bacteria (expressed as percent of TBD, log-transformed). All mixed effects were modelled in SAS version 9.3.

**RESULTS**

*Patient characteristics and clinical outcomes of participants*

A total of 41 neonates were recruited between March 2014 and October 2015 for this stool analysis sub-study as part of the single-centre nested study in the N3RO RCT. There were 21 infants in the intervention group and 20 in the control group (Figure 1). A total of 221 stool samples were collected from these infants for analysis. Postnatal day of collection ranged from day four to day 87. An average of five samples was collected from each infant (range: one to nine samples). Baseline patient characteristics recorded in the CRF are detailed in Table 3 and were not significantly different between intervention and control groups. The clinical outcomes for the N3RO nested study participants at study end were also obtained from the CRF and are detailed in Table 4. These characteristics were not significantly different between intervention and control groups.
Figure 1. Flow diagram for the infants on whom stool analysis was conducted in the N3RO nested study according to the CONSORT statement.
Legend to Figure 1. Abbreviations: docosahexaenoic acid (DHA), postmenstrual age (PMA).
Table 3. Baseline patient characteristics of neonates enrolled in the N3RO nested study

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>Intervention (DHA) (n=21)</th>
<th>Control (no DHA) (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>10/11</td>
<td>11/9</td>
</tr>
<tr>
<td>Average gestational age</td>
<td>26 27/7</td>
<td>26 47/7</td>
</tr>
<tr>
<td>Gestational age &lt; 27 weeks</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Singleton/multiple birth</td>
<td>14/7</td>
<td>14/6</td>
</tr>
<tr>
<td>Mode of delivery (vaginal/C-section)</td>
<td>14/7</td>
<td>10/10</td>
</tr>
<tr>
<td>Maternal steroids</td>
<td>20</td>
<td>17</td>
</tr>
</tbody>
</table>

Legend to Table 3. There were no differences in baseline characteristics between groups. Abbreviations: docosahexaenoic acid (DHA).

Table 4. Clinical outcomes of neonates enrolled in the N3RO nested study

<table>
<thead>
<tr>
<th>Outcome at study end</th>
<th>Intervention (DHA) (n=21)</th>
<th>Control (no DHA) (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent (%) compliance (total doses received/total possible doses)</td>
<td>90.2%</td>
<td>90.5%</td>
</tr>
<tr>
<td>Postnatal steroids</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Necrotising enterocolitis</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Sepsis</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Bronchopulmonary dysplasia</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Surgery</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Days of parenteral nutrition</td>
<td>29</td>
<td>24.5</td>
</tr>
<tr>
<td>Days of intravenous lipids</td>
<td>21.5</td>
<td>18.3</td>
</tr>
<tr>
<td>Days to reach full enteral feeds</td>
<td>22.0</td>
<td>19.3</td>
</tr>
<tr>
<td>Type of enteral feed at discharge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d. breast milk</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>e. formula</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>a. both</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Legend to Table 4. There were no differences in clinical characteristics between groups. Abbreviations: docosahexaenoic acid (DHA).

Changes in total bacteria, Staphylococcus spp. and carriage of mecA gene during the neonatal period

Mean TBD as a composite of data collected from all infants, increased from the first week to a maximum at approximately postnatal day 21 (Figure 2A). After three weeks, an overall decrease in mean TBD was seen resulting in approximately half of the level at postnatal day 21 by study end. Individual data from five infants with more than five samples collected in each group was selected as a representation of the diversity in TBD, Staphylococcus spp. and mecA+.
bacteria yield from stool samples between infants (Supplementary Figure S4). Of the 35 infants that had a sample collected both before and after postnatal day 21, 22 infants’ peak TBD occurred on or before postnatal day 21.

Mean *Staphylococcus* spp. yield and *meca*+ bacteria as a composite of data from all infants also reached a peak around postnatal day 21 before decreasing until study end (Figure 2B and 2C). Of the 35 infants who had a sample collected both before and after postnatal day 21, 22 infants’ peak *Staphylococcus* spp. levels and 27 infants’ *meca*+ bacteria levels reached a maximum before postnatal day 21. *meca*+ bacteria levels closely mirrored that of *Staphylococcus* spp. (r=0.84, P<0.0001, Supplementary Figure S2 and S3) with the exception of some instances where *meca*+ bacteria levels were greater than *Staphylococcus* spp. levels.
Figure 2. Yield of total bacteria, *Staphylococcus* spp. and *mecA*+ bacteria in stool samples collected from 41 infants.

Legend to Figure 2. Yield of A) Total bacteria detected by the universal 16S primer, B) *Staphylococcus* spp., and C) *mecA*+ bacteria in stool samples. Samples were collected between postnatal day 4 and 87. Yield= ng DNA/ng faecal material
Effect of omega-3 on Staphylococcus spp. and mecA+ bacteria levels in faecal samples over time

Within both intervention and control groups, a significant effect over time was observed for relative abundance (% of TBD) of both Staphylococcus spp. and mecA+ bacteria (Table 5). There was approximately 21% lower relative abundance of Staphylococcus spp. with each seven day increment in the control group and 29% in the intervention group. For measures taken one week apart, mecA+ bacteria levels significantly decreased by approximately 55% in the control group and 40% in the intervention group (Table 5). Rate of change over time in relative abundance of Staphylococcus spp. and mecA+ bacteria was not significantly different between groups (Table 5).

Table 5. Estimated weekly change in relative abundance of Staphylococcus spp. and mecA+ bacteria in faecal samples

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Adjusted estimate (95% CI)</th>
<th>Adjusted P value</th>
<th>Adjusted ratio (Intervention/Control)</th>
<th>Adjusted interaction P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Relative abundance (% of TBD) of <em>Staphylococcus</em></strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intervention</td>
<td>0.71 (0.58, 0.86)</td>
<td>0.001</td>
<td>0.90 (0.67, 1.20)</td>
<td>NS</td>
</tr>
<tr>
<td>Control</td>
<td>0.79 (0.64, 0.98)</td>
<td>0.031</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Relative abundance (% of TBD) of mecA+ bacteria</strong>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intervention</td>
<td>0.60 (0.48, 0.75)</td>
<td>P&lt;0.001</td>
<td>1.07 (0.77, 1.50)</td>
<td>NS</td>
</tr>
<tr>
<td>Control</td>
<td>0.56 (0.44, 0.71)</td>
<td>P&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legend to Table 5. Relative abundance refers to percent (%) of total bacteria detected in faecal samples collected from intervention (DHA) and control (no DHA) groups. Adjusted estimates give the estimated change in outcome corresponding to a seven day increase in postnatal days (ratio of medians). The adjusted ratios present the estimated difference between treatment groups in change over time (ratio of ratios). Abbreviations: docosahexaenoic acid (DHA), total bacteria detected (TBD).
The effect of birth mode and enteral feeding on trajectory of Staphylococcus spp. and mecA+ bacteria over time

Decrease in bacterial carriage of the mecA gene over time was significantly higher (P<0.001) in infants that were delivered vaginally compared to Caesarean section. Birth mode did not have a significant effect on staphylococci levels over time. There was no significant interaction observed between treatment group and birth mode. Time to reach full enteral feeds significantly influenced trajectory of mecA+ bacteria in the control group (P<0.05) but not intervention. Time to reach full enteral feeds was not significantly associated with changes in staphylococci trajectory over time.

Antibiotic, probiotic and antifungal medication exposure

All but two infants received probiotics during at least one postnatal week. Days of antibiotic, probiotic and antifungal medication exposure are detailed in Supplementary Table S1. Table S1 details exposure from postnatal week one to six only because less than 10 infants received antibiotics after postnatal week six. All but one infant had at least one episode of antibiotics over the course of the study; most commonly within the first days to week of life and most often a combination of penicillin/gentamycin. Depending on their medical condition thereafter, infants received additional course(s) of antibiotics, most commonly a combination of either vancomycin/cefotaxime or penicillin/cefotaxime. Distribution of antibiotic types administered to infants (as detailed in Table 2) were consistent between intervention and control groups for each week.

Patterns of Staphylococcus spp. and mecA+ bacteria recovery following antibiotic administration were not consistent between infants. Antibiotic exposure appeared to exert little to no obvious effect on Staphylococcus spp. and mecA+ bacteria in some infants and caused drastic shifts in others within days. For example, infant 2 received antibiotics on postnatal day 1-8 (penicillin, gentamycin, flucloxacillin), day 18-23 (piperacillin+tazobactam, vancomycin),
day 29-31 (piperacillin+tazobactam, vancomycin), day 36-38 (gentamycin, flucloxacillin), day 46-48 (ampicillin, gentamycin) and day 53-56 (flucloxacillin). No obvious transitions in *Staphylococcus* spp. or mecA+ bacteria levels were observed on/around these particular postnatal days (Figure S4). Infant 5 received antibiotics on postnatal day 2-13 (penicillin, gentamycin, cefotaxime, ampicillin, vancomycin, flucloxacillin), day 27-29 (gentamycin, flucloxacillin), day 40-42 (gentamycin, vancomycin). An overall reduction in *Staphylococcus* spp. and mecA+ is seen after postnatal day 30 in infant 5 (Figure S4). Similar inconsistencies in antibiotic exposure and *Staphylococcus* spp. and mecA+ bacteria levels were observed for other infants and no obvious patterns were identified.

**DISCUSSION**

Results of the present study show that while preterm infants experience a significant decrease in relative abundance of *Staphylococcus* and mecA+ bacteria between the first days of life and 36 weeks PMA, the change over time was not affected by omega-3 LCPUFA. A high degree of diversity was observed in the levels of *Staphylococcus* spp. and mecA+ bacteria in stool samples collected from infants in this study. Our data are consistent with reports that preterm infants exhibit a high prevalence of *Staphylococcus* and that the highest incidence of mecA gene carriage is in very low birth weight infants. In many infants, staphylococci accounted for a large proportion of the total bacterial detected early in the postnatal period and declined with time. This decline over time likely reflected an increased colonisation with other bacterial species not assessed as part of this study.

We hypothesised that omega-3 LCPUFA may act to reduce inflammation in the gut, which could favour adherence of non-pathogenic bacteria to mucosal surfaces and reduce levels of opportunistic pathogens such as *Staphylococcus* and mecA+ bacteria. In adults and animals, non-pathogenic bacterial flora are known to inhibit binding to epithelial cells and colonisation by pathogenic bacteria. Animal and *in vitro* studies highlight the potential role of omega-
3 LCPUFA in the maintenance of gut integrity and epithelial barrier function. \(^{178, 192, 193}\) LCPUFA in the diet have also been reported to influence the diversity of the microbiome in adults, \(^{258}\) infants, \(^{190, 191}\) and in animals. \(^{145, 192}\) Neutral and negative effects on microbial colonisation patterns in response to omega-3 LCPUFA supplementation have also been reported. In a mouse model, omega-3 LCPUFA has increased survival in *Staphylococcus aureus*-induced sepsis and decreased bacterial load. \(^{268}\) While omega-3 LCPUFA did not influence the colonisation patterns of staphylococci or *meca*+ bacteria in our sample, the positive results reported in other studies in the literature \(^{190, 191}\) may have been due to omega-3 LCPUFA-induced promotion of an environment more favourable to commensal bacteria. Further investigation of the relationship between omega-3 LCPUFA and other bacterial species is warranted to confirm any protective effects.

Early introduction of enteral feeds, with breast milk as the preferred source, is standard practice in NICUs worldwide. Breast milk has a substantial influence on patterns of bacterial colonisation because it contains its own microbial flora. \(^{184, 258, 269}\) Furthermore, breast milk possesses anti-infective properties and can differentially effect the bacterial composition in the gut compared to infant formula. \(^{254, 269}\) The average number of days to reach full enteral feeds in this population was 21 days, coinciding with the timeline on which both staphylococci and *meca*+ bacteria levels reached a maximum and subsequently declined. Consistent with other reports of feeding-influenced changes in gut bacteria profile \(^{270, 271}\), our data suggest an inverse association between enteral feeding and levels of both staphylococci and *meca*+ bacteria.

It is the abundance and virulence potential of *Staphylococcus* that determines its pathogenicity, not mere presence alone. \(^{254}\) Colonisation of *Staphylococcus* and *meca*+ bacteria are risk factors for systemic infection \(^{263, 265}\), but the relationship between abundance and infection risk has not been fully described in a preterm infant. \(^{272}\) *Staphylococcus* colonisation can be disrupted by factors associated with preterm birth such as exposure to antibiotics. \(^{23, 254, 273}\) In a preterm infant, disruptions in the establishment of the microbiome as a whole can result in failure to
achieve immune homeostasis and can also affect intestinal mucosal barrier function \(^{189}\). The type of antibiotic, dose, duration and timing of introduction can all result in differential effects on bacterial diversity \(^{271, 274}\). A wide variation in antibiotic exposure was observed for the neonates in the current study and data did not reveal a clear pattern between antibiotic exposure and staphylococci or carriage of the meCA gene.

To our knowledge, this is the first study to assess the effect of supplemental omega-3 LCPUFA on levels of *Staphylococcus* spp. and bacteria carrying the meCA gene in preterm infants < 29 weeks gestation. A strength of this study is the high-quality design of the RCT and the collection of weekly stool samples allowing detection of early changes in colonisation patterns. While sample sizes for analyses of microbial species in the gastrointestinal tract range from a single participant \(^{275}\) to several hundred participants \(^{276}\), our sample size is comparable to recent studies assessing microbial species in preterm and/or very-low-birthweight infants \(^{263, 277, 278}\). A limitation of this sub-study is the evaluation of only a single genus which does not allow for interpretation of how the microbiome as a whole is influenced by omega-3 LCPUFA. Outside of the context of this manuscript, our group is currently collaborating to examine the effect of omega-3 LCPUFA supplementation on transitions in overall species composition over time in this group of infants. Secondly, a limitation of collecting the sample from the nappy is that it has been exposed to the infants’ perineal skin flora. However, collecting a sample that has not been exposed to the perineal area is not practical for preterm infants and is therefore a limitation of many microbial analysis studies using faecal matter.

Lack of research to map the gut microbiome in preterm infants makes it difficult to compare the results of this study with the “normal” flora of a preterm infant \(^{279}\). While such a high proportion of meCA+ bacteria was an unexpected finding, it does not necessarily confirm the presence of a methicillin-resistant bacterial infection, rather it eludes to the potential for such an infection. It is important to note that the expression of meCA is inducible and that it is also not uncommon for populations of *Staphylococcus* and other bacteria to express this gene \(^{280}\).
The diversity and abundance of antibiotic resistant genes, the antibiotic resistome, is not well characterised in preterm infants \(^{281, 282}\). However, even healthy preterm infant gut microbiomes has been found to harbour a wide diversity of antibiotic resistant genes, including \(\text{mecA}^{282}\). The antibiotic resistome observed in the early postnatal period likely reflects the exposure of bacteria to other habitats, such as bacteria acquired by the mother from her surroundings \(^{281}\) and the effect of nutrition in the postnatal period on this pre-established antibiotic resistome is yet unknown.

Interactions between host and bacterial species in the gut in the early postnatal period are critical for proper immune system development and maturation \(^{189, 274, 283-285}\). Nutritional modulation of microbial species directly or indirectly via a modulation to the mucosal environment in the neonatal gastrointestinal tract is a promising approach to the prevention and treatment of clinical conditions with various aetiologies \(^{286}\). While plausible biological mechanisms exist for LCPUFA to effect change in the gastrointestinal environment, our data did not reveal an effect of enteral supplementation with omega-3 LCPUFA on \(\text{Staphylococcus} \text{spp.} \text{ or meca}^{+}\) bacteria in preterm infants. Mechanistic studies of other dietary interventions and their impact on the relationship between the composition of the gut microbiome, immune development and health outcomes in the preterm infant are important next steps.
SUPPLEMENTARY DATA

Supplementary Figure 1 (S1). Total bacteria detected and *Staphylococcus* spp. in stool samples collected from 41 infants.
Legend to Figure S1. Samples were collected between postnatal day 4 and 87. DNA yield = ng DNA/ng faecal material.

Supplementary Figure 2 (S2). *Staphylococcus* spp. and *mecA*+ bacteria in stool samples collected from 41 infants.
Legend to Figure S2. Samples were collected between postnatal day 4 and 87. DNA yield = ng DNA/ng faecal material.
Supplementary Figure 3 (S3). Correlation between *Staphylococcus* spp. and meca+ bacteria yield in stool samples collected from 41 infants.

Legend to Figure S3. Samples were collected between postnatal day 4 and 87. DNA yield = ng DNA/ng faecal material. Spearman $r=0.84$ and $P<0.0001$
Supplementary Figure 4 (S4). Profiles of total bacteria, *Staphylococcus* spp. and *mecA*+ bacteria yield in stool samples collected from 10 infants.

**Infant 2: Intervention**

**Infant 3: Intervention**
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Infant 5: Intervention

Infant 9: Control
Infant 10: Control

Infant 13: Intervention
**Infant 23: Control**

![Graph 1](image1)

**Infant 25: Control**

![Graph 2](image2)
Legend to Figure S4. Samples were collected between postnatal day 4 and 87 from 10 infants; five in each of the intervention (DHA) and control (no DHA) groups. DNA yield = ng DNA/ng faecal material. “*” = axis was extended to account for higher values.
Supplementary Table 1 (S1). Antibiotic, probiotic and antifungal medication exposure in infants in intervention and control groups between postnatal weeks one to six

<table>
<thead>
<tr>
<th>Postnatal week (n Intervention/n Control)</th>
<th>Days antibiotic exposure Mean, (95% CI)</th>
<th>Days probiotic exposure Mean, (95% CI)</th>
<th>Days antifungal exposure Mean, (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intervention</td>
<td>Control</td>
<td>Intervention</td>
</tr>
<tr>
<td>1 (n=20/n=19)</td>
<td>4.71 (3.77, 5.66)</td>
<td>4.89 (4.06, 5.73)</td>
<td>2.62 (1.64, 3.60)</td>
</tr>
<tr>
<td>2 (n=13/n=11)</td>
<td>2.55 (1.32, 3.78)</td>
<td>2.47 (1.16, 3.79)</td>
<td>5.00 (3.60, 6.40)</td>
</tr>
<tr>
<td>3 (n=9/n=8)</td>
<td>1.74 (0.67, 2.80)</td>
<td>1.21 (0.24, 2.18)</td>
<td>5.68 (4.58, 6.79)</td>
</tr>
<tr>
<td>4 (n=7/n=7)</td>
<td>1.32 (0.21, 2.42)</td>
<td>1.32 (0.30, 2.33)</td>
<td>5.84 (4.77, 6.91)</td>
</tr>
<tr>
<td>5 (n=8, n=6)</td>
<td>1.47 (0.37, 2.58)</td>
<td>1.21 (0.19, 2.23)</td>
<td>6.00 (4.95, 7.05)</td>
</tr>
<tr>
<td>6 (n=8, n=4)</td>
<td>1.72 (0.59, 2.85)</td>
<td>0.89 (-0.07, 1.85)</td>
<td>4.83 (3.58, 6.09)</td>
</tr>
</tbody>
</table>

Legend to Table S1. Mean days of exposure to antibiotic, probiotic and antifungal medication exposure in the intervention (DHA) and control (no DHA) groups. Abbreviations: docosahexaenoic acid (DHA).
CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

Preterm infants are immunologically immature and have an increased risk of developing inflammatory disorders in the postnatal period. Importantly the omega-3 LCPUFA DHA has been shown to attenuate inflammation in both adult and animal models. Supplementation of the diet with omega-3 LCPUFA may also provide an immunomodulatory benefit in preterm infants, but data is lacking. To address this gap in knowledge, this thesis examined the immunomodulatory potential of supplemental DHA in preterm infants born < 29 weeks as part of a single-centre nested study in the N3RO RCT.

5.1. Main findings reported in this thesis

In type II AECs:

An in vitro assessment of the effect of enteral DHA versus LA emulsions on cytokine release revealed that DHA attenuated the release of some of the pro-inflammatory cytokines associated with the development of BPD (IL-1β, IL-8 and IFNγ) compared with LA and ALA in soy-based emulsions.

