Effects of iron and omega-3 supplementation on the immune system of iron deficient children in South Africa: a randomised controlled trial

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Preface

“Nobody can go back and start a new beginning, but anyone can start today and make a new ending."

-- Maria Robinson,
American author

And acknowledgements

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Abstract

Background

Iron deficiency (ID) is the world's most prevalent micronutrient deficiency and predominantly affects developing countries, also South Africa. In areas with low fish consumption and high n-6 PUFA vegetable oil intake, there is a risk for having inadequate n-3 PUFA status. Both iron and n-3 PUFA play important roles in the immune response, and supplementation is a strategy to alleviate deficiencies. However, little is known about potential interactive effects between concurrent iron and n-3 PUFA supplementation on the immune system. This is also important in the context that iron supplementation may be unsafe and may increase morbidity and mortality.

Aim

The overall aim of this thesis was to assess the effects of iron and docosahexaenoic (DHA)/eicosapentaenoic acid (EPA) supplementation, alone and in combination, on the immune system of ID children. More specifically, these effects were investigated on the occurrence and duration of illness and school-absenteeism due to illness, peripheral blood mononuclear cell (PBMC), red blood cell (RBC) and plasma total phospholipid fatty acid composition, iron status, fatty acid-derived immune modulators and targeted PBMC gene expression. Furthermore, association of PBMC, RBC and plasma total phospholipid fatty acid composition with allergic disease, were also examined.

Design

In a 2-by-2 factorial, randomised, double-blind, placebo-controlled trial, South African children (n = 321, aged 6–11 y) were randomly assigned to receive oral supplements of either 1) iron (50 mg as ferrous sulphate) plus placebo; 2) DHA/EPA (420/80 mg) plus placebo; 3) iron plus DHA/EPA (420/80 mg); or 4) placebo plus placebo for 8.5 mo, four times per week. Absenteeism and illness symptoms were recorded and biochemical parameters for compliance as well as parameters fundamental to immune function were assessed at baseline and endpoint. Furthermore, in a cross-sectional design, associations of allergic disease with baseline fatty acid composition of PBMC, RBC and plasma were examined.

Results

The combination of iron and DHA/EPA significantly attenuated respiratory illness caused by iron supplementation. DHA/EPA supplementation alone improved respiratory symptoms at school, but increased headache-related absenteeism. DHA/EPA and iron supplementation individually tended to increase and decrease anti-inflammatory DHA and EPA-derived mediators,

respectively. Furthermore the anti-inflammatory DHA-derived immune mediator, 17HDHA was higher in the DHA/EPA plus placebo and iron plus DHA/EPA groups than in the iron plus placebo group. Also, the pro-inflammatory arachidonic acid (AA)-derived modulators (5- and 15-hydroxyeicosapentaenoic acid) were significantly lower in the iron plus DHA/EPA group compared to the placebo plus placebo groups.

In the study population, 27.2% of the children had allergic disease and AA in PBMC phospholipids was significantly lower in the allergic children than in the non-allergic children. In RBC phospholipids dihomo-gamma-linolenic acid (DGLA) and the ratio of DGLA: linoleic acid (LA) correlated negatively and the n-6:n-3 PUFA ratio positively with total immunoglobulin E (tIgE). Furthermore, trans-C18:1n-9, tended to be higher in the allergic group.

Conclusion

DHA/EPA prevented respiratory illness caused by iron supplementation and although DHA/EPA on its own reduced respiratory morbidity when the children were present at school, surprisingly it increased the likelihood of being absent with headache and fever. The biochemical findings compliment the clinical results and support previous observations about DHA/EPA supplementation to reduce inflammation, but add to the current knowledge base that a relatively high oral dose of non-haem iron modulates circulating lipid-derived immune modulators and related gene expression. Furthermore, when supplementing with iron and DHA/EPA combined, in this ID population with low fish intake, the anti-inflammatory effect of DHA/EPA is maintained concurrently with attenuation of respiratory morbidity. This finding support the notion that excess iron (probably as non-transferrin bound iron) becomes available for pathogens and is probably why we found that iron increased respiratory infectious morbidity. The improved clinical outcome with combined supplementation seems to be related to increased lipid-mediator synthesis gene expression and the availability of DHA/EPA, leading to a more pro-resolving profile and enhanced immune competence.

Overall these results give better insight into immune function and infectious morbidity in relation to n-3 PUFA and iron status and treatment, as well as the possible association of fatty acid status with allergic disease in young South-African school children.

Agtergrond

Ystertekort is die wêreld se mees algemene mikro-nutriënt tekort en beïnvloed oorwegend ontwikkelende lande, ook Suid-Afrika. In gebiede met 'n lae inname van vis en 'n hoë n-6 poli-onversadigde vetsure (POVS) groente-olie-inname, ontstaan daar 'n risiko vir onvoldoende n-3 POVS status. Beide yster en n-3 POVS speel 'n belangrike rol in die immuunrespons, en aanvulling is 'n effektiewe strategie om die nutriënt-tekorte te verlig. Daar is egter min inligting bekend oor die potensiële interaktiewe effekte op die immuunstelsel tussen gelyktydige yster en n-3 POVS aanvulling. Dit is ook belangrik in die konteks dat yster aanvullings onveilig kan wees en morbiditeit en mortaliteit kan verhoog.

Doel

Die oorkoepelende doel van hierdie tesis was om die effek van yster en ‘n mengsel van dokosaheksanoësuur (DHS) en eikosapentanoësuur (EPS) aanvulling, individueel en in kombinasie op die immuunstelsel van ystertekort kinders te evalueer. Meer spesifiek, hierdie effekte is ondersoek op die voorkoms en duur van die siekte en die skool-afwesigheid weens siekte, perifere bloed Mononukleêre sel (PBMS), rooibloedsel (RBS) en plasma totale fosfolipied vetsuur samestelling, ysterstatus, vetsuur-afgeleide immuun modulators en geteikende PBMS geenuitdrukking. Verder is die assosiasies tussen PBMS, RBC en plasma totale fosfolipied vetsuur samestelling met allergiese siekte, ook ondersoek.

Studie ontwerp

In 'n 2-by-2 multifaktoriale, gerandomiseerde, dubbel-blinde, plasebo-gekontroleerde intervensie studie, is Suid-Afrikaanse kinders (n = 321, tussen die ouderdomme 6-11 jaar) ewekansig toegewys aan groepe om mondelinge aanvullings te ontvang, van óf 1) yster (50 mg as ferrosulfaat) plus plasebo; 2) DHS/EPS (420/80 mg) plus plasebo; 3) yster plus DHS/EPS (420/80 mg); of 4) plasebo plus plasebo vir 8.5 maande, vier keer per week. Afwesigheid en siekte simptome is aangeteken en biochemiese parameters om nakoming van die intervensie te bevestig, sowel as parameters wat fundamenteel is aan immuun funksie, is geëvalueer op die basislyn en eindpunt. Verder is die assosiasie van allergiese siekte in 'n deursnee-ontwerp, met die basislyn vetsuursamestelling van PBMS, RBS en plasma ondersoek.

Resultate

Die kombinasie van yster en DHS/EPS het die effek van yster aanvulling, om respiratoriese siekte te vermeerder, verkom. Individuele DHS/EPS aanvulling het respiratoriese simptome by die skool verbeter, maar het hoofpyn-verwante afwesigheid vermeerder. Individuele DHS/EPS en yster aanvullings was geneig om anti-inflammatoriese DHS en EPS-afgeleide modulators,

onderskeidelik, te verhoog of te verlaag. Verder was die anti-inflammatoriese DHS-afgeleide immuun modulator, 17-HDHS hoër in die DHS/EPS plus plasebo en yster plus DHS/EPS groepe as in die yster plus plasebo groep. Addisioneel, was die pro-inflammatoriese aragidoonsuur (AS) -afgeleide modulators (5- en 15-hidroksieeikosapentanoësuur) aansienlik laer in die yster plus DHS/EPS groep in vergelyking met die placebo plus placebo groep.

In die studie bevolking, het 27,2% van die kinders aan allergiese siekte gelei en AS in PBMS fosfolipiede was aansienlik laer in die allergiese kinders as in die nie-allergiese kinders. In RBC fosfolipiede was dihomo-gamma-linoleensuur (DGLS) en die verhouding van DGLA: linoleïensuur (LS) negatief en die n-6:n-3 POVS verhouding positief, met 'n totale immunoglobulien E (tIgE) gekorreleer. Verder, was trans-C18: 1n-9, geneig om hoër in die allergiese groep te wees.

Gevolgtrekking

DHS/EPS voorkom respiratoriese siekte wat veroorsaak word deur yster aanvullings en hoewel DHS/EPS op sy eie respiratoriese morbiditeit verminder het terwyl die kinders by die skool was, het dit die waarskynlikheid van afwesigheid met hoofpyn en koors verhoog. Die biochemiese bevindings komplimenteer die kliniese resultate en ondersteun vorige waarnemings oor DHS/EPS aanvulling om inflammasie te verminder. Dit dra egter ook by tot die huidige kennisbasis deur aan te dui dat 'n relatiewe hoë dosis nie-heem yster sirkulerende lipied-afgeleide immuun modulators en verwante geenuitdrukking reguleer. Verder, wanneer yster en DHS/EPS aanvulling gekombineer word, in hierdie ystertekort populasie met 'n lae vis inname, word die anti-inflammatoriese effek van DHS/EPS gehandhaaf. Dit gebeur gelyktydig saam met attenuasie van respiratoriese morbiditeit. Hierdie bevinding ondersteun die idee dat oortollige yster (waarskynlik as NTBI) beskikbaar raak vir patogene en is waarskynlik die rede waarom ons gevind het dat yster aansteeklike respiratoriese morbiditeit verhoog het. Die verbeterde kliniese uitkoms met gekombineerde aanvulling blyk te wees met betrekking tot verhoogde lipied-modulator sintese, verwante geenuitdrukking en die beskikbaarheid van DHS/EPS, wat gepaard gaan met ‘n meer pro-opruimende profiel en verbeterde immuun reaksie.

In geheel gee hierdie resultate ‘n beter insig in immuun funksie en aansteeklike siektes in verhouding tot n-3 POVS en yster status sowel as hul aanvulling, asook die moontlike assosiasie van vetsuurstatus met allergiese siekte in jong Suid-Afrikaanse skoolkinders.

Key terms

ARA / AA	arachidonic acid (20:4n-6)
ALA	alpha-linolenic acid (18:3n-3)
ALOX	arachidonate lipoxygenase
ATP	adenosine triphosphate
BHR	bronchial hyper responsiveness
CoA	coenzyme A
COX	cyclooxygenase
CD	cluster of differentiation
$cPLA_2$	cytosolic phospholipase 2
CREB	cAMP response element binding protein
CRP	C-reactive protein
CVD	cardiovascular disease
D5D	delta-5 desaturase
D6D	delta-6 desaturase
DAG	diacylglycerol
Dcytb	duodenal cytochrome b
DGLA	dihomo-γ-linolenic acid (20:3n-6)
DHA	docosahexaenoic acid (20:6n-3)
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
DOE	Department of Education
DOH	Department of Health
DPA	docosapentaenoic acid (22:5n-3 and 22:5n-6)
ECP	eosinophylic cationic protein
EFA	essential fatty acid
EMB	erythrocyte membrane
EPA	eicosapentaenoic acid (20:5n-3)
ER	endoplasmic reticulum
ETHZ	Eidgenoessische Technische Hochschule Zurich
FADS	fatty acid desaturase gene
FAO	Food and Agriculture Organisation of the United Nations
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
Fer	ferritin

FDA	Food and Drug Administration
GLA	γ-linolenic acid (18:3n-6)
GMT	geometric mean titre
GPX	glutathione peroxidase
GRAS	generally regarded as safe
HAV	hepatitis A virus
HAZ	height-for-age
Hb	haemoglobin
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroxyperoxyeicosatetraenoic acid
HIV	human immunodeficiency virus
HODE	hydroxyoctadecadienoic acid
HOTE	hydroxyoctadecatrienoic acid
ID	iron deficiency / iron deficient
IDA	iron deficiency anemia
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRE	iron response element
IP_3	inositol-1,4,5-triphosphate
$iPLA_2$	calcium-independent phospholipase A_2
ISAAC	International Study on Asthma and Allergy in Childhood
LA	linoleic acid (18:2n-6)
LCPUFA	long-chain polyunsaturated fatty acid
LOQ	limit of quantitation
LOX / LO	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene
LX	lipoxin
LXR	liver X receptor
MHCI	major histocompatibility complex 1
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid
MT	metallothionein
n-3 DPA	omega-3 docosapentaenoic acid (22:5n-3)
n-6 DPA	omega-6 docosapentaenoic acid (22:5n-6)
n-3 PUFA	omega-3 polyunsaturated fatty acid
NADP	nicotinamide adenine dinucleotide phosphate

NICD	National Institute for Communicable Diseases
NF-κB	nuclear factor kappa-B
NFCS-FB	National Food Consumption Survey Fortification Baseline
NK	natural killer
NOS	nitric oxide synthase
NRAMP	natural resistance to infection with intracellular pathogens
NTBI	non-transferrin bound iron
PBMC	peripheral blood mononuclear cell
PC	phosphatidylcholine
PCR	polymerase chain reaction
PD1	protectin D1
PEA	phosphatidylethanolamine
PG	prostaglandin
PGC1α	peroxisome proliferator-activated receptor-gamma coactivator-1 alpha
PI	phosphatidylinositol
PL	phospholipase
PLA_2	phospholipase A_2
PPAR	peroxisome-proliferator activated receptor
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
qPCR	quantitative polymerase chain reaction
RBC	red blood cell
Rv	resolvin
RCT	randomised controlled trial
RNA	ribonucleic acid
ROS	reactive oxygen species
SAE	serious adverse event
SD	standard deviation
SF	serum ferritin
SFA	short chain fatty acid
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
SLC	solute family carrier
SREBS	sterol regulatory element-binding proteins
$sPLA_2$	secretory phospholipase A_2
SZn	serum zinc
TAG	triacylglycerides

TB	(*mycobacterium) tuberculosis*
TCR	T cell antigen receptor
TfR	transferrin receptor
TGF	transforming growth factor
tIgE	total Immunoglobulin E
Th	T helper
TLR	toll-like receptor
TNFα	tumour necrosis factor-alpha
WAZ	weight-for-age
WHO	World Health Organisation
ZnPP	zinc protoporphyrin

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Chapter 1 Introduction

1.1 Rationale of the study

Iron deficiency (ID) is the world's most prevalent micronutrient deficiency and predominantly affects developing countries (Zimmermann & Hurrell, 2007). Due to rapid expansion of red blood cell mass in young children, their dietary iron requirements are very high (estimated at 6.9 mg per day for a 6-12 month old infant) and puts them at a high risk for developing ID and ID anaemia (IDA) (Hellwig *et al.*, 2006). ID and IDA affect immune function (Beard, 2001), lead to a greater vulnerability to infections (Tansarli *et al.*, 2013) and are associated with deficits in cognitive abilities, psychomotor skills and neurophysiology in later life (Georgieff, 2011; Lozoff, 2007). Iron supplementation is currently one of the strategies advocated to address ID and IDA (Pasricha *et al.*, 2013; Stoltzfus & Dreyfuss, 2006). However, it has been suggested that ID may be protective of some infectious diseases, particularly malaria (Jonker *et al.*, 2012), therefore cautioning us about the safety of iron supplementation, especially in areas with malaria and a high infectious disease burden (Roth *et al.*, 2010). Furthermore, since fat intake may be low in low-income countries, polyunsaturated fatty acid (PUFA) intake may be limited (Briend *et al.*, 2011). Moreover, higher availability and intake of oils high in the n-6 PUFA precursor, linoleic acid (LA), may cause the conversion of n-3 PUFA precursor to be compromised and further reduce n-3 long-chain PUFA (LCPUFA) blood levels (Brenna *et al.*, 2009; Briend *et al.*, 2011). Low n-3 PUFA status, particularly n-3 LCPUFA, is associated with several health outcomes, such as impaired immune function and brain metabolism as well as increased risk of cardiovascular disease (Calder, 2013b; McNamara & Carlson, 2006; Nicholson *et al.*, 2013). In settings with low fish intake and no change in diet, supplementation with docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) is recommended as a means of improving n-3 LCPUFA status (Brenna *et al.*, 2009; Briend *et al.*, 2011)

Simultaneous ID and low n-3 PUFA status are found in certain populations (Baumgartner *et al.*, 2012c). Furthermore, nutrition is known to influence immune function (Calder, 2002; Field *et al.*, 2002) and both iron and n-3 PUFA play important roles in the immune response (Beard, 2001; Calder, 2013b). Interactions between iron and n-3 PUFA have been demonstrated in animal models and human studies (Smuts *et al.*, 1995; Stangl & Kirchgessner, 1998; Tichelaar *et al.*, 1997) and can be explained to some extent at the molecular level (Brand *et al.*, 2008; Ober & Hart, 1998). Thus it is likely that the simultaneous deficiency of these nutrients may have synergistic effects and exacerbate deficits in immune function further than ID or low n-3 PUFA status individually (Baumgartner *et al.*, 2012a; Baumgartner *et al.*, 2012b; Baumgartner *et al.*, 2012c). Moreover, it is likely that combined supplementation with iron and n-3 LCPUFA might interact to influence immune function, as has been suggested by two animal studies (Clauss *et*

al., 2008; Rørvik *et al.*, 2003). It has also been demonstrated that supplementing a certain micronutrient where there is a background of another micronutrient deficiency, may show no effect or even cause further imbalance and have detrimental effects on functional outcomes, such as cognition (Baumgartner *et al.*, 2012b; Baumgartner *et al.*, 2012c). Very little data are available for the effects of iron supplementation in the presence of low n-3 PUFA status and *vice versa*, let alone the effect on the human immune function. Furthermore, there are no studies in children which have investigated potential interactive effects between concurrent iron and n-3 PUFA supplementation on immune function or on occurrence and duration of infectious morbidity.

This was the first human study to assess the interactions of iron and n-3 LCPUFA supplementation in ID children in a two-by-two factorial, randomized, double-blind, placebo-controlled trial. In populations of low socio economic status, such as found in rural South Africa, ID coexisting with a low intake of n-3 PUFA could have synergistic adverse effects on the immune function of children. Therefore, occurrence and duration of illness symptoms and absenteeism from school due to illness, as well as specific immune function modulators that have been found to be affected by iron and/or n-3 PUFA in previous studies, were assessed.

1.2 The study site

The study was conducted at four primary schools in the malaria-free rural area of a The Valley of a Thousand Hills, which is situated about 40 km northwest of the coastal city of Durban in the province of KwaZulu-Natal, South Africa (Figure 1). This is a low socio-economic area inhabited predominantly with Zulu-speaking people. The four schools are situated within a radius of 10 km of each other. Even though the schools are participating in the National School Nutritional Programme, providing the children with a cooked meal on school days, there was in 2005 still a prevalence of 15% stunting, 5% underweight and 1% wasting in 1-9 y-olds in KwaZulu-Natal (Labadarios *et al.*, 2008). Furthermore, in The Valley of a Thousand Hills, ID prevalence (serum ferritin < 15 mg/L) was subsequently found to be 20.6%, which is higher than estimated by the national survey (Baumgartner *et al.*, 2012c; Hoosain *et al.*, 2013). The plasma total phospholipid n-3 PUFA composition status of children living in inner KwaZulu-Natal, was estimated to be 4%, whereas it was about 6% and 8% for children living in Cape Town and directly at the sea (Smuts, 2011). Thus, it seems that children living further inland, also in KwaZulu-Natal, have a lower n-3 PUFA status than when living on the coast where fish is more easily accessible.

Figure 1: Map of South Africa and The Valley of a Thousand Hills in KwaZulu-Natal, northwest of Durban Central (http://www.stayinsa.co.za/themaps, last access 22.10.2014).

1.3 Aim

The aim of this study was to determine if providing iron and a mixture of DHA and EPA, alone and in combination, to children with ID and poor n-3 PUFA intake would improve their immune function and result in reduced illness and school-absenteeism due to illness.

1.4 Objectives

1) To determine the effects of iron and n-3 LCPUFA supplementation, alone and in combination on occurrence and duration of illness and school-absenteeism due to illness.

2) To determine the effects of iron and n-3 LCPUFA supplementation, alone and in combination, on peripheral blood mononuclear cell (PBMC) total phospholipid fatty acid composition and iron status.

3) To determine the effects of iron and n-3 LCPUFA supplementation, alone and in combination, on fatty acid-derived immune modulators.

4) To determine the effect of iron and n-3 LCPUFA supplementation, alone and in combination on targeted gene expression in PBMC.

5) To assess the allergic disease prevalence in the study population and determine the association of PBMC, red blood cell (RBC) and plasma total phospholipid fatty acid composition with allergic disease.

1.5 Ethical approval

The study was approved by the Ethical Committee of the ETH Zürich and the NWU Potchefstroom with ethics numbers EK 2008-33 and NWU-0061-08-A1, respectively (Annexure 1).

1.6 1.1 Research team

The research team and contribution made by each team member are described in Table 1.

Table 1: Research team and contribution

Team member	Affiliation	Contribution
Mrs L Malan 	Centre of Excellence for Nutrition, North-West University	Implemented and executed the intervention study with focus on immune function outcomes, developed LCMSMS method for eicosanoid analysis, and fatty acid mass spectrometry and quantitation method, supervised analysis of eicosanoids and fatty acids, planned and involved in qPCR experiment, analysed tIgE, performed statistics and wrote manuscripts.
Prof. CM Smuts 	Centre of Excellence for Nutrition, North-West University	Initiated and partly conceptualized and involved in all aspects of the intervention study. Supervised gas chromatography of fatty acid analyses and assisted with writing of manuscripts.
Dr. J Baumgartner 	Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, ETH Zürich, Switzerland and Centre of Excellence for Nutrition, North-West University	Co-investigator; implemented and executed the intervention study, with focus on cognitive outcomes. Assisted with writing of manuscripts.
Prof. DR. MB Zimmermann 	Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, ETH Zürich, Switzerland	Conceptualized the study and involved in some aspects of its execution. Assisted with finalisation of first manuscript.
Prof. PC Calder 	Human Development and Health Academic Unit, Faculty of Medicine, University of Southampton, Southampton, United Kingdom, the NIHR Biomedical Research Centre in Nutrition, Southampton University Hospital NHS Foundation Trust and University of Southampton, Southampton, United Kingdom and the Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia	Assisted with detailed planning of immune function study, and played a leading role in planning and finalisation of manuscripts.

1.7 Thesis outline

Chapter One is an introduction and Chapter Two consists of a literature review which focuses on the role of iron and n-3 PUFA in immune function, as well as an overview of intervention studies which have been performed with each nutrient with infectious morbidity as an outcome. Furthermore, immune function and the metabolism of iron and fatty acids, as well as their involvement in cell signalling and oxidative stress, are discussed. A section is attributed to allergic disease in relation to fatty acid composition. Lastly, interactive mechanisms and studies of the combined intervention with iron and n-3 PUFA on immune function, or the lack thereof, are deliberated.

Chapter Three is a manuscript entitled “N–3 long-chain polyunsaturated fatty acids reduce respiratory morbidity caused by iron supplementation in iron-deficient South African school children: a randomized, double-blind, placebo-controlled intervention”. The manuscript is written in American English and was accepted for publication in the American Journal of Clinical Nutrition in October 2014 (Annexure 2).

The manuscript that Chapter Four consists of, is named: “Iron and a mixture of docosahexaenoic and eicosapentaenoic acid supplementation, alone and in combination, affect bioactive lipid signalling and morbidity of iron deficient South African school children in a two-by-two randomised controlled trial”. It was submitted to the journal, Prostaglandins, Leukotrienes & Essential Fatty Acids (PLEFA).

Chapter Five is a manuscript entitled “Allergic disease is associated with alterations in long-chain polyunsaturated and *trans*-fatty acid composition of peripheral blood mononuclear cells, red blood cells and plasma in rural South African school children”. It was submitted to the journal, Clinical and Experimental Allergy.

In Chapter Six, the final conclusions are made and implications as well as perspectives for future research are discussed.

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Chapter 2 Literature review

2.1 The immune system

2.1.1 Immune response

The immune system protects against pathogens, identifies and eliminates tumour cells and responds to physical insults such as injury, surgery, burns and irradiation (Janeway *et al.*, 2005). Numerous biochemical mediators are produced during the response to an invading pathogen; while some are directly destructive to pathogens, others have a controlling or regulating capability and stimulate the activity of certain cell types (Mak & Saunders, 2005). Furthermore, some mediators act to end the immune response when the source that originally elicited the immune stimulation has been eliminated (Ariel & Serhan, 2007). The host's response to an invading pathogen is, therefore, protective and beneficial to health, nevertheless can cause perpetuating tissue damage if inappropriately activated or due to an inability to be switched off (Calder, 2013b).

The immune response to pathogens is divided into two types, namely innate and adaptive response (Janeway *et al.*, 2005). The innate or natural response consists of physical barriers, soluble factors and phagocytic cells and, due to its fast activation, provides the first line of defence against pathogens (Figure 2, 1 – 5). However, it is not improved by prior exposure to a pathogen (Janeway *et al.*, 2005). Innate immunity is directed against structures of pathogens necessary for their survival (can be common to numerous pathogens), for example the component of the cell wall of Gram–negative bacteria, lipopolysaccharide (LPS), which is recognised by Toll-like receptor (TLR)-4 on the surface of innate immune cells (Calder, 2013a; Janeway *et al.*, 2005). Phagocytic macrophages (differentiated from monocytes) and neutrophils are the main cells involved in innate immunity. These cells have surface receptors specific for common bacterial surface molecules, which when engaged, activate phagocytosis and destruction of the pathogen. Neutrophils destroy pathogens by releasing antimicrobial toxins (Figure 2.1), whereas macrophages can directly phagocytose pathogens, leading to production of cytokines and recruitment of more cells from the blood (Figure 2.2).

Cellular communication is fundamental to an effective immune system, making signalling molecules of utmost importance. Cytokines are one of the largest and most diverse families of signalling molecules in the body. Cytokines that usually circulate at very low concentrations can increase up to 1,000-fold during trauma or infection (Janeway *et al.*, 2005). They are released early in the immune response and cause numerous outcomes including increased major histocompatibility complex 1 (MHCI) expression and secretion of additional cytokines which

expand inflammatory responses. Cytokines bind to specific membrane receptors, triggering second messenger signalling and subsequent alterations in gene transcription. There are more than 50 known cytokines, often classified into pro-and anti-inflammatory families. The main pro-inflammatory cytokines responsible for early responses are interleukin (IL) 1-α, IL1-β, IL-6, and tumour necrosis factor (TNF)-α. Other pro-inflammatory cytokines include interferon (IFN)-γ, granulocyte macrophage colony-stimulating factor, IL-11, IL-12, IL-17, IL-18, and a variety of chemokines that act as chemo-attractants to inflammatory cells. Anti-inflammatory cytokines limit the potentially harmful effects of continued or excess inflammatory reactions. The key anti-inflammatory cytokines comprise of the IL-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13. Most of these anti-inflammatory cytokines have at least some pro-inflammatory properties. An extremely dynamic balance exists between pro- and anti-inflammatory cytokines. Furthermore, cytokine signalling can result in increased or decreased expression of membrane proteins, proliferation, and/or secretion of effector molecules. It is, therefore, often problematic to make generalizations about the roles of individual cytokines. To add to this complexity, combinations of cytokines can act either synergistically or antagonistically depending on the state of the target cells and the combinations, amounts, and chronological order of cytokine secretion (Janeway *et al.*, 2005).

Infected cells presenting low levels of MHCI on their surface are identified by natural killer (NK) cells, which release lytic enzymes causing the infected cell to die via apoptosis (Figure 2.3). The complement system can also recognize bacteria, resulting in their lysis (Figure 2.4).

Antigen presenting cells can be formed from macrophages by taking up peripheral antigens and migrating to lymph nodes to present antigen on their surface to naïve B and T cells (Figure 2.5) (Janeway *et al.*, 2005). The activation of an appropriate immune response is dependent on unique dendritic cells, the most professional antigen presenting cells which control the responses of several types of lymphocytes and play a central role in the transition between innate and adaptive immunity (Harizi & Gualde, 2005).

Increased secretion of inflammatory endogenous mediators such as cytokines and arachidonic acid (AA)-derived lipid mediators (eicosanoids) can activate antigen presenting cells, predominantly dendritic cells, which subsequently induce an adaptive immune response. There is collective evidence that eicosanoids play an important role in connecting innate and adaptive immunity by acting on cells of both systems (Harizi & Gualde, 2005). Eicosanoids are not stored in cells, but are synthesized as needed. They are formed from the LCPUFA that make up cell and nuclear membranes (Funk, 2001; Soberman & Christmas, 2003). Eicosanoid biosynthesis begins when a cell is activated by mechanical trauma, cytokines, growth factors or other stimuli. (The stimulus may even be an eicosanoid from a neighbouring cell; the pathways are complex.)