In neonates:

- At a supplemental dose of 60 mg/kg/day, DHA neither attenuated the release of pro-inflammatory cytokines nor upregulated regulatory cytokines in blood.
- DHA supplementation resulted in a significant reduction in immunoregulatory SP-D levels in plasma at study end compared to control.
- The levels of *Staphylococcus* and bacteria carrying the gene coding for methicillin resistance (*meca*) in stool were not affected by higher dose enteral DHA when compared with standard feeding practice.
Evidence suggests that feeding is an important contributor to the diversification of the bacterial composition in the gut. Marked reductions in *Staphylococcus* and *mecA*+ bacteria levels in stool coincided with the average number of days to reach full enteral feeds in the study population, a trend worthy of further investigation.

- Days of antibiotic exposure or type were not different between infants supplemented with DHA and those that were not.

### 5.2. General discussion

#### 5.2.1. Supplemental DHA did not reduce the burden of inflammatory mediators

The N3RO RCT revealed that in 1273 infants born < 29 weeks that a high DHA enteral emulsion increased the frequency of BPD compared with control (RR 1.13, 95% confidence interval 1.02 to 1.25; P=0.02, unpublished data). This outcome was despite the plausible biological mechanisms exist supporting the hypothesis that DHA may actually attenuate inflammation and thus protect against BPD in the preterm infant [10, 34, 95, 102, 105, 221, 287]. The unexpected increase in BPD resulting from DHA supplementation in preterm infants enrolled in the N3RO RCT was also not in accordance with results from other omega-3 LCPUFA intervention trials in preterm infants.

The extensive evaluation of immune markers undertaken in this thesis provide some insights into the mechanisms behind the unanticipated clinical outcome of the N3RO RCT. The results of the nested study in the N3RO RCT revealed that the mechanism underlying the increase in BPD was not due to an increase in pro-inflammatory mediators. The argument could be made that the high-dose DHA exerted an immunosuppressive effect [144, 288], but this is not supported by the absence of between-group differences in cytokine levels, especially the regulatory IL-10 or TFGβ1. Furthermore, an immunosuppressive effect of DHA would have been reflected in the incidences of other clinical outcomes (sepsis, NEC, ROP, overall mortality) which were also not different between groups. Hence, the mechanism underpinning the effect of high-dose DHA on BPD occurrence is more likely localised to the lung.
5.2.2. Surfactant protein D and immunoregulation

The significant decrease in SP-D at study end in the intervention group may be important when considering the BPD outcome of the N3RO RCT. Relevant to BPD and other respiratory disorders, SP have been reported to enhance pathogen clearance and regulate adaptive and innate immune cell functions 167, 170, 225, 289, 290. Surfactant proteins also have the potential to exert a systemic immunomodulatory effect as they are expressed in non-pulmonary sites 168, 250. The immunoregulatory potential of SP-D was not assessed as part of this thesis as the aim was to assess the effect of DHA supplementation on SP-D levels as an indicator of pathological lung changes. The decrease in SP-D levels observed in the intervention group in the nested study could have been indicative of impaired host defence mechanisms in the lung 167, 173, 248. The mechanism underlying the reduction in SP-D in the intervention group at study end is not clear. While there is no supporting data in the literature to suggest an association between omega-3 LCPUFA and SP levels, one plausible biological mechanism to explain the decrease in SP-D is via omega-3 LCPUFA-induced changes in the lipid composition of surfactant. In the case of a cell membrane, incorporation of certain LCPUFA into the membrane can result in an alteration in how proteins associate with the cell membrane 133, 291. Slight changes in the lipid composition of surfactant due to LCPUFA supplementation 292, 293 may also result in structural differences and an altered affinity and expression at the cell surface for certain SPs over others.

The relationship between supplemental LCPUFA and surfactant production was not assessed in this thesis but deserves further attention in light of the increase in BPD associated with DHA.

5.2.3. Enteral DHA does not appear to influence Staphylococcus or mecA+ bacteria levels

For omega-3 LCPUFA to elicit changes in the bacterial species present in the gut, literature suggests that it would likely act via an improvement in gut integrity and epithelial barrier function 178, 192, 193 or influence mucosal inflammation 294. This is important because these mechanism are of relevance for pathogen colonisation in the early postnatal period for preterm infants. Analysis of Staphylococcus and bacteria carrying the gene for methicillin-resistance in
stool samples in preterm infants did not reveal a benefit for DHA supplementation as DHA did not affect prevalence or abundance of these bacteria.

Omega-3 LCPUFA have been reported in the literature to exert a protective effect against sepsis and NEC. However, the incidence of sepsis, NEC and other clinical outcomes in which bacteria in the gastrointestinal tract could play a role in disease development and/or progression were not different between intervention and control groups in both the nested study and the N3RO RCT. DHA did not appear to exert a direct influence over *Staphylococcus* and mecA+ bacteria levels, which have been associated with neonatal inflammatory outcomes. Additionally, an indirect effect of DHA on these pathogens via a reduction in mucosal inflammation was unlikely to have occurred as inflammatory mediators measured in plasma were not different between groups. Further study is warranted on nutritional modification of the other species in the microbiome and resulting disease states; an emerging area of interest with important implications for preterm infants.

5.2.4. Comparing and contrasting the results from cell culture experiments and preterm infants
The results assessing the immunomodulatory capability of the DHA emulsion in A549 cells satisfied the hypothesis that if DHA was immunomodulatory, it was acting at least in part, via a reduction in pro-inflammatory mediators. While the immunomodulatory potential for the enteral emulsion preparations used in the N3RO RCT have not been assessed previously *in vitro*, the results in this thesis are consistent with other *in vitro* assessments of omega-3 DHA and omega-6 LA in A549 cells. It is important to discuss the limitations of cell culture experiments which may account for the differences observed between the *in vitro* and *in vivo* results in this study.

Firstly, *in vitro* experiments offer valuable mechanistic insight and allow for manipulations that are not possible *in vivo*. However, there are limitations in interpretation of cell culture experiments in that cell culture does not reflect the whole body environment. Secondly, the
dose administered enterally to N3RO RCT participants could not be mimicked in cell culture of type II AECs as the concentration of DHA in the lung environment in preterm infants after enteral supplementation is unknown. Instead, a defined, nontoxic dose was used in vitro. Thirdly, A549 cells were chosen primarily because type II AECs are affected in respiratory disease, and they produce SPs and cytokines in proportion to inflammatory stimuli \(^\text{17,298}\). While the A549 cells secreted cytokines in response to TNF\(\alpha\) stimulation, SP-D was not secreted in detectable limits. A lack of SP-D secretion has been previously reported in A549 cells \(^\text{17,299}\). The cytokine response to stimulation was of primary interest and was comparable to results reported in the literature for A549 cells and omega-3/omega-6 exposure \(^\text{207,220,297}\).

Lastly, although A549 cells have been used extensively as a model for type II AEC, they are not of neonatal origin. A comparable cell line in the neonatal stage of development was not commercially available. Thus, A549 cells represent the most appropriate model for this assessment. Overall, these are common limitations in cell culture experiments but do not negate the usefulness of the preliminary investigation in vitro to assess the immunomodulatory efficacy of enteral emulsions in A549 cells in this thesis. The cell culture analyses were conducted prior to any assessment of samples in preterm infants and therefore were useful to inform the choice of inflammatory biomarkers to be assessed in preterm infants in this thesis.

5.3. Strengths and limitations of the thesis

Based on projected recruitment rates at the WCH when the nested study was proposed, it was estimated that more infants would have been enrolled in the study. Several other studies commenced in the WCH NICU around the same time and this may have contributed to slower recruitment rates than anticipated. It could be argued that a limitation of this thesis is sample size, however, 51 infants is comparable to other studies in which cytokine responses \(^\text{10,217,227}\) and bacteria levels \(^\text{263,277,278}\) have been assessed in preterm infants.

With regards to stool analysis, it is recognised that *Staphylococcus* is just one constituent of a very complex and diverse microbiome. A limitation of examining only a single genus is that...
the effect of changes in the abundance of other bacterial species in the gut on *Staphylococcus* levels is not known. An investigation of the microbiome as a whole was not attainable in the time frame of this thesis and therefore is being conducted outside the context of this body of work. Given that *Staphylococcus* is predominant in the early postnatal period and a major pathogen in preterm infants, it represented a logical genus to examine as a first step within this thesis and the results will be used to inform next steps.

DHA has been postulated to interact with a wide variety of targets in adult and animal models. These targets include cytokine production and downstream signalling, gene expression, eicosanoid production, resolvins, and the microbiome \(^7, 221\). The targets with which DHA interacts in a preterm infant have not been well-described. Given the focus on inflammation and the role of pro-inflammatory and regulatory cytokines in the pathogenesis of neonatal inflammatory disorders in this thesis, an extensive evaluation of cytokines as a potential target for immunomodulation by DHA was undertaken in this thesis. While the aforementioned targets are deserving of further investigation and together would make for a broader understanding of the role of LCPUFA in infant nutrition, all could not be examined with such limited sample volume and within the time frame of the thesis. Thus, the small blood volume (0.5 mL) that could be safely and ethically obtained from preterm infants for research purposes is an unavoidable limitation.

However, the information generated from the small blood volume was maximised through the use of multiplex assays and a secondary whole blood stimulation experiment. The extensive immunological evaluation of a large number of pro-inflammatory mediators and regulatory factors afforded by multiplex assays is a major strength of this thesis. Further, collection of stool samples allowed for an examination of the effect of enteral LCPUFA supplementation on levels of a common pathogen in the gastrointestinal tract of preterm infants. This was a non-invasive approach to maximise the information that could be generated from the nested study. Longitudinal assessment of pro-inflammatory and regulatory factors in the blood as well as
bacteria in the stool is a strength of this thesis as both early changes and long-term effects of DHA supplementation could be examined.

A major strength of this study is the robust evidence provided due to the high-quality design of the RCT and the ability to confirm the success of the intervention via fatty acid profiles in the blood. Extensive clinical information was also recorded in the CRF alongside the collection of biological samples in the nested study. The clinical data along with the outcomes of the parent N3RO RCT allowed for the nested study results to be interpreted through the larger picture of inflammatory disorders in the postnatal period.

5.4. Research questions raised by this thesis and direction for future research
5.4.1. Does DHA have a dose-response effect in preterm infants?
Differences in response to supplementation with DHA and other omega-3 LCPUFA between adults and preterm infants are likely explained by fundamental differences in the immune stages between these age groups. It is most appropriate then, to compare results of this study only with others that have been conducted in preterm infants. Taking care not to ignore the beneficial effects reported for DHA supplementation in preterm infants in the literature, another plausible biological mechanism to explain differences in cytokine response between existing enteral supplementation studies 13, 14 and those presented in this thesis is a U-shaped dose-response effect for DHA.

The 60 mg/kg/day of DHA supplied in the N3RO RCT was in addition to the DHA that infants were receiving in breast milk, omega-3 LCPUFA-supplemented formula, and/or lipid emulsions. Infants received enteral DHA in formula or breast milk in the trial reported by Field et al. (2000) and Lopez-Alarcon et al. (2012) used 100 mg pure fish oil in addition to breast milk/formula/lipid emulsions in their trial. While exact estimates for mg/kg/day of DHA cannot be calculated, it would be expected that 100 mg pure fish oil in addition to other nutrition sources 14 would be slightly higher than the total DHA received by infants in the N3RO RCT and that DHA in breast milk and/or LCPUFA-supplemented formula 13 would be slightly less.
Some pro-inflammatory cytokines were reduced at a higher dose of DHA than in the N3RO RCT\textsuperscript{10,14} and the regulatory cytokine IL-10 was increased with a potentially lower dose\textsuperscript{13}.

Given that there are only a few studies that are suitable for comparison to the results in this thesis, this is merely a suggestion for a plausible biological mechanism to explain differences in cytokine response to DHA and further studies would be needed to test this hypothesis. While there may well be a niche for DHA to exert a beneficial effect in certain populations, it is evident that a supplemental dose of 60 mg/kg/day does not confer any benefit in preterm infants. The results of this nested study and the N3RO RCT clearly highlight a need to determine the threshold dose at which DHA exerts a negative effect on lung immunoregulation and/or lung development in a preterm infant to better inform recommended dietary intake during the postnatal period.

5.4.2. Can DHA influence the immunoregulatory capacity of surfactant?
Literature is lacking for the effect of LCPUFA supplementation on the lipid and protein composition of surfactant in preterm infants and resulting immunoregulatory properties. This is significant because decreased SP-D has been described as a predictor of BPD\textsuperscript{173} and the results presented in this thesis indicate that omega-3 LCPUFA-mediated effects on the preterm infant lung deserve more research. An examination of the effect of LCPUFA supplementation on other surfactant proteins and lipids that act as ligands within the innate immune system would provide more insight into this relationship\textsuperscript{164,213}. Other surfactant proteins, namely SP-A and more recently SP-B and SP-C, have also been reported to modulate the immune response in the lung to protect against heightened inflammatory responses that could damage the lung\textsuperscript{164,172,225,298}. Lipids in surfactant, in particular phosphatidyl glycerol\textsuperscript{301} and DPPC\textsuperscript{302,303} have demonstrated an ability to influence aspects of the innate immune response. In light of this data and other studies assessing PUFA on respiratory outcomes\textsuperscript{292,293}, it is possible that lipids such as omega-3 LCPUFA exert an effect on the composition of surfactant lipids as well. Given the high rate of BPD and requirement for respiratory support in preterm infants\textsuperscript{29,110}, determining
whether nutritional modifications can influence the lipid and/or protein composition of surfactant to protect against neonatal respiratory disease would be of benefit in clinical care.

5.4.3. Can DHA program the immune response?
Further work based on the outcomes of this thesis could focus on examining differences in gene expression and transcriptional reprogramming of immunity in immune cells in preterm infants. Investigation of the effect of DHA supplementation on peroxisome proliferator-activated receptor (PPAR) γ, G-protein coupled receptor (GPR) 120 and toll-like receptor (TLR) 2 and 4 would be appropriate starting points in preterm infants given the knowledge basis in adults and animals 7, 221. An understanding of whether DHA has the potential to alter pathways and transcriptional networks, and if so, which ones, would be useful for determining the effect of DHA on disease states common to preterm infants.

5.5. Concluding remarks
This thesis provides an overview of the current understanding of omega-3 DHA supplementation in the preterm infant. Clinical studies show mostly neutral or modest beneficial effects of DHA in nutrition regimens. The few studies reporting on LCPUFA-induced changes in inflammatory biomarkers also display a modest positive effect on the immune response and oxidative stress in preterm infants. Improving clinical outcomes through dietary modifications is an attractive, non-invasive approach in preterm infants. However, the results presented in this thesis do not support the use of supplemental DHA at 60 mg/kg/day to regulate inflammation in this population. Taken together with the results of the N3RO RCT in which DHA was found to be associated with an increased incidence of BPD, a this dose appears to interfere with aspects of host lung defence and/or immunoregulation.

The results presented here are useful to begin to understand the immunological effects of higher doses of DHA. It is likely that several factors are at play in mediating both the beneficial and negative effects reported for DHA supplementation. Until the effects DHA on the immune
system of preterm infants are fully understood, careful consideration should be given to the addition of DHA to infant nutrition sources at doses approaching or exceeding 60 mg/kg/day.
CHAPTER 6

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161. Elbarbary NS, Ismail EA, Farahat RK, El-Hamamsy M. Omega-3 fatty acids as an adjuvant therapy ameliorates methotrexate-induced hepatotoxicity in children and adolescents


APPENDIX 1

CONFERENCE ABSTRACTS/PRESENTATIONS ARISING FROM DATA PRESENTED IN THIS THESIS


2. 20th Annual Congress of the Perinatal Society of Australia & New Zealand (PSANZ). Townsville, Queensland. 2016. “Immunological manipulation of the respiratory epithelium with docosahexaenoic (DHA) and linoleic acid (LA) in commercial lipid emulsions” (short oral presentation and poster).

3. The Australian Society for Medical Research: SA Scientific Meeting. Adelaide, South Australia. 2015. “An examination of the mechanisms by which docosahexaenoic acid (DHA) can influence inflammation in the preterm infant. An introduction to a nested side study in N3RO” (oral presentation).

APPENDIX 2

PUBLICATION ARISING FROM THIS THESIS

Fink NH, Collins CT, Gibson RA, Makrides M, Penttila IA. Targeting inflammation in the preterm infant: The role of the omega-3 fatty acid docosahexaenoic acid. JNIM. 2016:5;55-60.
# Statement of Authorship

**Title of Paper**  
Targeting inflammation in the preterm infant: the role of the omega-3 fatty acid docosahexaenoic acid

**Publication Status**  
- Published
- Accepted for Publication
- Submitted for Publication
- Unpublished and Unsubmitted work written in manuscript style

**Publication Details**  

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## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

1. the candidate's stated contribution to the publication is accurate (as detailed above);
2. permission is granted for the candidate to include the publication in the thesis; and
3. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Targeting inflammation in the preterm infant: The role of the omega-3 fatty acid docosahexaenoic acid

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ARTICLE INFO
Article history:
Received 4 December 2015
Received in revised form
30 March 2016
Accepted 22 March 2016
Available online 21 April 2016

KEYWORDS:
Long chain polyunsaturated fatty acid
Docosahexaenoic acid
Preterm infant
Inflammation
Immuno-regulation

ABSTRACT
Long chain polyunsaturated fatty acids are critical for the normal growth and development of preterm infants. Interest in these compounds rests in their anti-inflammatory properties. Clinical conditions with an inflammatory component such as bronchopulmonary dysplasia, necrotising enterocolitis and sepsis are risk to the survival of these infants. Dysregulation of inflammatory responses plays a central role in the aetiology of many of these neonatal disorders. There is evidence to suggest that the omega-3 long chain polyunsaturated fatty acid docosahexaenoic acid (DHA) can down-regulate local and systemic inflammation in adults and animal models; however, very little is known about its protective effects in infants, especially preterm infants. Due to their immunological immaturity, preterm infants are particularly sensitive to diseases with an inflammatory aetiology in the early postnatal period. This makes DHA supplementation immediately after birth to combat neonatal inflammation an attractive therapy. Mechanistic data for DHA use in preterm infants is lacking and results from adult and animal studies may not be relevant to this population because of fundamental immune system differences. While there is increasing evidence from randomised controlled trials to support a beneficial effect of DHA for the preterm infant, more evidence is required to establish short and long-term effects of DHA on the immune status of preterm infants.

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1. Introduction

Preterm birth, defined as birth at less than 37 completed weeks gestation, occurs in around 12% of deliveries worldwide with major implications for the long term health of the child [1, 2]. Mortality rates of preterm infants have decreased substantially over the last few decades due to advancements in medical care [1]. However, morbidity rates, particularly in the very preterm infant (born less than 28 weeks gestation) have continued to rise [1]. Functionally and immunologically immature, the very preterm infant requires intensive support, and the medical interventions necessary for their survival can trigger a local or systemic inflammatory response [3].

Preterm infants have an under-developed immunoregulatory system, therefore there is the potential for chronic inflammation to develop [4]. Dysregulation of inflammatory responses plays a central role in the aetiology of many life-threatening neonatal disorders including bronchopulmonary dysplasia (BPD), necrotising enterocolitis (NEC) and sepsis [3–7] and presents a continuing challenge to clinicians involved in their care. Interest is intensifying in dietary compounds that promote resolution of inflammation and confer a protective effect against development of neonatal inflammatory disorders [8]. There is some controversy as to whether or not preterm infants can synthesise sufficient long chain polyunsaturated fatty acids (LCPUsA) such as docosahexaenoic acid (DHA) and arachidonic acid (AA) from essential fatty acid (EFA) precursors [9–13] because genetic variants in the fatty acid desaturase genes may affect rates of synthesis of LCPUsA [13, 14]. However, all infants receive an exogenous source of EPA and/or LCPUsA, via breast milk, lipid emulsions, formula or a combination of these sources [15]. Interest in DHA supplementation and its
effect on clinical outcomes in preterm infants has escalated because these sources do not provide sufficient levels of DHA for these infants [16]. This review will focus specifically on the potential for the omega-3 LCP FA DHA to act as an immunomodulatory agent to improve clinical outcomes in preterm infants.

2. Omega-3 LCP FA and their role in early immune development

Preterm infants have a fundamentally different immune system to that of an adult or even a term infant, making them especially susceptible to an exaggerated immune activation [3]. The preterm infant relies heavily on the non-specific innate immune response for defence [4,17]. The antigen-specific adaptive immune system of preterm infants is also underdeveloped at birth, particularly with regard to T cells mediating inflammatory responses (helper 1: Th1) and the important T cells involved in regulating the immune response (regulatory T cells) [13,18–20]. Ineffective early T cell function after birth, when the infant is exposed to a massive environmental antigenic onslaught from the birth process and neonatal intensive care unit (NICU) can result in excess inflammation and a lowered ability to down-regulate immune responses once initiated [4,43,44].

Breast milk has long been considered the gold standard for infant nutrition and is essential for promoting appropriate immune development in newborns [21]. In addition to a full complement of LCP FA, breast milk also contains a complex mixture of immunologically active components such as growth factors, lactoferrin, prostaglandins, immunoglobulins, cytokines and immune cells [22,23]. Together with LCP FA, the immunomodulatory bioactive components in breast milk such as interleukin (IL) 10, transforming growth factor (TGF) β and DHA serve as mediators to promote oral tolerance and they also modulate developing immune responses while the infant develops their own immunological maturity [3,19,24–28]. This immune maturation is crucial in order for a complex and dynamic relationship to develop between the innate and adaptive immune system [41,19,29], allowing infants to respond effectively and appropriately to self and pathogenic environmental stimuli [25,30]. Without appropriate regulation, an unchecked inflammatory pathophysiology can result, leading to many neonatal morbidities [3,41].

3. Inflammatory disorders in the neonate and the role of DHA

A heightened immune response leading to an exaggerated release of inflammatory mediators is a hallmark of BPD and other inflammatory disorders in the neonatal period, such as sepsis, NEC and retinopathy of prematurity (ROP) [3]. These disorders have a multi-factorial pathogenesis for which a single medication or comprehensive treatment is not available. Data from both preterm infant [431] and animal studies [32,33] support the potential for DHA to serve as a general preventative agent against inflammation without inhibiting development or function of underdeveloped organs.

3.1. Bronchopulmonary dysplasia

BPD is a disorder of prematurity characterised by the need for assisted ventilation or supplemental oxygen at 36 weeks postmenstrual age and signs of impaired alveolarisation and vascularity in the lungs [4,34]. BPD occurs in approximately 45% of infants born less than 28 weeks gestation that survive preterm birth [4,35]. Ongoing lung damage may be caused by the preterm infant’s inability to down-regulate and maintain control of the inflammatory immune response, leading to a chronic inflammatory state [35–38]. Decreased levels of DHA have been found to be associated with respiratory disease in preterm infants [39,40] and results from a prospective observational study in preterm infants supports this trend [41]. The best evidence for the ability of DHA to improve respiratory outcomes in preterm infants comes from our “Docosahexaenoic acid for Improvement in Neurodevelopmental Outcome (DINO) trial” in the subgroup of infants born weighing less than 1250 g, those who received higher-DHA breast milk or formula had a reduced rate of BPD [42]. A recent meta-analysis supports the potential for DHA as a preventative agent against adverse respiratory outcomes with administration early in life [43]. These data support the concept that there is an early window of opportunity for effective immune modulation with DHA; the critical period is when the immune system is still developing and before clinical phenotypes have been established in the infant [4,41].

3.2. Necrotizing enterocolitis

NEC is predominantly a disease of prematurity, it is the most common gastrointestinal illness in newborns and has a high mortality rate [44–46]. As the disease progresses, inflammation in the intestine worsens causing breakdown of the mucosal barrier and an escalating immune cascade leading to sepsis, shock and even death [4,44,45]. The role for developing NEC is strongly influenced by commensal bacteria, which exert metabolic, nutritional and immunological effects on the host [48]. A preterm infant has very low bacterial diversity and the establishment of a more complex microbiome is easily disrupted by events related to premature birth, for example, early antibiotic administration and Caesarean sections [49–51]. This process, termed dysbiosis, is implicated in the development of both sepsis and NEC [48].

Breast milk is the ideal choice for nutrition in the preterm infant and its early introduction is critical due to known gastrointestinal benefits [52]. Breast milk promotes bacterial colonisation of the gut, which in turn, is a major promoter of the development of immunomodulatory properties and functions required to elicit an immune response about immunological homeostasis [53]. Enteral feeding regimens for preterm infants consist of breast milk, preterm formula, or more commonly in the first few weeks, a combination of the two [54], both are sources of DHA which has been shown to influence the composition of the microbiome, albeit with controversial efficacy [32,54–56]. It has been proposed that fat intake and type of fat (saturated vs. unsaturated) influence the distribution of beneficial and protective bacteria in preterm infants [51]. A meta-analysis of trials in which NEC was reported has shown no benefit for omega-3 LCP FA [4]; however none of the included trials were specifically designed nor powered to determine the true effect of DHA on NEC. Data from neonatal animal models is promising, as it has been reported that omega-3 LCPFA enriched diets support the colonisation of beneficial bacteria and protect against growth of pathogenic bacteria [32] and are protective against NEC [57]. Further, large-scale studies are required to first determine if DHA can reduce the incidence of NEC, and secondly if it is through a direct anti-inflammatory action or if DHA influences microbial communities directly in the gut.

3.3. Sepsis

Sepsis is a systemic inflammatory condition caused by infection. Globally, sepsis is responsible for approximately 15% of neonatal deaths [58], with rates of infection dependent on the geographic region [5,32,60]. In preterm infants, sepsis is classified as either early-onset (<72 h of life) or late-onset (>72 h of life), with the latter being a common complication associated with prolonged admission to NICU [59,60]. The distinction between the two is of clinical importance, as early-onset sepsis usually results from exposure to
bacteria in utero or during delivery and late-onset sepsis is acquired from bacteria in the environment (ie, nosocomial infections) [59]. Different microorganisms are responsible for the pathogenesis of sepsis; bacterial infections are most common, but fungal, viral, and parasitic infections are also important as well [39]. Decreased DHA has been associated with an increased risk of late-onset sepsis in preterm infants [39]. An appropriate balance of omega-3/LCPFA has an impact on disease risk and alterations in these LCPFA could be responsible for immune dysregulation and subsequent increase in sepsis risk [39].

3.4. Retinopathy of prematurity

ROP is the second leading cause of childhood blindness worldwide [61,62]. Impaired vasculogenesis and improper retinal neuronal development are responsible for the pathogenesis of ROP [63]. Because DHA is an integral part of cell membrane phospholipids, it may protect against the processes that impair vascular formation in ROP and therefore help to ameliorate vascular abnormalities and disease development [64-67]. Although a recent meta-analysis has shown no clear trend in risk for ROP between groups supplemented with omega-3 LCPFA [4], a single study has reported that omega-3 LCPFA supplementation was associated with a reduction in the incidence of ROP in preterm infants [65]. Results from preclinical studies in preterm infants and animal studies support the idea that further large-scale randomized-controlled trials to determine the clinical efficacy of DHA supplementation to prevent inflammatory conditions in preterm infants are needed.

4. Potential mechanisms for Omega-3 LCPFA in the regulation of inflammation

The growing body of evidence suggests that dietary intake of LCPFA early in life could influence immune development and other health outcomes. While there is extensive literature on the mechanisms of action of DHA and inflammation in adult disease, the targets that DHA acts on to exert its influence during initial immune development and resulting clinical conditions remains unclear. Studies in adults and animal models show that DHA influences a wide variety of mechanisms; some of which may be relevant to the neonate. These mechanisms include alterations in cell signalling pathways via changes to lipid and cell membrane composition [68], modifications to receptor-mediated pathways such as PPAR, Nrf2 or GPR120 to inhibit or attenuate inflammation [69,70] and increases in anti-inflammatory prostaglandin synthesis [71]. DHA may also influence the gut microbiome to promote immune regulation [50,56], and decrease oxidative stress [72]. However, there is a large gap in the literature relating to which aspects of inflammation are responsive to DHA in a neonate, or more specifically, in a preterm infant [73]. Preterm infants are an immunologically unique population and therefore mechanistic data pertaining to DHA’s action in adults may not be appropriate to predict efficacy in this population. In the following sections we review the evidence for the immunomodulatory efficacy of DHA, the effect of downstream metabolites of omega-3 LCPFA and oxidative stress in preterm infants and preterm models (in vitro and animal studies). Important trends in data from studies utilising LCPFA supplementation in term infants is also included when relevant or when data in preterm infants are unavailable.

4.1. DHA and the immune response: cytokine synthesis and release

In preterm infants, LCPFA supplementation has been reported to modulate cytokine synthesis and immune cell phenotypes [34]. In a double-blind, randomised-controlled clinical trial in preterm infants a medium-chain triglyceride/omega-3 LCPFA emulsion was found to attenuate the inflammatory response compared to a soy/omega-6 emulsion [74]. Pro-inflammatory cytokines (IL-6 and IL-8) were significantly reduced at study-end in the omega-3 LCPFA group [75], highlighting the potential for omega-3 LCPFA to dampen the heightened immune response seen in preterm infants after birth. The addition of DHA and AA (omega-6 LCPFA) to standard infant formula has been shown to increase the production of immunoregulatory IL-10, reduce IL-2 and increase the proportion of antigen-mature memory (CD45RO+) CD4+ cells (important in adaptive immunity) in infants to levels comparable to those seen in the breast milk-fed group [74], indicating an effect of DHA on immune maturation [74]. Furthermore, Gold et al. (2000) observed that omega-3 eicosapentaenoic acid (EPA) and AA reduced antigen- and mitogen-stimulated TNF production in vitro in cord blood samples from a US birth cohort study (30-42 weeks gestation) [76]. These data support the potential for omega-3 LCPFA to inhibit release of pro-inflammatory cytokines by Th1 cells. Additionally, lymphocyte proliferation was reduced by EPA and AA, indicating an attenuation of the immune response by both omega-3 and 6 LCPFA [76].

While immune response data from preterm infants are limited, data from term infants are supportive of an immunoregulatory role for DHA. Cellular immune responses were compared in term neonates who received either standard formula, formula supplemented with DHA/AA or breast milk from 2 to 6 weeks of life [77]. Although results were not significantly different, the infants that received the formula supplemented with DHA/AA had similar cytokine and T cell responses to those in the breast milk group [77]. Cultured lymphocytes from both formula groups produced more pro-inflammatory cytokines than the human milk fed group. However at 6 weeks, an increased pro-inflammatory TNFα response was seen in the formula group but not for the formula + DHA/AA group [77]. In addition, the group without LCPFA supplementation exhibited less maturation of the adaptive immune response (lower CD4+CD28+ cells) and they had significantly fewer peripheral blood effector memory T cells (CD4+CD44+ cells), which suggests that these fatty acids influence the degree of T cell maturation and development of effector memory T cells [77,78]. A decrease in pro-inflammatory cytokines in response to in vitro stimulation with allergens (ovalbumin, house dust mite, cat hair, phytohaemagglutinin) was also reported for immune cells from term infants, whose mothers were supplemented with fish oil (3.7 g/day omega-3 LCPFA) during pregnancy [79]. The data supports a role for early dietary LCPFA in immune development and regulation.

Animal studies also reinforce the anti-inflammatory role of omega-3 LCPFA seen in preterm and term infants. A reduction in systemic inflammation would be reflected by a reduction in pro-inflammatory mediators and/or the increase in regulatory cytokines. In a neonatal piglet model, increased dietary DHA and decreased omega-6 LCPFA was associated with a reduction in systemic inflammation (as measured by C-reactive protein (CRP)) [73]. Mice receiving an omega-3 LCPFA supplemented diet produced offspring with significantly increased immunoregulatory IL-10 levels in the colon and spleen [84]. Importantly, variations in the local tissue cytokine milieu also have the potential to influence other immune cells, such as antigen presenting cells (APC) that are critical for initiating immune responses [80]. DHA-induced changes in the cytokine environment may have downstream effects on priming of APC and other immune cells for the promotion of an anti-inflammatory response during initial antigen encounter [8,80].
Appendix 2

4.2. Role of DHA in the resolution of inflammation: resolvin D1

Downstream metabolites of LCPUFA, such as resolvins and protectins, are also important in mediating an anti-inflammatory response. Resolvins are directly synthesised metabolites of omega-3 LCPUFA and play a key role in terminating inflammatory responses [81]. These metabolites promote resolution and tissue repair and importantly for the preterm infant this occurs without compromising host defence [81]. Much of the work that examines the interaction between LCPUFA, resolvins and inflammation has been conducted in vivo in samples obtained from adult and animal models, leaving a large gap in knowledge for the effectiveness in neonates. Results from neonatal animal models serve as the best indicator for effectiveness of LCPUFA’s ability to trigger resolution of inflammation. Martin et al. (2014) recently examined the effect of supplementation with a downstream metabolite of DHA, Resolvin D1 (RvD1), along with a downstream metabolite of AA, Lipoxin A4 (LXA4), in a neonatal mouse model of BPD [82]. The results demonstrate that these metabolites reversed the histologic and biochemical changes associated with lung injury in the model. Whilst RvD1 and LXA4 were shown to reduce alveolar damage when administered on their own, the most significant reduction in alveolar damage was seen when the two were combined [82]. Similarly, Vehmaa et al. (2014) also reported that prenatal DHA supplementation reduced neonatal lung inflammation in a mouse model [83]. These recent findings suggest biological plausibility for a mechanistic role of terminal metabolites of DHA in counteracting inflammatory lung damage.

4.3. DHA and the modulation of oxidative stress

Supplemental oxygen therapy, while vital to the survival of infants with BPD, also places a large oxidative stress burden on the infant [7]. Furthermore, if the infant is also receiving parenteral nutrition, the lipids are subjected to an increased risk for peroxidation while exposed to supplemental oxygen. DHA and its downstream metabolites have been reported to reduce oxidative stress [84]. A double-blind randomised controlled trial in preterm infants (n = 38) reports that a parenteral lipid emulsion containing omega-3 LCPUFA reduced oxidative stress compared to standard lipids (soy) [85]. Oxidative stress levels were determined by Vitamin A, E and total antioxidant potential. Positive results with respect to omega-3 LCPUFA and oxidative stress were also obtained when very preterm infants (n = 30) were randomised to either a lipid emulsion containing soy and olive oils (Clinilec®) or one containing soy, medium-chain triglycerides, olive oil and fish oil (SMOFlipid®) [86]. Vitamin E levels and plasma F2-isoprostanes (lipid peroxidation marker) were used to quantify levels of oxidative stress in the infants. Compared to Clinilec®, SMOFlipid® reduced oxidative stress in this high-risk infant population and was determined to be safe and well-tolerated [86].

Results from research conducted in children and adolescents with acute lymphoblastic lymphoma (ALL) are relevant as they add strength to the use of DHA as an adjuvant therapy to combat oxidative stress in preterm infants. In a randomised, double-blind, placebo-controlled trial, participants (n = 70) received either 100 mg/day fish or placebo oil in combination with their methotrexate regimen [87]. Methotrexate is a necessary treatment in patients with ALL but is known to induce hepatotoxicity, the pathological changes were attributed to oxidative stress. Omega-3 LCPUFA as an adjunct therapy in these patients ameliorated the hepatotoxicity compared to placebo and no adverse reactions to the fish oil were observed [87]. These results are promising; however, more in-depth studies are required in larger cohorts to establish the robustness of these findings and the mechanisms underlying the action of DHA against oxidative stress.

In a neonatal guinea pig model indicators of oxidative stress (peroxide and aldehyde concentrations) were reduced when guinea pigs were fed an omega-3 LCPUFA emulsion compared to an omega-6 LCPUFA emulsion [88]. Levels of pro-inflammatory IL-1 and tumour necrosis factor alpha (TNFα); mPGF1α and mPAF were also significantly reduced in the fish oil emulsion group [89]. Further research in humans to determine appropriate dose and timing regimens is required to reveal whether DHA would serve as an effective adjunct therapy to combat oxidative stress in infants the NICU.

5. Implications and conclusions

There is increasing experimental evidence and a convincing rationale for DHA supplementation to improve clinical outcome in preterm infants by targeting the critical window of time where immunomodulation has the greatest potential for benefit [4,89]. However, several areas require further consideration and research. First, further large-scale interventions are required in order to determine the clinical efficacy of DHA during the Nutritional period in this unique and vulnerable population. Second, to optimise DHA’s benefit on clinical outcomes, it is essential that the sites of action of both the fatty acid and its downstream mediators on the preterm infant’s immune system and their regulatory functions is better understood. This knowledge would allow clinicians to determine whether DHA would be best used as a preventative versus treatment option in preterm infants. Lastly, the mechanisms of action of DHA on the immune system in the short and long-term must be examined.

It is anticipated that the results from a large-scale randomised clinical trial of enteral DHA supplementation on respiratory outcomes and other parameters in preterm infants that is currently underway in Australia, New Zealand and Singapore will answer some of these questions and guide future research and recommendations. The N3RO trial (n-3 LCPUFA for the improvement in Respiratory Outcomes - ACTRN trial #12612005382020) will shed light on the mechanisms of action of omega-3 LCPUFA on the immune system while it is still functionally immature.

Author contributions

All authors contributed to conceptualisation and critical review of the literature. All authors were involved in the writing, editing and final review of the manuscript. All authors have read and approved the final version of the manuscript.

Acknowledgements

Research fellowships were provided by the National Health and Medical Research Council of Australia (MM Principal Research Fellow APP1047104 and RAG Senior Principal Research Fellow APP1049617), The Maitland McEvedy Research Fund, Women’s and Children’s Hospital Research Foundation (CTC). The N3RO trial is supported by Project Grant 1022112 from the NHMRC, Australia. Study product for the N3RO trial is donated by Clover Corporation Limited, Australia. The contents of the published material are solely the responsibility of the authors and do not reflect the views of the National Health and Medical Research Council of Australia.

CTC, RAG and MM have received non-financial support from Clover Corporation, Med Jackson Nutritional Services and MC. MC and MM hold stock options in companies outside of that of the submitted work. RAG serves on a scientific advisory board for NESTLE. MM serves on scientific advisory boards for Nestle, Fonterra and Nutricia. Associated honours for RAG and MM are paid to their respective institutions to support conference travel and continuing education for post graduate research.
APPENDIX 3

PROTOCOL FOR THE NESTED STUDY WITHIN THE N3RO RANDOMISED CONTROLLED TRIAL

Author: Naomi Fink

This section details the framework of the nested study within the N3RO randomised controlled trial (RCT) (Docosahexaenoic acid for the reduction of bronchopulmonary dysplasia in preterm infants born at less than 29 weeks gestational age: a randomised controlled trial: ACTRN 12612000503820) that forms the basis for this PhD project. Where appropriate, the published study protocol from the N3RO RCT (1) is referenced to avoid redundancy.