As shown in Figure 8, this activates the release of phospholipase (PL) enzymes from the cell membrane which subsequently catalyzes ester hydrolysis of fatty acids, among other molecules, which are then available for eicosanoid synthesis. The hydrolysis step appears to be the rate-determining step for eicosanoid production (Funk, 2001; Neitzel, 2010).The fatty acids may be hydrolyzed by any of several PL. Of these, group IV cytosolic PLA_2 ($cPLA_2$) is the key actor, as cells lacking $cPLA_2$ can generally not synthesize eicosanoids (Soberman & Christmas, 2003). $CPLA_2$ (Group IV PLA_2) is calcium-dependant and seems to have a preference for AA, whereas calcium-independent PLA_2 ($iPLA_2$) seems specific for the release of DHA (Dennis, 2000; Sun *et al.*, 2010).

Prostanoids, a main class of eicosanoids, have a vast impact on inflammatory and immune responses. Prostaglandin (PG) E_2 is one of the best known and most well-characterized prostanoids in terms of immunomodulation. Although cytokines are known as vital regulators of immunity, eicosanoids, including PGE_2, PGD_2, leukotriene (LT) B_4, and LTC_4, may also affect cells of the immune system by modifying cytokine release, cell differentiation, survival, migration, antigen presentation, and apoptosis. By influencing several aspects of immune and inflammatory reactions, these lipid mediators emerge as key regulators of the crosstalk between innate and adaptive immunity (Harizi & Gualde, 2005).

The adaptive immune response is more flexible and powerful than the innate response and comprises of B and T lymphocytes that constantly circulate through the body by means of the lymph and blood systems. These cells can recognize more than a thousand antigens. After recognition of one such antigen, by means of antigen presenting cell interaction with B- and T-cells in the lymph nodes, these activated B and T cells migrate to the periphery where they mediate adaptive immunity (Figure 2.6). Once activated, the T cell undergoes a process of clonal expansion in which it divides rapidly to produce numerous identical effector cells. Activated T cells then travel to the periphery in search of infected cells displaying similar antigen/MHCI complex (Figure 2.7). Peripheral antigen presenting cells induce helper T cells to release cytokines and recruit cytotoxic T cells (Figure 2.8). Activated antigen-specific B cells receiving signals from helper T cells differentiate into plasma cells and secrete antibodies or immunoglobulins (Ig). This is called humoral immunity (Figure 2.9). These can bind to pathogens, block pathogen invasion and enhance pathogen destruction, by binding to target antigens and creating immune complexes which can then activate complement or be taken up by macrophages through Fc receptors (Figure 2.10). Several Ig isotypes exist. IgM dominates the early humoral immune response, whereas IgG and IgA dominates later. IgE is prominent during allergic reactions (Janeway *et al.*, 2005). Furthermore, construction of cytotoxic T cell synapses leads to lysis of the infected cell (Figure 2.11).

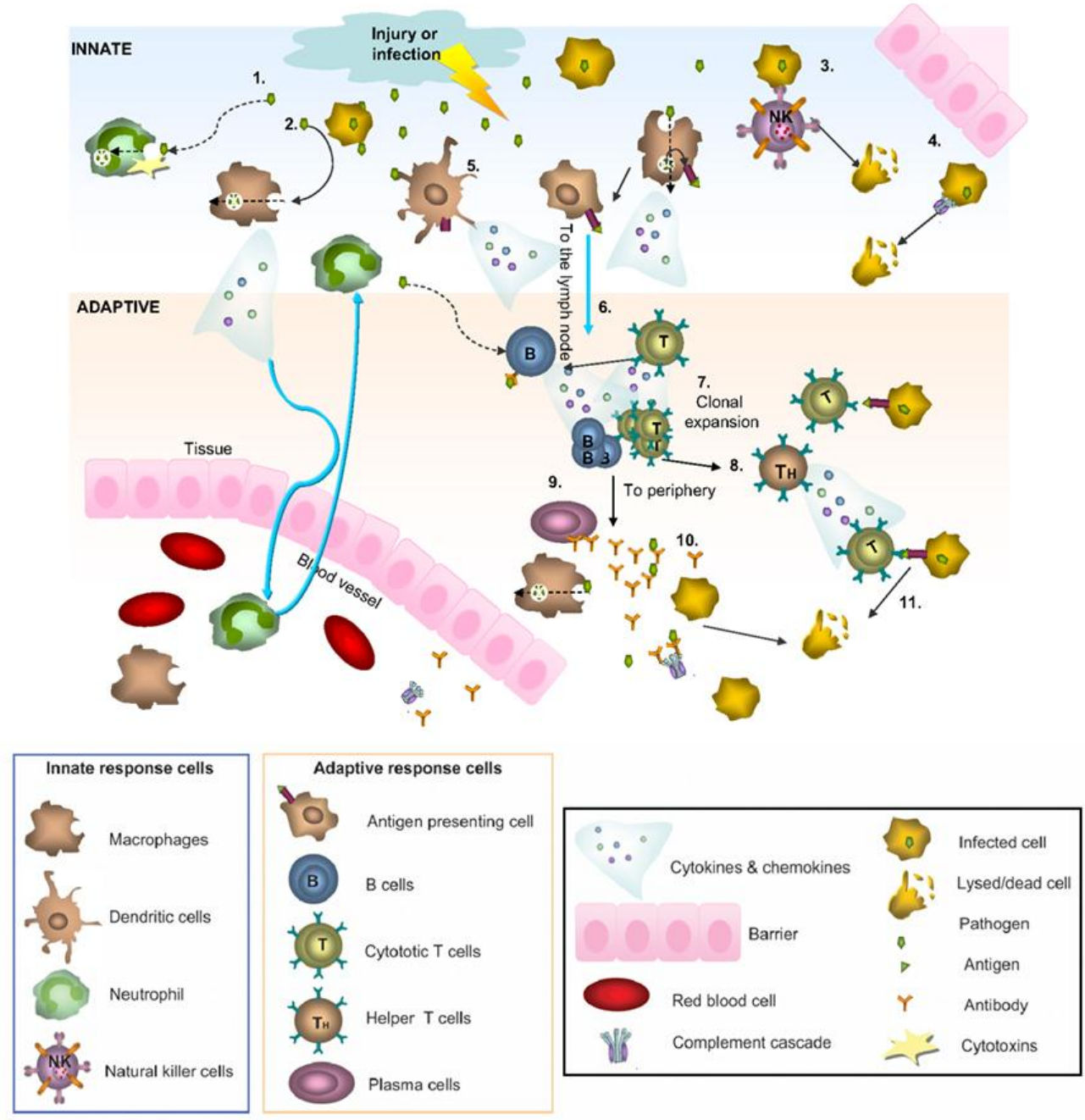

Figure 2: The innate and adaptive immune system. The innate response provides immediate defence against infection (1–5). The adaptive response confers the ability to recognize and remember specific pathogens to generate immunity (6–11) (Janeway *et al.*, 2005).

2.1.2 Immune cells

Immune cells (leukocytes) in circulating blood (peripheral leukocytes) consist of neutrophils, monocytes, lymphocytes, eosinophils and basophils (Figure 3). The concentrations of these vary widely depending on the health of an individual (Delves *et al.*, 2011). Healthy blood contains less than 107 leukocytes per ml, while blood from an infected person may have tenfold higher leukocyte concentrations. Naïve peripheral T cells, which are mature cells which have not been activated by contact with a pathogen, can survive for years in the blood and lymphatic system (Sprent, 1993). In contrast, activated (effector) leucocytes are short-lived (between one and six days), with the exception of memory cells (B and T lymphocytes) which could survive for years in humans (Pillay *et al.*, 2010; Tough & Sprent, 1995).

A PBMC is any blood cell having a round nucleus and PBMC include lymphocytes (T cells, B cells and NK cells), monocytes, macrophages (differentiated monocytes) and dendritic (antigen-presenting) cells (Delves *et al.*, 2011). PBMC are separated from whole blood by centrifugation in ficoll, which separates the blood into a top layer of plasma, followed by a layer of PBMC and a bottom fraction of polymorphonuclear cells (such as neutrophils and eosinophils) and erythrocytes. Lymphocytes within the PBMC population constitutes 70 – 90% and monocytes about 10 – 30%, while dendritic cells are rare, being only 1 – 2% of PBMC. The cell types within the lymphocyte population consist of 70 – 85% cluster of differentiation (CD)3+ T cells, 5 – 20% B cells, and 5 – 20% NK cells. The CD3+ cells are composed of CD4 and CD8 T cells, in a roughly 2:1 ratio. Both CD4 and CD8 T cells can be further divided into naïve, and the antigen-experienced central memory, effector memory, and effector subtypes that exist in resting or activated states.

CD4 T cells are known as helper T cells and can be further classified into various functional subtypes based on the expression profiles of specific cytokines, surface markers, or transcription factors (Calder, 2002; Mak & Saunders, 2005). These include regulatory T cells, TH1, and TH2 as well as other described subpopulations. The cytotoxic CD8 T cells have been shown to be extremely diverse in marker expression and function and may contain up to 200 functional phenotypes. Circulating B cells include transitional, naïve, and memory subtypes as well as plasma blasts. Circulating monocytes have been described as either being classical monocytes or non-classical CD16+ pro-inflammatory monocytes, which comprise of up to 10% of the monocytes in peripheral blood and have unique functions compared with classical monocytes (Delves *et al.*, 2011).

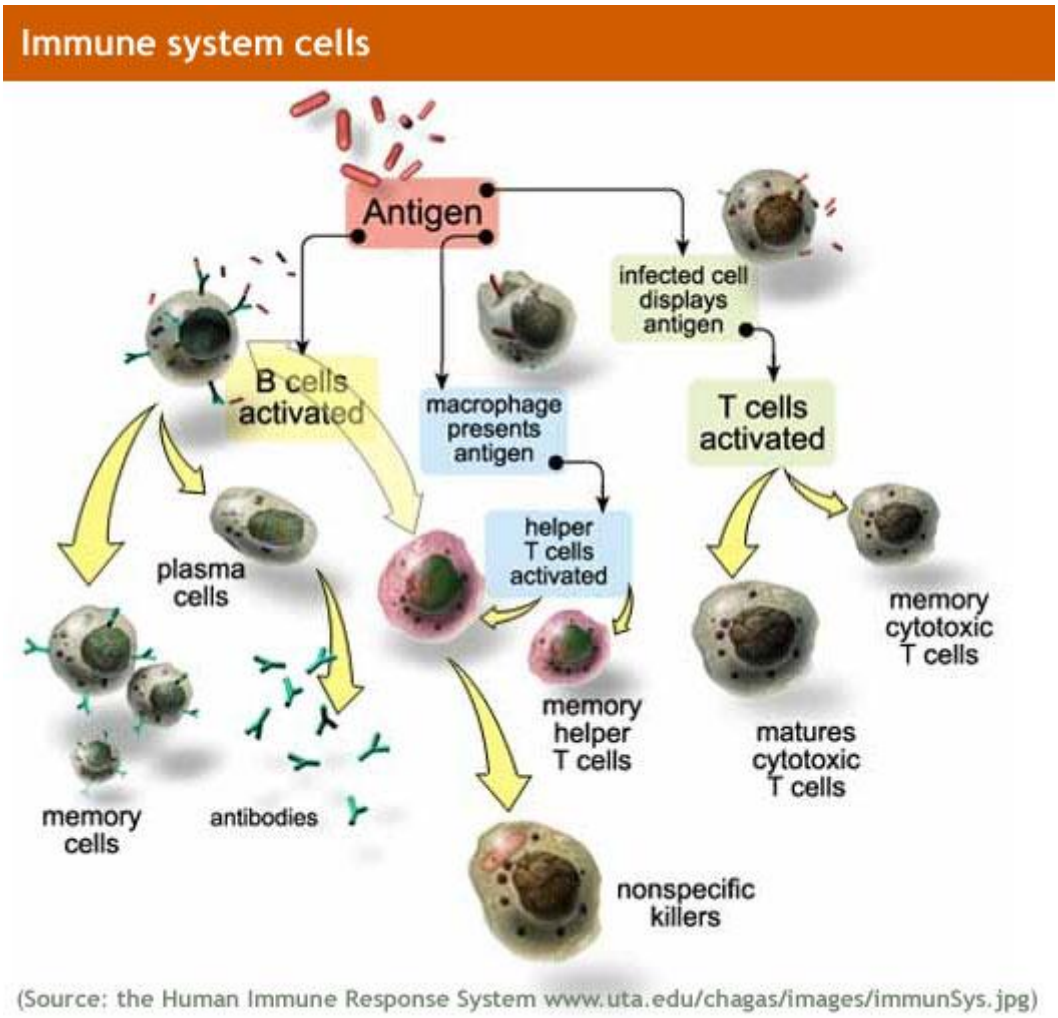

Figure 3: Immune cell interactions and functions (http://www.uta.edu/chagas-/html/biolImS1.html last access 01.09.2014).

2.1.3 Approaches to measure infectious morbidity and immune function

Infectious morbidity can be measured by clinical outcomes, such as incidence and duration of illness (Birch *et al.*, 2002; Birch *et al.*, 2010; Dalton, 2006; de Silva *et al.*, 2003; Gera & Sachdev, 2002; Minns *et al.*, 2010; Thienprasert *et al.*, 2009). Since no single immune function parameter allows conclusions to be drawn about the modulation of the whole immune system, clinical outcome of infection itself is still regarded as the best measure to do this (Albers *et al.*, 2007). Clinical endpoints such as mortality and morbidity from (common) infections provide the most appropriate indication of the host's ability to cope with common pathogens and thus reflect the overall balance between pathogen exposure and the integrated host defences. Ideally illnesses should be diagnosed by a medical professional, but this is not always possible in scientific studies. Infectious morbidity in school children is easier to measure than in smaller children, because of the possibility of self-reporting which can be combined with observation by study assistants. Furthermore, absenteeism from school due to illness can be a useful clinical

outcome if combined with reporting of symptoms (Dalton, 2006; Thienprasert *et al.*, 2009). With such an approach, effects on single symptoms or combinations of symptoms can be determined. Furthermore, symptoms can be scored into at least respiratory or gastric illness, which can be used to determine effects on these two categories of illness.

Immune function markers which are directly influenced by iron and DHA/EPA, such as the LCPUFA-derived immune modulators (eicosanoids and docosanoids) and gene expression of inflammatory and oxidative stress- associated genes can be related to clinical outcomes (Albers *et al.*, 2007; Calder, 2012). This will give valuable insight into mechanisms of immune function in relation to clinical outcomes.

2.2 Fatty acid metabolism and structural organisation

2.2.1 Terminology, synthesis and dietary sources of fatty acids

The human diet contains a variety of fatty acids, ranging from four-carbon fatty acids in milk to thirty-carbon fatty acids in some fish oils (Calder, 2002). These are usually straight chain fatty acids with an even number of carbon atoms. Monounsaturated fatty acids contain one double bond, while PUFA contains two or more double bonds. The systematic names signify the number of carbon atoms in the chain as well as the number of double bonds and position of each of the double bonds, e.g. 4, 7, 10, 13, 16, 19-DHA has twenty two carbon atoms and six double bonds starting from the third carbon from the carboxyl end. Trivial names, e.g. DHA and shorthand notations (22:6n-3) are often used. Shorthand notations indicate the number of carbons, the number of double bonds and lastly the position of the first double bond from the methyl-end (Calder, 2002; Sala-Vila *et al.*, 2008). Table 2 presents the fatty acids generally referred to in this thesis and Figure 4 depicts the structure and naming of some 18 carbon fatty acids (Neitzel, 2010).

Table 2: Fatty acid nomenclature

Systematic name	Trivial name	Shorthand notation
Octanoic	Caprylic	8:0
Decanoic	Capric	10:0
Dodecanoic	Lauric	12:0
Tetradecanoic	Myristic	14:0
Hexadecanoic	Palmitic	16:0
Octadecanoic	Stearic	18:0
cis-9-hexadecenoic	Palmitoleic	16:1n-7
cis-9-octadecenoic	Oleic	18:1n-9
trans-9-octadecenoic	Elaidic	18:1n-9 T
cis-9,cis12-octadecadienoic	Linoleic	18:2n-6
All cis-9,12,15-octadecatrienoic	α-Linolenic	18:3n-3
All cis-6,9,12-octadecatrienoic	γ-Linolenic	18:3n-6
All cis-8,11,14-eicosatrienoic	Dihomo-γ-linolenic	20:3n-6
All cis-5,8,11,14-eicosatetraenoic	Arachidonic	20:4n-6
All cis-5,8,11,14, 17-eicosapentaenoic	Eicosapentaenoic	20:5n-3
All cis-7,10,13,16,19-docosapentaenoic	n-3 Docosapentaenoic	22:5n-3
All cis-4,7,10,13,16-docosapentaenoic	n-6 Docosapentaenoic / Osbond	22:5n-6
All cis-4,7,10,13,16,19-docosahexaenoic	Docosahexaenoic	22:6n-3

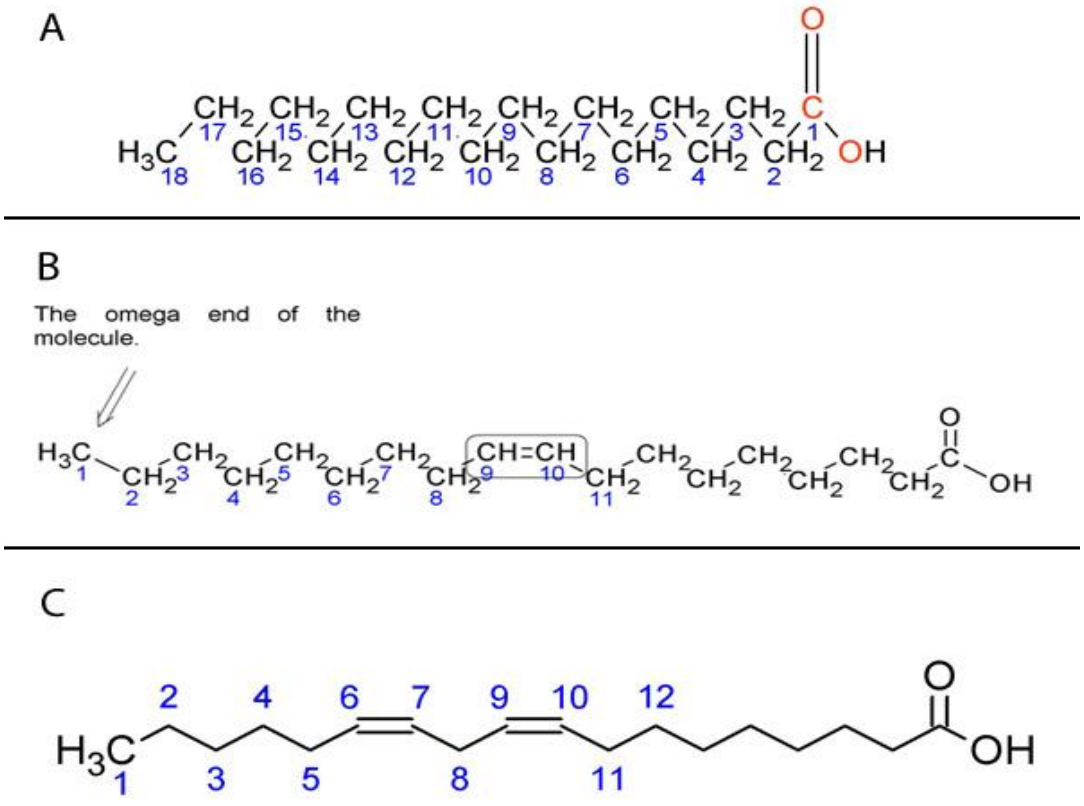

Figure 4: Naming of fatty acids. (A) Stearic acid (18:0), an 18-carbon saturated fatty acid. The systematic numbering system, starting with the carboxylic acid, is shown in blue. (B) Oleic acid (18:1n-9), an 18-carbon monounsaturated fat. (C) Linoleic acid (18:2n-6), an 18-chain n-6 polyunsaturated fatty acid (Neitzel, 2010).

Since humans are not able to introduce a double bond at the n-3 or n-6 position of the fatty acid chain, these PUFA are essential and must be consumed in the diet. Linoleic acid (LA) and α-linolenic (ALA) acid are the most common n-6 and n-3 PUFA consumed by most people. Elongation (addition of a 2-carbon unit) and desaturation (addition of a double bond) of these and other fatty acids are facilitated in the human liver by a series of enzymatic reactions depicted in Figure 5. AA and DHA are formed from LA and ALA, respectively. The bulk of the synthesis takes place in the cytosol. The pathway involves synthesis of 24:5n-3 and 24:4n-6 by elongation up to 24:5n-3 and 24:4n-6. These two fatty acids are desaturated at position 6 to yield 24:6n–3 and 24:6n–3, which are translocated to the peroxisomes where partial oxidation generates DHA (22:6n-3) and n-6 DPA (22:5n-6) (Innis, 2003). However, the extent to which the LCPUFA can be synthesized from essential PUFA in humans is relatively limited (Brenna *et al.*, 2009). Dietary intake of the longer chain PUFA, specifically DHA, EPA and AA, therefore, becomes important for their many functionalities, including in the immune system. Synthesis of DHA and AA is thought to use the same D6D and D5D enzymes. This can lead to competition

between LA and ALA and inhibition of the enzyme pathway by products of the same and the opposing n-3 and n-6 series of fatty acids (Brenna *et al.*, 2009; Innis, 2003).

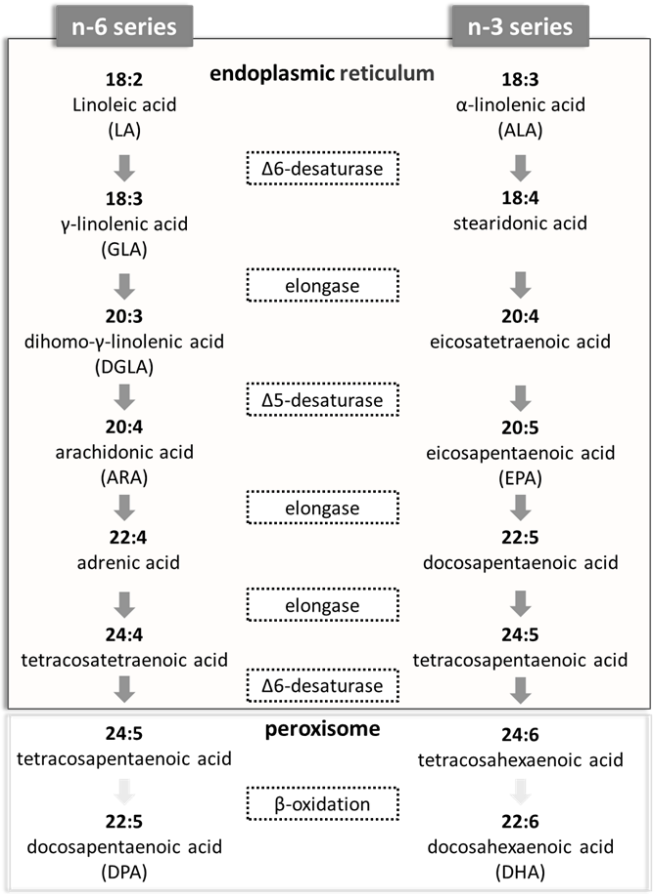

Figure 5: N-6 and n-3 LCPUFA synthesis. Abbreviations: ALA: α-linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DGLA: di-homo-γ-linolenic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; GLA: γ-linolenic acid; LA: linoleic acid (Edwards & O'Flaherty, 2008).

2.2.2 Cellular membranes and phospholipids

All cellular membranes consist of a lipid bilayer composed mainly of phospholipids, which are organised with the hydrophobic tails facing inwards and the hydrophilic heads facing outwards into the aqueous regions on either side (Figure 6). Phospholipids, sphingolipids, glycolipids and cholesterol are the main classes of membrane lipids and contain both polar and non-polar domains (amphipatic molecules). Many proteins, such as receptors, ion channels and transporters are imbedded into this membrane to a higher or lower degree of rigidity (Lodish, 2008).

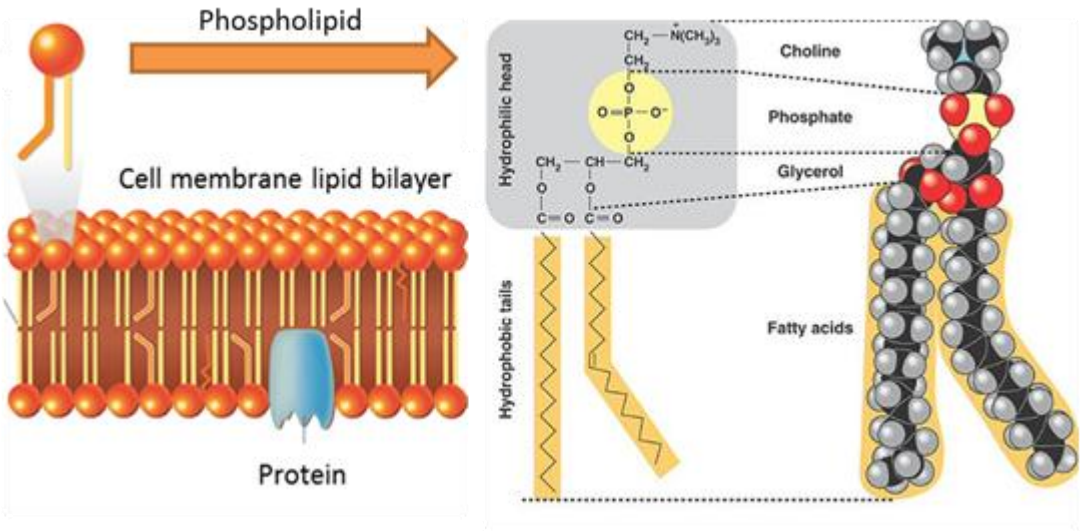

Figure 6: The phospholipid within a cell membrane. (Adapted from http://bioap–.wikispaces.com/Ch+5+Collaboration+2010last access 29.8.2014)

The most abundant lipid class in membranes is the phospholipid, consisting of a glycerol-3-phosphate backbone containing three adjacent carbon atoms, a polar head group and two fatty acids. The carbons are stereotypically numbered *sn*-1, *sn*-2 and *sn*-3. The *sn*-3 carbon atom is linked through a phosphate group to a polar head group which determines the nomenclature of the phospholipid, as such named phosphatidylcholine (PC), phosphatidylethanolamine (PEA), phosphatidylserine (PS) or phosphatidylinositol (PI). Two fatty acids are bound to the *sn*-1 and *sn*-2 positions, of which predominantly saturated fatty acids are at the *sn*-1 and unsaturated fatty acids at the *sn*-2 position, respectively. Furthermore, LCPUFA like DHA and AA are mostly incorporated into phospholipids rather than triacylglycerides (TAG), whereas ALA and LA are rather incorporated into TAG. Each type of cell membrane maintains a specific ratio of the phospholipid species as well as a specific set of membrane-bound proteins, depending on the function of the cell. The degree of membrane fluidity or membrane order is dictated by the saturation of the fatty acids incorporated into the phospholipid (Masi *et al.*, 2013). Furthermore, the different phospholipid species are unequally distributed between the outer and inner leaflet of the lipid bilayer. Sphingomyelin and PC are predominantly found in the exoplasmic leaflet, making it less fluid, whereas PEA, PS and PC mainly make up the inner leaflet and make it more fluid than the outer leaflet. One of the cell-signalling mechanisms involves the “flip-flop” exchange of the inner and outer leaflets, and reinstatement of this equilibrium. It has been shown that exposure of PS on the surface of a cell is an early event of apoptosis that leads to the recognition and removal of these cells by macrophages (Knapp & Wurtman, 1999). Furthermore, the flip-flop mechanism was shown *in vitro* to be induced by iron with the subsequent switch-on of anti-oxidative cellular machinery which was enhanced by DHA-composition of cell membranes (Schonfeld *et al.*, 2007). Fatty acid composition is distributed

with the species of phospholipid, since most DHA are incorporated into PEA, and to a lesser extent into PS. As such, the majority of AA is incorporated into PI and PEA (Knapp & Wurtman, 1999).