The following documents are associated with this section:

1. N3RO RCT consent form (Appendix 5)
2. N3RO RCT patient information sheet (Appendix 6)
3. N3RO nested study standard operating procedure (SOP) for blood sample collection (Appendix 7)
4. N3RO nested study SOP for stool sample collection (Appendix 8)
GLOSSARY OF ABBREVIATIONS

BPD      Bronchopulmonary dysplasia
CRF      Case report form
CNRC     Child Nutrition Research Centre
DHA      Docosahexaenoic acid
DMAC     Data Management and Analysis Centre
EFA      Essential fatty acid
GA       Gestational age
HREC     Human Research Ethics Committee
IFN      Interferon
IL       Interleukin
LA       Linoleic acid
LCPUFA   Long chain polyunsaturated fatty acids
LPS      Lipopolysaccharide
MIP      Macrophage inflammatory protein
MRN      Medical record number
NICU     Neonatal intensive care unit
PCR      Polymerase chain reaction
PMA      Postmenstrual age
RCT      Randomised controlled trial
SAE      Serious adverse event
SAHMRI   South Australian Health and Medical Research Institute
SCBU     Special care baby unit
SOP      Standard operating procedure
SP-D     Surfactant protein D
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<td>TNF</td>
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STUDY SYNOPSIS

Title
Examination of the mechanisms by which supplemental docosahexaenoic acid (DHA) exerts an immunomodulatory effect in the premature infant: a nested study in N3RO.

Objectives
The primary objective of this study is to determine if supplemental DHA has an immuoregulatory effect on the levels of pro-inflammatory and regulatory cytokines.

The secondary objectives of this study are to determine if supplemental DHA has an effect on a) levels of surfactant protein D as an indicator of pathological lung changes, and b) prevalence and abundance of *Staphylococcus* and bacteria carrying the methicillin resistance gene (*mecA*).

Design
A nested study within one centre of a randomised placebo-controlled multicentre in-patient trial of two parallel groups.

Location
Women’s and Children’s Hospital (WCH), Adelaide, South Australia

Outcomes
Primary outcome (laboratory): changes in immune markers indicative of a reduction in pro-inflammatory and an increase in anti-inflammatory markers in plasma.

Secondary outcome (laboratory): a) an increase in surfactant protein D levels in plasma, b) a decrease in *Staphylococcus* and bacteria carrying the methicillin resistance gene (*mecA*).

Intervention
**Study product:**

Intervention: Aqueous emulsion of DHA oil (tuna oil) containing 19.5% total fat (with 70% of total oil as DHA) that will deliver around 60 mg/kg/day of DHA.

Control: Placebo (soy oil) emulsion with no DHA.

**Dosing regimen:** Infants will be given 0.5 mL/kg/d in three divided doses three times daily (0.17 mL/kg/dose).

**Route:** Enteral

Population
Infants born at less than 29 weeks gestational age (GA)

Significance
The results of this project will provide evidence for the mechanisms of action by which DHA may be acting in a preterm infant to modulate the immune response.
1. INVESTIGATORS AND FACILITIES

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1.4. Study Management

Central coordination for the N3RO RCT is via the Child Nutrition Research Centre (CNRC), South Australian Health and Medical Research Institute (SAHMRI). The investigators at each
study centre are responsible for the conduct of the study at their centre including informed consent, recruitment, data collection and maintenance of study documentation.

1.5. **Independent Serious Adverse Event and Trial Monitoring Committees**

1.5.1. **Serious Adverse Event committee**

An independent blinded Serious Adverse Event committee has been established as part of the N3RO RCT to review serious adverse events (SAEs) \(^{(1)}\). Due to the low and negligible risk of the nested study, no additional committee will be established. Any SAEs occurring during the nested study will be reviewed by the N3RO RCT committee.

1.5.2. **Trial Monitoring Committee**

An independent Trial Monitoring Committee was set up for the parent N3RO RCT to review the yearly progress of the trial and provide feedback to the Trial Management Committee \(^{(1)}\). Any issues with trial monitoring in the nested study will be addressed by the N3RO RCT monitoring committee, therefore a separate Trial Monitoring Committee will not be required.

1.6. **Funding**

The costs associated with the laboratory analysis for this nested study will be financed through the Centre for Research Excellence “Foods for Future Australians” fund.
2. BACKGROUND

In preterm infants, the homeostatic mechanism that normally regulates an inflammatory response is impaired as a result of their immature immune system\(^2,3\). While the mechanisms of action in a preterm infant are not fully understood, there is evidence to suggest that DHA has the potential to attenuate inflammation and therefore influence clinical outcomes related to dysregulated inflammation\(^4\).

A preterm infant misses out on the *in utero* accretion of omega-3 long chain polyunsaturated fatty acids (LCPUFA) such as DHA during the third trimester. While there is some controversy surrounding whether or not a preterm infant can synthesise sufficient levels of LCPUFA from essential fatty acid (EFA) precursors\(^5,6\), the general consensus is that the infant must receive exogenous DHA via breast milk and/or infant formula after birth. Changes in maternal diet\(^7\), genetic variations\(^8\) and environmental factors\(^9\) can influence the amount of DHA in mother’s breast milk. Combined with delays in reaching full enteral feeds, DHA delivery to the preterm infant can be inconsistent. Enteral DHA supplementation is safe and well-tolerated in preterm infants and can ensure that the infant receives the target dose\(^10\).

The anti-inflammatory action of DHA and other omega-3 LCPUFA have been extensively reviewed in adult and animal models\(^11,12\). Adult and animal data reveal omega-3 LCPUFA can influence cell signalling pathways\(^13,14\), inhibit or attenuate inflammation by modifying receptor-mediated pathways\(^15\), decrease oxidative stress\(^16\) and increase production of anti-inflammatory prostaglandin synthesis\(^17\). Limited data exists in preterm infants regarding the effect of DHA and other omega-3 LCPUFA on inflammatory biomarkers. Cytokines and surfactant proteins participate in normal lung development but can also mediate lung injury\(^18,19\) and are potential targets for the immunoregulatory action of DHA.
Data from adult and animal models reveal that DHA may also influence the environment in the gastrointestinal system to promote immune regulation \(^{20-22}\). Accordingly, the gut environment influences prevalence and abundance of microbial species in the gut capable of activating and directing immune responses \(^{22, 23}\). The bacterial species in the gastrointestinal tract and the respiratory immune system interact via the gut-lung axis \(^{24}\). One of the first species to colonise the respiratory tract, and in parallel, the gut is \textit{Staphylococcus} \(^{24}\). \textit{Staphylococcus} is one of the most important human bacterial pathogens implicated in nosocomial and community infections \(^{25}\) and has been associated with the development of bronchopulmonary dysplasia (BPD) and other neonatal inflammatory disorders \(^{26-28}\). Methicillin-resistant staphylococci are known to cause significant morbidity and mortality in the neonatal intensive care unit (NICU) \(^{29}\). Diet can modulate the prevalence and abundance of bacterial species and thus has the potential to influence the immune response. The effect of LCPUFA on prevalence and abundance of bacterial species, specifically \textit{Staphylococcus}, in the gastrointestinal system of a preterm infant is not known.

Strong experimental evidence exists for DHA supplementation during the critical window of time when immunomodulation has the greatest potential for benefit \(^{30}\). Therefore, DHA supplementation in the early neonatal period could serve as a safe and cost-effective intervention to reduce inflammation associated with development of some neonatal inflammatory disorders.

\textbf{2.1. Rationale and aim}

Few trials to date have specifically investigated postnatal supplementation of preterm infants with DHA and resulting effects on biomarkers of inflammation. The primary outcome of the N3RO RCT is an inflammatory lung disorder of prematurity, BPD. The N3RO RCT provides an opportunity to nest a study to examine the immunological mechanisms by which DHA exerts an immunomodulatory effect on immune responses. The results of this project will provide
Appendix 3 | 177

evidence for how DHA may be acting in a preterm infant to regulate immune response and offer insight into the mechanisms by which it may be protective against BPD and other inflammatory conditions.

3. TRIAL FRAMEWORK

A randomised placebo-controlled multicentre in-patient trial of two parallel groups is being coordinated in South Australia by Dr Carmel Collins of the CNRC, Adelaide (a division of the SAHMRI; Healthy Mothers, Babies & Children) (1). Briefly, the N3RO RCT was designed to test whether DHA supplementation reduces the incidence of BPD in infants born less than 29 weeks GA. Infants were randomised to receive either a treatment or placebo emulsion from enrolment to 36 weeks postmenstrual age (PMA). This nested study within N3RO will be conducted at one participating N3RO RCT centre; the WCH, Adelaide, SA.

4. OBJECTIVE

4.1. Primary objective

The primary objective of this study is to determine if supplemental DHA has an immuoregulatory effect on the levels of pro-inflammatory and regulatory cytokines.

4.2. Secondary objectives

A secondary objective of this study is to determine if supplemental DHA has an effect on the levels of surfactant protein D (SP-D) in plasma, as an indicator of pathological lung changes. Furthermore, the influence of supplemental DHA on the prevalence and abundance of Staphylococcus and bacteria carrying the methicillin resistance gene (mecA) in the stool samples from preterm infants will be assessed.

5. HYPOTHESIS

5.1. Primary: DHA will have a reductive effect on pro-inflammatory cytokines and increase regulatory cytokines compared to control.
5.2. Secondary: a) DHA will increase SP-D levels compared to control, b) DHA will reduce relative abundance of *Staphylococcus* and bacteria carrying the methicillin resistance gene (*mecA*) compared to control.

6. **OUTCOME MEASURES**

6.1. *Primary outcome measure*

Assessment of:

a. Pro-inflammatory cytokine levels in plasma and or/supernatant from whole blood stimulation: Interleukin (IL) 1β, IL-6, IL-8, IL-12p70, IL-17A, IL-23, Tumor necrosis factor (TNF) α, Macrophage inflammatory protein (MIP) 1α and interferon (IFN) γ

b. Regulatory cytokine levels in plasma and or/supernatant from whole blood stimulation: IL-10 and Transforming growth factor (TGF) β

6.2. *Secondary outcome measures*

a. SP-D levels in plasma will be assessed.

b. Levels of *Staphylococcus* and *mecA*+ bacteria will be assessed in stool samples.

7. **LOCATION AND SETTING**

This study will be conducted at one participating centre, the WCH. Infants in the N3RO nested study will be cared for in either the NICU, a Level 6 facility with 14 beds, or the Special Care Baby Unit (SCBU), a Level 4-5 facility with 35 beds. The units are staffed with neonatologists, registrars, neonatal nurse practitioners, neonatal intensive care nurses, registered nurses and midwives, enrolled nurses and assistants in midwifery.

7.1. *NICU: Standard of care*
Other than the trial product, all infants will receive the same clinical care according to standard practise in the NICU at the WCH.

7.2. **Training and support of NICU staff**

Several in-service training sessions will be offered for NICU and SCBU staff in order to familiarise the clinical care team with the protocol for the nested study prior to its commencement. A brief outline of the study rationale, objectives and outcomes will be presented. Staff will be informed about the appropriate sample collection containers, bags, labels, cot cards and collection procedures. Clinical staff will be informed that copies of all SOP, contact information (ie. phone and pager number) and a summary of the study will be left in the NICU and SCBU nursing communication binders. Furthermore, due to the nature of the sample collection procedure, communication between nursing staff and the PhD candidate will occur on a regular basis during the sample collection phase of the trial.

8. **ETHICAL APPROVAL**

An ethics application to collect the additional samples required for the nested study was submitted in November 2013 and approved by the Human Research Ethics Committee (HREC) at the WCH on 27 November 2013 (HREC 2434/12/16). An amendment was submitted in February 2014 and approved on 4 March 2014 to collect an additional blood sample on day 14 of life to assess early changes in immune parameters and stool samples at one-week intervals. To avoid over-sampling of the infant, blood samples will only to be taken when regular clinical bloodwork has already been ordered by a physician or member of a clinical care team member.

9. **SERIOUS ADVERSE EVENTS**

The nested study will be assessed as part of the N3RO RCT by the Independent SAE and Trial Monitoring Committees (1).

10. **STUDY POPULATION**
Preterm infants born at less than 29 weeks GA comprise the study population.

11. ELIGIBILITY

11.1. Inclusion criteria

The inclusion criteria requires the infant is born at less than 29 weeks GA, is within three days of commencing enteral feeds and has a legally acceptable representative capable of understanding the informed consent document and providing consent on the infant’s behalf (1).

11.2. Exclusion criteria

1. Infants born with major congenital abnormalities

2. If the mother is already taking supplements that provide >250mg DHA per day and does not wish to cease her supplement regimen

3. Infants enrolled in another study focused on fatty acids

4. Infants receiving intravenous lipid support where fish oil is a component of the lipid emulsion (1).

12. ENROLMENT AND RANDOMISATION

12.1. Recruitment

A Chief Investigator or Associate Investigator (or nominee) approaches the parents/guardians of each eligible infant, ideally within 24-48 hours after birth. The information sheet (Appendix 6) is provided to detail the purpose, procedures and risks and benefits of the study prior to an informed consent discussion. Consent is to be voluntary and free from coercion. The N3RO RCT consent form (Appendix 5) gives parents the “yes/no” option to participate in the nested study. Trial data will be recorded in a separate case report form (CRF) for each infant enrolled in the N3RO RCT and this nested study (1).

12.2. Randomisation
Briefly, a computer generated randomisation schedule is generated by an independent statistician for the N3RO RCT. Stratification occurred for sex, study centre and GA (<27 completed weeks and 27 to < 29 completed weeks). Multiple births are randomised individually. Sequence generation, allocation and study ID generation are detailed in the study protocol (1). Importantly for this nested study, stratification occurs by centre and therefore all infants at WCH will receive a balanced randomisation.

12.3. **Blinding**

Participants and their family, care providers, outcome assessors and data analysts are all blinded to randomisation group.

12.4. **Participant withdrawal**

Parents/guardians are advised that they are free to withdrawal their child at any time from either the N3RO RCT or its nested study, or both. The reasons for withdrawal are to be recorded in the CRF.

12.5. **Study duration**

Recruitment is set to continue until the N3RO RCT stops recruiting or until sample size is reached, whichever comes first.

13. **INTERVENTION**

The aqueous emulsion of DHA (tuna oil) contains 19.5% total fat, 0.5 mL of emulsion will deliver 60 mg/kg/d of DHA and the placebo emulsion contains soy oil (linoleic acid (LA), no DHA). Infants are administered treatment or placebo oil enterally at a rate of 0.5 mL/kg/d in three equal doses daily (0.17 mL/kg/dose). Emulsions are administered through the naso-/oro-gastric tube immediately preceding a scheduled feed (1).

13.1. **Calculation and ordering of dose**
Dosage and route of administration, ordering procedure via medication chart and pharmacist preparation is detailed in the study protocol (1).

14. SAMPLE COLLECTION AND ANALYSIS

In addition to samples and information collected as part of the parent N3RO RCT, extra biological samples will be collected for the nested study. These additional samples consist of a 0.5 mL blood sample at baseline, two weeks of life and study end, and stool samples at four-week intervals and study end.

14.1. Baseline, mid, study end time points

“Baseline” sample collection refers to the first sampling time point after randomisation, which is approximately postnatal day 3-5. “Mid-point” sample collection refers to postnatal day 14 +/- 3 days. “End” sample collection refers to study end, 36 weeks PMA, discharge or transfer home, whichever occurs first. The N3RO trial follows infants until 36 weeks PMA even if the infant is transferred to another hospital. However, sample collection for this study is not feasible outside of the WCH and will therefore be collected before the infant is transferred or discharged prior to 36 weeks PMA. Samples will be collected Monday to Friday between the hours of 0800-1600. Scheduled postnatal days are allowed +/- a 3 day window due to weekends and/or attempts to coincide N3RO nested study sampling with clinical sampling events. Stool samples will be requested weekly from each infant.

14.2. Sample request procedure

A spreadsheet will be created for each recruited infant with a record of each day/week that a sample is due. These dates will be recorded on a calendar and biohazard bags/labels will be prepared ahead of time. A laminated cot card will be labelled with infant’s name, type of sample required, date and the PhD candidate’s pager number. This card will be attached to the infant’s cot on the day of or day prior to sample collection and cleaned with alcohol before next use.
Biohazard bags and blood collection microtainers will be left at bedside. Once the sample is collected, the clinical staff member responsible for the infant’s care on that day will be instructed to page the candidate to pick up the material immediately after collection.

14.3. Blood collection and processing

Blood will be collected according to standard clinical practise by clinical staff in the NICU or SCBU at the WCH at baseline, postnatal day 14 and upon completion (see SOP in Appendix 7). Briefly, 0.5 mL capillary blood will be collected into lithium heparin coated microtainers (Greiner Bio-One MiniCollect Capillary Blood Collection System 1 mL tubes). If the infant has an arterial line, clinical staff will be instructed to preferentially collect blood via this route. Samples will labelled, sealed in a biohazard bag and collected immediately from the NICU by the candidate.

14.3.1. Whole blood lipopolysaccharide (LPS) stimulation and analysis of inflammatory markers

Stimulation of whole blood obtained from participants will proceed according to a previously published method (31). Effect of DHA on cytokine secretion in response to endotoxin (E. coli LPS) will be assessed by BD Biosciences enhanced sensitivity human flex sets (BD Biosciences, CA, USA) and ELISA (R&D Systems, MN, USA). IL-6, IL-8, IL-10 and IL-12p70, IFNγ, TNFα (flex set) and TGFβ (ELISA) will be measured. Final concentrations will be reported in pg/mL or fg/mL, as appropriate.

14.3.2. Analysis of pro-inflammatory and regulatory cytokines in blood

After the whole blood aliquots are removed for LPS stimulation, the remaining blood sample will be centrifuged at 2000 g for 10 minutes to isolate plasma. Immune markers in plasma (IL-1β, IL-6, IL-8, IL-12p70, IL-17A, IL-23, TNFα, IFNγ, MIP-1α and IL-10) will be analysed.
using a Millipore High Sensitivity human T cell magnetic bead panel (Merck Millipore, Australia). Final concentrations will be reported in pg/mL or fg/mL, as appropriate.

14.3.3. Analysis of SP-D in plasma

SP-D levels will be assessed using a Quantikine Human SP-D ELISA (R&D Systems, MN, USA). Final concentrations will be reported in ng/mL.

14.4. Stool collection

Stool samples will be collected by clinical staff in the NICU and SCBU at the WCH. Using gloves, the clinical staff will be instructed to place nappies directly into a labelled biohazard bag and page for immediate pick up (see SOP in Appendix 7). At this point, the sample will be placed in the refrigerator (maximum four hours) or processed immediately.

14.4.1. Analysis of Staphylococcus and mecA+ bacteria in stool

In order to assess bacterial species, DNA will be extracted from stool samples using MoBio PowerSoil Powerlyzer DNA isolation kits (Mo Bio Laboratories, Inc., Carlsbad, USA). *Staphylococcus* and mecA+ species will be assessed from DNA extractions via real time quantitative polymerase chain reaction (PCR) using primers for the *tuf* and mecA genes respectively (Sigma-Aldrich, Sydney, Australia). Values will be reported as percent of total bacterial detected, which will be estimated from extracted samples by sequencing 16S rRNA with a universal bacterial primer via real-time PCR.

15. STATISTICAL ANALYSIS

15.1. Sample size calculation

Recent studies have detected clinically meaningful differences in cytokine expression in similar sample sizes in preterm infants (32, 33) with group sizes of n=12 (34), n=15 (35) and n=25 (36). Based on the projected N3RO recruitment rate of 4-6 infants/month at the WCH at the time that the side study was proposed, a sample size of n=100 has been decided.
15.2. **Data analysis**

All participants will be analysed according to the group to which they were randomised (intention-to-treat principle). Differences in cytokine and SP-D levels between groups at each time point and over time will be analysed using a longitudinal Tobit regression model. A generalised estimating equation will be used to account for repeated measures, with a time-by-treatment interaction term to test for differences between treatment groups in change over time. This model will take into account detection thresholds of the cytokine and SP-D assays. Outcomes (concentration of cytokines and SP-D) are not normally distributed and will be log transformed prior to analysis.

Descriptive analysis of bacterial species load over time will be conducted. Bacterial species outcomes will be analysed using mixed-effects linear regression models. Treatment group and time (days postnatal) will be modelled as fixed effects and repeated measures over time will be accounted for. Time will be modelled as a continuous variable. Outcomes (% of total bacterial detected that is a) *Staphylococcus* and b) *mecA*+) are not normally distributed and will be log transformed prior to analysis.

Differences between groups in baseline patient characteristics and other clinical outcomes will be assessed by an independent, two-tailed t-test.

**16. DATA MANAGEMENT**

16.1. **Data collection**

All data collected as part of the N3RO trial is recorded in the infant’s CRF and entered into a computer database (1). Relevant variables for each participant in this nested study will be collected and stored in separate spreadsheets specific to this nested study.

Information on antibiotic, antifungal and probiotic exposure will be recorded from each participant’s case note files. Date, type of medication, dose in mg/kg/day, final dose and time(s)
of administration will be recorded for each participant for each day between baseline and study end. This information will be stored in a spreadsheet with one sheet per participant.

16.2. Sample and storage log

Each sample will be recorded on a hard copy sample log-in sheet and kept in chronological order in a sample log book. Along with the barcode number for each tube of sample material, the participant’s name, study ID, date, gestation age, postnatal day, time of collection, medical record number (MRN) and date of birth will be recorded. Sample information from each log in sheet will be transferred to a spreadsheet with one sheet for each participant. The storage location (box number, position number, freezer number) will be included. Lastly, the same information will be logged into a freezer location database (ProbeFinder software).

16.3. Laboratory analysis data

Data obtained from laboratory analysis of blood and stool samples will be stored in a separate spreadsheet for each analysis (ie. plasma cytokines, whole blood stimulation cytokines, SP-D, etc). Data for each sample will identified by each participant’s study ID, date of birth, MRN and the barcode number from each storage tube.
REFERENCES


APPENDIX 4

DATA ANALYSIS PLAN FOR THE NESTED STUDY IN THE N3RO RANDOMISED CONTROLLED TRIAL

Author: Naomi Fink

1. PREFACE

This statistical analysis plan describes the planned analyses and reporting for the single-centre nested study in the N3RO RCT. This study is being conducted to assess the immunomodulatory efficacy of supplemental DHA in preterm infants < 29 weeks gestational age (GA). This plan was written in consultation with the independent statistician assigned to the N3RO RCT, Dr. Jennie Louise of the Data Management and Analytical Services (DMAC), University of Adelaide.

2. OUTCOME VARIABLES

2.1 Primary

- Pro-inflammatory cytokine levels in plasma and or/supernatant from whole blood culture: Interleukin (IL) 1β, IL-6, IL-8, IL-12p70, IL-17A, IL-23, Tumor necrosis factor (TNF) α, Macrophage inflammatory protein (MIP) 1α and interferon (IFN) γ
- Regulatory cytokine levels in plasma and or/supernatant from whole blood culture: IL-10 and Transforming growth factor (TGF) β

2.2 Secondary

- Surfactant protein (SP) D levels in plasma
- DNA yield (ng DNA/ng faecal material) of total bacteria detected, *Staphylococcus* and bacteria carrying the *mecA* gene
3. STRATIFICATION VARIABLES

Stratification occurred for infant sex and GA in the N3RO RCT and thus, the nested study.

Appendix 4 Table 1. Stratification variables for the analysis of data resulting from the nested study in the N3RO RCT

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant Sex</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td>Gestational age</td>
<td>&lt; 27 weeks</td>
</tr>
<tr>
<td></td>
<td>≤27 weeks</td>
</tr>
</tbody>
</table>

Legend to Appendix 4 Table 1. Multiple births were randomised individually and the first born infant was randomised first. Abbreviations: randomised controlled trial (RCT)

4. SEQUENCE OF PLANNED ANALYSES

4.1. Interim analyses

There are no planned interim analyses for the nested study.

4.2. Final analyses and reporting of outcomes

Once the study is completed, all samples collected and analysed and all data entered, a blinded review of the data will be conducted. Blinded treatment codes will be included in the database and analysis of primary outcomes will be performed blinded to treatment group. The blinding will be broken following the analysis of primary outcomes as outlined in the nested study protocol.

5. SAMPLE SIZE DETERMINATION

Recent studies have detected clinically meaningful differences in cytokine expression in similar sample sizes in preterm infants (1, 2) with group sizes of n=12 (3), n=15 (4) and n=25 (5). Based on the projected N3RO recruitment rate of 4-6 infants/month at the Women’s and Children’s Hospital at the time that the side study was proposed, a sample size of n=100 has been decided.
6. ANALYSIS METHODS

6.1. Software

All analyses will be performed using SPSS version 24 or SAS version 9.3 or later.

6.2. Approach

All participants will be analysed according to the treatment they were randomised to receive (intention-to-treat principle).

6.3. Withdrawal, outliers and missing data

Data collected on participants up to the point of withdrawal will be included in the analysis. Outliers will be queried during analysis and unless identified as a data entry error, will not be excluded from the primary analysis. Missing data will not be imputed.

6.4. Covariates

Both unadjusted and adjusted analyses will be performed in order to test each hypothesis. GA and sex were used as stratification variables during randomisation and all analyses will be adjusted for these factors.

6.5. Data transformations

Based on assumptions about the distribution of outcomes, cytokine, SP-D and Staphylococcus and meca DNA yield will be log transformed prior to analysis. Data will be assessed prior to analysis to ensure that the assumptions about the distribution of outcomes are valid. Untransformed data will be reported for ease of reader interpretation.

7. DESCRIPTIVE STATISTICS

7.1. Screened and randomised infants

Descriptive information relating to the screened and randomised population will be presented as specified by the CONSORT statement.
7.2. Baseline characteristics and clinical outcomes

Information on baseline characteristics and clinical outcomes will be obtained from the case report form (CRF) for each infant enrolled in the N3RO RCT. Means and standard deviations or medians and interquartile ranges (where appropriate) will be reported for continuous variables. Frequencies and percentages will be reported for categorical variables. This information will be presented by treatment group for the following parameters:

Baseline characteristics

- Sex (male/female)
- Mean GA
- Singleton/multiple birth
- Mode of delivery (vaginal/Caesarean section)
- Maternal steroids (yes/no)

Clinical outcomes

- Percent compliance (total doses received/total dose possible)
- Postnatal steroids (yes/no)
- Surgery (yes/no)
- Bronchopulmonary dysplasia (yes/no)
- Sepsis (yes/no)
- Necrotising enterocolitis (yes/no)
- Days of parenteral nutrition (number of days)
- Days of intravenous lipids (number of days)
- Days to reach full enteral feeds (number of days)
- Type of feed at discharge (breast milk, formula, breast milk+formula)
8. **STATISTICAL ANALYSES**

For each outcome variable, statistical significance will be assessed at the 0.05 level using a two-sided comparative test of treatment effect, unless otherwise specified.

8.1. *Baseline characteristics and clinical outcomes*

Between-group differences in baseline characteristics and clinical outcomes as described in section 6.1 will be assessed using a student’s t-test or ANOVA, as appropriate.

8.2. *Primary outcomes relating to the intervention*

Longitudinal Tobit regression models will be used to assess between group differences and changes over time in pro-inflammatory and regulatory cytokines in plasma and supernatants from whole blood culture following incubation with *E. coli* lipopolysaccharide. A generalised estimating equation will be used to account for repeated measures and a time-by-treatment interaction will be included to test for differences between groups in rate of change over time. Both unadjusted and adjusted analyses will be conducted. Adjusted analyses included gestational age group (< 27 weeks) and infant gender as covariates.

This analysis approach will not account for clustering due to multiple births. Sensitivity analyses will therefore be conducted using standard mixed models with random effects for infant and for multiple births.

8.3. *Secondary outcomes relating to the intervention*

8.3.1. **Surfactant protein (SP)-D**

Longitudinal Tobit regression models will be used to assess between group differences and changes over time in SP-D in plasma. A generalised estimating equation will be used to account for repeated measures and a time-by-treatment interaction will be included to test for differences between groups in rate of change over time. Both unadjusted and adjusted analyses
will be conducted. Adjusted analyses included gestational age group (< 27 weeks) and infant gender as covariates.

This analysis approach will not account for clustering due to multiple births and sensitivity analyses will therefore be conducted using standard mixed models with random effects for infant and for multiple births.

8.3.2. DNA yield from stool samples

Yield of total bacteria detected, *Staphylococcus* and *mecA* will be calculated by normalising amount of DNA (ng) to amount of faecal material (ng). For statistical analysis purposes *Staphylococcus* and *mecA* yield will be analysed as percent (%) of total bacteria detected by the universal 16S primer. Outcomes will be analysed using mixed-effects linear regression models, in which treatment group and time (days postnatal) will be modelled as fixed effects. Repeated measures over time will be accounted for by including a random slope and intercept for each infant. A treatment-by-time interaction term will be included in the model to test for differences between treatment groups in pattern of change over time. This model will allow for inclusion of a random effect to account for clustering due to multiple birth.

Sensitivity analyses will be performed using different covariance structures and random intercepts only for repeated measures.
REFERENCES


APPENDIX 5

N3RO RANDOMISED CONTROLLED TRIAL CONSENT FORM

Author: Carmel Collins and the N3RO RCT investigative team
LAY TITLE: n-3 fatty acids for improvement of respiratory outcomes – The N3RO trial

SCIENTIFIC TITLE: Docosahexaenoic acid for the reduction of bronchopulmonary dysplasia in preterm infants born at less than 29 weeks gestational age: a randomised controlled trial.

I _________________________________________________________________

hereby consent to my child's involvement in the research project entitled:

“Docosahexaenoic acid for the reduction of bronchopulmonary dysplasia in preterm infants born at less than 29 weeks gestational age: a randomised controlled trial.”

1. The nature and purpose of the research project described on the attached Information Sheet has been explained to me. I understand it and agree to my child taking part.

2. I understand that my child may not directly benefit by taking part in this study.

3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Information Sheet, have been explained to me.

4. I understand that I can withdraw my child from the study at any stage and that this will not affect medical care or any other aspects of my child’s relationship with this healthcare service.

5. I understand that there will be no payment to me or my child for taking part in this study.
6. I have had the opportunity to discuss taking part in this research project with a family member or friend, and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.

7. I am aware that I should retain a copy of the Consent Form, when completed, and the Information Sheet.

8. I consent to two specimens of blood being taken from my child, one at the start of the study and one at the end of the study and being used in the above project.

9. If breastfeeding I consent to providing a small sample (1-2 drops) of breast milk when my baby reaches 36 weeks post menstrual age.

10. I agree to the accessing of mine and my child’s medical records at the Women’s and Children’s Hospital and any other hospital my baby and I may be transferred to and from, for the duration of the study.

11. I understand that the alternate contacts I have provided may be used to contact me as explained in the information sheet for study related purposes.

12. I am aware that a study newsletter will be sent to me.

13. I understand that my (my child’s) information will be kept confidential as explained in the information sheet except where there is a requirement by law for it to be divulged.

14. I **do/do not** (please circle one) consent to the blood and breast milk samples being used in any other research project, provided the project has Human Research Ethics approval.

15. I am aware that I may be contacted regarding a future follow-up study.

16. I **do/do not** (please circle one) consent to the collection of three extra specimens of blood being taken from my child, one at the start of the study, on day 14 of life and at the end of the study; two cheek swab samples, one at the start and one at the end of the study; one stool sample at the beginning and one every week until the end of the study; one sample of fluid from the breathing tube (if my baby has a breathing tube).

Signed: .................................................................

Relationship to patient: .................................................................
Full name of patient: ..............................................................

Dated:.............................

I certify that I have explained the study to the parent and consider that he/she understands what is involved.

Signed: ....................................................  Title: ......................................................

Dated: ..............................

Dated: ..............................
APPENDIX 6

N3RO RANDOMISED CONTROLLED TRIAL PATIENT INFORMATION SHEET

Author: Carmel Collins and the N3RO RCT investigative team
N-3 fatty acids for improvement of Respiratory Outcomes – The N3RO trial

Scientific title: Docosahexaenoic acid (DHA) for the reduction of bronchopulmonary dysplasia in preterm infants born at less than 29 weeks gestational age: a randomised controlled trial.

You are invited to take part in a study to help us find out if giving preterm babies extra DHA improves important respiratory (lung) outcomes associated with preterm birth. This is a multi-centre study coordinated by the Women’s and Children’s Health Research Institute, Adelaide.

What is docosahexaenoic acid (DHA) and why might it be important for preterm babies?
DHA is a type of fat (sometimes called “omega 3 or n-3”) that is found in breast milk, fish and fish oil. Because babies can’t make much DHA they rely on the amount they get from their mother during pregnancy. Although preterm babies get a small amount of DHA from breast milk or formula, this is much less than they would have received from their mothers had they not been born early.

What studies have been done? Some years ago we tested the effect of increasing the supply of DHA to preterm babies. This was achieved by asking the mothers to take capsules that increased the natural level of DHA in their breast milk. When this high DHA breast milk was given to their babies we saw a modest benefit in the mental development of girls, but not boys, and it also suggested that fewer babies had lung disease.

However, the importance of both these effects was hard to judge. As a result at the WCH it was thought advisable to provide fish oil capsules to mothers who were providing breast milk for preterm girls. This practice was not adopted in any other centre in Australia or overseas because many people thought that it was possible that the benefits seen in the children who received high DHA breast milk arose by chance and testing child development at the age we did (18 months) is not always a good measure of longer term learning.

What is the new study? In order to resolve this important issue we have received support from the Australian Government to carry out a new study. The study will be larger and importantly, we have developed a new emulsion that allows us to get the DHA to the baby much sooner; we hope this will mean that we will see greater benefits to the health of preterm babies. Because a DHA or control solution will be given directly to the baby it doesn’t matter how much milk they are getting.

What lung conditions can preterm infants get? Because preterm infants are born before many of their organs are mature, about half develop a lung condition called ‘bronchopulmonary dysplasia’, or BPD. This means that they still need breathing support or extra oxygen when they reach 36 weeks ‘corrected’ age (which is 4 weeks before they were...
due to be born). It is now thought that inflammation is one of the factors that lead to BPD. DHA is a known anti-inflammatory so it is possible that if the infant gets an adequate supply of DHA, it could help protect the lungs.

**What does the study involve?** If you choose to participate, your baby will be randomly assigned (like tossing a coin to decide) to receive one of two solutions – either one that contains extra DHA (around 3 times more than breast milk or formula) or one that does not contain any DHA. All babies will still receive some DHA through breast milk or infant formula as at present. Neither you, nor the clinicians nor the research team will be able to choose the group, nor will anyone in the care team know the group.

The study solution will be given to babies through their feeding tube three times a day, just before a normal feed. We aim to commence the solution as soon as possible after the first milk feed. The study solution will be given until your baby reaches 36 weeks corrected age. During the study we will ask for 1-2 drops of blood to measure the amount of DHA in your baby’s blood. This will be done once at the start of the study and once at the end of the study. Whenever possible the blood samples will be collected at the same time as your baby is having other tests. The blood samples are taken carefully by experienced collectors. If your baby has a line in place the blood will be taken from this, otherwise the sample will be taken by a ‘heel prick’ and your baby will receive pain relief beforehand.

We will look to see if your baby has BPD when they reach 36 weeks corrected age or discharge home, whichever occurs first. If you are breastfeeding you will be asked to provide a small sample (1 - 2 drops) of breast milk when your baby is around 36 weeks corrected age.

One of the research staff working on the study will review your medical records to document any pregnancy complications and details of your child’s birth. Your baby’s medical records will also be reviewed for feeding and health information.

**Optional additional samples**

The following samples are optional, if you decide not to have these samples taken you can still participate in the study. For those who choose to have these samples taken they will be used to measure immune markers (substances like antibodies) that provide information about how the immune system is developing and will help us to understand how DHA may help BPD.

The samples include:

- An extra 0.5 mls of blood (this is the same as around 1/10th of a teaspoon or 10 drops of blood), at the start of the study, on day 14 of life and at the end of the study. This will be collected at the same time as your baby is having other routine blood tests

- A cheek swab, once at the start of the study and once at the end of the study. The inside of the cheek is gently rubbed with a swab similar to a ‘cotton bud’

- A stool sample at the start of the study and one sample every week until the end of the study. This will be collected from the nappy during a normal nappy change
• If your baby has a breathing tube one small amount of fluid (1-2 drops) from the tube. This will be taken during normal clinical care procedures or at the time your baby’s doctors say the tube is no longer needed and is removed.

**Future follow-up studies.** DHA has the potential to improve longer-term outcomes. We would like to see the babies who participate in this study at two years of age and again in the early school years. If you would like to be informed about any such further studies we would contact you to see if you were interested in receiving any information.

However, we recognise that people often change their telephone number and address, and therefore cannot be contacted by researchers. To help keep in contact with you we are asking you to provide us with the names and contact details of persons who would be able to let us know your new contact details; these people are usually relatives or friends and are called ‘alternate contacts’. If we needed to use one of the alternate contacts we would call them, explain who we are and that you were involved in a study and have given us their contact details so that they can put us in touch with you.

**Study progress.** When this study is completed we will send you a summary of the study findings. During the study we will also send you newsletters once or twice a year updating you on the progress of the study and keeping you informed of plans for future studies.

**Risks and benefits of the study.** There is no known increased risk to the health of your baby of giving extra DHA. In our previous trial of 657 preterm babies that were born at <33 weeks gestation, breast milk or formula with extra DHA was safely given to these infants. Blood tests will cause some temporary pain and may cause a short term bruise.

**Your rights.** It is entirely your decision to participate or not in this study. If you do decide to participate and are breast feeding we would ask that you don’t take any fish oil capsules.

You are free to withdraw from the study at any time without explanation of why you have chosen to do so and without prejudice to you and your baby’s current or future treatment. All information gathered will be treated with confidence and no information that could identify you or your baby will be released to any person not associated directly with the study, except in the case of a legal requirement to pass on personal information to authorised third parties.

This requirement is standard and applies to information collected both in research and non-research situations. Such requests to access information are rare; however we have an obligation to inform you of this possibility. The results of this trial may eventually be published in medical journals or presented at professional meetings, but you or your baby will not be identified in any way.

The blood and breast milk samples will be discarded at the end of this study unless you have consented to store them for future use. It is possible that new knowledge may become available to indicate that other nutrients or factors are important for breathing outcomes or child development relating to DHA supplementation. Stored samples would only be used by N3RO Investigators with the permission of the Human Research Ethics committee. Any stored samples will be identified by a study number only and will be discarded at the end of the proposed two year follow-up study. Stored samples will not be used for genetic testing.
If you are providing breast milk for a preterm girl and choose not to participate we can advise you about how to increase the amount of DHA in your breast milk if you would like to do this.

**Any questions?** If you would like further information about the study please contact Dr Carmel Collins (8204 5755), Dr Andrew McPhee (8161 7631) or Dr Michael Stark (8161 7631).

This study has been reviewed by the Human Research Ethics Committee of the Women’s and Children’s Health Network (WCHN). Should you wish to discuss the study with someone not directly involved, in particular in relation to matters concerning policies, or your rights as a participant, or should you wish to make a confidential complaint, you may contact the executive secretaries of the committee, Ms Brenda Penny, WCH, 8161 6521.
APPENDIX 7

STANDARD OPERATING PROCEDURE: BLOOD SAMPLE COLLECTION
Title: Blood Sample Collection in NICU for N3RO Side Study (N3RO (S))

Document ID: CNRC_N3ROS_Blood  Version : V3

Author(s): Naomi Fink

Effective Date: 27/02/2014

Department/Institution Name: Child Nutrition Research Centre

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<td>3</td>
<td>Addition of a pager number to contact details</td>
<td>13/05/2014</td>
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NOTE: This document becomes an uncontrolled version once printed.

Please verify current version in electronic SOP records when reading hard copy SOP’s. All Current SOP’s are filed on the Shared Drive in PDF format.
1. INTRODUCTION AND PURPOSE

The objective of this SOP is to document the procedure for performing blood collection from preterm infants for the nested side study in N3RO (Examination of the mechanisms by which supplemental oral docosahexaenoic acid exerts an immunomodulatory effect in the premature infant. *A nested side-study in N3RO*).