2.2.3 Fatty acids in cell signalling

2.2.3.1 Liberation of fatty acids from immune cell membranes: Phospholipase A2

Fatty acids can be hydrolysed from phospholipids by a superfamily of PL enzymes, releasing a free fatty acids and lysophospholipid, as mentioned in section 2.1.1 (Figures 7 and 8). These enzymes are known to play multiple roles for maintenance of membrane phospholipid homeostasis and for production of a variety of lipid mediators (Dennis, 2000; Dennis *et al.*, 2011). Despite their common function, they are diversely encoded by a number of genes and express proteins that are regulated by various mechanisms. PLA_2 is responsible for releasing fatty acids at the *sn*-2 position of membrane phospholipids and over 30 different types of PLA_2s are present in mammalian cells (Murakami *et al.*, 2011). PLA_2 are subdivided into six families based on their structure, catalytic mechanism, localization and evolutionary relationships (Quach *et al.*, 2014) These families include $cPLA_2$, $iPLA_2$, secretory PLA_2 ($sPLA_2$), lysosomal PLA_2, platelet activating factor acetyl hydrolases, and the recently discovered adipose specific PLA_2 (Quach *et al.*, 2014). The families consist of various isoforms that are similar in structure and function (Sun *et al.*, 2010).

Among the PLA_2, $cPLA_2$ seems to be the main component for production of eicosanoids, since cells lacking $cPLA_2$ can generally not synthesize eicosanoids (Soberman & Christmas, 2003). $CPLA_2$ are situated in the cytosol and are activated with phosphorylation and increased calcium. The activated molecule is translocated to an intracellular membrane where it will cleave the phospholipid to yield the lysophospholipid and (mostly) AA. The newly released AA will result in the production of eicosanoids (Dennis, 2000) or be recycled into phospholipid by an acyltransferase (Ryan *et al.*, 2014). $iPLA_2$ are situated either in cytosol, the inner side of the cell membrane, endoplasmic reticulum (ER) or mitochondrial membrane and the normal role of these phospholipases is to maintain homeostasis through remodelling of membrane phospholipids as well as mediating cell growth signalling (Quach *et al.*, 2014). Advances in technology have led to a better understanding of the distinct functions of $iPLA_2$ and $cPLA_2$. While $cPLA_2$ favours the release of AA, there is substantial evidence that $iPLA_2$ is responsible for release of DHA (Quach *et al.*, 2014; Ryan *et al.*, 2014). Secretory PLA_2, which were originally classified from snake and bee venom, function extracellularly and act on cellular membranes, non-cellular phospholipids (e.g. surfactant and lipoproteins) and foreign phospholipids (e.g. bacterial membranes and dietary phospholipids) (Murakami *et al.*, 2011; Quach *et al.*, 2014). $SPLA_2$ has been linked with rheumatoid arthritis, atherosclerosis, central

nervous system inflammation, inflammatory bowel diseases, skin inflammation, cancer, and asthma. The benefit of this enzyme is that it causes the lysis of gram positive bacteria during infection, and is found in tears (Menschikowski *et al.*, 2006). In peripheral systems $sPLA_2$, specifically $sPLA_2$-IIA is considered to be an important inflammatory protein and to play a major role in connecting the innate and adaptive immune systems (Murakami *et al.*, 2011).

In a cellular system with sufficient supply of adenosine triphosphate (ATP) and coenzyme A (CoA), the released free fatty acids are readily converted to acyl-CoA and afterwards returned to membrane phospholipids through lysophospholipid acyltransferases. Since AA and DHA are major fatty acids in the *sn*-2 position of phospholipids, the deacylation–reacylation system plays a critical role in controlling the metabolic activity of these LCPUFA (Sun *et al.*, 2010). In addition to being returned to the cellular membrane, LCPUFA can also be metabolized to lipid mediators (eicosanoids and docosanoids) (Dennis, 2000) or degraded by beta-oxidation or peroxidation (Yavin *et al.*, 2002).

Therefore, phospholipids and their metabolites are not only cell building blocks, but are involved in a large number of important cellular control systems. This means that PLA_2 are also important in systems such as signal transduction and eicosanoid production. Through these systems, the actions of PLA_2 affect a wide range of physiological functions and diseases including asthma and allergy, sepsis, inflammatory bowel disease, arthritis and other inflammatory diseases (Dennis, 2000).

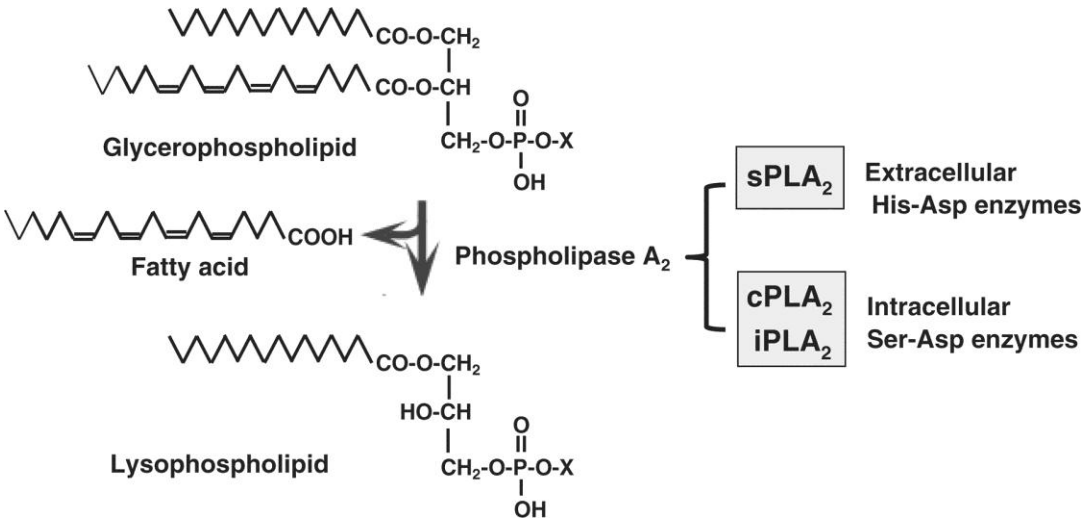

Figure 7: Actions of phospholipase A 2 (PLA_2). Intracellular cytosolic phospholipase 2 ($cPLA_2$), calcium-independent PLA_2 ($iPLA_2$) and extracellular secretory PLA_2 ($sPLA_2$) are shown (Murakami *et al.*, 2011).

2.2.3.2 Early signal transduction events

As mentioned before, $cPLA_2$ is activated by a number of exogenous stimuli, including receptor agonists and oxidant compounds (Sun *et al.*, 2010). This forms part of a complicated signalling system, including PLC, cross-talk between the families of PLA_2 as well as transcriptional and translational processes take part in the activation of inflammation and resolution thereof when stimulated (Figure 8). A variety of extracellular ligands binds to and activates specific receptors, such as receptor tyrosine kinases and G protein-coupled receptors. In G protein-coupled receptor signalling, PLC is activated and subsequently generates two second messengers, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3), by hydrolysing phosphatidylinositol-4,5-bisphosphate. DAG and IP_3-induced calcium release from the endoplasmic reticulum (ER) can activate protein kinase C, which stimulates PLD activity in a phosphorylation-dependent or a phosphorylation-independent manner. Calcium binding and phosphorylation by extracellular signal-regulated kinase can fully activate $cPLA_2$. Intracellular $iPLA_2$ is phosphorylated by protein kinase C. $cPLA_2$ and $iPLA_2$ can hydrolyse various phospholipids, including PC, PS and PA, to release AA and DHA, respectively, which are further converted into various lipid mediators (Ariel & Serhan, 2007). $SPLA_2$ further enhances signalling to initiate translational/posttranslational events that result in the activation of $cPLA_2$ (Park *et al.*, 2012). Thus, membrane phospholipids are substrates for the release of (non-esterified) PUFA intracellularly – the released PUFA can act as signalling molecules, ligands (or precursors of ligands) for transcription factors, or precursors for biosynthesis of lipid mediators such as eicosanoids and docosanoids (Calder, 2010).

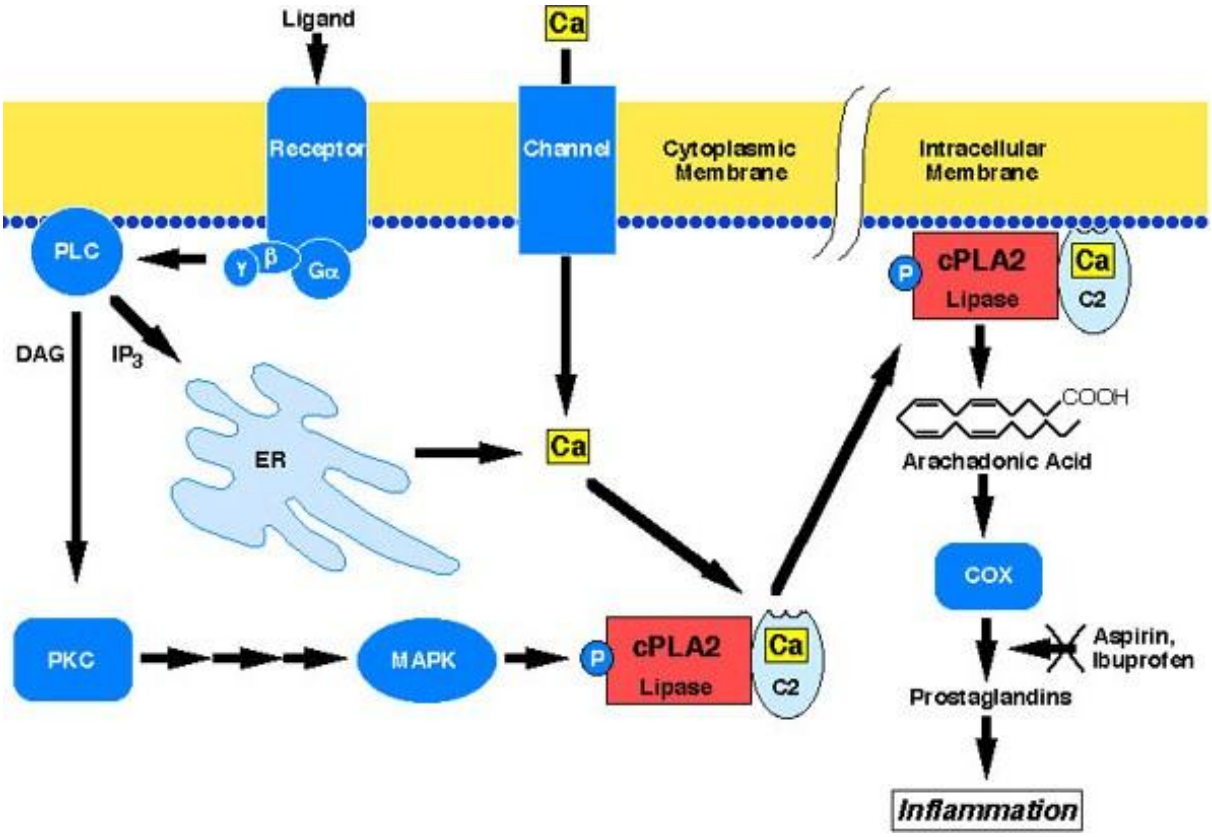

Figure 8: Phospholipase C and A signalling networks (http://chem35132007.pb-works.com/w/page/15648433/Phospholipase%20Inhibitors last access 1.9.2014). Abbreviations: COX: cyclooxygenase; cPLA2: cytosolic phospholipase A2; DAG: diacylglycerol; ER: endoplasmic reticulum; IP3: inositol-1, 4, 5-trisphosphate; MAPK: mitogen-activated protein kinase; PLC: phospholipase C; PKC: protein kinase C.

2.2.3.3 Lipid mediator synthesis

Among the first signalling events after infection or tissue injury is the release of pro-inflammatory lipid mediators that launch a series of signalling cascades with the final goal of destroying the invading pathogens and repairing the damaged tissue (Calder, 2010; Serhan & Petasis, 2011). AA are released from cellular stores and serve as a substrate for cyclooxygenase (COX) and lipoxygenase (LOX or LO) enzymes to form several bioactive eicosanoids and docosanoids, namely PG, LT, thromboxanes and lipoxins (LX) as seen in Figure 9 (Calder, 2010). These lipid mediators exert specialized inflammation-related actions by acting on specific G-protein coupled receptors (Serhan & Petasis, 2011). Activation of these receptors directly affects the expression levels of numerous enzymes, chemokines, cytokines, and growth factors that are important in inflammation and the resolution thereof (Serhan & Petasis, 2011). Most of the AA derived mediators have a pro-inflammatory function (Calder, 2010). There are at least 16 different 2-series PG and these are formed in a cell specific manner. For example monocytes and macrophages produce large amounts of PGE_2 and $PGF_{2;}$ neutrophils produce moderate

amounts of PGE_2 and mast cells produce PG_2. The 5-LOX enzymes are found mainly in mast cells, monocytes, macrophages and granulocytes and the 12- and 15-LOX primarily in epithelial cells. Metabolism of AA by the 5-LOX pathway results in hydroxyl and hydroxyperoxy derivatives, namely 5 hydroxyeicosatetraenoic acid (HETE) and 5 hydroxyperoxy-eicosatetraenoic acid (HPETE) as well as the 4-series LT. Eicosanoids (especially PGE_2 and 4-series LT) are responsible for the intensity and duration of inflammatory and immune responses. (Tilley, Coffman et al. 2001). LTB_4, which is a very effective chemotactic agent, promotes the recruitment of neutrophils to the inflamed tissue, while the formation of PGE_2 and PGD_2 further speed up the inflammatory process, ultimately resulting in the condition of acute inflammation (Serhan & Petasis, 2011). However, PGE_2 has been demonstrated to suppress lymphocyte proliferation and natural killer cell activity and inhibits production of TNF-α, IL-1, IL-6, IL-2 and IFN-γ and so therefore has also some anti-inflammatory effects. PGE_2 also increases immunoglobulin E (IgE) production by B lymphocytes. The 4-series LTs regulate the production of pro-inflammatory cytokines, e.g. LTB_4 enhances production of TNF-α, IL-1, IL-6, IL-2 and IFN-γ. 15 HETE inhibits lymphocyte proliferation, whereas 5-HETE improves it (Calder, 2010).

Initially it was assumed that these same mediators were responsible for the initiation and termination of acute inflammation, as well as the transition from acute to chronic inflammation. However, it is now well established that there are several counter-regulatory substances that are generated during the resolution of acute inflammation to produce a healthy termination of an acute inflammatory response. These substances include some LX, glucocorticoids and their product annexin-1-adenosine, PGE_2 and PGD_2 and its breakdown product PGJ_2, and transforming growth factor (TGF) β (Ariel & Serhan, 2007).

More recently, a group of endogenous local mediators possessing stereo specific and potent anti-inflammatory properties have been identified (Ariel & Serhan, 2007; Serhan & Petasis, 2011). EPA and DHA are substrates of COX and LOX enzymes and produce the newly described resolvins, protectins and maresins (Serhan & Petasis, 2011).

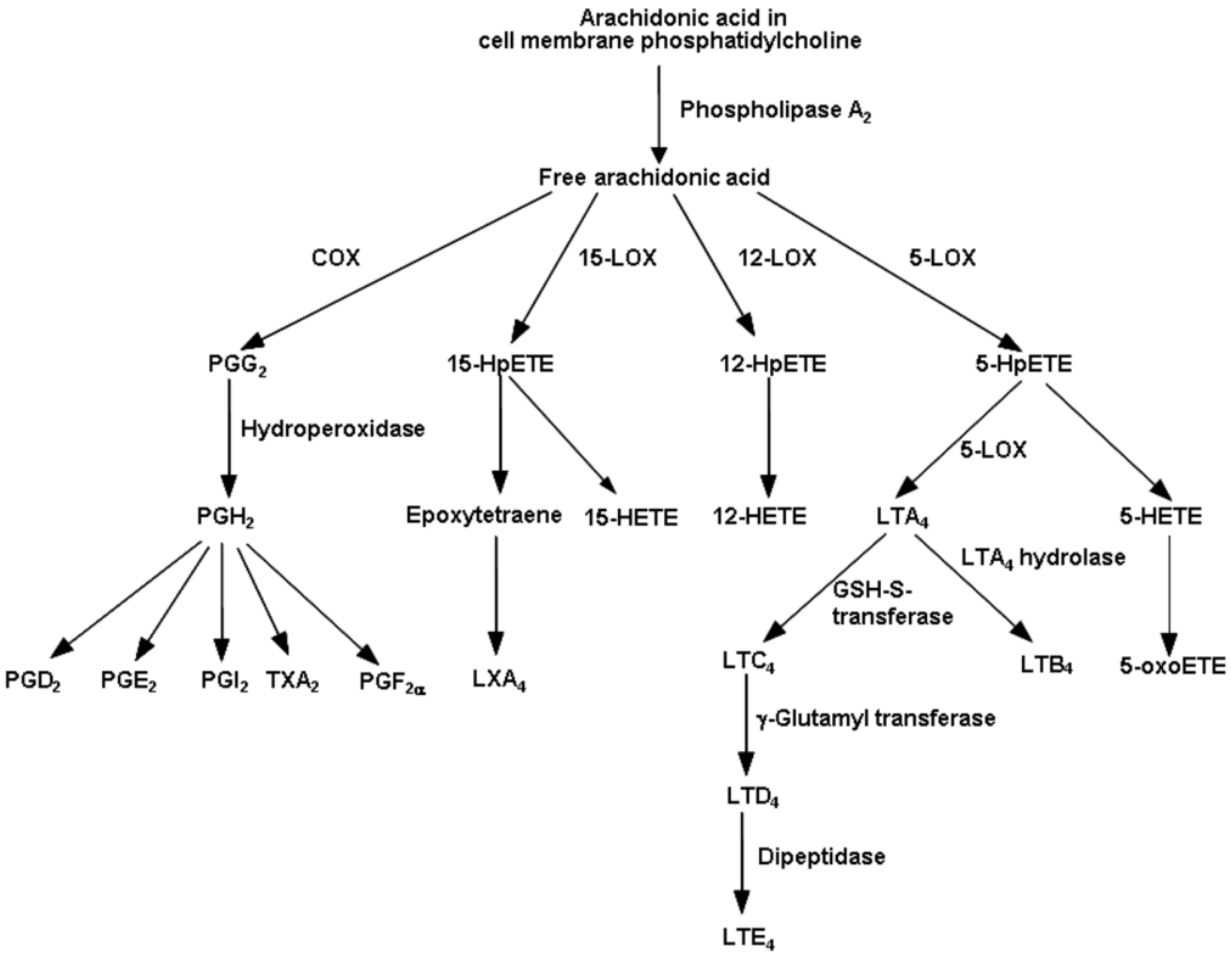

Figure 9: Eicosanoid biosynthesis from arachidonic acid. Abbreviations: COX: cyclooxygenase; HETE: hydroxyeicosatetraenoic acid; HpETE: hydroperoxyeicosatetraenoic acid; LOX: lipoxygenase; LT: leukotriene; LX: lipoxin; oxoETE: oxoeicosatetraenoic acid; PG: prostaglandin; TX: thromboxane. (Calder, 2010).

2.3 Polyunsaturated fatty acids and immune function

The impact of PUFA on immune function lies mostly within the effect of PUFA on membrane composition of the immune cells, which is influenced by fatty acid intake (Calder, 2013b; Fritsche, 2006). The immune response greatly depends on the phagocytic and cell signalling ability of the immune cells, which in turn are affected by the fatty acid composition of the phospholipid bilayer. PUFA intake and availability also directly interact with specific transcription factors and thus regulate gene expression (Jump *et al.*, 2013). Another aspect influenced by the PUFA composition of membranes, is the biosynthesis of eicosanoids and other lipid mediators, which mediates immune cell function and inflammation (Calder, 2002). Furthermore, fatty acids also seem to modulate cytokine biosynthesis, which directly influences immune responses (Fritsche, 2006).

2.3.1 Omega-3 polyunsaturated fatty acid status

The World Health Organisation (WHO) recommends a daily dietary intake 1-2% of energy for n-3 PUFA and the Food and Agriculture Organisation of the United Nations (FAO) recommends a DHA and EPA intake of 250–500 mg per day for 6–10 y-old children (Global Organization for EPA and DHA Omega-3s). Many national authorities have also recommended intake levels for their own countries (Flock *et al.*, 2013; Harris *et al.*, 2009a). Based on mostly cardiovascular disease (CVD) and cognitive outcomes, recommendations for DHA and EPA have been made by a number of international organisations and expert committees, with a consensus minimum daily intake of about 250 – 500 mg DHA and EPA or at least two servings of fatty fish per week for healthy adults (Flock *et al.*, 2013; Kuratko *et al.*, 2014; Smuts & Wolmarans, 2013). A useful blood indicator for n-3 PUFA status, the n-3 index, is the sum of EPA and DHA composition in RBC, and a percentage above 8% seems to be optimal (Harris & von Schacky, 2004; Luxwolda *et al.*, 2011), with high, intermediate, and low risk levels for CVD of the omega-3 index being < 4%, 4% – 8%, > 8%, respectively (Harris *et al.*, 2009b). Unfortunately, the use of the n-3 index as a fatty acid status marker is not yet implemented everywhere and epidemiological data are thus difficult to obtain and interpret. Other frequently used n-3 fatty acid status markers are plasma and RBC membrane phospholipids (Fekete *et al.*, 2009). Plasma total phospholipid DHA and EPA levels vary widely from about 1.3% – 7.7% and 0.5% – 2.4% respectively, depending on the population. Likewise, total RBC DHA varies generally between 2.4 – 6.6% (Fekete *et al.*, 2009). In South Africa data are scarce. In a comparative study between different geographical areas the RBC total phospholipid DHA varied from 3.5% to 4.6%, and, surprisingly, an inland area with very little fish intake (median intake of 18 g of fish per consumer) had levels (4.6%) comparable to the coastal area (4.2%) with moderate fish intake of 69 g per consumer (Ford, 2013). In contrast, previous data show that inland areas in South Africa have a lower total n-3 PUFA status than coastal areas as depicted in Figure 10 (Smuts, 2011). In the Valley of a Thousand Hills in KwaZulu-Natal (the current study's site), the RBC total phospholipid DHA composition of a population of 6–11 y-old ID school children was 3.0%, which could be described to be at the lower spectrum of DHA status (Baumgartner *et al.*, 2012c; De Meester *et al.*, 2012)

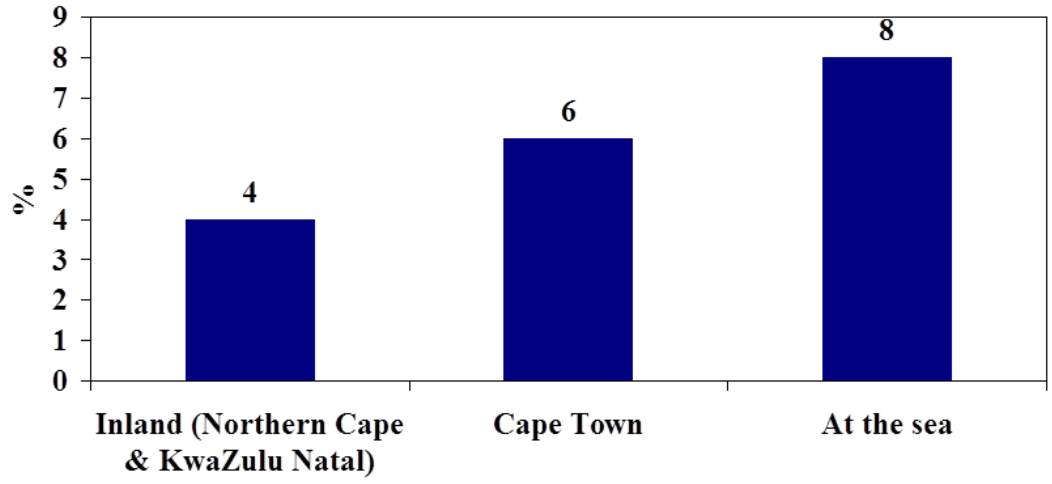

Figure 10: Plasma total phospholipid n-3 fatty acids of children living at various distances from the sea in South Africa (Smuts, 2011).

2.3.2 Polyunsaturated fatty acid interventions and infectious morbidity

Limited controlled intervention trials have thus far investigated the effects of dietary PUFA on clinical outcomes in school-age (Dalton, 2006; Thienprasert *et al.*, 2009; Thomas *et al.*, 2011) and younger children (Birch *et al.*, 2002; Minns *et al.*, 2010; Pastor *et al.*, 2006). These studies are shown in Table 3. Three of these described favourable effects of fish oil-enriched milk, fish flour spread and 900 mg ALA plus 100 mg DHA, respectively, on respiratory illness in 7–12-year-old school children (Dalton, 2006; Thienprasert *et al.*, 2009; Thomas *et al.*, 2011), while three described beneficial effects of DHA only, or DHA and AA-enriched formula on upper respiratory tract infections, bronchiolitis and asthma/wheezing in under 3-year-olds and infants (Birch *et al.*, 2002; Minns *et al.*, 2010; Pastor *et al.*, 2006). In a controlled study in Thailand, increased n-3 PUFA blood status had no immunosuppressive effects and reduced episodes and duration of illness. However, circulating concentrations of most plasma cytokines measured in this study were not significantly changed (Thienprasert *et al.*, 2009). In South African schoolchildren (6–9 y), there was a significant difference in absenteeism between the experimental group, who received a daily fish spread with ~190 mg DHA and ~ 82 mg EPA and the control group, receiving a similar daily spread with only ~36 mg DHA and ~14 mg EPA. The greatest impact was on respiratory illnesses, with 14.3% of subjects absent due to respiratory illness during the intervention, compared to 27.7% in the control group. Furthermore, the children were significantly less days absent due to any illness and respiratory illness in the intervention group. However, there was no significant intervention effect on IL-6 and TNF-α (Dalton, 2006). In Bangalore, India, 6–10 y-old school children received foods fortified with either high (100% recommended dietary allowance) or low (15% recommended dietary allowance) micronutrients, combined with either high (900 mg ALA plus 100 mg DHA) or low

(140 mg ALA) n-3 PUFA for 1 year. Combined with the micronutrient powder, children taking high n-3 PUFA had significantly fewer episodes and shorter duration of upper respiratory tract infections, less gastro-intestinal upset and less general symptoms compared with the children who received only ALA. The high micronutrient intervention also reduced the duration of general symptoms (Thomas *et al.*, 2011).

Birch *et al* reviewed medical charts of 3 y-olds that had been supplemented with DHA (0.32% – 0.36% of total fatty acids) and AA (0.64% – 0.72% of total fatty acids) during their first year of life and found that the supplemented children had significantly lower odds for developing upper respiratory tract infections, wheezing/asthma, wheezing/asthma/atopic dermatitis or any allergy when compared to controls (Birch *et al.*, 2002). A multiple-centre, open-label, 12 month observational study in infants receiving similar DHA/AA-dose supplemented formulas as in the study by Birch *et al*, also found significantly less incidence of bronchiolitis at ages 5, 7 and 9 months versus controls, who received none or lower levels of DHA/AA (Pastor *et al.*, 2006). Supplementation of 130 mg DHA per day for 60 days in 18 – 36 month-old children in a randomized controlled trial also resulted in lower incidence of respiratory illness (Minns *et al.*, 2010).

Thus, all these studies showed evidence of benefit relating to respiratory illness, with a daily dose ranging between about 130 – 1000 mg DHA and additionally in 4 of the 5 studies, 82 – 570 mg EPA. Two studies also included AA.

Since n-3 LCPUFA and their derivatives have anti-inflammatory effects (Calder, 2010) and the inflammatory response is a critical component of the immune system's response to pathogens, large doses of n-3 LCPUFA may impair defence against infections. This effect appears to be dose-dependent. Supplementation with high levels (>10% of total fat) of n-3 PUFA (compared to diets high in n-6 PUFA) to healthy animals or human subjects resulted in suppression of lymphocytic response to mitogen stimulation, NK cell activity and delayed-type hypersensitivity reactions (Field *et al.*, 2002). In contrast, supplementation with moderate amounts of n-3 PUFA (<10% of fat in animals and <1 g EPA + DHA/day in humans) seems not to be immunosuppressive and appear to enhance immune functions, including lymphocyte proliferation/activation, NK cell activity, macrophage activation and IL-1, IL-2, and TNF-α production after mitogen stimulation (Field *et al.*, 2002; Robinson *et al.*, 2001).

Although many studies are published on the function of n-3 PUFA to decrease inflammatory disease, the possible of improvement of immune response against infectious disease has received little attention. Some recent animal studies have demonstrated improved host resistance with n-3 PUFA supplementation (Maroufyan *et al.*, 2012; Sargi *et al.*, 2012). In bursal disease challenged chickens, Newcastle disease antibody titres as well as IL-2 and IFN-γ

concentrations were elevated as n-3 PUFA enrichment was elevated from 0.5% to 16.5% of the diet (Maroufyan *et al.*, 2012). In an *ex vivo* study of mice macrophages, a diet with 44.6–51.6% ALA were shown to improve the microbicidal activity of the macrophages by inducing the production of cytokines and metabolites such as nitrous oxide and hydrogen peroxide subsequent to infection with the fungus, *Paracoccidioides brasiliensis* (Sargi *et al.*, 2012).

Further strengthening the hypothesis that a moderate level of n-3 PUFA intake improves immune response, are the findings around the use of parenteral nutrition with n-3 PUFA in post-surgical and critically ill patients (Calder, 2007; Fullerton *et al.*, 2014; Pradelli *et al.*, 2012). A recent meta-analysis confirmed earlier findings, that n-3 PUFA-enriched parenteral nutrition regimens are safe and effective in lowering the infection rate and hospital or intensive care unit stay in surgical and intensive care unit patients. (Pradelli *et al.*, 2012). Furthermore, the propensity of the critically ill patient, to be more vulnerable to contracting secondary infections, might be due to the failure of the resolution of inflammation (Calder, 2007; Fullerton *et al.*, 2014). Moreover, resolution therapies which involve manipulation of lipid mediator levels, show promising results in reducing susceptibility to hospital-acquired infections (Eisen *et al.*, 2012; O'Neal Jr *et al.*, 2011; Otto *et al.*, 2012; Sossdorf *et al.*, 2013; Winning *et al.*, 2009).

Table 3: Randomised controlled trials of n-3 PUFA intervention in children with immune or morbidity outcomes

Author, year	Age	Duration	Type	Dose of fatty acid different from controls (mg/day)					Immune function	Findings: Respiratory morbidity and immune function parameters
				DHA	EPA	ALA	LA	AA		Respiratory outcomes: Upper respiratory infection (URI), otitis media (OM), sinusitis, bronchitis, bronchiolitis, and pneumonia
Dalton, 2006	7–9 y	6 mo	Fish flour spread	192	82	335	1567	23.2	Improved	14.3% subjects absent with respiratory illness vs 27.7% in control group; 0.04 days absent with respiratory illness vs 0.84 in control group
Thienprasert, 2008	9–12 r	6 mo	Fish oil in milk	1000	200				Improved	54.3% ill vs 67.4% in control group; 28.7% ill with fever vs 37.2% in control group; same episodes of illness; 2 days ill/subject vs 4 days in control group; illness = rhinitis, cold, influenza and diarrhoea; absent at least once: 54.3% vs 67.4% in control group
Hamazaki, 2008	8–14 y	3 mo	Fish oil capsules	650	100			39	Improved	Fewer absent days: 27 vs 49 in control group
Birch, 2010	3 y	12 mo at 0.5–12 mo of age	Formula	0.32%–0.36% of total fatty acids (17 mg/100 kcal)				0.64-0.72% of total fatty acids (34 mg/100 kcal)	Improved	79% vs 84% in controls of all respiratory illness, 45% URI vs 76% in controls, 76% OM vs 71% in controls; 34% of any allergy vs 61%, 16% wheezing vs 35%

Minns, 2010	18–36 mo	60 days	Formula	130					Improved	17% vs 46% in controls of participants experiencing one or more respiratory illnesses
Mazurak, 2008	5–7 y	7 mo	Supplement drink	14–21				20–30	Improved	IL-2, IL-10 and CD54↑, Expression of antigens on cells ↓
Pastor, 2006	0–12 mo	12 mo	Formula	0.32% of total fatty acids				0.64% of total fatty acids	Improved	5.8% vs 11.3% bronchitis /bronchiolitis, 16.2% URI vs 24.2 % at 12 mo, 3.0% vs 6.7% rhinitis at 1mo
Damsgaard, 2007	9–12 mo	3 mo	Fish oil	381	571					IFN-γ ↑, IL-10 ↓
Imhoff-Kunsch, 2011	1–6 mo	6 mo	Fish oil to mothers before birth	400 to mothers						Shorter duration of cold at 1 month, of fever and other illness at 6 mo, 14% less ill at 3 mo, longer rash at 1 mo
Lauritzen, 2005	2.5 y	4 mo during lactation	Fish oil to lactating mothers	1500 to mothers						IFN-γ ↑
Thomas, 2012	6–10 y	1 year	Supplement	100		900			Improved	fewer episodes of URTI/child/year (relative risk (RR)=0.88, 95%confidence interval (CI): 0.79, 0.97) and significantly shorter duration/episode of URTI (RR=0.81, 95% CI: 0.78, 0.85), LRTI (RR=0.91, 95% CI: 0.85, 0.97), compared with children who received low n-3 fatty acid intervention

2.3.3 Mechanisms of the effect of polyunsaturated fatty acids on immune function

The effects of PUFA on immune function are closely related to composition of these fatty acids in the phospholipids of inflammatory cell membranes (Figure 11. Here they are central in assuring the correct environment for membrane protein function, maintaining membrane order ("fluidity") and influencing lipid raft formation (Calder, 2010). Membrane phospholipids are substrates for the production of second messengers like DAG and it has been shown that the fatty acid composition of such second messengers, which is determined by that of the precursor phospholipid, can influence their activity. Furthermore, membrane phospholipids are substrates for the release of intracellular PUFA, which can then act as signalling molecules, ligands (or precursors of ligands) for transcription factors, or precursors for biosynthesis of lipid mediators (eicosanoids and docosanoids) which play a role in the regulation of many cell and tissue responses, including inflammation and immunity. Thus, the functions of immune and inflammatory cells can be influenced by alterations in membrane phospholipid fatty acid composition via:

- alterations in the physical properties of the membrane such as membrane order and raft structure;
- effects on cell signalling pathways, either through modifying the expression, activity or avidity of membrane receptors or altering intracellular signal transduction mechanisms that lead to altered transcription factor activity and changes in gene expression;
- changes in lipid mediators produced (eicosanoids, resolvins, etc.) with the different mediators having different biological activities and potencies (Calder, 2010).

Free or unbound PUFA can also directly associate with and influence receptors (Calder, 2012).

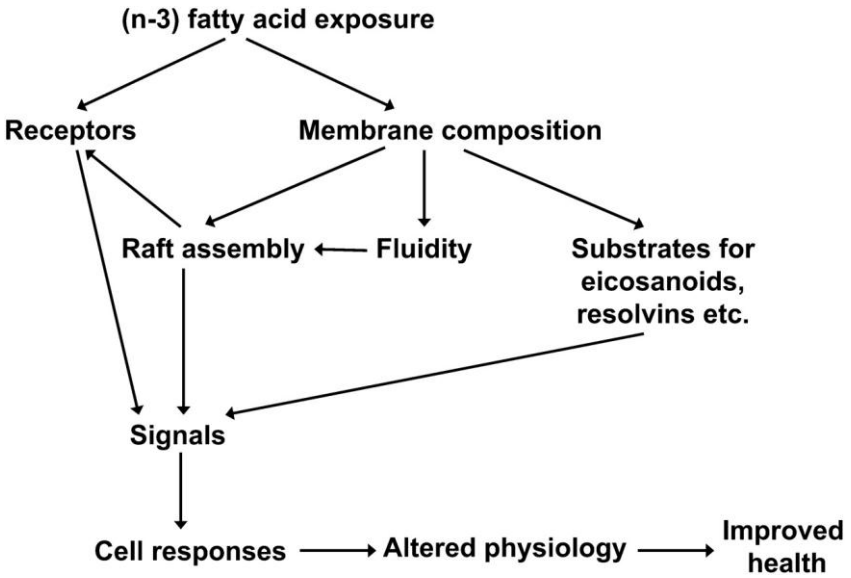

Figure 11: Overview of the mechanisms by which n-3 PUFA can influence cell function (Calder, 2012).

2.3.3.1 Immune cell polyunsaturated fatty acid composition and alteration of membrane order (fluidity).

The phospholipids of human PBMC contain 6 – 10% LA, 1 – 2% DGLA and 15 – 25% AA (Covar *et al.*, 2010; Damsgaard *et al.*, 2008; Gibney & Hunter, 1993). Relative to the n-6 composition, percentages of n-3 FA are low. ALA is usually only found in trace amounts, EPA at 0.1 – 0.8% and DHA at around 2 – 4% (Covar *et al.*, 2010; Damsgaard *et al.*, 2008; Yaqoob *et al.*, 2000). Availability of essential PUFA and their long chain metabolites influence membrane phospholipid composition of immune cells (Calder, 2008) and dietary intake is, therefore, reflected in the composition of immune cell membranes (Damsgaard *et al.*, 2008). Furthermore, when fish oil is supplemented in humans, the n-6/n-3 PUFA ratio is decreased in a dose-dependent manner in immune cells (Calder, 2002; Gibney & Hunter, 1993; Thies *et al.*, 2001; Yaqoob *et al.*, 2000). AA is at least partly replaced in immune cells by supplementation with n-3 LCPUFA (Covar *et al.*, 2010; Damsgaard *et al.*, 2008; Yaqoob *et al.*, 2000). However, the incorporation of, for example DHA, into PBMC seems not to be linear above 4.2 g per day (Witte *et al.*, 2010). This observation might indicate that there could be a limit to the amount of DHA that will be incorporated and is optimal for immune cell membranes. Another interesting observation that may indicate a more controlled incorporation of fatty acids into PBMC than into RBC, is that increasing the LA intake in healthy young men did not have any pronounced effect on incorporation of n-3 LCPUFA in PBMC, either alone or with simultaneous fish oil supplementation (Damsgaard *et al.*, 2008). In contrast, high LA intake has been demonstrated to impair hepatic synthesis, decrease n-3 LCPUFA incorporation into tissues (Gibson *et al.*, 2011) and lower plasma and erythrocyte n-3 LCPUFA levels (Makrides *et al.*, 2000). These findings are explained by the competition between n-6 and n-3 precursors for the desaturase enzymes (Brenna, 2011; Gibson *et al.*, 2011). These observations suggest that incorporation of DHA into PBMC may have a maximum threshold and that the amount of LA in the diet may affect n-3 LCPUFA incorporation into PBMC membranes less than in other membranes. It might point to a more regulated incorporation of fatty acids into immune cells, due to a balance needed between n-6 and n-3 PUFA for inflammatory and anti-inflammatory processes.

The time it takes for incorporation of n-3 LCPUFA into leucocytes has been reported to range from one week to several months, with four weeks being the near maximum time point for incorporation of both DHA and EPA (Browning *et al.*, 2012; Faber *et al.*, 2011; Yaqoob *et al.*, 2000). However, in a study by Faber *et al*, improved *ex vivo* production of pro-inflammatory cytokines was described after only one week of supplementation providing 2.4 g EPA and 1.2 g DHA daily to twelve healthy Caucasians aged >55 y (Faber *et al.*, 2011). After reviewing recent data from two studies, Calder concluded that near maximum incorporation of DHA and EPA into

human mononuclear cells occurs at about one week, given at the dose of Faber *et al*. (Calder, 2013b).

The incorporation of PUFA into cell membranes influences the fluidity or lipid order of the membranes, specifically influencing areas called lipid rafts. Lipid rafts are areas of membranes with distinct characteristic organisational composition (Pike, 2003; Simons & Toomre, 2000). They contain high concentrations of cholesterol and glycosphingolipids, and the side chains of the phospholipids are generally enriched in SFA (Calder, 2013b), but all rafts are not identical in terms of either the proteins or the lipids that they contain. An assortment of proteins, especially those involved in cell signalling, has been shown to be imbedded in lipid rafts. These include the Src family kinases, G proteins, growth factor receptors, mitogen-activated protein kinases, and protein kinases C. As a result, lipid rafts are thought to be involved in the regulation of signal transduction. Experimental evidence suggests that there are probably a number of different mechanisms through which rafts control cell signalling. For example, rafts may contain partial signalling pathways that are activated when a receptor or other essential molecule is engaged into the raft (Pike, 2003). Signal transduction is initiated by complex protein–protein interactions between molecules like ligands, receptors and kinases. Lipid rafts comprising of a given set of proteins can change their size and composition in response to intra- or extracellular stimuli. This favours particular protein–protein interactions, causing the activation of signalling cascades (Simons & Toomre, 2000).

For example, proposed initial lipid raft-associated signalling events for T cell antigen receptor (TCR)-mediated signalling are shown in Figure 12. For clarity, only a small subset of involved proteins is shown. A probable sequence of the basic initial events is shown numerically. 1) Ligand-induced receptor dimerization of the TCR (CD3) probably improves its raft association, which leads to 2) phosphorylation of the receptors' immune receptor tyrosine-based activation motifs by Src-family protein tyrosine kinases (for example, Lyn, Lck and Fyn). 3) Phosphorylated immune receptor tyrosine-based activation motifs act as a membrane docking site for cytoplasmic ZAP-70; these are also tyrosine kinases and are activated in the raft by tyrosine phosphorylation. 4) ZAP-70 can, in turn, activate other proteins such as LAT, a raft-associated adaptor. Through crosslinking, LAT can recruit other proteins into the raft and further amplify the signal. The complex cascade of later downstream signalling events is not shown. 5) One possible way of down regulating the signal may occur by binding of the cytosolic kinase to the raft-associated protein CBP. Cytosolic kinase may then inactivate the Src-family kinases through phosphorylation (Simons & Toomre, 2000).

Cell culture studies and feeding studies in mice have demonstrated that exposure to n-3 PUFA modifies raft formation of T cells (Calder, 2013b). Membrane order or fluidity directly influences

important immunological mechanisms like phagocytosis, which is improved by n-3 PUFA supplementation (Yaqoob, 2010). Cell culture experiments have shown that increased n-3 PUFA in membranes of leukocytes result in increased membrane fluidity and improved phagocytosis (Calder *et al.*, 1994). This effect was confirmed *in vivo* by a study where the oral intake of 1.5 g EPA plus DHA per day for 6 months by healthy human volunteers increased neutrophil phagocytic activity by about 40% and monocyte phagocytic activity by about 200% (Calder, 2008).

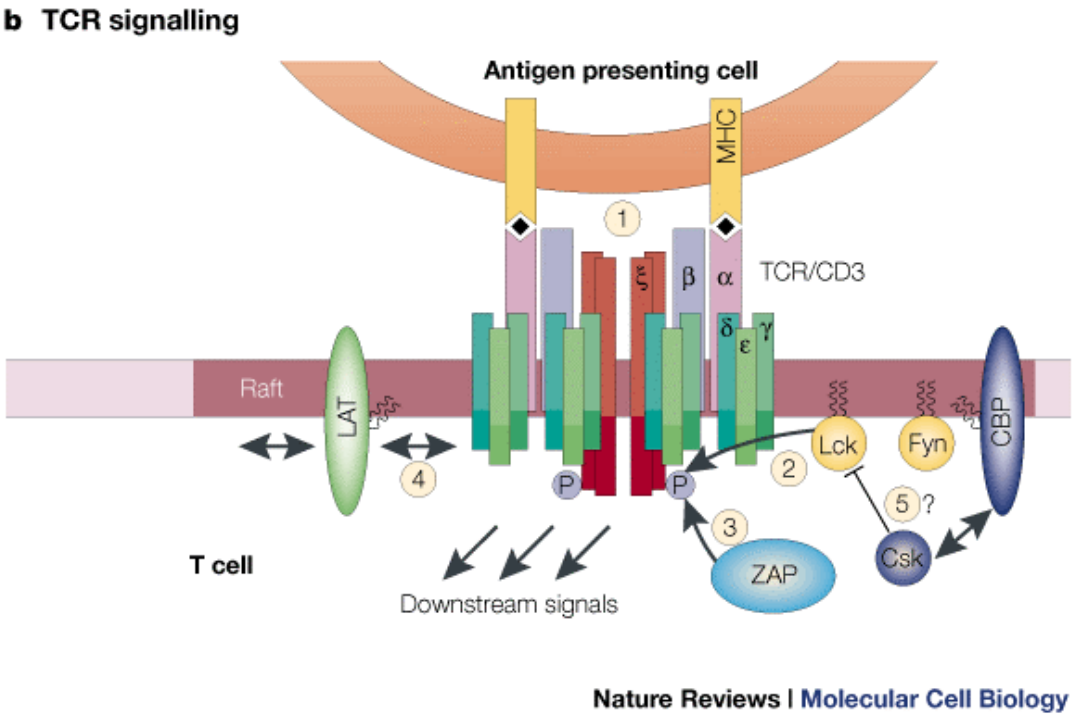

Figure 12: Initial lipid raft–associated signalling events for T-cell antigen receptor-mediated signalling. Abbreviations: CBP: raft-associated protein; Csk: cytosolic kinase; Fyn, LcK and ZAP: Src-family protein tyrosine kinases; LAT: transmembrane receptor; MHC: major histocompatibility complex; TCR/CD3: T-cell antigen receptor (Simons & Toomre, 2000).

The functionality of many membrane-associated proteins in immune cells has been demonstrated to be modulated by membrane lipid changes, for example the binding of cytokines to their receptors as well as the positioning of some cell surface molecules. Many of these molecules are involved in co-stimulation processes necessary for lymphocyte activation (Calder 2002).

Many cell signalling molecules are generated directly from membrane phospholipids, e.g. IP_3, DAG, phosphatidic acid, choline ceramide, platelet-activating factor and AA. These have important roles in regulating the activity of proteins involved in immune-cell response (Calder, 2002).

2.3.3.2 Alteration of gene expression

Fatty acids, especially PUFA, are known to control the expression of many genes involved in numerous metabolic pathways (Bouwens *et al.*, 2009; Calder, 2013b; Clarke & Jump, 1994; Sampath & Ntambi, 2004) and more than 5,000 papers dealing with fatty acid–regulated gene transcription have appeared in PubMed (Jump *et al.*, 2013). Fatty acids exert much of their effects by regulating the activity and nuclear abundance of transcription factors, such as peroxisome-proliferator activated receptors (PPAR), nuclear factor kappa-B (NF-κB), sterol regulatory element-binding proteins (SREBP) and liver X receptors (LXR) (Mathews *et al.*, 2000; Sampath & Ntambi, 2004). This regulation is accomplished by direct fatty acid binding to the transcription factor (acting as a ligand) or by indirect mechanisms where fatty acids regulate signalling pathways controlling the expression of transcription factors or the phosphorylation, ubiquitination, or proteolytic cleavage of the transcription factor (Jump *et al.*, 2013).

Evidence exists that n-3 PUFA inhibit the activation of NF-κB, which is a common transcription factor for several genes involved in inflammation, e.g. the *COX-2* (*PTGS2*) gene as well as genes coding for inflammatory cytokines, adhesion molecules and inducible nitric oxide synthase and thus have an anti-inflammatory and antioxidative gene expression effects (Bouwens *et al.*, 2009; Calder, 2013b; Lo *et al.*, 1999). NF-κB is a trimer in the cytosol and is activated by a signalling cascade started by extracellular inflammatory stimuli which involves phosphorylation of an inhibitory subunit of NF-κB. This subunit dissociates from the trimer, which allows the translocation of the remaining NF-κB dimer to the nucleus (Perkins, 2007). LPS (as well as some inflammatory cytokines and UV irradiation) induces inflammation by triggering NF-κB. EPA or fish oil has been shown to reduce LPS-induced activation of NF-κB in human monocytes and this was associated with diminished inhibitory subunit NF-kB phosphorylation (Novak *et al.*, 2003; Zhao *et al.*, 2004). Two other newly demonstrated mechanisms whereby EPA and DHA supress inflammatory signalling via NF-kB includes interference with early membrane events involved in activation of NF-kB via TLR-4 and action via G-protein receptor 120 which initiates an anti-inflammatory signalling cascade that inhibits signalling leading to NF-κB activation (Calder, 2013b).

Another transcription factor that is directly activated by n-3 PUFA, and one of the first and most researched in relation to fatty acids, is PPAR (Jump & Clarke, 1999; Masi *et al.*, 2013). In turn, PPAR is able to regulate inflammatory gene expression directly by binding to DNA (Calder, 2013b; Jump *et al.*, 2013). Furthermore, PPAR has also been demonstrated to interact physically with NF-κB preventing its nuclear translocation and, therefore, preventing its activation (Berghe *et al.*, 2003). PPAR alpha has been shown to regulate expression of various genes and is expressed in monocytes/macrophages, endothelial cells, and vascular smooth

muscle cells, where it is associated with decreased production of inflammatory cytokines (Wang *et al.*, 2012).

PBMC generally refer to monocytes and lymphocytes and have been demonstrated as a suitable sample to determine gene expression changes specifically related to immune function in humans (Bouwens *et al.*, 2007; de Mello *et al.*, 2012). They also seem to reflect the effects of dietary changes at the level of gene expression (de Mello *et al.*, 2012). Recently, a double blind trial in healthy elderly Dutch subjects (> 65y) proved that the supplementation of 1.8 g EPA and DHA for a 26 week period can alter the global gene profiles of PBMC *in vivo* to a more anti-inflammatory and anti-atherogenic status. Genes involved in inflammatory and atherogenic related pathways, such as NF-κB signalling, eicosanoid synthesis (such as arachidonate 5-lipoxygenase; *ALOX-5*), scavenger receptor activity, adipogenesis and hypoxia signalling showed decreased expression after DHA and EPA supplementation. The study of Bouwens *et al*. was the first *in vivo* microarray study on the effect of long term n-3 PUFA intervention on gene expression alteration in humans and showed that PBMC profiling can also reflect gene expression changes in healthy subjects (Bouwens *et al.*, 2009).

In this study, the antioxidant enzymes glutathione peroxidase (*GPx*) *3* and superoxide dismutase (*SOD*) *2* were down-regulated by DHA/EPA supplementation (unpublished data). Furthermore, metallothionein (*MT*) *1E* expression was increased by n-3 LCPUFA supplementation (unpublished data). MT is a small, cysteine-rich, metal binding protein that can bind heavy metals with high affinity and are, therefore, seen as a non-enzymatic antioxidant (Vergani *et al.*, 2011).

Recently, some mechanisms of expression regulation by the DHA and EPA derived resolvins and protectins were identified. *C-C chemokine receptor 5* expression was up regulated *in vivo* by LXA_4, resolvin E1 and protectin D1 and facilitated the scavenging of C-C chemokine receptor 5 ligands, which is followed by clearance through macrophage engulfment. This mechanism for removing pro-inflammatory chemokines from the site of inflammation would further limit leukocyte recruitment, prevent excessive immune response and tissue damage and promote return to homeostasis (Ariel & Serhan, 2007).

There has been some evidence that DHA/EPA affects PLA_2 expression (Bouwens *et al.*, 2009; Quach *et al.*, 2014). Activation of NF-kB, can enhance $sPLA_2$ expression, while activation of PPAR (concomitant with increased DHA) can suppress its expression at the mRNA level (Quach *et al.*, 2014). Furthermore, DHA has been shown to decrease levels and activity of AA-specific $cPLA_2$ *in vivo* (Martin, 1998). As seen in Figure 13 $sPLA_2$ contribute to inflammation by releasing free fatty acids, including AA, thus initiating the biosynthesis of pro-inflammatory lipid mediators (Dennis *et al.*, 2011). In addition, the hydrolytic product, lysophospholipid, is also a

pro-inflammatory lipid mediator. A possible mechanism for this effect could be linked to $sPLA_2$ and lower levels of AA due to its replacement with DHA. As seen in Figure 13, signalling messengers (PG and LT) of AA trigger cell signalling pathways by activating GPCR and cytokine receptors. These events can result in amplification of pro-inflammatory cytokine production and intensification of inflammation (Quach *et al.*, 2014), which would be lowered by AA being less available. Furthermore, *PLC beta 1* (phosphoinositide specific) was down regulated by DHA supplementation in the study by Bouwens *et al*, which could in turn reduce $cPLA_2$ expression/levels (Figure 8) (Afman, 2012).

Divalent metal transporter 1 (DMT1) is encoded for by two genes. These proteins encoded by the solute carrier family (*SLC) 11A1* and *SLC11A2* genes function as ion transporters also referred to as *DMT1*. The genes are also named natural resistance to infection with intracellular pathogens (*NRAMP1* and *NRAMP2*, respectively) (Andolfo *et al.*, 2010; Lam-Yuk-Tseung & Gros, 2006). *SLC11A1* is intimately involved in the innate immunity and have roles in phagosome maturation and cell activation (Hedges *et al.*, 2013).

NRAMP2 or *SLC11A2* is a commonly expressed metal-iron transporter gene with four variant mRNA transcripts, differing for alternative promoter at 5' (DMT1 1A and 1B) and alternative splicing at 3' untranslated region, differing by a sequence that includes or lacks an iron response element (IRE+ and IRE–, respectively) (Andolfo *et al.*, 2010; Lam-Yuk-Tseung & Gros, 2006). Isoform I (IRE+) is expressed at the plasma membrane of some epithelial cells such as the enterocytes of the duodenum, where it is critical for the apical absorption of dietary iron. Isoform II (IRE–) is expressed in most cell types and is needed for the uptake of transferrin-bound iron from acidified endosomes (Hubert & Hentze, 2002; Lam-Yuk-Tseung & Gros, 2006; Mackenzie *et al.*, 2007). A DHA-dependent over-expression of *DMT1*, together with elevated intracellular iron levels, has been demonstrated in oligodendrial cells recently after acute and long-term treatments with divalent iron (Brand *et al.*, 2008). This outcome was specific to DHA-enriched cells while addition of AA did not exert any changes (Brand *et al.*, 2008). The finding demonstrates an interaction between the presence of the n-3 PUFA DHA and iron handling that might be important in the contexts of immunity, inflammation and infection.

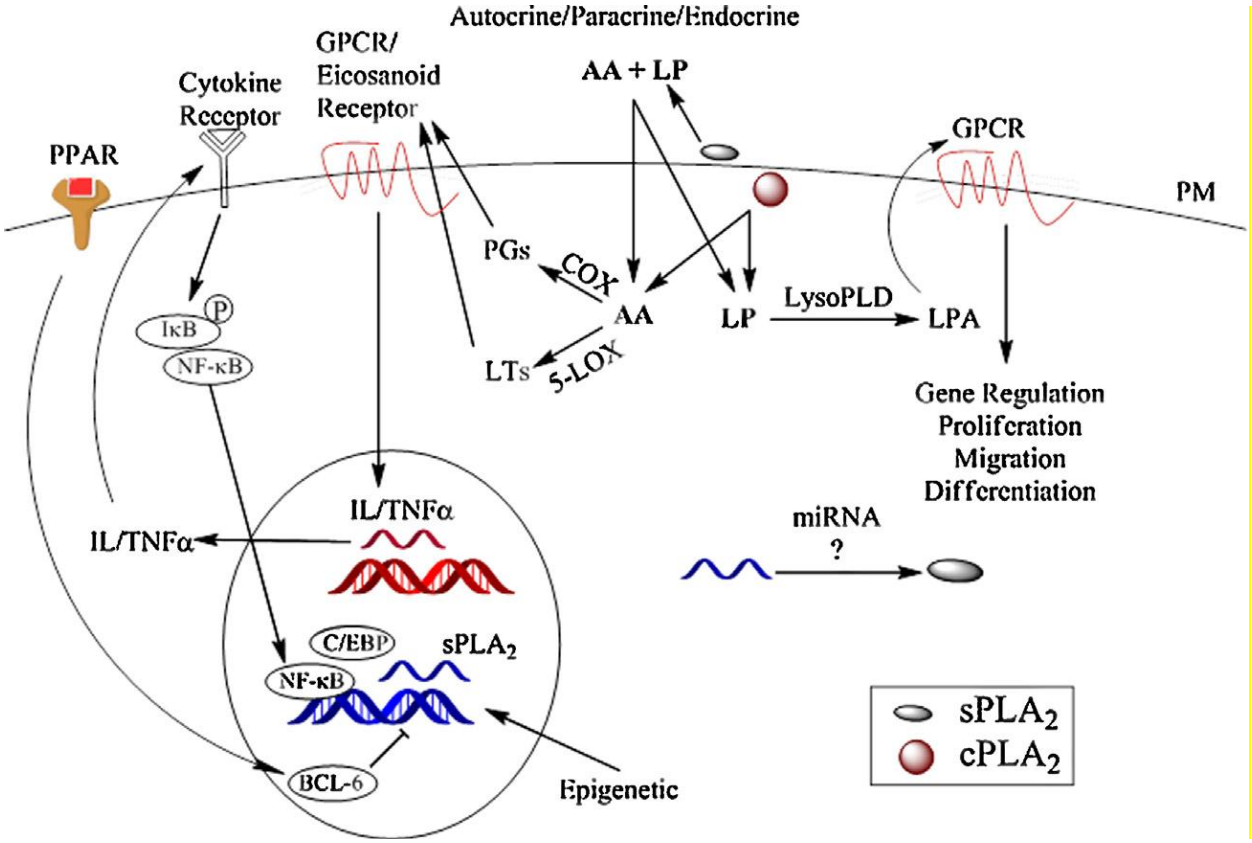

Figure 13: Regulation of *sPLA$_2$* expression by cell signalling and gene regulation. Abbreviations: BCL-6: B-cell lymphoma 6; C/EBP: Ccaat-enhancer-binding proteins; COX: cyclooxygenase; cPLA$_2$: cytosolic phospholipase 2; GPCR: G-coupled protein receptor; IL: interleukin; LOX: lipoxygenase; LP: lysophospholipid; LysoPLD: lysophospholipase D; LTs: leukotrienes; mi-RNA: micro-ribonucleic acid; NF-κB: nuclear factor kappa B; PGs: prostaglandins; PM: plasma membrane; sPLA$_2$: secretory phospholipase 2; TNFα: tumour necrosis factor alpha (Quach *et al.*, 2014)

2.3.3.3 Effects of n-3 PUFA on lipid-derived mediators and inflammation

The identification of resolvins and protectins supports the hypothesis that resolution is an active process, which terminates inflammation and returns the local tissues to homeostasis (Serhan & Petasis, 2011). Very few human interventions have so far reported data on circulating n-3 LCPUFA derived lipid-mediators in the plasma of supplemented subjects (Dawczynski *et al.*, 2013; Mas *et al.*, 2012). Table 4 shows data from Mas *et al.* of plasma and serum samples taken from 50–67-yr-old healthy volunteers after 3 weeks of supplementation with 4 g fish oil/day (35% EPA and 25% DHA) and Table 5 depict data from Dawczynski *et al.* who supplemented mildly hypertriacylglycerolemic subjects with yoghurt enriched with 3 g n-3 LCPUFA/d for a period of 10 wks.