2. SCOPE/ APPLICABILITY

This SOP applies to nursing staff, registrars, residents, nurse practitioners, fellows and consultants in the neonatal intensive care units (NICU) at the Women’s and Children’s Hospital (WCH).

3. PROCEDURE FOR 0.5 mL COLLECTION IN NICU

3.1. Samples will be collected Monday-Friday, where applicable, between the hours of 0800-1600.

3.2. Samples will be collected at baseline, which is to be the first scheduled blood collection event after randomization, on day 14 of life and at 36 weeks gestation (upon study completion).

3.3. Blood is only to be collected for this study during routine clinical bloodwork and/or N3RO blood collection events.

3.4. Samples will only be collected from infants where parental consent has been obtained.

3.5. Blood collection should proceed according to the following established guidelines:
   Sampling from arterial lines:
   a. For arterial lines: “Care of the neonate with arterial catheters and sampling from arterial lines – Neonatal Intensive Care” Handbook. Women’s and Children’s Hospital Adelaide: August 2012
   b. For capillary blood collection: “WCHN Multidisciplinary Clinical Guideline – Capillary Blood Collection”

   *Note: if the infant has an arterial line, blood should preferentially be collected from here before a capillary collection.*


3.7. Green top, lithium-heparin (no gel) tubes will be supplied at bedside with labels for each infant enrolled in the study. Fill tubes to 0.5 mL volume (approximately half the tube).
3.8. All specimen containers should be labelled **AFTER** sample collection and details matched with the CRF and any printed labels, as per Sample Management SOP.

3.9. Write the first name, surname, date of collection, sex and study ID on the labels provided and attach outer edge of the label to the specimen container.

3.10. Place specimen containers in a biohazard bag and inform one of the contacts in “Section 3.13” that the specimen is ready for pick-up. Keep the specimen at bedside until it is collected.

3.11. Discard used items into the appropriate category of waste and perform hand hygiene again.

3.12. In the event of an adverse reaction from the patient:

   a. Stop the procedure
   b. Call for assistance within the team
   c. Follow the hospital’s emergency procedure – call 33#
   d. Notify OH&S and complete incident report as required

3.13. Sample collection contact 1: Naomi Fink
      WCHRI, Level 7, Rieger Building
      Pager 4413
      8161 6848 or 0498 136 437

Sample collection contact 2: Dr Adaweyah Donato
      Pager 4413
      WCHRI, Level 7, Rieger Building
      8161 7073 or 0402 220 725

Sample collection contact 3: Irene Kanter
      Pager 4413
      WCHRI, Level 7, Rieger Building
      8161 7073 or 0438 847 017

3.14. If no contact can be made with sample collector please contact WCHRI reception on 8161 7443.

**3.2. Potential Hazards**

Body excretion - infectious.
4. READ RECORD

By signing this read record I certify that I have read the **CNRC_SOP_N3ROS_Blood** and have understood the procedure.

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APPENDIX 8

STANDARD OPERATING PROCEDURE: STOOL SAMPLE COLLECTION
**Title:** Collection of Infant Stool Samples for N3RO Side Study

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<td><strong>Author:</strong> Adaweyah Donato</td>
<td></td>
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<tr>
<td><strong>Effective Date:</strong> 02/2014</td>
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### Document Revision History

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**NOTE:** This document becomes an uncontrolled version once printed.

Please verify current version in electronic SOP records when reading hard copy SOP's. All Current SOP's are filed on the Shared Drive in PDF format.
1. INTRODUCTION AND PURPOSE

The objective of this SOP is to document the procedure for collection of stool samples from preterm infants for the N3RO Side Study.

2. SCOPE/ APPLICABILITY

This SOP applies to nursing staff, registrars, residents, nurse practitioners, fellows and consultants in the neonatal intensive care units (NICU) at the Women’s and Children’s Hospital (WCH).

3. PROCEDURE

All human material must be treated as infectious

3.1 Personal Protective Equipment Required

3.1.1 Gloves

3.2 Sample Collection

3.2.1 Samples will be collected Monday-Friday, where applicable, between the hours of 0800-0230.

3.2.2 Samples will only be collected from infants where parental consent has been obtained.

3.2.3 Gloves must be worn. This is to protect the sampler and to avoid sampler DNA contamination of the specimen. For each infant, a new pair of gloves must be used to minimise this risk.

3.2.4 Use the specimen bag provided to collect and store the infant’s nappy.
3.2.5 Write the infant’s name, date, time (in 24 hours), study ID, gestational age at birth and postnatal age (in days) of the infant on the label provided and attach to the specimen container.

3.3 Contact Details for sample collection

3.3.1 Sample collection contact 1: Naomi Fink
WCHRI, Level 7, Rieger Building
Pager 4413
8161 6848 or 0498 136 437

3.3.2 Sample collection contact 2: Dr Adaweyah Donato
WCHRI, Level 7, Rieger Building
Pager 4413
8161 7073 or 0402 220 725

3.3.3 Sample collection contact 3: Irene Kanter
WCHRI, Level 7, Rieger Building
Pager 4413
8161 7073 or 0438 847 017

3.3.3 If no contact can be made with either sample collector please contact WCHRI reception on 8161 7443.

3.3.4 For general questions regarding the N3RO side study please contact Naomi Fink

3.4 Potential Hazards

3.4.1 Body excretion - infectious.

3.4.2 Splash hazard from placing stool sample in solution – infectious.
4. READ RECORD

By signing this read record I certify that I have read the “CNRC_SOP_N3ROS_Stool” and have understood the procedure.

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## APPENDIX 9

### MATERIALS AND METHODS

#### MATERIALS

All materials, unless otherwise specified, were of analytical reagent grade.

*Processing and collection of biological samples collected as part of the N3RO nested study*

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<td>Interpath Services Pty Ltd.; VIC, Australia</td>
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<td>Greiner Bio-One, MiniCollect 1 mL lithium heparin</td>
<td>450477</td>
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*A549 cells and cell culture*

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<td>Dimethyl sulfoxide (DMSO)</td>
<td>D2650</td>
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<td>Trypan Blue Solution, 0.4%</td>
<td>T8154</td>
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<td>T-75 flasks</td>
<td>156499</td>
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<tr>
<td>Fetal bovine serum (FBS) - heat inactivated for 30 mins at 56°C, frozen at -20°C, stored at 4°C once thawed</td>
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<td>Ham’s F-12K (Kaigh’s) Medium</td>
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<td>10 000 U/mL penicillin + streptomycin (100x)</td>
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<td>Soy oil emulsion (for enteral use)</td>
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### Appendix 9

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<td>Chloroform</td>
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Flow cytometric analysis of cytokines in supernatant

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Determination of SP-D and TGFβ concentration in supernatant and plasma by ELISA

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**Determination of cytokine concentration in plasma samples: high sensitivity magnetic bead panel**

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<td>Milliplex human high sensitivity T Cell magnetic bead panel</td>
<td>HSTCMAG-28SK</td>
<td>Merck Millipore; VIC, Australia</td>
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<td>Sheath fluid</td>
<td>4050000</td>
<td>Thermo Fisher Scientific; Sydney, NSW, Australia</td>
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<tr>
<td>Affymetrix hand-held magnetic plate washer</td>
<td>EPX-55555-000</td>
<td>Jomar Life Research; VIC, Australia</td>
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**PCR and qPCR analysis**

<table>
<thead>
<tr>
<th>MATERIAL</th>
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<tr>
<td>Mo Bio PowerSoil Powerlyzer DNA isolation kit</td>
<td>12855</td>
<td>Mo Bio Laboratories Inc.; Carlsbad, CA, USA</td>
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<tr>
<td>Qubit dsDNA HS Assay Kit</td>
<td>Q32851</td>
<td>Life Technologies; Carlsbad, CA, USA</td>
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<td>Platinum® SYBR® Green qPCR SuperMix-UDG</td>
<td>11733038</td>
<td>Thermo Fisher Scientific; Sydney, NSW, Australia</td>
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<tr>
<td>KAPA Taq PCR kit (KAPA hot start DNA polymerase, Magnesium chloride (MgCl$_2$), 5X hot start buffer, deoxynucleoside triphosphates (dNTP))</td>
<td>BK1001</td>
<td>KAPA Biosystems; Wilmington, MA, USA</td>
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<td>Primers</td>
<td>See Appendix 9, Table 2 for primer information</td>
<td>Sigma-Aldrich; Sydney, NSW, Australia</td>
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<td>AMRESCO E2 Vision Three loading dye</td>
<td>N313-KIT</td>
<td>VWR International; Pty Ltd. VIC, Australia</td>
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<tr>
<td>Promega 100bp DNA ladder</td>
<td>G2101</td>
<td>Promega Australia; Alexandria, NSW, Australia</td>
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Media, buffers and solutions

<table>
<thead>
<tr>
<th><strong>A549 COMPLETE GROWTH MEDIUM IN HAM'S F-12K</strong></th>
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<tbody>
<tr>
<td><strong>COMPOUND</strong></td>
</tr>
<tr>
<td>Penicillin</td>
</tr>
<tr>
<td>Streptomycin</td>
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<td>FBS</td>
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**PHOSPHATE BUFFERED SALINE (PBS) 20X**

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<tr>
<th><strong>COMPOUND</strong></th>
<th><strong>QUANTITY</strong></th>
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<tr>
<td>NaCl</td>
<td>80g</td>
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<tr>
<td>KCl</td>
<td>2g</td>
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<tr>
<td>Na₂HPO₄</td>
<td>11.5g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2g</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>500 mL (final)</td>
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</tbody>
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METHODS

Centrifugation of all samples was performed in either a Sigma 3-16K or an Eppendorf 5415R centrifuge at room temperature, unless otherwise stated.

1.1. **A549 CELLS AND CELL CULTURE**

A549 cells at passage 12 (passage range 12-20) were used throughout these experiments as a model of alveolar type II epithelial cells \(^1\)\(^,\)\(^2\). The cells were cultured in growth media containing Ham's F-12K (Kaighn's) Medium supplemented with 5% heat-inactivated FBS, penicillin (50 U/mL) and streptomycin (50 µg/mL) unless otherwise indicated. All media were purchased endotoxin free. The cells were maintained at 37°C and in a 5% CO₂ concentration in humid conditions.

1.1.1. **A549 subculturung**

A549 cells were passaged according to American Type Culture Collection (ATCC) recommended procedures. Culture medium was removed aseptically using a serological pipette and discarded. Cells were washed by rinsing with 10 mL warm PBS to remove any trypsin inhibitor present from the media. Next, 2-3 mL of trypsin-EDTA (0.05%, 0.02%) was added to the flask and the cells incubated in 5% CO₂ at 37°C until detached. Growth media was added
to the flask (6-8 mL), drawn off using a serological pipette and aliquoted to new T-75 flasks at between 2 x 10^3 and 1 x 10^4 cells/cm^2 (subcultivation ratios between 1:3 and 1:8). Media was renewed 2 – 3 times per week until cells were subcultured.

1.1.2. Cryopreservation of A549 cells
For cryopreservation, A549 cells were recovered from the tissue culture flask following the procedure as described above. The cell suspension was placed into a 15 mL conical tube, centrifuged at 400g for 5 minutes to pellet the cells, the media was then discarded. After washing, the cells were resuspended in media to produce a cell suspension between 2 x 10^3 and 1 x 10^4 cells/mL. A 15 mL conical tube was placed on ice and 20% DMSO solution was prepared in FBS. DMSO was added slowly (drop by drop) to the cell suspension and constantly agitated to ensure equal distribution. The final DMSO concentration was 10%. 1 mL of the cell suspension was added to each cryotube on ice. Cryotubes were immediately placed in a Mr. Frosty™ Freezing Container at -80°C overnight and subsequently transferred to liquid nitrogen for long-term storage.

1.1.3. Optimisation of cell culture conditions
1.1.3.1. Development of A549 cell growth curves
A549 cells were resuspended in standard culture media and seeded at four densities (1, 2, 3 and 4 x 10^5 cells/well) into 6-well plates which were then incubated at 37°C with 5% CO_2. Proliferation of the cells was assessed at day 1, 2, 3 and 4. Wells were counted (in triplicate) and assessed for viability using trypan blue dye in a Neubauer improved cell counting chamber. Approximate confluency was recorded, as well as average cell count and viability for each day (Appendix 9 Figure 1). Percent increase in cell number was calculated per day (Appendix 9 Figure 2).
Appendix 9 Figure 1. A549 cellular growth curves.
Legend to Appendix 9 Figure 1. Average cell count per day (cells/well) for A549 cells (n=3) seeded at four densities (1x10^5 – 4x10^5 cells/well).

Appendix 9 Figure 2. Percent confluency by day for A549 cells.
Legend to Appendix 9 Figure 2. Average percent confluency by day for A549 cells (n=3) seeded at four densities (1x10^5 – 4x10^5 cells/well).
1.1.3.2. Assessment of cytotoxicity of lipid emulsions in A549 cells

Using a pre-mixed WST-1 Cell Proliferation Reagent (Takara Bio Inc., Japan), a range of concentrations of docosahexaenoic acid (DHA) and linoleic acid (LA) (0-400 µM) \(^{(3,4)}\) enteral lipid emulsion preparations were tested to determine the threshold concentration at which cellular proliferation of A549 cells was inhibited. The WST-1 reagent allows for rapid colorimetric measurement of cell proliferation and viability and can be used to assess cytotoxicity as a function of decreased dye production. Certificate of analysis for high-DHA fish oil emulsion and soy oil emulsions (see Appendix 10) were used to calculate the molarity of DHA and LA in each solution. Serial dilutions from pure emulsion to 50-400 µM DHA or LA of each lipid emulsion in Ham’s F-12K were prepared prior to each experiment.

A549 cells were resuspended in standard culture media and incubated at 37°C with 5% CO\(_2\). After 24 hours, DHA and LA (high-DHA fish oil and soy oil emulsion) were added at different concentrations (0-400 µM each). \(2.12 \times 10^4\) cells in 200 µl of culture media+DHA/LA were added to wells in a 96-well plate. After incubation for 24 hours, 20 µL of WST-1 cellular proliferation reagent was added to the wells and the plate incubated at 37°C with 5% CO\(_2\). A second 96 well plate containing 0-400 µM DHA and LA without cells included as a control for each oil. Proliferation was assessed over 4 hours (at 30 min and 1, 2, 3, 4 hours and absorbance read at 450 nm with a reference wavelength of 595 nm using a multi-well spectrophotometer (Victor™ x4 Multilabel reader, Perkin Elmer, Singapore). Reference values were subtracted from the readings at 450 nm. Results of the cellular proliferation assay determined that both 25 µM and 50 µM DHA and LA did not inhibit growth compared to 0 µM (Appendix 9 Figure 3 and 4).
Appendix 9 Figure 3. A549 cellular proliferation in the presence of 0-400 µM docosahexaenoic acid. Legend to Appendix 9 Figure 3. Absorbance (nm) following WST-1 cellular proliferation assay in A549 cells (n=3) treated with 0-400 µM docosahexaenoic acid (DHA) in high-DHA fish oil emulsion for 24 hours.

Appendix 9 Figure 4. A549 cellular proliferation in the presence of 0-400 µM linoleic acid. Legend to Appendix 9 Figure 4. Absorbance (nm) following WST-1 cellular proliferation assay in A549 cells (n=3) treated with 0-400 µM linoleic acid (LA) in soy oil emulsion for 24 hours.

Different concentrations (0-400 µM DHA or LA) were also assessed in the same manner as above for commercially available lipid emulsions for parenteral use; SMOFlipid, ClinOleic, Intralipid, Omegaven. For emulsions containing DHA (SMOFlipid and Omegaven), the molarity was calculated for 50 µM DHA solutions and for 50 µM LA solutions without DHA (ClinOleic and Intralipid). Product inserts for SMOFlipid, ClinOleic, Intralipid, Omegaven...
were used to approximate the molarity of DHA and LA in each solution (Appendix 10). Serial
dilutions from pure emulsion to 0-400 µM of each lipid emulsion in Ham’s F-12K were
prepared prior to each experiment. Results of the cellular proliferation assay determined that
both 25 µM and 50 µM DHA and LA in these emulsions also did not inhibit growth compared
to 0 µM. Consistent with literature (3, 4), 50 µM DHA/LA was used in all further cell culture
assays.

1.1.3.3. Cytokine stimulation of A549 cells in the presence of DHA/LA
A549 cells were stimulated with TNFα (2 ng/mL) as per the protocol of Bryan et al. (2006) (2)
in the presence of 50 µM DHA and LA. Culture media (2 mL) containing 1.25 x 10^5 cells/mL
of culture media were added to 24 well plates and incubated for 24 h at 37°C and 5% CO₂. For
all experiments (n=3-4), cells were between passage 16 and 20. After a 24 h incubation period,
cells were washed with warm PBS and cultured with/without 2 ng/mL TNFα. Media was
replaced with serum-free enteral and parenteral media preparations: a) 50 µM DHA (high-
DHA fish oil emulsion), b) 50 µM soy (soy oil emulsion) and serum-free parenteral media
preparations, c) 50 µM DHA (Omegaven), d) 50 µM DHA (SMOFlipid), e) 50 µM soy
(ClinOleic), f) 50 µM soy (Intralipid). Serum-free Ham’s F-12K media served as the no-
LCPUFA control. After a 24 hour incubation period with/without TNFα and respective media
preparation, aliquots of cell culture supernatant were immediately frozen at -80°C until cytokine
analysis. Culture plates containing the remaining A549 cells in media were then frozen at -80°C
for a single freeze-thaw cycle to lyse the cells.

For lipid analysis, cells were incubated in Ham’s F-12K media for 24 hours in 6-well plates,
followed by a 24 hour incubation with each serum-free emulsion preparation and Ham’s F-12K
media alone. After 24 hours, the post-incubation media was removed and frozen at -80°C. Each
well was rinsed with 2 mL warm PBS to remove lipids and media and 1 mL warm 1x
trypsin/EDTA was added to each well. Plates were incubated for 5-10 mins or until cells
detached and 4 mL of media was added to each well. The contents of each set of triplicate wells
were combined into one 15 mL polypropylene tube and centrifuged at 400g to pellet cells. Media was decanted, the pellet resuspended in 1 mL PBS and transferred to an Eppendorf tube. The tube was then centrifuged at 400 g for a final wash, PBS drawn off, and the pellet resuspended in 50 µL PBS and frozen at -80°C. Samples of each pre-incubation media preparation were frozen at -80°C for later fatty acid analysis.

1.1.3.4. Flow cytometric analysis of cytokines in A549 cell culture supernatants

Cell culture supernatants before and after culture with TNFα and lipid emulsions were assayed for inflammatory cytokines using an enhanced sensitivity human cytokine bead array according to the manufacturer’s protocol. All reagents used were supplied with the enhanced sensitivity flex set and master buffer kit. Unstimulated cell culture supernatants from A549 experiments were assayed undiluted and at a 1:10 to 1:100 dilution after TNFα-stimulation. Cytokines analysed included interleukin (IL)-1β, IL-6, IL-8, IL-10 and interferon (IFN) γ. The minimum concentration detected for each cytokine was 0.64 pg/mL (IL-1β), 6.21 pg/mL (IL-6), 0.89 pg/mL (IL-8), 0.93 pg/mL (IL-10) and 0.81 pg/mL (IFNγ).

Protocol was as follows: mixed capture antibody beads for each cytokine were vortexed and 10 µL was added to each FACS tube containing 25 µL of sample and gently mixed. Samples were incubated at room temperature for 2 hours in the dark. Human detection antibodies for each cytokine (10 µL) were added and gently mixed. Samples were incubated for a further 2 hours at room temperature in the dark. Wash buffer (1 mL) was then added to each FACS tube and centrifuged for 5 minutes at 200 g. Supernatants were carefully aspirated and discarded, at which point 50 µL of the enhanced sensitivity detection reagent was added and gently mixed. Samples were incubated at room temperature for 1 hour in the dark. After a second wash with 1 mL of wash buffer and a 5 minute spin at 200 g, supernatants carefully aspirated and discarded, and 150 µL wash buffer added. FACS tubes were stored at 4°C in the dark overnight until flow cytometry analysis the following morning.
Cytokines in supernatants were analysed on a BD Biosciences FACS Canto flow cytometer. Briefly, BD instrument set-up beads supplied with the master buffer kit were used to adjust detector voltages and set fluorescence compensation. A singlet gate was selected based on forward and side-scatter characteristics and events within this gate were analysed (Appendix 9 Figure 5).