Table 4: Mean concentration (pg/mL) of lipid mediators in human blood following n-3 fatty acid supplementation (Mas *et al.*, 2012).

Lipid mediator (pg/mL)	Serum	EDTA	Heparin	Citrate
RvD1	24.4 (2.5)[a]	31.4 (4.6)	33.0 (4.0)	40.6 (7.3)
RvD2	26.6 (4.7)	26.4 (3.6)	29.9 (3.8)	32.1 (4.9)
17R-RvD1	55.3 (6.0)	60.8 (7.3)	73.8 (7.4)	70.2 (4.5)
10S,17S DiHDHA	<LOQ[b]	<LOQ	<LOQ	<LOQ
PD1	<LOQ	<LOQ	<LOQ	<LOQ
18R/S-HEPE	190.8 (16.6)	385.7 (52.6)	310.0 (22.8)	367.8 (28.0)
17R/S-HDHA	175.3 (32.2)	364.7 (65.0)	319.6 (64.5)	486.2 (227.3)

a Values are given as mean (SE).
b<LOQ = concentration below criteria for the limit of quantification (25pg) in 1 mL of serum or plasma.
Abbreviations: DiHDHA: dihydroxydocosahexaenoic acid; HDHA: hydroxydocosahexaenoic acid; HEPE: hydroxyeicosapentaenoic acid; LOQ, limit of quantitation; PD: protectin D; RvD: resolvin D

The production of AA derived eicosanoids in human immune cells (*ex vivo*) has been shown to decrease with fish oil supplementation (Calder, 2008) and is one of the contributing factors for the anti-inflammatory effects seen with DHA and EPA supplementation. The decrease in AA content of cell membrane phospholipids that happens with incorporation of EPA and DHA decreases the availability of the normal eicosanoid substrate. Thus, incorporation of more n-3 fatty acids into cell membranes is associated with decreased production of the major 2-series PG and 4-series LT, as has been demonstrated by Dawczynski *et al* in Table 4 for PGE_3, PGD_2, LTB_4 (lower, but not significantly) and some HETE (Dawczynski *et al.*, 2013). This is a key anti-inflammatory effect of n-3 fatty acids, and has been long acknowledged (Calder, 2013b).

Table 5: Plasma lipid mediators observed before and after supplementation with 3 g n-3 LCPUFA enriched yoghurt. Adapted from (Dawczynski *et al.*, 2013).

Eicosanoids (pg/µl)	Group receiving 3 g n-3 LCPUFA/d		
	0 weeks	10 weeks	P-value
5-HEPE	6.16 ± 6.80	3.19 ± 4.02	0.063
8-HEPE	0.20 ± 0.21	0.18 ± 0.24	0.646
12-HEPE	0.76 ± 0.82	1.01 ± 0.83	0.312
18-HEPE	0.58 ± 0.41	0.70 ± 0.73	0.406
5-HETE	22.68 ± 19.96	11.60 ± 27.82	0.030
8-HETE	0.97 ± 0.77	0.57 ± 1.16	0.030
9-HETE	0.82 ± 0.90	0.53 ± 1.18	0.041
11-HETE	0.89 ± 0.78	0.52 ± 1.29	0.030
12-HETE	2.92 ± 2.70	2.24 ± 1.62	0.305
15-HETE	1.26 ± 0.97	0.87 ± 1.68	0.427
PGE_3	0.01 ± 0.00	0.08 ± 0.19	0.008
PGE_2	0.07 ± 0.05	0.06 ± 0.07	0.148
PGD_2	0.52 ± 0.52	0.29 ± 0.79	0.041
LTB_4	0.18 ± 0.39	0.07 ± 0.05	0.176

Abbreviations: HEPE: hydroxyeicosapentaenoic acid; HETE: hydroxyeicosatetraenoic acid; LT: leukotriene; PG: prostaglandin

For example, the effect of AA-derived PGD_2 and EPA-derived PGD_3 on neutrophil adhesive interactions with endothelial cells was examined recently in an *in vitro* setting (Tull *et al.*, 2009). EPA as well as PGD_3 prevented neutrophil transmigration through the endothelial cell monolayers, an effect which could be inhibited by either AA or PGD_2. An antagonist to the PGD receptor DP1 also inhibited transmigration, while a DP1 agonist overcame the inhibitory effect of EPA. The authors concluded that PGD_2 acts to up-regulate neutrophil transmigration, similar to neutrophilic penetration into inflammatory sites, acting through DP1 while PGD_3 acts to prevent this effect of PGD_2 again acting at DP1. The observation that PGD_3 can successfully compete with PGD_2 is supported by the findings of another group (Wada *et al.*, 2007) that DP1 has a greater affinity for PGD_3 than for PGD_2 (Tull *et al.*, 2009).

It seems, however, that the major anti-inflammatory effects of DHA and EPA may be mediated by the formation of resolvins, protectins and maresins which actively regulate and activate the resolution process of inflammation (Ariel & Serhan, 2007; Calder, 2013b; Serhan & Petasis, 2011). Circulating n-3 LCPUFA–derived mediators have been measured in humans after supplementation with DHA and EPA (Table 5, (Mas *et al.*, 2012) and precursors (HEPE) of these have been increased in circulatory human plasma (Table 4) (Dawczynski *et al.*, 2013). These mediators have been demonstrated in the last 10 years or so to be anti-inflammatory and inflammation resolving in cell cultures and animal models (Calder, 2008). As seen in Figure 14, resolvins originating from EPA are called E-series while those produced from DHA are named D-series. Related compounds termed protectins and maresins also originate from DHA (Calder, 2013b; Serhan *et al.*, 2009). Even more recently an n-3 DPA-derived modulator with its own specific actions has also been identified (Dalli *et al.*, 2013). Specific anti-inflammatory mechanisms of these mediators include reducing of trans endothelial migration of neutrophils, preventing the infiltration of neutrophils into sites on inflammation, cytokine and reactive oxygen species regulation and lowering the magnitude of the inflammatory response. Recent results also indicate a novel function of these mediators in removing chemokines transported from tissue by apoptotic neutrophils and T cells during the resolution of inflammation (Ariel & Serhan, 2007). Furthermore, significant concentrations of resolvins E1 and D1 were recently found in the plasma of healthy human volunteers after supplementation with fish oil for 3 weeks (Mas *et al.*, 2012). Aspirin triggers specific epimers of the resolvin and protectin families from DHA and EPA. As seen in several murine models, these epimers share the typical structural features and actions with the LOX and COX-generated resolvins and protectins (Serhan *et al.*, 2002).

During the course of the inflammatory response, the synthesis of PUFA derived lipid mediators increases with time (Figure 15). The early mediators (PG and LT) are synthesized within seconds and minutes and control the oedema and post-capillary events at the site of infection subsequent to the recruitment of neutrophils (Serhan & Petasis, 2011). After some time (hours up to days), leukocyte recruitment and arrival of monocytes and macrophages lead to a growing amount of mediators (Figure 15). Thus during initial infection, mostly pro-inflammatory mediators (LT and PG) are produced, while at a later time, a lipid mediator class switching leads to the production of pro-resolving mediators (LX, resolvins, and protectins) that promote resolution. The release of AA and mobilization of EPA and DHA from circulation (Kasuga *et al.*, 2008) and the synthesis of the pro-resolving mediators from these substrates, also follows a time course that favours an initial inflammatory response and host defence, followed by resolution and return to homeostasis (Serhan & Petasis, 2011). It seems that $cPLA_2$ plays a major role in eicosanoid class switching (Norris *et al.*, 2014). The acute inflammatory process stays unresolved and develops into chronic inflammation when the pro-resolving mediators are not formed or are not functioning correctly. This pro-inflammatory situation causes cell damage

and is associated with the pathogenesis of many diseases (Libby *et al.*, 2014; Serhan & Petasis, 2011).

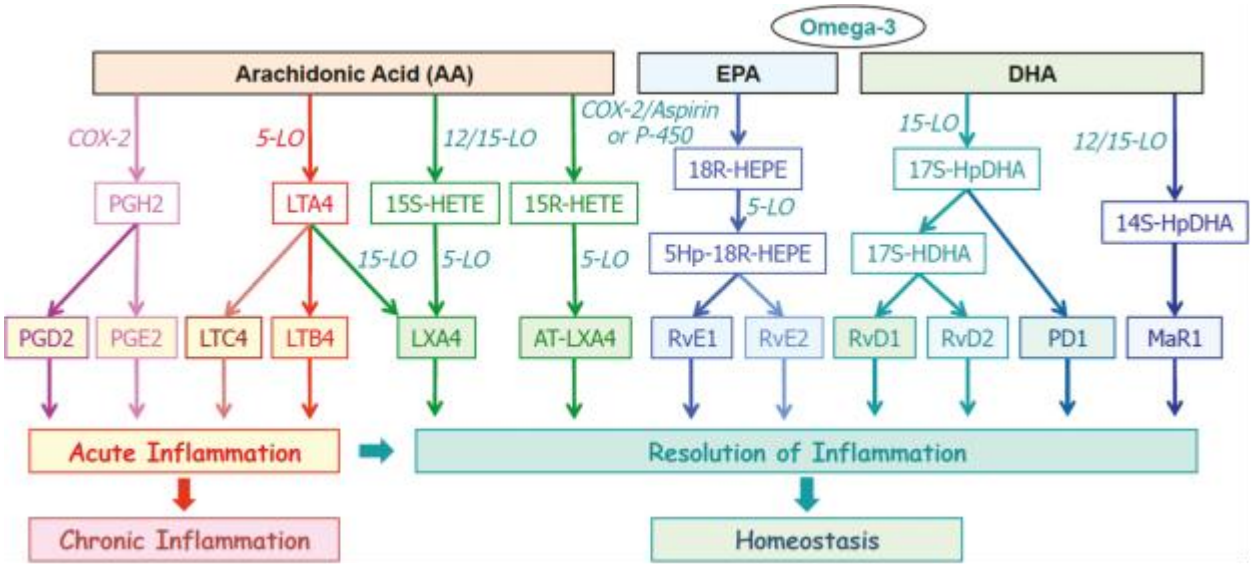

Figure 14: Biosynthetic cascades and actions of selected lipid mediators derived from arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Abbreviations: COX: cyclooxygenase; HpDHA: hydroperoxydocosahexaenoic acid; HEPE: hydroxyeicosapentaenoic acid; HpEPE: hydroperoxyeicosapentaenoic acid; LOX: lipoxygenase; LT: leukotriene; MaR: maresin; PG: prostaglandin; Rv: resolvin; TX: thromboxane (Serhan & Petasis, 2011).

The biological potency and probable pathogenicity of dysregulation of lipid mediators have been demonstrated by the abuse of these molecules by some pathogens (Fullerton *et al.*, 2014). Certain pathogens, like *Mycobacterium tuberculosis* (TB) and *Pseudomonas aeruginosa* have been demonstrated to exploit lipid mediators, for example by either their own synthesis and secretion or the stimulation of excessive host anti-inflammatory mediators (such as LX), thereby blocking dendritic cell activation and preventing their phagocytosis. Findings like these emphasize that we still have much to learn about the role lipid mediators (pro-and anti-inflammatory) plays in the immune response and host defence (Aliberti & Bafica, 2005; Fullerton *et al.*, 2014; Vance *et al.*, 2004).

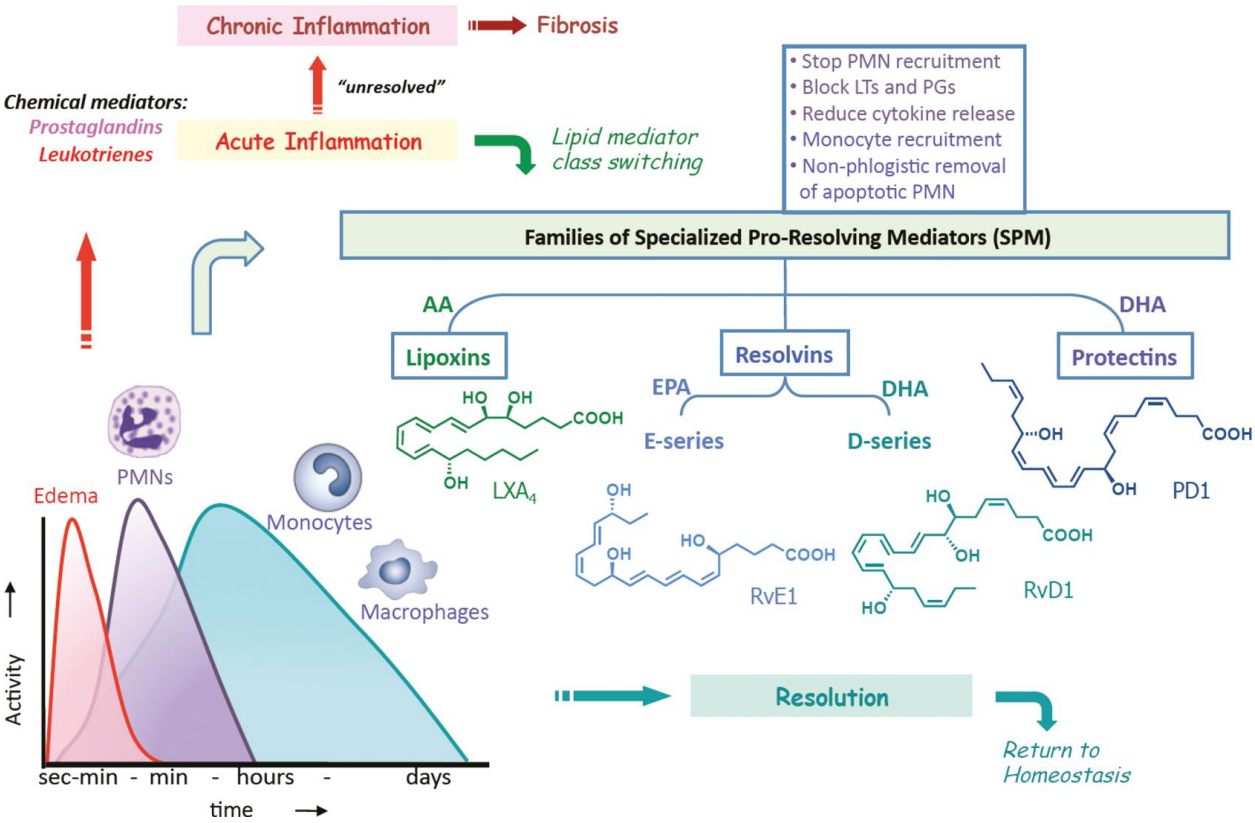

Figure 15: Inflammatory response and resolution time course: Roles of pro-resolving lipid mediators. Abbreviations: LT: leukotriene; LX: lipoxin; PD: protectin; PMN: neutrophils; Rv: resolvin. (Serhan & Petasis, 2011).

2.3.4 Omega-3 polyunsaturated fatty acids and oxidative stress

Metabolic regulation and cell signalling go along with, and are dependent on, the production of minute concentrations of superoxide and nitrogen monoxide (Martins de Lima *et al.*, 2007). Furthermore, the innate immune response makes use of oxidative reactions to combat pathogens (Janeway *et al.*, 2005). For example, NADPH oxidase, a multicomponent superoxide generating enzyme complex, is well known for its role in oxidative killing of micro-organisms by neutrophils. After activation, granules containing NADPH oxidase components fuse with phagocytic vacuoles and generate superoxide. Also, granules may migrate to the cell surface and release superoxide into the extracellular space (Babior, 1999). The levels of these highly reactive species that may cause oxidative stress and cell damage are kept within a narrow range. This is achieved by keeping the rate of production and removal in balance, by means of enzymatic antioxidants such as SOD, GPX and glutathione catalase, or non-enzymatic antioxidants, such as ascorbic acid, α-tocopherol, glutathione, urate, carotenoids, cysteine, bilirubin and flavonoids (Koskenkorva-Frank *et al.*, 2013).

N-3 PUFA are theoretically more susceptible to oxidation because oxygen easily attacks double bonds, producing lipoperoxides (Giordano & Visioli, 2014). In this respect, there is general

concern that high intakes of n-3 LCPUFA, such as taken in with supplementation, might increase circulating concentrations of lipoperoxides. There is some evidence that there might be a threshold below which PUFA supplementation acts as an anti-oxidant and above which pro-oxidative effects are seen (Driss *et al.*, 1984; Guillot *et al.*, 2009; Lagarde *et al.*, 2013).

However, numerous studies have demonstrated anti-oxidative effects for n-3 PUFA (Ali *et al.*, 2014; Barden *et al.*, 2004; Brand *et al.*, 2008; Kones, 2010; Pacheco *et al.*, 2014). Several mechanisms have been proposed, such as DHA acting as a quencher or ”sink” for reactive oxygen species (ROS) (Massaro *et al.*, 2002), direct inhibition of NADPH oxidase 4 (Giordano & Visioli, 2014) and up regulation of antioxidant cellular machinery like superoxide dismutase (SOD) and glutathione peroxidase (GPX) (Brand *et al.*, 2008). A contributing factor is probably n-3 PUFA ability to resolve inflammation, which is usually associated with oxidative stress (Reuter *et al.*, 2010). An extreme example is in the case of traumatic insult, which is associated with hyper-inflammation (including oxidative stress), subsequently followed by an immunosuppressed state which increases susceptibility to infection (Calder, 2007). A range of nutrients, including n-3 PUFA are able to curb inflammation (and the associated oxidative stress) and to maintain or improve immune function (Calder, 2007).

Reliable methods to measure oxidative stress *in vivo* are very limited (Giordano & Visioli, 2014). Free radicals are extremely reactive molecules that are impossible to quantify *in vivo* and consequently, we can only evaluate the damage they cause to macromolecules, namely lipids, proteins, DNA and sugars (Davies & Roberts, 2011; Halliwell, 2009). One such accepted biomarker of lipid peroxidation is the formation of isoprostanes, namely F2-isoprostanes such as 8-iso-PGF2α (Davies & Roberts, 2011) and these have been lowered in neonates with high atopic risk by fish oil supplementation during pregnancy (Barden *et al.*, 2004).

2.4 Allergic disease

2.4.1 Allergic disease prevalence

Allergic disease, including rhinitis, asthma and eczema/dermatitis, has increased worldwide over the last few decades, both in industrialized and developing countries (Enke *et al.*, 2008; Potter, 2008; Prescott, 2013). It is estimated that around 500 million people worldwide and over 30 million in Africa have allergic rhinitis and that about 200 million also have asthma as a co-morbidity (Bousquet *et al.*, 2008). The third phase of the International Study on Asthma and Allergy in Childhood (ISAAC III) found a prevalence for allergic rhinitis, asthma and eczema of 18–20% in 13–14 year olds in South Africa, (Ait-Khaled *et al.*, 2007). Furthermore, in South Africa, there has been an increase in bronchial hyper responsiveness (BHR), a marker for asthma, in urban, peri-urban and rural children between 1979 and 2003 (Steinman *et al.*, 2003).

In this study, a high prevalence of sensitization, especially to mites, and increased BHR were reported in a rural community where there has been no significant change in the last 20 years in basic hygiene. These findings do not support the “hygiene hypothesis”, which implies that early exposure to endotoxins exerts a sustained protection against the development of allergic disease or increased BHR (Steinman *et al.*, 2003).

2.4.2 Polyunsaturated fatty acids and allergy

Among the leading diet-related hypotheses relating to allergic disease, is that the altered intake of n-6 and n-3 PUFA in the modern Western diet might be involved (Tricon *et al*., 2006). In particular a higher intake of n-6 PUFA (Blasbalg *et al.*, 2011) and lower relative intake of n-3 PUFA is thought to be important (Kremmyda *et al.*, 2011; Sala-Vila *et al.*, 2008). As evidence to this end, the n-6:n-3 PUFA ratio was positively associated with the risk for asthmatic symptoms in 6–8 y-old asthmatic children (Oddy *et al.*, 2004). Furthermore, n-6 fatty acid-derived inflammatory mediators in subjects with asthma were markedly increased, and opposing n-3 fatty acid-derived anti-inflammatory mediators decreased, in sputum and breath condensate samples (Gonzalez-Reche *et al.*, 2006; Levy *et al.*, 2007; Pavord *et al.*, 1999). For example, Levy *et al.* found lower levels of protectin D1, a potent DHA derived anti-inflammatory mediator, in exhaled breath condensates of patients with exacerbated asthma compared to healthy subjects. (Levy *et al.*, 2007).

In accordance with such observations, there is some mechanistic evidence to suggest how the n-6:n-3 PUFA ratio could be causally associated with the development of allergic disease (Levy *et al.*, 2007; Pavord *et al.*, 1999; Phipps *et al.*, 2014). As such, DHA derived 17-hydroxydocosahexaenoic acid and resolvin D1 strongly reduced the production of IgE in human B cells (Phipps *et al.*, 2014) and are associated with reduction of the transcription factor, NF-κB (Weise *et al.*, 2011). Furthermore, when protectin D1 was administered to a murine asthmatic model before aeroallergen challenge, airway eosinophil and T lymphocyte recruitment, airway mucus, levels of specific pro-inflammatory mediators (IL-13, cysteinyl LT and PGD_2) and airway hyper responsiveness to inhaled methacholine were decreased (Levy *et al.*, 2007). Moreover, PD1 treatment after aeroallergen challenge markedly accelerated the resolution of airway inflammation in this model (Levy *et al.*, 2007).

Eicosanoids seems to play a significant role in allergic disease and asthma. PGD_2, LTC_4, LTD_4 and LTE_4 are produced in cells that mediate pulmonary inflammation in asthma, such as mast cells, and are believed to be the major mediators of asthmatic bronchoconstriction (Brightling *et al.*, 2000). Of particular interest for allergic disorders, is the ability of PGE_2 to inhibit the production of Th1-type cytokines IL-2 and IFN-γ without affecting the production of the Th2-type cytokines IL-4 and IL-5, and to stimulate B-cells to produce IgE. These observations suggest

that PGE_2 promotes the development of allergic disease and that n-3 PUFA supplementation could be of value for allergy and asthma sufferers (Pavord *et al.*, 1999).

Although other pro-inflammatory conditions, especially rheumatoid arthritis, have been reported to benefit from n-3 PUFA supplementation (Calder 2001), studies of fish oil supplementation in older human children and adults with a predisposition to allergic disease found only limited benefits (Sala-Vila *et al.*, 2008; Thien *et al.*, 2011). It remains unclear if the anti-inflammatory properties of n-3 fatty acids benefit allergic individuals after maturation of the immune system has occurred (Anandan *et al.*, 2009). Currently it seems that earlier intervention, such as in gestation and early infancy, is more efficacious. PUFA and their derivatives influence both early immune development as well as maturation of the immune system (Covar *et al.*, 2010; Enke *et al.*, 2008). Infants are born with an immature immune system and supplementation of n-3 LCPUFA has been shown to speed up the maturation process and has also been hypothesized to protect against allergic disease (Dunstan *et al.*, 2003).

A Th2 polarized immune response is believed to be important against helminthic infections (Calder, 2002). If, however, Th2 type immune response is inappropriately directed towards harmless antigens, such as allergens, tissue damage and inflammation may develop. Th2 response produce a group of biological mediators which is characteristic of allergy and helminthic infections, namely high levels of circulating IgE, and elevated mast cells and tissue eosinophils. Inappropriate Th2 biased immune response, therefore, seem to underlie allergic disease (Abbas *et al.*, 1996; Romagnani, 1991).

A possible reason for the dampening effect n-3 PUFA has on development of allergic disease during gestation and infancy, could be the effect of polarization of the Th lymphocytes towards a Th1 polarization, therefore, improving the capacity to produce cytokines such as IFN-γ and IL-2 (Damsgaard *et al.*, 2007). Th1 cells are essential for an antigen-specific phagocytic mediated immune response against microorganisms, primarily bacteria, fungi and some parasites (Calder, 2002). Early studies indicate that DHA modulates T cell function to favour a Th2 phenotype *ex vivo* in a murine model (Arrington *et al.*, 2001). One of the mechanisms proposed that reduced oxidative stress could have been involved. Evidence exists that an oxidative environment stimulates antigen presenting cells to produce Th2 type cytokines, whereas n-3 PUFA supplementation to mothers with neonates at risk to develop allergic disease, reduced oxidative stress in neonates and altered antigen presentation to a Th1 type system (Dunstan *et al.*, 2003).

Despite all the evidence that increased dietary n-6 PUFA may be causally involved in allergic disease, some studies report that lowered AA may be associated with allergic disease (Calder, 2009; Sala-Vila *et al.*, 2008). Likewise, intervention with a combined DHA- and AA-supplemented formula has shown delayed onset and reduced incidence of allergic disease in

children up to three years of age (Birch *et al.*, 2010). Birch *et al*. reviewed medical charts of 3 yr olds that were supplemented with DHA (0.32%–0.36% of total fatty acids) and AA (0.64%–0.72% of total fatty acids) during their first year of life and found that the supplemented children had significantly lower odds for developing upper respiratory tract infections, wheezing/asthma, wheezing/asthma/atopic dermatitis or any allergy (OR, 0.28) when compared to controls (Birch *et al.*, 2010). The involvement of both n-3 and n-6 LCPUFA might suggest that the synthesis pathway of these, of which both n-3 and n-6 PUFA utilises the same enzymes, might be causal.

2.4.3 Genetic variability of fatty acid desaturases and phospholipases and allergic disease

Susceptibility to allergic disease and asthma are thought to be genetically inherited (Postma *et al.*, 1995). Furthermore, the genetic component of allergic disease has been extended to include genetic variation in the fatty acid desaturation pathways and the release of fatty acids from phospholipid membranes (Glaser *et al.*, 2011; Sokolowska *et al.*, 2007). There are indications that single nucleotide polymorphisms (SNPs) in the genes encoding for delta-5 and delta-6 desaturases (D5D and D6D), fatty acid desaturase (FADS) 1 and 2, respectively, modulate risk for allergic disease (Glaser *et al.*, 2011; Lattka *et al.*, 2009). Impaired D5D and D6D activity causes increased precursor fatty acids and reduced product fatty acids of the specific desaturase involved (Figure 4).

For example, a reduced ratio of DGLA to AA was found by Lindskou *et al,* in mononuclear cells of patients with atopic dermatitis (Lindskou & Hølmer, 1992). Rzehak *et al* found an association of D5D polymorphisms with eczema, but could not find any differences in fatty acid composition between eczema and control groups, perhaps because they only analyzed plasma and PBMCs are likely to be a more sensitive matrix for immune-related functions (Rzehak *et al.*, 2010). However, Bokor *et al* have also seen this effect in serum (Bokor *et al.*, 2010). Early findings indicated that inherited inadequate delta-6 desaturation might be associated with eczema (Horrobin, 1993). Later it was noted that certain SNPs in FADS2 specifically affected D6D and were associated with eczema, confirming the observations made earlier (Ferreri *et al.*, 2005).

The altered release of fatty acids from phospholipids has also been implicated in allergic disease. $CPLA_2$ promoter polymorphisms associated with induced transcription of $cPLA_2$ (which release primarily AA from phospholipid membranes) were found in 18-75 y-old patients with severe asthma (Sokolowska *et al.*, 2007). Furthermore, DHA has been shown to decrease levels and activity of AA-specific $cPLA_2$ *in vivo* (Lattka *et al.*, 2009).

2.4.4 Methods to assess allergic disease

Serum total IgE can give an indication of allergic disease (Høst *et al.*, 2003). However, as tIgE could be elevated due to either allergic disease or helminthic infections (Lynch *et al.*, 1998), more specific testing (e.g. specific IgE-sensitisation against particular antigens) should ideally be performed to identify allergic disease (Høst *et al.*, 2003). Clinical allergic symptom assessment has been used very successfully to determine allergic disease prevalence worldwide by the International Study of Asthma and Allergies in Childhood (ISAAC) (Asher *et al.*, 1995; Asher *et al.*, 2006).

The ISAAC questionnaires were aimed at age groups, 6–7 y and 13–14 y (Asher *et al.*, 1995). The questionnaire for 6–7 y-olds is addressed to the children's parents or caretakers, whereas the one for the older children is addressed to the children themselves. The questions are formulated as such to determine if the child has asthma (wheezing), rhinitis, and/or eczema and they are asked about symptoms over the previous 12 mo. A child is considered to have allergic disease if question 2 from the wheezing module and/or questions 2 and 3 from the rhinitis module and/or questions 2 and 3 from the eczema module are answered positively (Asher *et al.*, 2006). The questionnaire modules used for our study are available in English and IsiZulu in Annexure 3. When translated to another language, the ISAAC questionnaires must be validated (Solé *et al.*, 1997). Acceptable validation methodologies include consultation with the local community, speaking the applicable language, and back translation into English by an independent translator (Zhao *et al.*, 2004).

2.5 Iron metabolism

2.5.1 Iron absorption and homeostasis

Iron is an essential nutrient and is mainly utilized in the body as an essential part of haemoglobin to carry oxygen, thus most of the body's iron is contained in the RBC or erythrocytes (Perutz, 1976). It also acts as a co-factor for numerous proteins and enzymes, such as other haem proteins, iron-sulphur cluster proteins and ribonucleotide reductases (Mathews *et al.*, 2000). These perform vital roles in iron regulation, energy production, DNA replication and repair and cell cycle progression (Mathews *et al.*, 2000). Furthermore, iron acts as a co-factor for enzymes involved in fatty acid and eicosanoid metabolism, such as fatty acid desaturases (Guillou *et al.*, 2010; Nakamura & Nara, 2004) as well as COX (Helmer *et al.*, 1976; Hemler & Lands, 1976) and LOX (Gilbert *et al.*, 2011b).

Iron homeostasis is tightly controlled to ensure coordinated iron absorption, utilization, recycling and storage (Figure 16) and is primarily maintained to ensure adequate plasma iron levels

(Hentze *et al.*, 2010). Absorption of iron is depicted in Figure 16. Enterocytes have villi (and microvilli) that protrude into the intestinal lumen to maximise the absorptive surface area (Andrews, 2008). Dietary non-haem iron is generally in the ferric form (Fe^{3+}) and needs to be reduced to the ferrous form (Fe^{2+}) before absorption (McKie *et al.*, 2001). This happens through dietary reducing agents and the membrane ferric reductase duodenal cytochrome b (McKie *et al.*, 2001). Iron is transported into the enterocyte through DMT1 (SLC11A1) and is then stored inside the enterocyte bound to ferritin or transported to the blood stream through ferroportin, where it is oxidized and incorporated into transferrin (Hentze *et al.*, 2010). Transferrin bound iron can be taken up by hepatocytes and stored as ferritin until needed. In adults non-haem iron absorption is about 1.0–4.3% and haem iron about 15–35% (Monsen *et al.*, 1978; Zimmermann *et al.*, 2005).

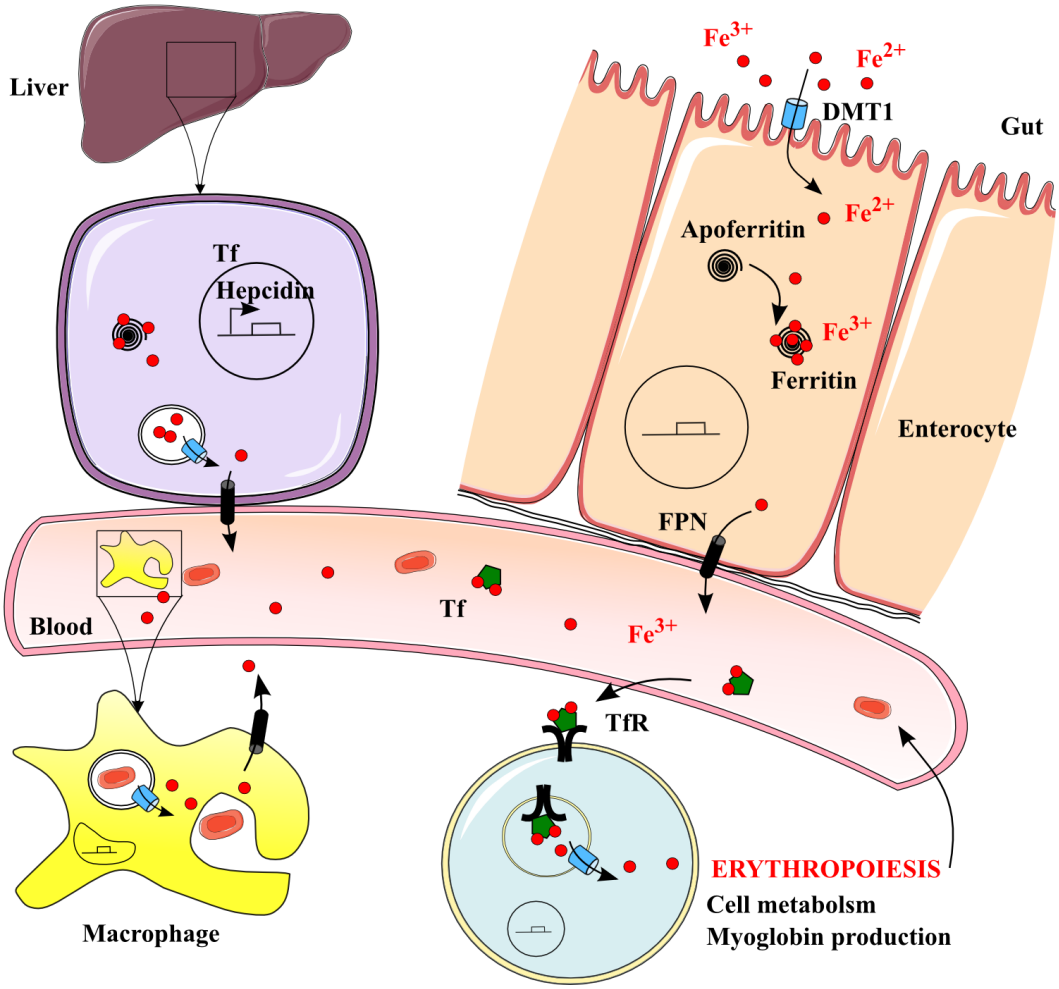

Figure 16: Iron absorption, utilization, storage and recycling. Abbreviations: DMT: divalent metal transporter; Tf: transferrin; TfR: transferrin receptor (Curis, 2013).

Body iron needs to be bound to a carrier for three reasons: first, it is an extremely reactive electron donor and causes oxidative stress by the generation of free radicals (Touati, 2000); second, it may precipitate at neutral pH of body fluids and third, it becomes accessible for

pathogens if it is in an unbound form (Drakesmith & Prentice, 2012). Circulating iron is bound to the glycoprotein transferrin (also to lactoferrin in neutrophils and human secretions), which keeps iron soluble and delivers it to cells via transferrin receptors. Transferrin receptors are highly expressed on the cell surfaces of haemoglobin-synthesizing erythroblasts. When transferrin saturation surpasses 60%, non-transferrin-bound iron (NTBI) accumulates, becomes available for pathogens and causing the generation of free radicals with concurrent cell damage (Hentze *et al.*, 2010).

Iron metabolism is balanced by two controlling systems – systemic iron is regulated by the hormone hepcidin and the iron exporter ferroportin, while cellular iron is controlled by iron-regulatory proteins that bind iron-response elements (IRE) post-transcriptionally in messenger RNAs (Hentze *et al.*, 2010). Hepcidin is secreted by hepatocytes, which also store the bulk of iron in the form of ferritin. Furthermore, hepcidin regulates systemic iron by binding to and activating the degradation of ferroportin on the cell surfaces of iron–releasing cells, thus decreasing iron transfer to transferrin (Hentze *et al.*, 2010). Furthermore, hepcidin is able to integrate signals from iron status and the warning signals of infection, causing an overall decrease in iron levels upon pathogenic invasion (Diaz-Ochoa *et al.*, 2014).

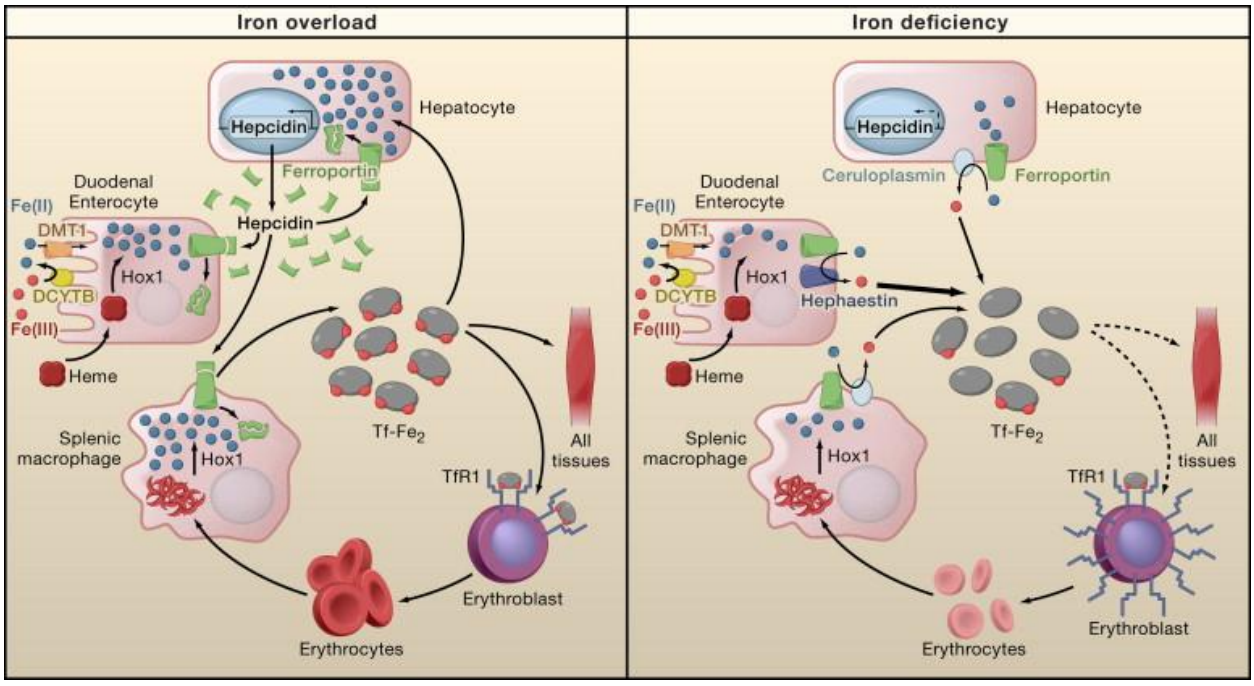

Figure 17: Regulation of iron homeostasis in iron deficiency and overload. Red dots = iron-loaded transferrin (Tf-Fe_2), blue dots = ferritin. Abbreviations: DCYTB: duodenal cytochrome b; DMT1: divalent metal transporter; Hox1: hemoxygenase-1; TfR-1: transferrin receptor 1 (Hentze *et al.*, 2010).

The cellular system decreases cellular ferritin and increases transferrin receptor expression in ID and exerts the opposite effect in iron overload (Kuhn, 1998). Regulation of systemic iron homeostasis during ID and iron overload is shown in Figure 17.

In iron overload, high hepcidin expression and levels degrade ferroportin and keep iron sequestered within cells (mainly hepatocytes, macrophages and duodenal enterocytes), thus reducing transferrin saturation (Figure 17). In contrast, iron is released by ferroportin into the circulation in ID, when hepcidin expression is low. Haemoglobin-derived haem is degraded by hemoxygenase-1 to recycle iron by macrophages, and released through ferroportin.

2.6 Iron and Immune function

2.6.1 Epidemiology of iron deficiency

ID is the most prevalent nutritional deficiency worldwide (WHO, 2008) and causes about 50% of the world's anaemia (WHO, 2001). An increase in anaemia prevalence from 30% to 46% has been reported from 1995 to 2011 in southern Africa (Stevens *et al.*, 2013) and estimated from anaemia prevalence, most preschool children in developing countries are considered to be ID (WHO, 2001). ID and IDA are associated with deficits in areas such as cognition, motor skills and neurophysiology in later life (Georgieff, 2011; Lozoff, 2007). ID also plays an essential role in impairing immune function (Beard, 2001) and leads to a higher susceptibility to infections (Tansarli *et al.*, 2013). Even though infants and pre-school children are the most vulnerable to develop ID due fast growth and development, ID is also very prevalent in young school children, including in South Africa (Labadarios, 2007). This is partly caused by monotonous grain-based diets containing poorly absorbable non-haem iron (Cook *et al.*, 1997; Zimmermann & Hurrell, 2007). In South Africa, even though the prevalence of anaemia in children under five has improved from 28.9% to 10.7% from 2005 to 2012, iron depletion (Hb ≥ 11 g/dL and ferritin < 12 ng/mL) has stayed the same (7.8% in 2005 and 8.1% in 2012) and is worse than in 1994 (4.8%) (Hoosain *et al.*, 2013). Furthermore, in some rural areas, ID prevalence was subsequently found to be much higher than estimated by the national survey, for example 20.6% in The Valley of a Thousand Hills in KwaZulu-Natal (Baumgartner *et al.*, 2012c). As one of the strategies to address the poor iron status of children and women in South Africa, iron supplementation was recommended by the executive summary of the National Food Consumption Survey Fortification Baseline (NFCS-FB) of South Africa in 2005 (Labadarios, 2007).

2.6.2 Iron deficiency and supplementation

ID is defined as a decrease in total body iron to such a degree that body iron stores are depleted and that tissue iron deficiency is also present (Cook, 2005). When ID leads to

insufficient haemoglobin levels in the blood, it is termed IDA (Stoltzfus *et al.*, 1998). Co-deficiencies with other micronutrients, infections such as malaria and anaemia of chronic disease may contribute substantially to the burden of ID and anaemia in developing settings. (Pasricha *et al.*, 2013). There is a pressing need to clarify the contribution of these conditions to the overall burden of ID and anaemia. Another big concern, recently brought to the forefront by increased mortality of iron-supplemented children in a malaria-endemic area (Sazawal *et al.*, 2006) is the safety of iron supplementation (Roth *et al.*, 2010) and even the safety of fortification of foods with iron (Soofi *et al.*, 2013; Zlotkin *et al.*, 2013).

Iron supplementation is one of the strategies to improve iron status and is advocated in ID and IDA children (Stoltzfus & Dreyfuss, 2006), together with nutritional education and food fortification (Berger *et al.*, 2011; Zimmermann & Hurrell, 2007). The advantage of supplementation is that it can be implemented quickly and is likely to increase iron stores rapidly (Pasricha *et al.*, 2013), whereas fortification sometimes fails to improve iron status (Rohner *et al.*, 2010) and nutritional education takes time and good iron-providing food could be too expensive or not accessible (Zimmermann & Hurrell, 2007). The WHO recommends 30-60 mg elemental iron per day (e.g. 300 mg ferrous sulphate ($7H_2O$) is equal to 60 mg elemental iron), together with anthelminthic and anti-malarial treatment to prevent or treat ID anaemia (Stoltzfus & Dreyfuss, 2006). Most studies of oral iron supplementation in deprived populations support the value of iron supplementation in improving long-term health (Field *et al.*, 2002; Oppenheimer, 2012; Oppenheimer, 2001; Vucic *et al.*, 2013), even though some gastrointestinal upset may be experienced (Gera & Sachdev, 2002). However, in malaria-endemic and high infection-burdened areas supplementation with iron remains problematic and controversial due to strong evidence of increased morbidity and mortality (English & Snow, 2006; Ojukwu *et al.*, 2010; Roth *et al.*, 2010; Sazawal *et al.*, 2006).

Untargeted daily supplementation of iron and folic acid in preschool children (1-36 months) increased morbidity and mortality in malaria-endemic Pemba, Zanzibar (Sazawal *et al.*, 2006). Although malaria was identified as the major risk factor, the co-supplemented folic acid was also a confounding factor. Folic acid can increase the risk for plasmodial infections and cause treatment failure of sulphadoxine-pyrimethamine, which was used to treat malaria in this setting (Gregson & Plowe, 2005). Even though a Cochrane review, evaluating 68 trials, concluded that iron supplementation is safe in malaria areas where surveillance and treatment are adequate (Ojukwu *et al.*, 2010), this might not be the case in many developing countries. Therefore, supplementation with iron should be treated with caution (Hurrell, 2011; Roth *et al.*, 2010) and untargeted supplementation is no longer advised by the WHO (de Benoist & Fontaine, 2007). Safety of iron supplementation in HIV and TB has also not been proven (Adetifa & Okomo, 2009; Oppenheimer, 2001). In addition, even certain iron fortification studies have reported

increased severe and bloody diarrhoea and increased hospitalization (Soofi *et al.*, 2013; Zlotkin *et al.*, 2013).

Some studies have reported immediate immune function benefits with iron supplementation, such as reduced morbidity from upper respiratory tract infections (de Silva *et al.*, 2003) and probable protection against diarrhoea, dysentery (Ekiz *et al.*, 2005) and acute respiratory illness (Sangeetha & Premakumari, 2010; Tielsch *et al.*, 2006). Other studies, as reviewed by Ojukwu, reported that iron supplementation does not increase the risk for respiratory infections, but more episodes of diarrhoea were seen when iron and zinc were administered together (Ojukwu *et al.*, 2010). In contrast, less episodes of diarrhoea and lower respiratory infections have also been reported with simultaneous supplementation of iron and zinc (Baqui *et al.*, 2003).

2.6.3 Mechanisms of the effect of iron in immune function

Iron plays an important role in immune function and the mechanism thereof has been investigated extensively (Beard, 2001; Ekiz *et al.*, 2005; Field *et al.*, 2002; Oppenheimer, 2001).

2.6.4 Cellular iron mechanisms in immune function

It is known that ID impairs lymphocyte activation and proliferation, reduces bactericidal killing by neutrophils and macrophages and affects certain B cell functions (Beard, 2001; Brock & Mulero, 2007). Humoral immunity may be less affected by ID, because antibody production in response to immunization with most antigens is maintained in humans with poor iron status (Field *et al.*, 2002). However there are some studies that suggest otherwise (Ekiz *et al.*, 2005). In most studies, a deficiency in iron results in impaired cell-mediated immunity and may also delay development thereof. Neutrophil function and NK cell activity are impaired, but macrophage phagocytosis seems not to be affected by ID. Neutrophil killing of bacteria is specifically compromised due to the reduced activity of myeloperoxidase, which is dependent on iron for its function. Myeloperoxidase produces hypochlorous acid from hydrogen peroxide and a chloride anion (or the equivalent from a non-chloride halide) during the neutrophil's respiratory burst. It requires haem, and thus iron, as a cofactor. It oxidizes tyrosine to tyrosyl radical using hydrogen peroxide as oxidizing agent. Hypochlorous acid and tyrosyl radical are cytotoxic, so they are used by the neutrophil to kill bacteria and other pathogens (Hampton *et al.*, 1998).

Iron absorption capacity likely accounts for the gastric upset and possible gastric morbidity experienced during supplementation. Residual iron that is not absorbed in the proximal duodenum, was demonstrated to be detrimental to the gut microbiome by promoting the growth of pathogenic enterobacteria and decreasing the growth of beneficial lactobacilli and bifidobacteria (Coray *et al.*, 2012; Jaeggi *et al.*, 2014; Kortman *et al.*, 2012; Zimmermann *et al.*,

2010). Another likely reason for stomach upset with iron supplementation could be increased gut inflammation due to the high propensity of ferrous iron to produce free radicals and, therefore, generating oxidative stress and subsequent inflammation (Gutteridge *et al.*, 1990).

Iron status with respect to iron regulation during inflammation is also of essential importance in immune function. ID promotes recycling of iron by macrophages (through phagocytosis of senescent RBC) and uptake by enterocytes in order to increase circulating iron (Cherayil, 2010). Iron is released into the circulation via the transporter ferroportin on the surfaces of these cells (Hentze *et al.*, 2010). During inflammation, hepcidin (produced mostly in the liver) is increased through IL-6 and CRP and in turn brings about degradation of ferroportin and decreases the efflux of iron from macrophages and duodenal epithelial cells (Drakesmith & Prentice, 2012). Iron is, therefore, sequestered by these cells and withheld from the circulation (Cherayil, 2010). This mechanism, also called the hypoferremia of inflammation, is thought to benefit the host in the early, acute stages of infection by limiting iron supply to microorganisms and therefore diminishing their growth capacity (Drakesmith & Prentice, 2012). In the case of iron deficiency, however, the excess sequestration of iron away from other immune cells could diminish host resistance (Fleming, 2008). If continued long term, hypoferremia of inflammation could escalate into anaemia of inflammation or chronic disease (Wessling-Resnick, 2010). In addition to hepatic hepcidin, small amounts are also produced by monocytes and macrophages (Weiss, 2009). Monocyte/macrophage hepcidin is primarily regulated by local inflammatory stimuli and exerts local, and not systemic, actions (Zhang & Rovin, 2013). Furthermore, DMT1-mediated uptake of NTBI and its retention via down-regulation of ferroportin have also been described in monocytes (Ludwiczek *et al.*, 2003). This happened in response to pro-inflammatory cytokines, whereas anti-inflammatory cytokines (IL-10) resulted in TfR-mediated iron uptake by activated monocytes (Ludwiczek *et al.*, 2003). Iron uptake could thus be impaired and iron kept out of circulation in circumstances where people bear a high infectious burden (Hurrell, 2011). On the other hand, low iron status has been proposed as a protective mechanism against infection (Gwamaka *et al.*, 2012; Jonker *et al.*, 2012).

Malaria is the most prominent disease worsened by iron supplementation (Hurrell, 2011; van Santen *et al.*, 2013). The mechanism whereby iron supplements increase the severity of malarial infections relates to the formation of NTBI, which happens when a large quantity of supplemental iron is consumed without food (Hurrell 2007). The existence of NTBI has been described recently and was first noticed where the binding capacity of transferrin had been surpassed, as in beta-thalassemia (Hershko *et al.*, 1978). However it was since also observed in subjects without transferrin saturation who were supplemented with iron (Schümann *et al.*, 2012). A plausible reason is that a high oral dose of iron exceeds the body's capacity to bind to free iron and, therefore, increases iron available for pathogens. Malaria parasites seem to be

unable to utilize haem iron, and are dependent on labile iron, such as NTBI (Scholl *et al.*, 2005). Furthermore, malarial disease, among others, is associated with enhanced RBC phagocytosis by macrophages, enhancing their iron content and thereby also the survival of intracellular species, for example Salmonella (van Santen *et al.*, 2013). It has also recently been shown that pathogens, including Salmonella, increased considerably in the stool of children treated with iron-fortified biscuits (Zimmermann *et al.*, 2010). Furthermore, research has shown increased iron absorption after malaria treatment and it seems likely that all diseases and inflammatory conditions which increase hepcidin could decrease iron absorption and thus also the efficacy of iron interventions (Hurrell, 2011). It is, therefore, evident that, although supplementation with iron is effective in increasing iron status, a safer method needs to be found especially in malaria-endemic areas. Current approaches to improve safety are using lower iron doses and improving malaria control (Stoltzfus, 2012).

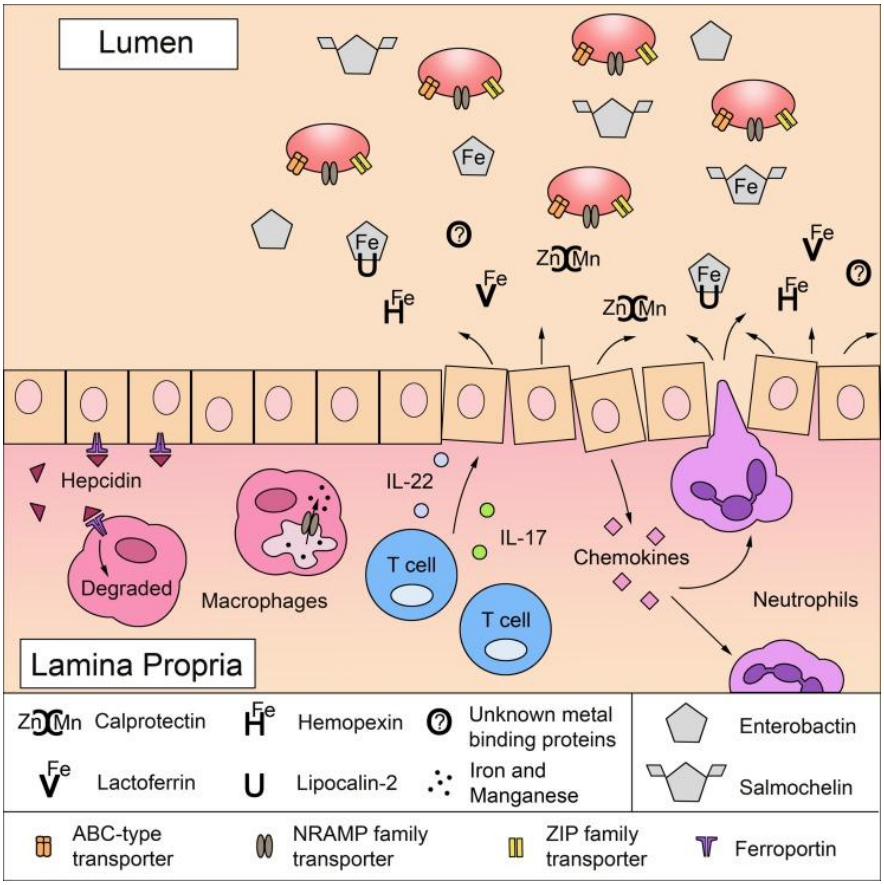

Figure 18: Competition for metals between host and pathogen in the intestinal mucosa. Abbreviations: ABC: ATP binding cassette; NRAMP: natural resistance-associated macrophage protein; ZIP: Zinc-regulated transporters, iron-regulated transporter-like Proteins (Diaz-Ochoa *et al.*, 2014).

It appears that whether a pathogen is intra- or extracellular may be significant in its relation to iron status. As such, TB and HIV are also affected by host iron status and safety of iron

supplementation in these infectious diseases has not been proven (Adetifa & Okomo, 2009; Jonker & van Hensbroek, 2014; McDermid *et al.*, 2013; Oppenheimer, 2001; Solomons & Chomat, 2014). Very few studies of iron supplementation in HIV have been performed (McDermid & Prentice, 2006). However, at least two studies did not show increased viral load with relatively low-dose supplementations (Esan *et al.*, 2013; Olsen *et al.*, 2004). Furthermore, in HIV-infected Malawian children, improved respiratory morbidity was seen with iron supplementation (Esan *et al.*, 2013).

TB within the phagocytic vacuoles of the macrophages where the maximum concentration of Fe^{3+} is only between 1 and 10 ng/ml and has developed a variety of circumvention mechanisms to acquire host iron. Apart from adapting to host-limiting iron circumstances, TB bacteria has also developed a coping mechanism for increased iron due to host sequestering, by binding iron to bacterioferritin, which acts like transferrin in humans (McDermid & Prentice, 2006). Although ID, among other micronutrient deficiencies and malnutrition, is associated with the development of TB, iron supplementation in TB, also in latent TB, might be unsafe (Adetifa & Okomo, 2009; Franke *et al.*, 2014).