Appendix 9 Figure 5. Scatter plot of singlet gate applied to the top standard (200 000 fg/mL).
Legend to Appendix 9 Figure 5. Scatter plot based on forward (FSC) and side scatter (SSC) properties in order to exclude populations such as cellular debris from the flow cytometric analysis.

Standards were assayed from lowest (0 pg/mL) to highest (200 pg/mL) concentrations prior to the samples. Mean fluorescence intensity was generated by BD FacsDiva™ software version 6.1.3 (BD Biosciences, San Diego, USA, 2009). Standard curves were generated to model the protein concentration as a function of mean fluorescence intensity using Microsoft Excel. Standard curves were accepted if the $R^2$ value of the curve was greater than 0.98.

1.1.3.5. Determination of SP-D concentration in A549 cell culture lysates
Cell culture lysates before and after culture with TNFα and lipid emulsions from A549 experiments were analysed for SP-D using a Surfactant protein D Human Quantikine ELISA kit according to the manufacturer’s protocol. The SP-D ELISA is a solid phase sandwich ELISA
with an assay range of 0.6 - 40 ng/mL. Standards were prepared as outlined in the protocol and assayed in duplicate. Cell lysate samples were assayed undiluted in single detection and intra-assay controls were included. Assay diluent (100 µL) and 50 µL sample/standard were added to each well and incubated for 3 hours at room temperature. Each well was aspirated/washed four times with wash buffer. Human SP-D conjugate (200 µL) was added to each well and incubated for 2 hours at room temperature. The aspiration/wash step was repeated and 200 µL substrate solution (1:1 tetramethylbenzidine/H₂O₂) was added to each well and incubated for 30 minutes in the dark. Stop solution (50 µL- 2N H₂SO₄) was added to each well prior to measuring absorbance.

Absorbance was measured on a multi-well spectrophotometer (Victor™ x4 Multilabel reader, Perkin Elmer, Singapore) at 450 nm with a reference wavelength of 595 nm. Reference values in each well at 595 nm were subtracted from the readings at 450 nm. The average reading of the blank standard replicates (0 ng/mL) were subtracted from all standard and sample values to account for background. Standard curves were generated to model the protein concentration as a function of mean absorbance using Microsoft Excel. Standard curves were accepted if the $R^2$ value of the curve was greater than 0.98.

1.1.3.6. Fatty acid extraction and methylation

After cell culture, fatty acids were extracted from supernatant and cell pellets and methylated according to a previously published procedure (5). Saline (0.9%) was added to media samples (1 mL) and pellet samples (50 µL) to reach a total volume of 1.5 mL in 12 mL glass culture tube. Isopropanol (2 mL) was added to each sample and tube was vortexed and allowed to stand for at least 5 minutes. Chloroform (4 mL) was added to each tube, vortexed and allowed to stand for at least 5 minutes. Tubes were centrifuged at 3000 rpm for 10 minutes. Organic phase was subsequently removed with a glass pipette and transferred to a scintillation vial. Samples were evaporated to dryness under a stream of nitrogen. Sulfuric acid (1%, 2 mL) was added to vials and heated for 3 hours at 70°C to methylate the fatty acids. Samples were vortexed every
30 minutes and lids were loosened at first vortex to relieve pressure. After cooling, water (0.25 mL) and heptane (0.75 mL) were added to vials and vortexed. After a 5 minute settling period, the organic layer containing the fatty acid methyl esters (FAME) was transferred to GC vial containing anhydrous sodium sulfate. Samples were stored at -20°C until GC analysis.

1.1.3.7. Analysis of fatty acids by gas chromatography (GC)
FAME were separated and identified using a Hewlett-Packard (HP) 6890 gas chromatograph fitted with a BPX70 capillary column 50 m x 0.32 mm, film thickness 0.25 mm (SGC Pty Ltd., Victoria, Australia), and a flame ionisation detector (FID) as previously described (5). Briefly, the temperature ramp was programmed to increase from 140-240°C with a constant injector temperature of 250°C and FID temperature of 300°C. The carrier gas was helium and the injection mode was set at a split ratio of 20:1. FAME in unknown samples were identified by comparing to commercial lipid standards using the HP ChemStation software. Results were reported as percent of total fatty acids.
Legend to Appendix 9, Figure 6. Abbreviations: docosahexaenoic acid (DHA), arachidonic acid (AA), long-chain polyunsaturated fatty acid (LCPUFA), tumor necrosis factor alpha (TNFα).
1.2. COLLECTION AND PROCESSING OF BIOLOGICAL SAMPLES FROM PRETERM INFANTS

Refer to the clinical protocol (Appendix 3) for the trial framework of the nested study.

1.2.1. Peripheral blood sample

1.2.1.1. Collection

Blood was collected according to standard clinical practise by clinical staff in the neonatal intensive care unit (NICU) or special care baby unit (SCBU) at the Women’s and Children’s Hospital (WCH) at baseline, postnatal day 14 and at study end (see SOP in Appendix 7). Briefly, 0.5 mL capillary blood was collected by clinical staff into lithium heparin coated tubes. If the infant had an arterial line, blood was preferentially collected via this route. Samples were labelled appropriately, sealed in a biohazard bag and collected immediately by the candidate.

1.2.1.2. Stimulation of whole blood with E. coli LPS

LPS stimulation of whole blood cultures was carried out according to a previously described method (6). Briefly, heparinised whole blood from N3RO trial participants, was diluted 6.25 X in RPMI 1640 culture medium containing 0.1% FBS. Duplicate 40 µL aliquots of whole blood with added LPS (from E. coli, final concentration 1µg/mL) were set up in sterile 96-well round bottom culture plates and incubated at 37°C in the presence of 5% CO₂. Whole blood was cultured in media alone as the control. Whole blood cultures were incubated for 21-24 hours. Supernatants were subsequently removed by pipette and stored in 60 µL aliquots at -80°C for later batch analysis of cytokines by flow cytometry.

1.2.1.3. Isolation and cryopreservation of supernatants from whole blood culture and plasma

After the whole blood aliquots were removed for LPS stimulation, the remaining blood sample was centrifuged at 2000 g for 10 minutes to isolate plasma. If sample volume was >120 µL, 60 µL aliquots were pipetted off into barcoded cryotubes and stored at -80°C until batch analysis of immune markers.
1.2.2. Faecal matter samples

1.2.2.1. Collection

Stool samples were requested weekly for each infant and collected by clinical staff in the NICU and SCBU at the WCH (see SOP in Appendix 8). Using gloves, the clinical staff were instructed to place nappies directly into a labelled biohazard bag and the candidate was paged for immediate pick up. At this point, the sample was placed in the refrigerator (maximum 4 hours) or processed immediately.
1.2.2.2. Cryopreservation
Stool was transferred aseptically from the nappy into a barcoded cryotube in 150-250 mg aliquots using a sterile pipette tip. Care was taken to avoid dragging fibres from the nappy. Samples were frozen immediately at -80°C for later DNA extraction.

1.3. ANALYSIS OF IMMUNE MARKERS IN PLASMA AND WHOLE BLOOD
For all immune marker analyses, intra- and inter-assay controls were included for each batch analysis and results were accepted if differences between assays were less than 1 SD, unless otherwise stated.

1.3.1. Determination of cytokine concentration in plasma samples
Cytokines in plasma samples obtained at baseline, postnatal day 14 and study end were assayed using a Millipore Human High Sensitivity T Cell Magnetic Bead Panel (Merck Millipore, VIC, Australia) according to the manufacturer’s protocol. IL-1β, IL-10, IL-12p70, IL-17A, IL-23, TNFα, MIP1α and IFNγ were assessed in an 8-plex and run on a Luminex 200. IL-6 and IL-8 were assessed in a 2-plex and run on a Luminex MAPGPIX®. Each respective software calculated the true limits of detection (minimum Detectable Concentration-MinDC) by mathematically determining what the empirical MinDC would be if an infinite number of standards were run under the same conditions for that assay. Cytokines assayed and their respective limit of detections are outlined in Appendix 9 Table 1.
Appendix 9 Table 1. Limit of detection and range of standards for each cytokine assessed via the Millipore Human High Sensitivity T Cell Magnetic Bead Panel

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Minimum detectable concentration (pg/ml)</th>
<th>Range of standards (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>0.19</td>
<td>0.49-2000</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.12</td>
<td>0.18-750</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.14</td>
<td>0.31-1250</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.64</td>
<td>1.46-6000</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.19</td>
<td>0.49-2000</td>
</tr>
<tr>
<td>IL-17A</td>
<td>0.75</td>
<td>0.73-3000</td>
</tr>
<tr>
<td>IL-23</td>
<td>12.94</td>
<td>7.93-32.50</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.15</td>
<td>0.43-1750</td>
</tr>
<tr>
<td>MIP1α</td>
<td>2.00</td>
<td>0.31-1250</td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.59</td>
<td>0.61-2500</td>
</tr>
</tbody>
</table>

Seven standard samples and two quality controls were added to wells in duplicate and samples were assayed in a single detection. When sample volume allowed, samples were assayed in duplicate. Plates were incubated overnight at 4°C on a plate shaker, protected from light.

Plates were washed three times with wash buffer and using a handheld magnetic plate. Detection antibody (50 µL) specific to each cytokine was added to each well and plates were agitated for 1 hour on a plate shaker at room temperature, protected from light. Strepavidin-Phycoerythrin (50 µL) was added to each well and plates incubated for 30 minutes on a plate shaker at room temperature, protected from light. Contents of the well were aspirated and washed three times with wash buffer using a handheld magnetic plate. Sheath fluid was added to each well and beads were resuspended for 5 minutes on a plate shaker.

The mean fluorescence value was detected and plasma concentration was calculated according to the standard curve generated by the software. Coefficients of variation between assays were low (< 1.5%) for standards, and the data between plates was combined and extrapolated from the same standard curve.
1.3.2. Flow cytometric analysis of cytokines in supernatant from whole blood culture

Supernatants from whole blood culture after LPS stimulation were assayed for inflammatory cytokines using an enhanced sensitivity human cytokine bead array and according to the manufacturer’s protocol (and as described in Appendix 9, Section 1.1.3.4). All reagents used were supplied with the enhanced sensitivity flex set and master buffer kit. Supernatants from whole blood stimulation experiments were assayed undiluted. Cytokines analysed included IL-6, IL-8, IL-10 and IL-12p70, IFNγ and TNFα. Detection limits for each cytokine were 0.64 pg/mL (IL-1β), 6.21 pg/mL (IL-6), 0.89 pg/mL (IL-8), 0.93 pg/mL (IL-10), 0.81 pg/mL (IFNγ) and 1.41 pg/mL (TNFα).

1.3.3. Determination of TGFβ concentration supernatant from whole blood culture

Supernatants from whole blood culture after LPS stimulation were analysed for TGFβ using a Human TGF-beta 1 DuoSet ELISA according to the manufacturer’s protocol. The TGFβ ELISA is a solid-phase sandwich ELISA with an assay range of 31.20 - 2,000 pg/mL. Standards were prepared as outlined in the protocol and added to each well in duplicate. Samples were assayed in single detection and where sample volume allowed, in duplicate. Plates were coated with 100 µL capture antibody (final concentration 2.0 µg/mL) and incubated at room temperature overnight. Plates were washed five times with wash buffer (0.05% Tween20 in PBS: PBS-T) and blocked with block buffer (5% Tween20 in PBS) for a minimum of 1 hour. To activate latent TGFβ to its immunoreactive form, 1N HCl was added to each sample and incubated at room temperature for 10 minutes. Each sample was subsequently neutralised with 1.2N NaOH/HEPES and diluted with reagent diluent (1.4% FBS in PBS-T) to a final dilution of 1:6. Plates were aspirated/washed as previously described and 100 µL sample/standard was added to each well and incubated for 2 hours at room temperature. Aspiration/wash step was repeated and 100 µL of detection antibody (final concentration 300 ng/mL) was added to each well and incubated for 2 hours at room temperature. Aspiration/wash step was repeated and 100 µL of streptavidin-HRP was added to each well and incubated for
20 mins at room temperature, protected from light. Aspiration/wash step was repeated and 100 µL of 1:1 tetramethylbenzidene/H$_2$O$_2$ added and incubated for 20 minutes at room temperature, protected from light. Prior to measuring absorbance, 50 µL 2N H$_2$SO$_4$ was added to each well.

Absorbance was measured with a Victor™ x4 Multilabel reader at 450nm with a reference wavelength of 595 nm (as described in Appendix 9, Section 1.1.3.5).

### 1.3.4. Determination of SP-D concentration in plasma samples

Plasma samples from the neonates in the N3RO trial were obtained at baseline, postnatal day 14 and study end and analysed for SP-D using a Surfactant protein D Human Quantikine ELISA according to the manufacturer’s protocol. All reagents used were supplied with the kit. The SP-D ELISA is a solid phase sandwich ELISA with an assay range of 0.6 - 40 ng/mL. Standards were prepared as outlined in the protocol and assayed in duplicate. Plasma samples were assayed at a final dilution of 1:5 or 1:10 in single detection.

Assay diluent (100 µL) and 50 µL sample/standard were added to each well and incubated for 3 hours at room temperature. Each well was aspirated/washed four times with wash buffer. Human SP-D conjugate (200 µL) was added to each well and incubated for 2 hours at room temperature. Aspiration/wash step was repeated and 200 µL substrate solution (1:1 tetramethylbenzidene/H$_2$O$_2$) was added to each well and incubated for 30 minutes, protected from light. Stop solution (50 µL- 2N H$_2$SO$_4$) was added to each well prior to measuring absorbance.

Absorbance was measured with a Victor™ x4 Multilabel reader at 450nm with a reference wavelength of 595 nm (as described in Appendix 9, Section 1.1.3.5).

### 1.4. ANALYSIS OF STAPHYLOCOCCUS AND MECA+ BACTERIA IN FAECAL SAMPLES

#### 1.4.1. DNA extraction and quantification

Total DNA was extracted from the faecal samples using MoBio PowerSoil Powerlyzer DNA isolation kits according to the manufacturer’s instructions with slight modifications. Briefly,
approximately 0.15g of stool sample was added to the Bead Solution and samples were mechanically lysed with an MP Biomedicals FastPrep-24 (Jomar Life Research, Welland, Australia) with two pulses of maximum speed for 60s. Eluted DNA concentration was quantified fluorometrically with a Qubit dsDNA HS Assay kit.

1.4.2. In silico primer analysis
Primers from previous studies were used to test their suitability and specificity in detecting staphylococci and methicillin resistance from clinical samples. Ten primers sets in total were used from Xu et al. (2012)\(^7\), Mason et al. (2001)\(^8\), Vannuffel et al. (1995)\(^9\), Martineau et al. (2001)\(^10\) and Denman et al. (2006)\(^11\). Their specificity were tested against sequences available at the National Centre for Biotechnology Information (NCBI) via a primerBLAST search to ascertain primer specificity\(^12\).

1.4.3. Assessment of specificity of primer sets
Specificity of these primers was assessed against pure reference bacteria by PCR. Briefly, a PCR reaction contained 2.5 µL KAPA hot start buffer 5x, 1.25 µL MgCl\(_2\) (25 mM), 0.25 µL dNTP (10 mM), 0.25 µL each forward and reverse primer (10 µM), 1.0 µL DNA, 0.1 µL Taq polymerase and 6.9 µL water. The PCR reaction was performed on a Veriti 96-well Thermal Cycler (Life Technologies) using the following program: 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, a range of temperatures from 50°C to 64°C (to test optimal temperature for each primer) for 30 seconds, and 72°C for 60 seconds and a final extension step of 72°C for 5 minutes.

On completion of PCR reactions, 5 µL of sample was added to 1 µL of loading dye. A 1.5% agarose gel was prepared in TAE buffer and loaded into a Bio-Rad electrophoresis tank. Molecular marker (3 µL) and 2 µL water were added to the first and last well. The gel was electrophoresed at 100V for 1 hour. PCR products were visualised under UV light used GeneGenius software (Syngene, USA).
1.4.4. qPCR primer design

Determination of total bacterial load was performed by quantitative PCR (qPCR) using primers specific to conserved regions of the 16S ribosomal RNA gene (11). Enumeration of staphylococci was achieved using primers specific to the *tuf* gene (10), while copies of the methicillin resistance determinant, *mecA*, were quantified using a separate qPCR assay with a primer specific to the *mecA* gene (9, 13) (Table 2).

### Appendix 9 Table 2. PCR primers for qPCR assay

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal amplification</td>
<td>5'-CGG CAA CGA GCG CAA CCC-3'</td>
<td>130</td>
<td>Denman, 2006</td>
</tr>
<tr>
<td></td>
<td>5'-CCA TTG TAG CAC GTG TGT AG CC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus specific</td>
<td>5'-CGT GTT GAA CGT GGT CAA ATC A-3'</td>
<td>370</td>
<td>Martineau, 2001</td>
</tr>
<tr>
<td></td>
<td>5'-CAT TTC AGT ACC TTC TGG TAA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin resistance</td>
<td>5'-TGG CTA TCG TGT CAC AAT CG-3'</td>
<td>310</td>
<td>Vannuffel, 1995</td>
</tr>
<tr>
<td></td>
<td>5'-CTG GAA CTT GTT GAG CAG AG-3'</td>
<td></td>
<td>and Ryffel 1990</td>
</tr>
</tbody>
</table>

1.4.5. PCR-based enumeration of total bacteria, staphylococci, and *mecA*+ bacteria

PCR reactions were performed in a total volume of 10 µL, and contained 1 µL DNA (1-100 ng DNA/µL), 0.8 µL forward and reverse 16S universal primer (0.23 µM) or 0.91 µL forward and reverse *tuf* or *mecA* primers (0.26 µM) and 5 µL SYBR Green (company). PCR reactions were performed using a QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Sydney, Australia) with the following parameters: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C (*tuf*) or 58°C (*mecA*) or 60°C (16S) for 60 seconds. The final melt curve program was 95°C for 15 seconds, 55°C (*Tstag*) or 58°C (*mecA*) or 60°C (16S) for 60 seconds and 95°C for 15 seconds. Samples and standards were assayed in triplicate. Results were normalised to mg of faecal material and expressed as percent of total bacterial load.
1.4.6. Standard curve and DNA yield

Standard curves were generated by plotting the observed C<sub>t</sub> values against the logarithm of the number of femtograms (fg) of DNA in the standard. Standard curve slopes are presented in Appendix 9, Table 3. Amount of DNA was calculated using the following equation:

\[
\text{DNA (fg) = 10}^{(\text{slope x Ct value} + \text{y-intercept})}
\]

DNA yield was calculated by normalising values for femtograms of DNA to the amount of faecal material (fg) used for extraction. For statistical analysis purposes (see Appendix 4) Staphylococcus and meca levels were expressed as percent (%) of total bacteria detected by the universal 16S primer. Cycle threshold (C<sub>t</sub>) values for intra- and inter-assay controls were all less than 1 SD different between assays.