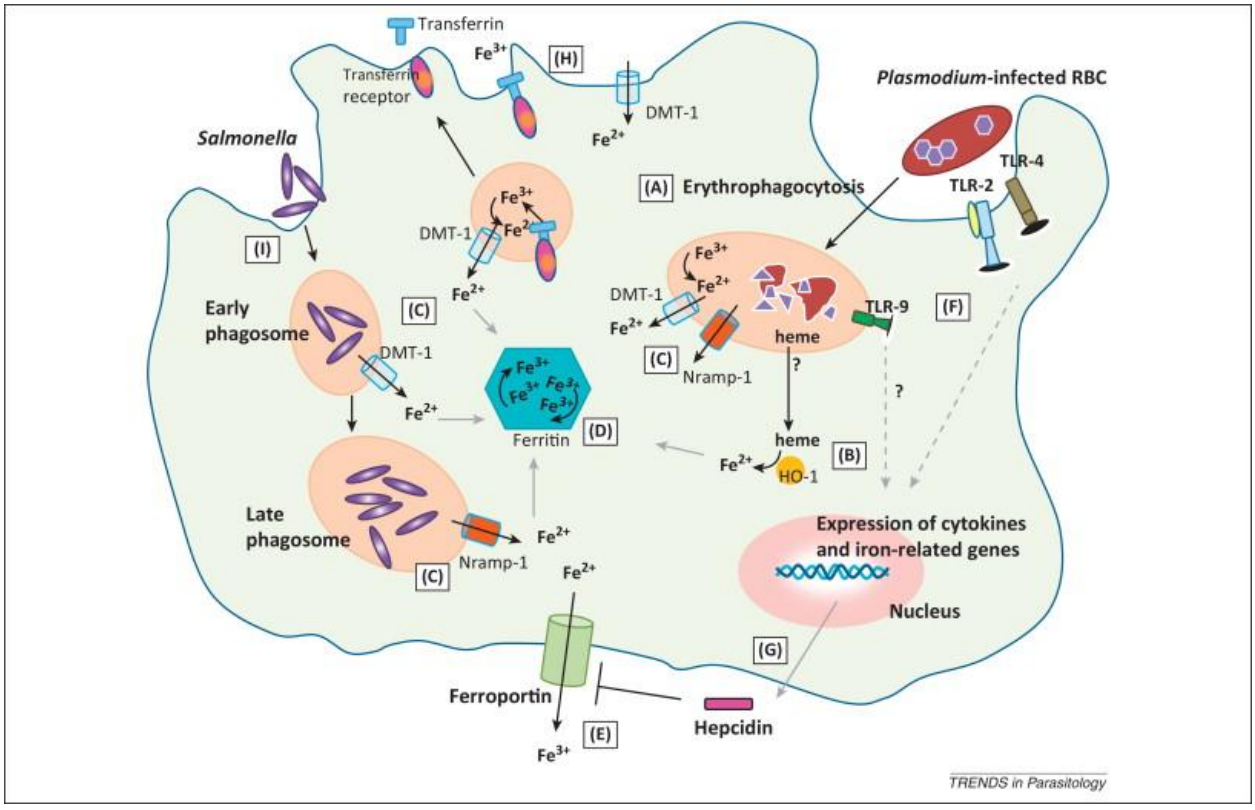

Figure 19: Proposed interaction of malaria and Salmonella with macrophage iron. Malaria infection causes sequestering of RBC (infected and uninfected), which probably benefits phagocytosed Salmonella. Abbreviations: DMT: divalent metal transporter, Nramp: natural resistance-associated macrophage protein; TLR: toll-like receptor (van Santen *et al.*, 2013).

2.6.4.1 Iron and gene expression

Hepcidin is predominantly expressed in the liver and is markedly induced during infections and inflammation, which leads to the sequestering of iron in macrophages, hepatocytes and enterocytes (Ganz, 2005). Even though the expression of hepcidin is regulated by iron, certain cytokines, particularly IL-6, can up-regulate its expression regardless of iron status and, therefore, decrease iron bioavailability during inflammation and infection (Dunn *et al.*, 2007).

As discussed before, one of the DMT1 proteins (*NRAMP2* or *SLC11A2*) is a commonly expressed metal transporter in many cell types (Andolfo *et al.*, 2010; Lam-Yuk-Tseung & Gros, 2006), while *SLC11A1* is mainly expressed in immune cells and involved in innate immunity (Forbes & Gros, 2001; Hedges *et al.*, 2013). Isoform I of *SLC11A2* is located at the plasma membrane of enterocytes and isoform 11 in most cell types in the cytoplasma. Isoform 1 has an IRE-encoding sequence in its 3' untranslated region and is regulated by iron (Hubert & Hentze, 2002; Lam-Yuk-Tseung & Gros, 2006; Mackenzie *et al.*, 2007). Recently, Isoform I have also been demonstrated to be expressed in monocytes (Ludwiczek *et al*., 2009).

Microarray data show that the expression of various other genes, some of which are not involved in the iron metabolism like inducible nitric oxide synthase, is also affected by iron status (Zimmermann & Hurrell, 2007). Furthermore, arachidonate 12-lipoxygenase gene (*ALOX-15*) was strongly up-regulated in ID rats in two separate studies, in brain, intestine and liver tissue (Baumgartner, 2011; Collins *et al.*, 2008). The increased expression in brain tissue was also evident in rats with combined ID and n-3 fatty acid deficiency (ID, fold change=27.6; ID+n-3 PUFA deficiency, fold change=30.9) (Baumgartner, 2011). Collins *et al.* suggested that the induction of *ALOX-15* mRNA expression may be the key event in the change in lipid metabolism that happens during iron deficiency (Collins *et al.*, 2008). Furthermore, they reported increased levels of biologically active lipid-derived mediators (12-HETE, 13-HODE, and 13-HOTE) in the intestinal tissue indicative of ALOX-15 activity, which were suggested to result in increased iron and/or nutrient uptake due to structural changes in enterocyte cell membranes (Collins *et al.*, 2008).

Expression of the non-enzymatic antioxidant heavy metal scavenger, MT, is induced by heavy metals such as iron and zinc (Vergani *et al.*, 2011). This up-regulation is part of cellular antioxidant machinery for protection against oxidative stress and occurs in many cell types, including PBMC, together with enzymatic antioxidant defences, such as SOD and GPx (Cao & Cousins, 2000; Vergani *et al.*, 2011).

Unbound iron may induce oxidative stress, which triggers cellular anti-oxidative stress mechanisms (Koskenkorva-Frank *et al.*, 2013). The transcriptional co-activator peroxisome

proliferator-activated receptor-γ coactivator-1 α (PGC-1α) has a dominant regulatory function in keeping the balance between the production and the scavenging of pro-oxidant molecules by synchronising mitochondrial biogenesis and the expression of antioxidants. It has been demonstrated that nitric oxide-induced *PGC-1α* up-regulation consequently up-regulates the expression of *SOD2*, an important part of the cell's antioxidative machinery (Aquilano *et al.*, 2013; Borniquel *et al.*, 2006). This observation is further strengthened by the finding that, due to *PGC-1α* down-regulation in most cell types with frataxin deficiency, patients with Friedreich ataxia suffer from a blunted response to iron-induced oxidative stress (Marmolino *et al.*, 2010). Furthermore, frataxin-deficient cells not only produce more free radicals, they also show a reduced ability to mobilize antioxidant defences, especially to induce *SOD2* expression subsequent to exposure to oxidants such as hydrogen peroxide and iron (Jiralerspong *et al.*, 2001).

2.6.5 Iron and oxidative stress

NTBI also plays a role in increasing oxidative stress (Crist *et al.*, 2013; Koskenkorva-Frank *et al.*, 2013). Metabolic regulation and cell signalling go along with, and are dependent on, the production of minute concentrations of superoxide and nitrogen monoxide (Mathews *et al.*, 2000). The concentrations of these radicals can, however, drastically increase under pro-inflammatory conditions causing antioxidant defences to become overwhelmed. The consequences of this redox imbalance are oxidative or nitrosative stress, causing a Fenton-type reaction that generates the highly toxic hydroxyl radical and subsequent biological damage (Brissot *et al.*, 2012; Papanikolaou & Pantopoulos, 2005). Iron metabolism, superoxide, hydrogen peroxide and nitrogen monoxide are complexly linked. Iron is involved in both the production and the scavenging of these compounds and IDA is also associated with oxidative stress. Likewise, oral as well as intravenous iron preparations used for the treatment of ID may induce oxidative and /or nitrosative stress which could be due to the formation of NTBI (Hurrell, 2011; Vergani *et al.*, 2011). Further supporting this notion, is the finding that the probability of oxidative and nitrosative stress upon intravenous iron administration increases as the amount of labile (or weakly-bound) iron present in the complex increases (Koskenkorva-Frank *et al.*, 2013). Very recently it was demonstrated that T lymphocytes are able to take up and store NTBI in the same way hepatocytes do. Moreover, it was shown that both hepatocytes and T lymphocytes favoured the uptake of oligomeric Fe3Cit3 above other iron-citrate species, signifying the possible existence of a selective NTBI carrier (Arezes *et al.*, 2013).

The NTBI concept may be extended to include intracytosolic iron forms which are not linked to ferritin, the major storage protein which gives the same protection inside the cell as transferrin gives in the plasma (Brissot *et al.*, 2012). The intracellular NTBI consists of the iron species

moving between vesicular, storage, and functional iron and is termed the transit or labile iron pool. Thus elevated levels of NTBI are potentially responsible for cellular damage both at the cellular surface and inside cells (Brissot *et al.*, 2012).

2.7 Interactions between iron and omega-3 polyunsaturated fatty acids

The preceding review of the current literature on n-3 PUFA and iron and their individual roles in immune function demonstrate several points of possible interaction. The most obvious of these is that iron is a co-factor for the desaturases in LCPUFA synthesis (Figure 4) and that uptake of cellular iron as well as handling of intracellular iron will be influenced by membrane order or fluidity (Figure 19). Other likely mechanistic interactions might involve oxidative stress and cellular response, acting on signalling and involving gene expression. It is, therefore, likely due to these shared mechanisms, that the availability of each nutrient may influence the other's mechanistic performance and that combined deficiency will have additive effects on functional outcomes, particularly immune function.

2.7.1 The role of iron in omega-3 polyunsaturated fatty acids status

2.7.1.1 Animal studies

Several animal studies have demonstrated a relationship between ID and fatty acid status (Cunnane & McAdoo, 1987; LeBlanc *et al.*, 2009b; Oloyede *et al.*, 1992; Rao & Larkin, 1984; Stangl & Kirchgessner, 1998; Tichelaar *et al.*, 1997). Iron is a structural component of the catalytic centre of the fatty acid desaturase enzymes and, therefore, it is generally accepted that ID reduces the activity of these enzymes (Brash, 1999; Nakamura & Nara, 2004; Rao & Larkin, 1984; Shanklin *et al.*, 1994). Some investigations have clearly demonstrated that, even though percentages of the precursors were increased, the long chain products were decreased in ID subjects compared to iron sufficient controls (Cunnane & McAdoo, 1987; Oloyede *et al.*, 1992; Stangl & Kirchgessner, 1998).

In contrast, this effect of ID could not be confirmed, or could even be interpreted as the opposite, by others (LeBlanc *et al.*, 2009a; Tichelaar *et al.*, 1997). In one case DHA in the brains of pups from ID guinea pigs was significantly higher than in pups with maternal iron sufficiency. The authors suggested that the transfer of DHA from the mother to the foetus might have been enhanced by ID (LeBlanc *et al.*, 2009a). Furthermore, Tichelaar and colleagues demonstrated that DHA and DGLA were increased in ID rat plasma phospholipids, concomitant with decreased LA but no change in AA compositions. Furthermore, in RBC phospholipid fractions, ID was associated with reduced ALA and increased DPA and DHA (Tichelaar *et al.*,

1997). Even though alterations in fatty acid composition were observed, they could not be explained by impaired desaturation.

2.7.1.2 Human studies

ID was shown to impair D5D and D6D activities in some human studies as well (Fogerty *et al.*, 1984; Krajcovicova-Kudlackova *et al.*, 2004; Smuts *et al.*, 1995). It was demonstrated that the conversion of ALA into EPA as well as of DPA into DHA was impaired in adults with low serum iron. This result indicated reduced activity for both D5D and D6D. Furthermore, a greater affinity of D6D for n-3 PUFA compared to n-6 PUFA was also suggested by this study, because the conversion index for all the steps of n-3 LCPUFA synthesis was reduced, but the AA to LA ratio stayed the same for both groups. However, the authors reasoned that the finding could have been made due to dietary consumption of AA and no dietary consumption of fish (Krajcovicova-Kudlackova *et al.*, 2004). The same authors have previously also reported inhibited n-3 LCPUFA synthesis in vegetarians with IDA, mainly affecting DHA (Krajčovičová-Kudláčková *et al.*, 1997; Krajcovicova-Kudlackova *et al.*, 1999). This finding indicates that n-3 PUFA metabolism might be affected to a greater extent than n-6 PUFA metabolism by iron status, possibly due to desaturase enzymes having a greater affinity for ALA (Williams & Burdge, 2006; Williams & Burdge, 2007). Another group reported a significant association between iron levels and DHA composition in the livers of sudden infant death syndrome neonatal infants (Fogerty *et al.*, 1984).

Three studies also showed evidence that desaturases has a higher affinity for n-3 PUFA or that iron could be involved in the preferential incorporation of n-3 PUFA into membranes (Ciappellano *et al.*, 2009; Smuts *et al.*, 1995; Zhou *et al.*, 2011). Horse meat generally has high iron content and is a good source of both n-3 and n-6 EFA (~200 mg ALA and ~400 mg LA per 100 g). The authors found that consumption of 175 g horse meat per week for 90 days increased only total RBC n-3 PUFA and DHA composition, and not n-6 PUFA composition (Ciappellano *et al.*, 2009). Furthermore, Zhou and colleagues showed a moderate positive, but highly significant, association between ferritin and the highly unsaturated n-3 PUFA in a cross sectional study of 1511 Canadian Inuits, even though only 13.7% of the survey participants were ID (Zhou *et al.*, 2011). Together with an effect of ID to reduce desaturase activity, Smuts and colleagues also reported a greater effect of ID on n-3 PUFA than on n-6 PUFA incorporation into RBC membranes (Smuts *et al.*, 1995). The RBC fatty acid composition of ID primary school children (n=15) was compared to matched controls (n=15) with normal iron status before and after a 15-week intervention with soup fortified with 20 mg iron. Before intervention, the percentage of total n-3 PUFA (and ALA, EPA, DPA and DHA individually) in RBC phospholipid PC and PEA fractions were significantly lower in ID children. Of the n-6

PUFA, only PEA DGLA was significantly lower in the ID group. The finding that not only the longer chain n-3 PUFA, but also the essential n-3 PUFA, ALA, was significantly reduced in ID, gave rise to the theory that iron could also be involved in the incorporation of PUFA into membranes and that ID may affect n-3 PUFA incorporation more than n-6 PUFA incorporation. Furthermore, even though a decrease in n-3 PUFA intake was seen towards the end of the study, the overall n-3 PUFA status in both RBC PC and PEA in the ID children improved to percentages comparable to the control group's status after the iron intervention. Although the n-6 PUFA intake was almost double the recommended intake for this age group and the n-6 to n-3 intake ratio increased during the study, n-3 PUFA incorporation into membranes was still preferential to that of n-6 PUFA (Smuts *et al.*, 1995). A possible explanation could be that the children could have had a higher relative intake of AA to EPA/DHA, which could cause a negative feedback for the n-6 desaturases, as also concluded by a similar previous study (Krajčovičová-Kudláčková *et al.*, 1997). The authors suggested that lower iron status prevented the RBC membrane phospholipids from incorporating n-3 PUFA, leading to a greater incorporation of saturated fatty acids (Smuts *et al.*, 1995). Alternatively, it is also known that if there is a shortage of n-3 LCPUFA, n-6 LCPUFA (especially n-6 DPA) replace them in membranes to maintain polyunsaturation and fluidity (Connor *et al.*, 1990; Elizondo *et al.*, 2007). More iron could mean improved desaturation and, therefore, more available n-3 LCPUFA to incorporate into membranes. Thus, in this case it is likely that a balance was restored by correction of a confounding factor (iron), leading to increased availability of n-3 LCPUFA for incorporation into membranes.

These studies provide evidence that iron availability influences fatty acid metabolism, and that more mechanisms than desaturase activity may be involved. Furthermore, is not clear what role these interactions play in the immune response.

2.7.2 The role of omega-3 polyunsaturated fatty acids in iron status

2.7.2.1 Animal and *in vitro* studies

Dietary fat type and levels alter iron absorption and utilization in animal models (Miret *et al.*, 2003; Rao & Larkin, 1984; Rodriguez *et al.*, 1996; Shotton & Droke, 2004). Generally, animal studies have demonstrated that dietary saturated fat promotes iron absorption and increases iron stores in ID, while diets high in unsaturated fats rather reduce iron retention and promote development of ID (Rao & Larkin, 1984; Shotton & Droke, 2004). In a study performed by Shotton *et al.*, no significant differences in Hb changes were found between rats fed safflower oil (n-6 PUFA), flaxseed oil (n-3 PUFA), olive oil (n-9 MUFA) and beef tallow (SFA), although a tendency towards increased Hb and haematocrit values was found in the animals fed low iron together with olive oil or beef tallow compared to the other two groups. In accordance with

others, the authors concluded that low iron status react on different fat diets, whereas normal iron status shows no effect (Shotton & Droke, 2004). Since ID seems to result in abnormal shifts in cellular membranes, affecting n-3 PUFA mostly (Krajčovičová-Kudláčková *et al.*, 1997; Smuts *et al.*, 1995), correcting of membrane composition would be expected to take preference for n-3 PUFA and, therefore, that the impact of feeding n-3 PUFA rich oils should be the greatest. This effect was seen in some animal studies, and the mechanism was also demonstrated by *in vitro* studies (Miret *et al.*, 2003; Mitjavila *et al.*, 1996; Rodriguez *et al.*, 1996).

Even though it seems that SFA improves absorption/storage/basic functions of iron, PUFA is likely necessary in cell membranes to react to oxidative stress caused by NTBI (Brand *et al.*, 2008; Schonfeld *et al.*, 2007). This group demonstrated *in vitro* that DHA supplemented pheochromacytoma cells had increased transferrin receptors, increased iron uptake and that this caused lipid peroxidative cell damage and accelerated apoptotic death (Schonfeld *et al.*, 2007). Furthermore, in reaction to DHA treatment, the ubiquitous *DMT1 (SLC11A2)* was over-expressed, accompanied by increased intracellular iron levels. However, these effects were in a cell culture setting and seem to be dose-dependent (Schonfeld *et al.*, 2007). It might, therefore, be translated differently to an *in vivo* situation using more physiological levels. It was subsequently demonstrated by the same group that DHA enrichment of oligodendroglia cells caused esterification into ethanolamine esters which are incorporated into the inner leaflet of the phospholipid bilayer. Iron added to these cells in low concentrations stimulated a temporary translocation of the PEA to the outer membrane and given adequate time for preconditioning, cellular antioxidant mechanisms were up-regulated and the cells became resistant to subsequent lipid peroxidation-induced stress (Brand *et al.*, 2008). Thus, these two studies demonstrated DHA modulated iron uptake and the signal-response to oxidative stress in these cells. Similar findings and conclusions were made in endothelial cells (Ober & Hart, 1998). An elevated ferritin concentration has been demonstrated after short exposure of endothelial cells to DHA.

Brand *et al.* argued that the increase in ferritin is the cause for the ability of DHA to elevate simultaneously cellular iron content and reduce iron concentration-dependent hydrogen peroxide-mediated cell injury. They argued that this could be the mechanism involved not only in brain cells, but also in other cells, which could be the key to the interactive observations made around iron and n-3 PUFA in nutritional deficiencies, losses of PUFA in aging and excessive iron accumulation in degenerative disorders (Brand *et al.*, 2008).

In contrast, two studies also suggested that higher iron absorption in rats fed with fish oil was caused by increased lipid peroxidation and, therefore, increased RBC turnover (Miret *et al.*, 2003; Rodriguez *et al.*, 1996). Miret *at al.* fed a fish oil supplemented-diet (with ~9 g/kg EPA and

~4 g/kg DHA) to rats, resulting in decreased total non-haem iron levels in the livers and spleens of these rats compared to olive oil supplemented rats. The mechanism involved was assumed to be an increased PUFA content in RBC causing the RBC to be more susceptible to lipid peroxidation. The probability of these oxidatively modified RBC to be removed from circulation would then increase causing the requirement of new RBC and, therefore, also an increase in the percentage of reticulocytes. The formation of new reticulocytes requires iron and will thus extract it from the stores in the liver and spleen. The mechanism is supported by the observation that the percentage reticulocytes in rats fed with fish oil increased compared to those fed with olive oil, but anti-oxidative mechanisms were not measured in these studies. (Miret *et al.*, 2003). Similar findings and conclusions were made by Rodriquez *et al*., and the authors of both publications cautioned against very high doses of fish oil supplementation.

2.7.2.2 Human studies

A study performed on the cognitive effects of n-3 PUFA supplementation in 6–10 y-old South African school children in KwaZulu-Natal illustrated an improvement in iron status (Smuts, 2012). The children received 11 ml of oil (a combination of evening primrose, sunflower and high DHA fish oil and vitamin E), whereas the control group received 11 ml sunflower oil, for 6 months. All children also received 5 mg iron (ferrous bisglycinate) and 2.1 mg beta-carotene in the form of a biscuit. A significant increase was found in the RBC total phospholipid DHA composition of the experimental group whereas it declined in the control group. Furthermore, the prevalence of children with Hb <11 g/dl declined in the experimental group (7.69% to 5.38%) and stayed the same in the control group (11.28% to 11.19%). Likewise, the prevalence of children with ferritin <20 ng/ml declined from 34.53% to 26.87% in the experimental group whereas it increased from 32.61% to 35.56% in the control group (Smuts, 2012). It seems thus that iron could be utilized better in the children receiving the mix of oils compared to n-6 PUFA (sunflower oil) alone.

In accordance with this, two other human studies supported these findings, but also those in animals, that higher PUFA intake (but only the n-6 PUFA, LA) is associated with lower iron status (Lukaski *et al.*, 2001; van Dokkum *et al.*, 1983). An increase in LA intake from 4 to 16 energy % (at a constant level of fat intake of 42 energy %) caused a decrease in iron balance from 3.3 to 2.3 mg/day and a decrease in haemoglobin from 9.6 to 9.1 mmol/l in a small group of 12 young men (van Dokkum *et al.*, 1983). Furthermore, in three endurance athletes in a cross-over design, less iron was retained and ferritin tended to decrease with high (50% of energy intake) polyunsaturated (predominantly LA) intake, indicating impaired iron absorption and utilization (Lukaski *et al.*, 2001).

2.7.3 Combined effects of polyunsaturated fatty acids and iron on functional outcomes

Data on the combined effects of PUFA and iron on functional outcomes are scarce. Some cognitive effects were noticed by Baumgartner and colleagues in an animal model and human randomised controlled trial (Baumgartner *et al.*, 2012a; Baumgartner *et al.*, 2012b; Baumgartner *et al.*, 2012c). They found that combined deficiency of iron and n-3 PUFA in male rats disturbed brain monoamine metabolism and created larger memory shortfalls than ID or n-3 PUFA deficiency alone (Baumgartner *et al.*, 2012a). Moreover, supplementation with either of these nutrients individually further exacerbated the cognitive deficits (Baumgartner *et al.*, 2012b). The human study echoed these effects. In children with poor iron and n-3 PUFA status, although iron supplementation enhanced verbal and nonverbal learning and memory (mainly in anaemic), DHA/EPA supplementation impaired working memory in anaemic children and long-term memory and retrieval in girls with ID (Baumgartner *et al.*, 2012c). These findings demonstrated that supplementation with single nutrients in doubly-deficient subjects may be further detrimental and might be due to an adaptation by the programming or epigenetic effect (Roth & David Sweatt, 2011).

2.7.4 Combined effect of polyunsaturated fatty acids and iron deficits on immune function

Very few studies have investigated the role of PUFA and iron interactions in immune function. Apart from each influencing the other, iron deficiency further alters lipid-associated immune response by being part of the catalytic centre of the COX and LOX enzymes and, therefore, ID will likely also reduce the production of the lipid-derived immune mediators, eicosanoids and docosanoids. (Gilbert *et al.*, 2011a; Kuhn *et al.*, 2005). Another known mechanism whereby iron status influences the availability of fatty acid-derived immune mediators (as well as protein kinase C), involves the hydrolysis of cell-membrane phophatidylinositol-4, 5-biphosphate by PLC (Kuvibidila *et al.*, 1998). The end products of this enzymatic reaction, IP_3 and DAG, regulate protein kinase C activity. Both protein kinase C activation and the hydrolysis of cell-membrane phospholipids are crucial for signal transduction that leads to T-cell proliferation and many subsequent immune functions. ID impairs this event in early T-cell activation and may lead to impaired immune response in ID humans (Kuvibidila *et al.*, 1998).

Two animal studies have investigated immune outcomes related to iron and n-3 PUFA interactions (Clauss *et al.*, 2008; Rørvik *et al.*, 2003). It was noticed that the black rhinoceroses in captivity, when their diet was low in ALA, made them prone to iron-storage diseases and inflammatory diseases. An adjustment of their diet to contain similar levels of ALA as the typical free range diet, corrected the situation (Clauss *et al.*, 2008). It was also found that survival

increased during a natural outbreak of cold water vibriosis and of furunculosis in farmed Atlantic salmon, when given a diet of low iron combined with high DHA/EPA levels (Rørvik *et al.*, 2003).

The only human study that investigated combined intervention effects of micronutrients and n-3 PUFA on infectious morbidity, was performed with a micronutrient fortification of food, including 18 mg/day iron but also many other micronutrients (Muthayya *et al.*, 2009; Thomas *et al.*, 2011). The results are, therefore, not directly comparable to intervention with iron in combination with n-3 PUFA. In a two-by-two factorial RTC, n-3 PUFA (900 mg ALA and 100 mg DHA per day) reduced the duration of upper respiratory tract infections (Thomas *et al.,* 2012); and, in agreement with our study, they discovered an antagonistic interaction effect of micronutrient treatment, containing 18 g iron per day, to increase the duration of upper respiratory tract infection in 6–10 y-old Indian school children (Muthayya et al., 2009; Thomas et al., 2011).

The research planned for this thesis was guided by the preceding review of the literature. Three articles were formulated to reach the aim and objectives of this study. First, the effects of the supplementation of iron and a mixture of DHA/EPA, alone and in combination, on the occurrence and duration of infectious morbidity, in ID children with low n-3 PUFA intake, were evaluated. Efficacy indicators, including iron status parameters and PBMC fatty acid composition, were included in this publication.

The second publication aimed to answer some mechanistic questions and to investigate biochemical and molecular outcomes that could be influenced by iron and n-3 PUFA supplementation and are related to immune function. The effect of the supplementation regime on circulating pro- and anti-inflammatory AA, DHA and EPA-derived mediators in plasma as well as a marker of oxidative stress (isoprostane), was assessed. Molecular interactions of selected mechanisms, iron regulatory, inflammatory and oxidative stress-related, were also tested on gene expression level.

Lastly, as part of investigating immune modulation of n-3 PUFA, associations of fatty acid composition with allergic disease were tested, using the baseline cross-sectional data and a questionnaire designed and validated to identify allergic disease in young children.

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Chapter 3 Manuscript 1

N–3 long-chain polyunsaturated fatty acids reduce respiratory morbidity caused by iron supplementation in iron-deficient South African school children: a randomized, double-blind, placebo-controlled intervention[1–4]

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Running head: N–3 LCPUFAs reduce morbidity caused by iron.

Registered at clinicaltrials.gov as NCT01092377

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[5]Abbreviations used: BAZ, BMI-for-age z score; CRP, C-reactive protein; DHA/EPA, mixture of docosa–hexaenoic acid and eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl ester; HAZ, height-for-age z score; ID, iron deficiency; IDA, iron-deficiency anemia; LCPUFA, long-chain polyunsaturated fatty acids; NTBI, non-transferrin-bound iron; PBMC, peripheral blood mononuclear cell; RBC, red blood cell; RCT, randomized controlled trial; SF, serum ferritin; TB, tuberculosis; TfR, transferrin receptor; WAZ, weight-for-age z score; ZnPP, zinc protoporphyrin.

ABSTRACT

Background: Although iron supplementation in malaria-free areas mostly reduces infectious morbidity, it can sometimes increase morbidity from infections as a result of the dependence of pathogenic microorganisms on iron. Supplementation with n–3 long-chain polyunsaturated fatty acids (LCPUFAs) improved morbidity in several human studies. However, information on the combined effect of iron and n–3 LCPUFA supplementation on infectious morbidity is limited.

Objective: To determine whether n–3 LCPUFA and iron supplementation, alone or in combination, affect absenteeism and illness in iron-deficient (ID) school children with low fish intake.

Study design: 321 South African children (aged 6–11 y) with ID were randomly divided into four groups to receive: 1) iron plus placebo, 2) docosahexaenoic and eicosapentaenoic acid (DHA/EPA) plus placebo, 3) iron plus DHA/EPA, or 4) placebo plus placebo as oral supplements 4 times per week for 8.5 months. Morbidity was recorded and iron status indices were measured. Total phospholipid fatty acid composition of peripheral blood mononuclear cell membranes were analyzed in a subsample (n = 130).