Appendix 9 Table 3. Standard curve line fit and slope for PCR assays

<table>
<thead>
<tr>
<th>Primer</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal amplification (16S)</td>
<td>0.9970</td>
<td>-0.2810</td>
</tr>
<tr>
<td></td>
<td>0.9989</td>
<td>-0.2691</td>
</tr>
<tr>
<td>Staphylococcus specific (tuf gene)</td>
<td>0.9979</td>
<td>-0.2651</td>
</tr>
<tr>
<td></td>
<td>0.9916</td>
<td>-0.2567</td>
</tr>
<tr>
<td>Methicillin resistance (meca gene)</td>
<td>0.9971</td>
<td>-0.2689</td>
</tr>
<tr>
<td></td>
<td>0.9979</td>
<td>-0.2702</td>
</tr>
</tbody>
</table>

1.4.7. Antibiotic and probiotic exposure data

Information on antibiotic, antifungal and probiotic was recorded from the WCH case note files for participants. Antibiotic, probiotic and antifungal exposure information was not available for research purposes for one participant. Date, type of medication, dose in mg/kg/day, final dose and time(s) of administration were recorded for each participant for each day between baseline and study end. The type of probiotic used in the NICU at the WCH is standard; Infloran supplying supplying Bifidobacterium bifidum and Lactobacillus acidophilus.
REFERENCES


CERTIFICATES OF ANALYSIS FOR LIPID EMULSIONS USED IN CELL CULTURE

1. High-DHA fish oil emulsion (Nu-Mega Ingredients Pty, VIC, Australia)
2. Soy oil emulsion (Nu-Mega Ingredients Pty, VIC, Australia)
3. ClinOleic (Baxter Healthcare; Old Toongabbie, NSW, Australia)
4. SMOFlipid (Fresenius Kabi; Mount Kuring-Gai, NSW, Australia)
5. Intralipid (Fresenius Kabi; Mount Kuring-Gai, NSW, Australia)
6. Omegaven (Fresenius Kabi; Mount Kuring-Gai, NSW, Australia)
Appendix 10 Figure 1. High-DHA fish oil emulsion certificate of analysis

Certificate of Analysis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Units</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Fat</td>
<td>%</td>
<td>22.6</td>
</tr>
<tr>
<td>Docosahexanoic Acid (DHA)</td>
<td>mg/mL</td>
<td>123</td>
</tr>
<tr>
<td>Eicosapentaenoic Acid (EPA)</td>
<td>mg/mL</td>
<td>8.8</td>
</tr>
<tr>
<td>Vitamin-C</td>
<td>mg/mL</td>
<td>19</td>
</tr>
<tr>
<td>Vitamin-E (α-tocopherol)</td>
<td>mg/100mL</td>
<td>21</td>
</tr>
<tr>
<td>Protein</td>
<td>%</td>
<td>6</td>
</tr>
<tr>
<td>Moisture</td>
<td>%</td>
<td>58</td>
</tr>
</tbody>
</table>

Nu-Mega Ingredients certifies that this product has been packaged by Syphamu Pty Ltd in accordance with the environmental specification of Grade-A work-zone as outlined in Annex 1 of PICS Guide for Good Manufacturing Practices for Medicinal Products 2009.

This product is to be used for clinical trial purposes only.

This product has undergone microbiological analysis to confirm acceptability for infants.

Glenn Elliott  
Quality & Regulatory Affairs Manager

Date: 26 May 2014 (re-issued)
Appendix 10 Figure 2. Soy oil emulsion certificate of analysis

Certificate of Analysis

<table>
<thead>
<tr>
<th>Product:</th>
<th>N3RO Trial - Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product Description:</td>
<td>Soy Oil Emulsion</td>
</tr>
<tr>
<td>Batch No:</td>
<td>290414</td>
</tr>
<tr>
<td>Filling Date:</td>
<td>30 Apr 2014</td>
</tr>
<tr>
<td>Retest Date:</td>
<td>29 Jan 2015</td>
</tr>
<tr>
<td>Date of Release:</td>
<td>22 May 2014</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>UNITS</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Fat</td>
<td>%</td>
<td>22.7</td>
</tr>
<tr>
<td>Alpha-linolenic (ALA)</td>
<td>mg/1mL</td>
<td>13.0</td>
</tr>
<tr>
<td>Linoleic Acid (LA)</td>
<td>mg/1mL</td>
<td>125</td>
</tr>
<tr>
<td>Vitamin-C</td>
<td>mg/mL</td>
<td>19</td>
</tr>
<tr>
<td>Vitamin-E (a-tocopherol)</td>
<td>mg/100mL</td>
<td>2.5</td>
</tr>
<tr>
<td>Protein</td>
<td>%</td>
<td>6</td>
</tr>
<tr>
<td>Moisture</td>
<td>%</td>
<td>68</td>
</tr>
</tbody>
</table>

Nu-Mega Ingredients certifies that this product has been packaged by Sypharma Pty Ltd in accordance with the environmental specification of Cradle2Cradle work zone as outlined in Annex 1 of PICs Guide for Good Manufacturing Practices for Medicinal Products 2009.

This product is to be used for clinical trial purposes only.

This product has undergone microbiological analysis to confirm acceptability for infants.

Glenn Elliott
Quality & Regulatory Affairs Manager

Date: 28 May 2014 (re-issued)
Appendix 10 Figure 3. ClinOleic product insert

**CLINOLEIC**

**Name of the medicine**

**Composition:**

- Stable fat emulsion containing a mixture of refined olive oil (approximately 69%) and refined soy oil (approximately 20%), 200 g.
- Egg lecithin (purified egg phosphatides) 12 g, glycerol 22.5 g.
- Sodium citrate 0.3 g and Water for injections to 1,000 mL. (Test pH between 6.3 - 8.0).

One of the active ingredients, soy oil, contains acetylsalicylic acid as an antioxidant (the radical scavenger) in the concentration of 0.15 mg of oil.

**Description**

CLINOLEIC 20% is a milk-like homogeneous liquid. CLINOLEIC 50% is an isotonic emulsion. It has an osmolality of approximately 345 mOsm/L and a water and energy content of 3,860 Kcal/L. The relative density of CLINOLEIC 20% is in the range of 0.935 - 0.998.

**Pharmacology**

**Pharmacological actions:**

CLINOLEIC 20% provides a moderate proportion of essential fatty acids (EFA), which probably facilitates their utilization. The combination of olive and soy oils allows a content of fatty acids in an approximate ratio of:

- Saturated fatty acids: 16% (SFA)
- Monounsaturated fatty acids: 65% (MUFA)
- Essential Polyunsaturated fatty acids: 29% (PUFA)

For patients requiring complete parenteral nutrition, complementary carbohydrates, amino acids, electrolytes, vitamins, and trace elements supplements are required.

CLINOLEIC 20% is a source of energy; the high-energy content of the emulsion allows the administration of a large quantity of calories in a small volume. CLINOLEIC 50% also contains glycerol for isotonicity. Egg lecithin supplies phosphatides and choline.

**Pharmacokinetic properties:**

In CLINOLEIC 20% most of the lipid particle sizes are in the range of chylomicrons (0.08 - 0.5 µm) with the mean diameter of less than 0.45 µm. However, they may contain a small fraction (up to 2.5%) of particles having a diameter of more than 1 µm.

**Clinical Trials**

CLINOLEIC has been used in a number of small clinical trials generally using intralipid as a comparative agent. The numbers enrolled in these trials were small and they are not suitable for data pooling or meta-analysis or for demonstrating non-inferiority to the comparator. The studies were of variable duration. The studies usually measured fatty acid composition of plasma lipid fractions. Of the population studied, 52% of the adults were aged over 65 years old.

The 2 pivotal trials enrolled 59 infants and children aged under 11 years old.

- Study CT 2402/P61/34/51 ("Ricor Study"). double-blind, randomised, parallel group, measured the level of fatty acids in plasma phospholipids (primary efficacy variable) and compared the long-term efficacy and safety of CLINOLEIC 20% (p=0.0) to Intralipid 20% (p=0.0) in children and infants who needed prolonged Parenteral Nutrition (PN) at home or hospital. Seventeen patients aged from 1 to 3 years old were exposed for 2 months and 1 patient was exposed for one month.

The results of the study are shown in the following table:

| Table 1: Primary efficacy variable: fatty acids in plasma phospholipids |
|-----------------------------|-----------------------------|
|                            | Day 0 | Day 9 |
| Oleic acid (C18:1 n-9) (p=0.022) | 18.6 | 14.9 |
| CLINOLEIC                   | 10.7  | 14.5  |
| Intralipid                  | 8.2   | 9.3   |
| Linoleic acid (C18:2 n-6) (p=0.0001) | 18.6 | 15.9 |
| CLINOLEIC                   | 17.8  | 30.2  |
| Intralipid                  | 17.2  | 15.9  |
| C20:4n-6 CL (C18:2 n-6) (p=0.0001) | 5.98 | 9.76 |
| CLINOLEIC                   | 5.85  | 5.48  |
| Intralipid                  | 5.85  | 5.48  |
| n-6 metabolites (C16:2 n-6) (p=0.0001) | 6.22 | 3.96 |
| CLINOLEIC                   | 6.03  | 3.34  |
| Intralipid                  | 6.03  | 3.34  |

- Study CT 2402/P61/34/51 ("Ricor Study"). double-blind, randomised, parallel group, compared the efficacy and safety of CLINOLEIC 20% (n=22) to Intralipid 20% (n=20) in premature infants requiring lipid based Total Parenteral Nutrition (TPN) for 7 days. Forty-two premature infants aged —gestational age: 30 to 36 weeks a 6 days were exposed.

The results are shown in the following table:

| Table 2: Primary efficacy variable: n-6 and n-3 metabolites fatty acids; mean acid arachidonic acid |
|-----------------------------|-----------------------------|
|                            | Day 0 | Day 9 |
| n-6 metabolites (p=0.10)    | 16.8  | 13.3  |
| CLINOLEIC                   | 17.7  | 12.0  |
| Intralipid                  | 17.7  | 12.0  |
| n-3 metabolites (p=0.7)     | 5.28  | 2.73  |
| CLINOLEIC                   | 3.28  | 2.73  |
| Intralipid                  | 3.28  | 2.73  |
| Mead acid (C20:3 n-9) (p=0.003) | 1.15 | 1.04 |
| CLINOLEIC                   | 1.15  | 1.04  |
| Intralipid                  | 1.15  | 1.04  |
| Arachidonic acid (C20:4n-6) (p=0.0) | 13.3 | 9.2 |
| CLINOLEIC                   | 13.3  | 9.2   |
| Intralipid                  | 13.3  | 9.2   |

**Biodisibility of the emulsion in the treatment control groups was similar.**

**Indications**

Parenteral nutrition when oral or enteral nutrition is impossible, inadequate or contraindicated.

**Contraindications**

- Known hypersensitivity to egg or soybean proteins or to any of the ingredients, inducing the induction and/or exceptions.
- Severe hyperlipidaemia or severe disorders of lipid metabolism characterized by hypercholesterolaemia and non-correctional metabolism disorders including lactic acidosis and uncompensated diabetes.
- Severe renal disease.
- Blood coagulation disorders, thrombophlebitis.
- Acute and chronic renal failure. In absence of specific studies, there is insufficient data to justify its use in acute/chronic renal failure.
- Myocardial Infarction.
Appendix 10 Figure 3. continued

Ingredients
Active ingredients:
mixture of refined olive oil (approximately 80%) and refined soya oil (approximately 20%), 200 g of mixture/litre.
Other ingredients:
- egg lecithin (12 g);
- glycerol (22.5 g);
- sodium citrate (0.3 g);
- water for injections to 1000 ml.
and
- ascorbyl palmitate (0.15 mg per gram of oil).

Storage
CLINOLEIC will be stored in the pharmacy or the hospital ward. It is recommended that the product be stored in a cool dry place, protected from light, where the temperature stays below 25°C.

Where can you get more information?
You can get more information from your doctor or pharmacist.

Name and Address of Sponsor
Baxter Healthcare Pty Ltd.
1 Baxter Drive
81 Tempe Street, NSW 2146
Australia

Australian Registration number:
CLINOLEIC 20% bottle
AUST R 97538
CLINOLEIC 20% bag
AUST R 97537

Date of preparation:
February 2014

Clototec and Baxter are trademarks of Baxter International Inc
Appendix 10 Figure 4. SMOFlipid product insert

PRODUCT INFORMATION

SMOFlipid® 20% emulsion for infusion

Each 1000mL contains:

- Soya Oil: 60 g
- Medium-chain Triglycerides: 60 g
- Olive oil: 50 g
- Fish oil: 30 g

Excipients includes:

- Glycerol: 25 g
- Egg Leithin: 12 g
- dl-alpha-Tocopherol: 165 - 225 mg
- Sodium Hydroxide: pH approx. 8
- Sodium Oleate: 500 mg
- Water for injections: to 1000mL

Total energy: 3400 kJ (800 kcal)

PHARMACOLOGY

The fat emulsion has a particle size and biological properties similar to those of endogenous chylomicrons. The constituents of SMOFlipid: Soya oil, Medium-chain Triglycerides, Olive Oil and Fish Oil have their own pharmacodynamic properties in addition to their energy contents.

Soya Oil has a high content of essential fatty acids. The omega-6 (ω6) fatty acid linoleic acid is the most abundant (approx. 55-60%). Alpha-linolenic acid, an omega-3 (ω3) fatty acid, constitutes about 6%. This part of SMOFlipid provides essential fatty acids.

Medium-chain fatty acids are rapidly oxidised.

Olive Oil mainly provides energy in the form of mono-unsaturated fatty acids.

Fish Oil is characterised by a high content of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). DHA is an important structural component of cell membranes, whereas EPA is a precursor of eicosanoids as prostaglandins, tromboxanes and leucotrienes.

Vitamin E protects unsaturated fatty acids against lipid peroxidation.
Appendix 10 Figure 5. Intralipid product insert

**PACKAGE INSERT**

**Intralipid®**
Lipid Injectable Emulsion, FS Std
Soybean Oil 10%, 20%, 30% w/v

**THERAPEUTIC CLASSIFICATION**
Lipid Emulsion for Intravenous Nutrition

**ACTION**
Intralipid acts as an energy source in patients for whom the usual intravenous therapy would not be adequate and as a source of essential fatty acids to prevent essential fatty acid deficiency.

Providing sufficient amounts of calories to satisfy basal metabolic requirements plus the additional needs imposed by disease and/or surgical stress can be difficult and sometimes even impossible. If the intravenous route has to be used and only carbohydrates are given as an energy source large amounts of fluid or very hypertonic solutions must be employed. Fat has an energy value a little more than twice that of carbohydrates, and is therefore an excellent source of energy for use in parenteral nutrition. By including fat emulsion in the nutritional programme a balanced intravenous nutrition can be achieved.

Moreover, INTRALIPID is practically isotonic with blood which makes it possible to infuse large amounts of energy providing substrate in a small volume of fluid via peripheral veins. This property makes possible peripheral vein infusion of solutions that otherwise have to be administered by central veins. (See ADMINISTRATION).

Fat emulsions may be used to supply up to 40% of the non-protein energy requirements of the patient. Each mL of Intralipid 10% contains 4.5 kJ (1.1 kcal), each mL of Intralipid 20% contains 8.4 kJ (2.0 kcal) and each mL of Intralipid 30% contains 12.6 kJ (3.0 kcal). Half a litre of Intralipid 10%, Intralipid 20% and Intralipid 30% thus contains 2.5 MJ (650 kcal), 4.2 MJ (1000 kcal) and 6.3 MJ (1500 kcal), respectively. Particle size and biological properties are similar to those of natural chylomicrons.

The intravenously administered fat is utilized as an energy source by the organism in the same manner as orally ingested fat; as demonstrated in a number of investigations and by different methods e.g. growth experiments. Parenterally administered fat is utilized rapidly by the body for energy purposes.

The elimination of fat from the blood stream after intravenous administration has been studied in the dog, rabbit and in man by determination of the plasma triglyceride content.
Appendix 10 Figure 5. continued

Molecular Mass: approximately 870 g/mol

Physical Form: Liquid

Melting Point/Freezing Point (range): -19° to +2°C / -10° to -25°C

Composition:

<table>
<thead>
<tr>
<th>1000 mL contain:</th>
<th>Intralipid 10%</th>
<th>Intralipid 20%</th>
<th>Intralipid 50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified soybean oil</td>
<td>100 g</td>
<td>200 g</td>
<td>300 g</td>
</tr>
<tr>
<td>Purified egg phospholipids</td>
<td>12 g</td>
<td>12 g</td>
<td>12 g</td>
</tr>
<tr>
<td>Glycerol monohydrate</td>
<td>22.0 g</td>
<td>22.0 g</td>
<td>16.7 g</td>
</tr>
<tr>
<td>Water for injection q.s. aq</td>
<td>1000 mL</td>
<td>1000 mL</td>
<td>1000 mL</td>
</tr>
<tr>
<td>pH is adjusted with sodium hydroxide</td>
<td>to pH approx 8</td>
<td>to pH approx 8</td>
<td>to pH approx 8</td>
</tr>
<tr>
<td>Energy content/ litre</td>
<td>4.6 MJ (1100 kcal)</td>
<td>8.4 MJ (2000 kcal)</td>
<td>12.6 MJ (3000 kcal)</td>
</tr>
<tr>
<td>Osmolarity (approx.)</td>
<td>300 mOsM/kg water</td>
<td>350 mOsM/kg water</td>
<td>310 mOsM/kg water</td>
</tr>
<tr>
<td>Osmolarity (approx.)</td>
<td>260 mOsM/L</td>
<td>260 mOsM/L</td>
<td>260 mOsM/L</td>
</tr>
</tbody>
</table>

Fatty Acid Pattern of Intralipid (%):

Considerable variation in the pattern can occur as a result of utilizing biological sources.

<table>
<thead>
<tr>
<th></th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>13</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>4</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>22</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>52</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>8</td>
</tr>
<tr>
<td>Others</td>
<td>1</td>
</tr>
</tbody>
</table>

Packaging (Biofine and Excel):

The packaging consists of an inner bag (primary package) with an oxygen barrier over-pouch. An oxygen absorber and an integrity indicator (Oxalert™) are placed between the inner bag and the over-pouch.
Appendix 10 Figure 6. Omegaven product insert

### Package Insert - Instructions for Use. Read Carefully

**Omegaven**  
Emulsion for Infusion

**Qualitative and Quantitative Composition**

100 ml emulsion contain:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highly refined fish oil containing</td>
<td>10.0 g</td>
</tr>
<tr>
<td>eicosapentaenoic acid (EPA)</td>
<td>1.25 - 2.82 g</td>
</tr>
<tr>
<td>docosahexaenoic acid (DHA)</td>
<td>1.44 - 3.08 g</td>
</tr>
<tr>
<td>myristic acid</td>
<td>0.1 - 0.6 g</td>
</tr>
<tr>
<td>palmitic acid</td>
<td>0.25 - 1.0 g</td>
</tr>
<tr>
<td>palmitoleic acid</td>
<td>0.3 - 0.9 g</td>
</tr>
<tr>
<td>stearic acid</td>
<td>0.05 - 0.2 g</td>
</tr>
<tr>
<td>oleic acid</td>
<td>0.6 - 1.3 g</td>
</tr>
<tr>
<td>linoleic acid</td>
<td>0.1 - 0.7 g</td>
</tr>
<tr>
<td>linolenic acid</td>
<td>≤ 0.2 g</td>
</tr>
<tr>
<td>octadecatetraenoic acid</td>
<td>0.05 - 0.65 g</td>
</tr>
<tr>
<td>eicosanoic acid</td>
<td>0.05 - 0.3 g</td>
</tr>
<tr>
<td>arachidonic acid</td>
<td>0.1 - 0.4 g</td>
</tr>
<tr>
<td>docosanoic acid</td>
<td>≤ 0.15 g</td>
</tr>
<tr>
<td>docosapentaenoic acid</td>
<td>0.15 - 0.45 g</td>
</tr>
<tr>
<td>dl-α-Tocopherol (as antioxidant)</td>
<td>0.015 - 0.0296 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Purified egg phosphatide</td>
<td>1.2 g</td>
</tr>
</tbody>
</table>

**Total energy:** 470 kJ/100 ml = 112 kcal/100 ml  
**pH value:** 7.5 to 8.7  
**Titration acidity:** < 1 mmol HCl/l  
**Osmolarity:** 308-376 mosm/kg

For excipients, see section list of excipients.

**Pharmaceutical Form**

Emulsion for infusion  
White homogenous emulsion