Results: Iron supplementation increased the number of days with illness when all symptoms were considered (B value: 0.87; 95% CI: 0.71, 1.03) as well as illness specifically due to respiratory symptoms (B value: 1.45; 95% CI: 1.21, 1.70), whereas DHA/EPA reduced the number of days with illness at school (B value: -0.96; 95% CI: -1.33, -0.59). The increases caused by iron were reduced to the levels seen in the placebo plus placebo group when iron was provided in combination with DHA/EPA as indicated by significant iron x DHA/EPA interactions (both $P < 0.001$).

Conclusion: Iron supplementation increased morbidity (mostly respiratory) in ID South African school children with low DHA/EPA intake, but when iron was given in combination with DHA/EPA this effect was prevented.

The trial was registered at clinicaltrials.gov as NCT01092377.

INTRODUCTION

Both iron and polyunsaturated fatty acids (PUFAs) are important for immune function (1-4). Iron deficiency (ID) impairs lymphocyte activation and proliferation, reduces bactericidal killing by neutrophils and macrophages, and affects certain B-cell functions (1). As a result, ID can increase susceptibility to infections (4). Several studies reported immune function benefits with iron supplementation, such as reduced morbidity from upper respiratory tract infections (5), and probable protection against diarrhea, dysentery and acute respiratory illness (6, 7). However, other studies have found that iron supplementation increased morbidity from acute and chronic infections, especially malaria, HIV and tuberculosis (TB) (8-12). It is well accepted that almost all microorganisms are dependent on iron and that iron overload, caused by supplementation among other reasons, can increase morbidity. Therefore, supplementation with iron remains controversial, particularly in areas with a high burden of infectious disease.

Availability of essential PUFAs and their long-chain metabolites (LCPUFAs) influences the fatty acid (FA) composition of the phospholipid bilayer of membranes of immune cells, such as the monocytes and lymphocytes in peripheral blood mononuclear cells (PBMCs) (13). The FA composition of these membranes affects fluidity, cell signalling and the type and quantity of immune mediators derived from the FAs incorporated into immune cell membranes (13). Only a few randomized controlled trials thus far investigated the effects of dietary PUFA intervention on clinical outcomes in school-age (14, 15) and younger children (16-18). Two of these reported beneficial effects of fish oil-enriched milk and fish flour spread, respectively, on respiratory illness in 7–12-year-old school children (14, 15), while three reported beneficial effects of DHA only- or DHA and arachidonic acid-enriched formula on upper respiratory tract infections, bronchiolitis and asthma/wheezing in under 3-year-olds and infants (16-18).

There is evidence that iron and PUFAs interact *in vivo*. Iron is a structural component of the catalytic centre of the FA desaturase enzymes responsible for the conversion of the essential FA into their respective LCPUFAs (19). Several studies demonstrated a relationship between iron and FA status (20-22). Furthermore, ID may alter the immune response due to iron being part of the catalytic centre of the lipoxygenase and cyclooxygenase enzymes, which are both involved in the synthesis of the eicosanoids derived from LCPUFAs (23-25).

The aim of the study reported here was to evaluate for the first time, the effect of iron and n–3 LCPUFA supplements, individually and combined, on school absenteeism and illness, as well as the FA composition of immune cells, in ID South African children.

SUBJECTS AND METHODS

Study site

The study was conducted at four primary schools in a malaria-free rural area in the province of KwaZulu-Natal, South Africa. As seen in **Figure 1**, 926 children were screened of whom 349 were included – and 321 finally started the study – according to the following inclusion criteria: 1) age 6–11 y; 2) hemoglobin (Hb) > 8 g/dL; 3) ID, defined as either serum ferritin (SF) < 20 µg/L, serum transferrin receptor (TfR) > 8.3 mg/L (26), or zinc protoporphyrin (ZnPP) > 70 µmol/mol heme in washed erythrocytes (27); 4) apparently healthy, with no chronic illness; and 5) no consumption of iron or n–3 FA-containing supplements. Written informed consent was obtained from the parents or guardians and verbal assent was obtained from the children who joined the study. The children were dewormed at baseline and after 4 mo with an oral dose of 400 mg mebendazole [Worm-Stop; Be-Tabs Pharmaceuticals (Pty) Ltd].

Study design

The study used a randomized, placebo-controlled, double-blind, 2-by-2 factorial design to determine effects of supplementation on cognitive performance as described previously by Baumgartner et al. (28). Morbidity was a planned secondary outcome of this study. Children were allocated randomly (stratified by school and by grade) to one of four groups to receive 1) iron and placebo (*n* = 81), 2) DHA/EPA and placebo (*n* = 81), 3) iron and DHA/EPA (*n* = 79), or 4) placebo and placebo (*n* = 80) as seen in Figure 1. With the exception of a few classes, individuals were assigned to all four treatment groups within each classroom. The number of children who fulfilled the study criteria and were finally included in the study, ranged between one and 14 per class. One iron tablet (Lomapharm, Paul Lohmann GmbH) and two DHA/EPA capsules (Burgerstein AG) were given orally on four days per week and contained 50 mg iron as iron sulfate, and in total 420 mg DHA and 80 mg EPA, respectively. Supplementation took place from mid-February to November 2010 and was interrupted by holidays for 2 wk in March and April, for 5 wk in June and July, and for 1 wk in September. Supplementation was further interrupted for 4.5 wk by a national strike of teachers in August and September. To make up for the unexpected loss of intervention days, supplementation was increased from 4 to 5 d/wk for 8 wk, which compensated for 2 wk of supplementation. Thus in total, supplements were provided for 105 d over a period of 8.5 mo. The mean total supplement intake during the trial was 4.8 g iron in the groups that received iron tablets; and 41.2 g DHA and 7.8 g EPA in the groups that received DHA/EPA capsules. The investigators and fieldworkers were blinded by assigning subjects to four group colors, with each color representing a combination of treatment codes. These colors and codes were used on supplement containers and forms throughout the trial. The treatment codes were kept secret by a member of an independent safety monitoring board

until after completion of the data analysis. The placebos were identical in appearance to their respective verums and the FA placebo capsules (RP Scherer GmbH) contained medium-chain triglycerides with the same total fat content as the DHA/EPA capsules. The iron tablets were given in the morning when the children arrived at school together with a vitamin C drink (~10 mg per serving) at least two hours before the DHA/EPA capsules, which were given just before the first break, when they received a meal at school. To ensure compliance the tablets and capsules were provided by the fieldworkers and they also observed that the tablets or capsules were swallowed. In total, supplements were provided for 105 days. All remaining ID children at the end of the study received additional iron supplementation. The ethical committees of North-West University, Potchefstroom, South Africa and ETH Zürich, Switzerland, approved the study protocol.

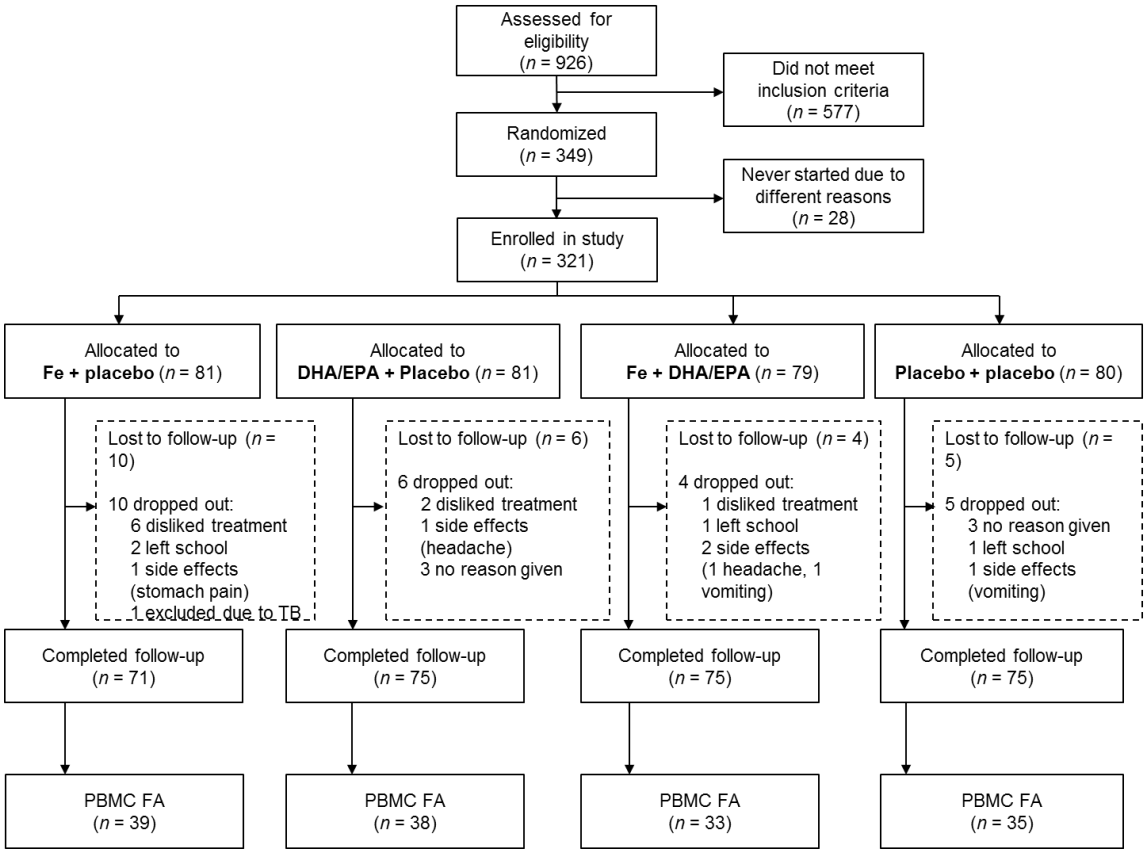

FIGURE 1: Participant flow chart. DHA/EPA, mixture of docosahexaenoic acid and eicosapentaenoic acid; TB, tuberculosis.

Morbidity

Morbidity data were collected for 1) days absent and 2) days at school with illness. The sum of these was evaluated for total days of illness. In total, morbidity data were recorded for 176 d over a period of 8.5 mo (66% of total study days). Symptoms were allocated to respiratory illness – namely, chest pain, cough, ear pain, runny nose, shortness of breath and sneezing -

and gastric illness – namely, diarrhea, stomach pain and vomiting. “All symptoms” included respiratory and gastric illness, together with the other symptoms observed, namely eye infection, fever, headache, muscle ache and sores in the mouth and nose. Fieldworkers, each assigned to a specific treatment group of children per school (n ~25) for the total duration of the study, asked the children which symptoms they experienced as soon as they were back at school after being absent. They also observed symptoms while the children were ill at school. Data were coded, double entered into Excel© in calendar format and programmed by two different programmers for calculation of duration and number of episodes. Data for weekends were collected on Mondays and included in illness data, but not in absenteeism data.

Biochemical measurements

Venous blood was collected at baseline and endpoint, and iron status indices (ZnPP, SF and TfR), as well as Hb and C-reactive protein (CRP) were measured as described previously (28). Blood for the preparation of PBMCs was collected in 4 ml cell preparation tubes (CPT) from Becton Dickenson, prepared according to the manufacturer’s instructions and stored until analysis at −80ºC for less than 6 months. Phospholipids were extracted from PBMC membranes in a randomly selected subsample (*n* = 130) with chloroform:methanol (containing 0.01% butylated hydroxytoluene) by using a modification of the method of Folch et al. (29), as described previously (28). External calibration was used for each compound and corrected with a weighed heptadecanoic acid internal standard. The relative composition (weight %) of FAs was reported as previously described (28).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics (version 21; IBM Corporation). The primary power analysis was based on the cognition outcomes and a sample size of 86 children per group was calculated to be necessary for these outcomes (28). Based on the results of a study by Dalton, a sample size of 144 subjects per group would give the study 80% power to detect a significant difference ($\alpha = 0.05$) in days absent due to illness (14). The sample size needed for this study was calculated with a two tailed, two proportions Z test based on the proportions of children who were absent with respiratory illness in the experimental (14.3%) and placebo (27.7%) groups of the Dalton study. All data were checked for the presence of outliers (± 3SD from the mean). A per protocol analysis was followed as the children who dropped out of the study were excluded from the data analysis (Figure 1). Differences in baseline characteristics between the subgroup for total phospholipid FA analysis in PBMC membranes and the rest of the children (non-PBMC subgroup) were examined by using independent *t*-tests for continuous variables and by using the chi-square test for categorical variables.

Estimated intervention effects of iron and of DHA/EPA and their interaction on FA composition and duration of each episode of illness and absenteeism were analyzed by using two-way ANCOVA on the endpoint measurement, using baseline values (only for FA composition), gender, age, school and grade as individual level covariates. Non-parametric data for FAs were transformed before performing the ANCOVA. Estimated intervention effects of iron and of DHA/EPA and their interaction on other morbidity data were analyzed with Poisson regression using a log linear link, adjusting for gender, age, school and grade. For duration and number of episodes of illness and absenteeism, only ill children were included. When a significant interaction was obtained, differences between groups were analyzed with Poisson regression using a log linear link with intervention group as between-subject factor and pairwise comparison using Bonferroni adjustment and including gender, age, school and grade as covariates. To examine whether iron or DHA/EPA supplementation increased or decreased the odds ratio (OR) for being absent or ill at school, we used binary logistic regression analyses, adjusting for gender, age, school and grade and including a DHA/EPA x iron interaction term. If there was a significant interaction, groups were compared using chi-square tests with Bonferroni correction. Kaplan-Meier curves were constructed and evaluated for DHA/EPA or iron effects and DHA/EPA x iron interactions on the fraction of children falling ill at least once (excluding recurrent illness and duration of illness) using Cox regression models including gender, age, school and grade as covariates, and analyzed for differences in the fraction of ill children between groups with the log-rank test. *P*-values < 0.05 were considered significant.

RESULTS

Subjects

At baseline, median serum zinc concentration ($P = 0.048$) and ID prevalence based on serum ferritin ($P = 0.030$) were higher, and zinc deficiency prevalence was lower ($P = 0.039$) in the group in whom PBMCs were studied (PBMC group) compared to those in whom PBMCs were not studied (**Table 1**). There were no differences between the four supplementation groups at baseline, within the total group (28) or the PBMC group, with respect to all baseline characteristics as shown in Table 1, except for ID prevalence based on serum transferrin receptor that was higher in the iron-supplemented group ($P = 0.020$) in the PBMC group (data not shown). A total of 25 children (7.8%) did not complete the study and there was no significant difference in drop-out rates between treatment groups (Figure 1). Compliance was high; the mean adherence to treatment (observed capsules and tablets swallowed) was 95.4% (< 80% for only 8 children) and did not differ between treatment groups. Iron supplementation significantly improved iron status indices (SF, TfR, body iron and ZnPP) and Hb (all $P < 0.001$) as reported by Baumgartner et al. (28).

TABLE 1

Baseline characteristics of all children and subgroups for total phospholipid fatty acid analysis of peripheral blood mononuclear cell membranes[1,2]

	All children (*n* = 236–322)	PBMC subgroup (*n* = 114–151)	non-PBMC subgroup (n = 120–171)
Age (y)	8.9 ± 1.3[3]	8.9 ± 1.3	8.9 ± 1.4
Ratio male:female (%)	51:49	51:49	51:49
Height (m)	1.28 ± 0.09	1.28 ± 0.09	1.28 ± 0.08
Weight (kg)	27.8 (17.9–48.1)[4]	28.0 (18.5–48.1)	27.7 (17.9–48.1)
Anthropometric indices [*n* (%)]			
Stunting (HAZ < –2SD)	18 (5.8)	11 (7.4)	7 (4.4)
Mildly stunted (HAZ < –1SD ≥ –2SD)	99 (32.1)	49 (32.9)	50 (31.4)
Underweight (WAZ < –2SD)	7 (2.9)	4 (3.5)	3 (2.5)
Overweight (BAZ > 1SD < 2SD)	64 (21.1)	32 (21.8)	32 (20.4)
Obese (BAZ ≥ 2SD)	21 (6.9)	10 (6.8)	11 (7.0)
Blood hemoglobin (g/dL)	12.1 ± 0.8	12.1 ± 0.8	12.1 ± 0.8
Serum ferritin (μg/L)[5]	19.1 (3.1–73.1)	18.1 (3.1–73.1)	19.4 (3.8–63.5)
Serum transferrin receptor (mg/L)	5.7 (2.4–11.8)	6.0 (2.5–11.8)	5.9 (2.4–11.1)
Body iron stores (mg/kg)	3.0 ± 2.5	2.9 ± 2.6	3.2 ± 2.4
Zinc protoporphyrin (μmol/mol heme)	75.0 (33.0–215.0)	75.0 (33.0–215.0)	74.0 (35.0–162.0)
C-reactive protein (mg/L)	0.4 (0.0–17.8)	0.3 (0.0–17.6)	0.4 (0.0–17.8)
Serum zinc (μg/L)	73.3 (45.8–106.2)	75.7 (51.1–106.2)[6]	71.0 (45.8–104.3)
Deficiencies [*n* (%)]			
Anemia (Hb < 11.5 g/dL)	65 (20.6)	34 (22.5)	31 (18.7)
Iron deficiency based on SF (<15.0 μg/L)	78 (28.1)	40 (31.0)[7]	38 (25.5)
Iron deficiency based on TfR (>8.3 mg/L)	36 (11.3)	18 (11.9)	18 (10.8)
Iron deficiency based on ZnPP (>70 μmol/mol heme)	197 (62.7)	94 (63.1)	103 (62.4)
Shortage of body iron (negative values)[8]	33 (12.0)	17 (13.2)	16 (11.0)
Iron deficiency anemia (Hb < 11.5 g/dL and SF < 15 μg/L)	25 (9.2)	10 (7.8)	15 (10.3)
Zinc deficiency (SZn <65 μg/L)	70 (24.8)	28 (21.7)[9]	42 (27.5)
Acute-phase protein [*n* (%)]			
C-reactive protein (>5 mg/L)	22 (7.2)	15 (10.3)	7 (4.4)

[1]Differences between the PBMC and non-PBMC subgroups were examined by using independent *t* tests for continuous variables and by using the chi-square test for categorical variables ($P<0.05$). [2]HAZ, height-for-age ȥ score; WAZ, weight-for-age ȥ score; BAZ, BMI-for-age ȥ score; Hb, hemoglobin; SF, serum ferritin; TfR, transferrin receptor; ZnPP, Zinc protoporphyrin. [3]Mean ± SD (all such values). [4]Median; minimum to maximum in parentheses (all such values). [5]Serum ferritin values of all children with CRP>5 mg/L were excluded. [6]Significantly different from non-PBMC subgroup, $P = 0.048$. [7]Significantly different from non-PBMC subgroup, $P = 0.030$. [8]Total body iron was calculated on the basis of Cook et al. (41). [9]Significantly different from non-PBMC subgroup, $P = 0.039$.

PBMC membrane total phospholipid FA composition

DHA/EPA supplementation increased the relative composition of EPA and DHA in PBMCs, and consequently also increased total n–3 PUFAs and total n–3 LCPUFAs (all $P < 0.001$) as shown in **Table 2.** DHA/EPA supplementation decreased both the total n–6:n–3 PUFA and n–6:n–3 LCPUFA ratios (both $P < 0.001$). There were no significant effects of iron supplementation on PBMC membrane total phospholipid FA composition and no interactions between iron and DHA/EPA supplementation.

TABLE 2

Effects of intervention with iron and DHA/EPA, alone and in combination, on the total phospholipid fatty acid composition of peripheral blood mononuclear cell membranes over 8.5 months[1]

	Group				Estimated intervention effect[2]		
	Iron + placebo (*n* = 39)	DHA/EPA + Placebo (*n* = 38)	Iron + DHA/EPA (*n* = 33)	Placebo + Placebo (*n* = 35)	Iron	DHA/EPA	Iron x DHA/EPA *P*-value
EPA (*% of total fatty acids*)							
Baseline	0.06 ± 0.07[3]	0.05 ± 0.11	0.06 ± 0.07	0.05 ± 0.06	0.025 (–0.017, 0.068)[4]	0.109 (0.066, 0.153)	0.439
Endpoint	0.09 ± 0.08	0.16 ± 0.11	0.17 ± 0.09	0.07 ± 0.07			
DHA (*% of total fatty acids*)							
Baseline	0.91 ± 0.38	1.03 ± 0.43	0.92 ± 0.30	0.93 ± 0.45	0.034 (–0.169, 0.236)	0.67 (0.462, 0.878)	0.327
Endpoint	1.07 ± 0.37	1.64 ± 0.50	1.57 ± 0.47	1.02 ± 0.41			
ARA (*% of total fatty acids*)							
Baseline	18.86 ± 5.34	20.59 ± 4.15	19.25 ± 3.33	19.80 ± 4.61	–0.240 (–2.005, 1.525)	–0.600 (–2.412, 1.212)	0.778
Endpoint	19.60 ± 4.67	18.96 ± 4.19	18.63 ± 3.29	19.7 ± 3.89			
Total n–3 PUFA (*% of total fatty acids*)							
Baseline	1.74 (0.51–2.98)[5]	1.86 (0.78–3.32)	1.85 (0.96–3.03)	1.58 (0.75–3.85)	0.020 (–0.087, 0.128)[6]	0.220 (0.110, 0.330)	0.373
Endpoint	2.10 (0.85–3.49)	2.42 (1.58–4.40)	2.49 (0.98–3.36)	1.80 (0.88–4.01)			
Total n–6 PUFA (*% of total fatty acids*)							
Baseline	30.2 (16.06–17.11)	32.49 (20.92–24.14)	30.98 (20.7–38.97)	33.4 (18.70–42.41)	–0.076 (–0.273, 0.122)[6]	–-0.094 (–0.297, 0.108)	0.851
Endpoint	31.27 (20.55–37.93)	29.43 (19.97–36.68)	29.21 (22.96–35.28)	32.3 (15.79–41.86)			
Total n–6:n-3 PUFA ratio							
Baseline	17.12 (9.75–35.93)	17.14 (8.81–35.08)	16.93 (9.70–30.95)	18.6 (10.72–43.70)	–0.035 (–0.093, 0.024)[7]	–0.162 (–0.222,–0.103)	0.142
Endpoint	15.85 (9.40–26.78)	11.64 (5.91–17.80)	11.96 (8.23–25.57)	16.10 (8.11–29.25)			
Total n–3 LCPUFA *(% of total fatty acids*)							
Baseline	1.74 (0.51–3.75)	1.86 (0.76–3.32)	1.85 (0.92–3.03)	1.58 (0.75–3.85)	0.018 (–0.089, 0.125)[6]	0.221 (0.112, 0.330)	0.392
Endpoint	2.10 (0.85–3.49)	2.40 (1.56–4.40)	2.44 (0.98–3.36)	1.79 (0.86–4.01)			
Total n–6 LCPUFA (*% of total fatty acids*)							
Baseline	22.9 ± 6.27	25.12 ± 4.63	23.52 ± 3.81	24.2 ± 5.24	–0.285 (–2.379, 1.809)	–1.427 (–3.522, 0.668)	0.731
Endpoint	23.7 ± 5.14	22.34 ± 4.53	22.21 ± 3.45	23.9 ± 4.28			
Total n–6:n–3 LCPUFA ratio							
Baseline	12.80 (6.67–24.59)	13.46 (6.69–28.77)	13.31 (7.38–24.25)	13 (8.02–31.63)	–0.020 (–0.078, 0.038)[7]	–0.160 (–0.220, –0.101)	0.338
Endpoint	12.43 (6.46–22.02)	9.01 (3.81–13.97)	9.24 (5.93–18.70)	12.6 (6.56–22.88)			

[1]Fatty acids measured in the total phospholipid fraction of peripheral blood mononuclear cell membranes. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid; LCPUFA. long-chain polyunsaturated fatty acids. [2]Intervention effects estimated by two-way ANCOVA adjusted for respective baseline values, gender, age, school and grade. [3]Mean ± SD (all such values). [4]Total dose; 95% CI in parentheses (all such values). [5]Median; minimum to maximum in parentheses (all such values). [6]Total n–3 PUFA, total n-6 PUFA and total n–3 LCPUFA were square root transformed for performing two-way ANCOVA analysis. [7]Total n–6:n-3 PUFA ratio and total n–6:n–3 LCPUFA ratio were log transformed for performing two-way ANCOVA analysis.

Morbidity

Illness (days absent and days ill at school combined per episode)

In total, 65% of the children were ill at least once during the study. As shown in **Table 3,** iron supplementation increased the number of days of illness (*P* < *0.001*), the duration of each episode of illness (*P* = 0.045) and the number of such episodes (*P* = 0.035). Furthermore, the number of days ill in the iron plus placebo group, when considering respiratory and all illness, was significantly higher than in the three other groups. Iron increased the number of days of illness (*P* < *0.001*) and tended to increase the number of episodes (*P* = 0.072) with respiratory illness. Furthermore, there were significant iron x DHA/EPA interactions, indicating that these increases in the number of days with all illness, and respiratory illness caused by iron supplementation, were attenuated by 106% and 94% respectively (i.e. essentially back to the level seen in the placebo plus placebo group) when iron was provided in combination with DHA/EPA (both *P* < 0.001).

With all symptoms considered (**Figure 2A**), the fraction of children falling ill at least once when receiving iron was significantly increased (*P* < 0.001) and there was a significant iron x DHA/EPA interaction (*P* = 0.009), indicating that this increase, caused by iron, was attenuated by DHA/EPA. Furthermore, the fraction of ill children in the iron plus placebo group was significantly higher (*P* = 0.001) than in the placebo plus placebo group at the end of the study, while it tended to be higher (*P* = 0.054) in the DHA/EPA plus placebo group. When only respiratory illness was considered **(Figure 2B)**, the fraction of children falling ill at least once when receiving iron was significantly increased (*P* = 0.001), but the iron x DHA/EPA interaction was not significant (*P* = 0.157). The fraction of ill children in the iron plus placebo group was significantly higher than in all the other groups (all *P* < 0.004) and the iron plus DHA/EPA group also differed significantly from the DHA/EPA plus placebo group (*P* = 0.010).

Absenteeism

Irrespective of intervention group, 59% of children were absent at least once due to all symptoms considered, 27% due to respiratory symptoms and 30% due to gastric symptoms. The most common symptoms reported to be the reasons for absenteeism were headache (22%), stomach pain (18%), cough (17%), diarrhea (14%) and (reported) fever (11.2%). Iron supplementation increased the number of days absent with respiratory symptoms (*P* < *0.001*) as seen in **Table 3**. Furthermore (**Table 4)**, iron supplementation increased the odds for being absent with respiratory symptoms (*P* = 0.020).

Both iron (P = 0.003) and DHA/EPA (P = 0.031) supplementation increased the number of days absent and the odds ratio (OR) for being absent when all symptoms were considered, as seen in Tables 3 and 4 (for OR, iron: P = 0.015; DHA/EPA: P = 0.019). However, there was a significant DHA/EPA x iron interaction, indicating that the increased days absent were attenuated when iron and DHA/EPA were provided in combination (P = 0.004). The significant OR interaction (P = 0.019) indicates that iron and DHA/EPA in combination attenuates the increased odds of being absent by 3 times (OR: 0.30, 95% CI: 0.11, 0.82). The single symptoms contributing to the increases were however different for the two supplements. Iron increased absenteeism with respiratory symptoms (P < 0.001), but none of the single respiratory symptoms reached significance (runny nose, P = 0.084; cough, P = 0.111). In contrast, DHA/EPA supplementation increased the number of days absent with headache (P = 0.001), fever (P = 0.003) and stomach pain (P < 0.001). However, respiratory-related absenteeism was attenuated by 86% with the addition of DHA/EPA and the days absent with headache were attenuated by 147% with the addition of iron.

Illness at school

The most common symptoms observed while ill at school were runny nose (18%) and cough (10%). Iron supplementation increased (P < 0.001) and DHA/EPA decreased (P < 0.001) the number of days of illness at school (Table 3). Iron also increased days with respiratory symptoms (P < 0.001), specifically runny nose (P < 0.001) and cough (P = 0.002). Furthermore, there were significant iron x DHA/EPA interactions, indicating that these increases when all symptoms were considered (P = 0.001) as well as with respiratory symptoms and runny nose (both P < 0.001), caused by iron supplementation, were attenuated when iron was provided in combination with DHA/EPA. DHA/EPA also reduced days at school with runny nose (P = 0.036). The group who received iron alone (iron plus placebo) had significantly more days of illness at school with respiratory symptoms (notably, runny nose) and when all symptoms were considered, than the three other groups. Furthermore, as seen in Table 4, iron supplementation increased, while DHA/EPA supplementation decreased the odds for being ill at school when all symptoms were considered (iron: P = 0.003; DHA/EPA: P = 0.001) and for being ill with respiratory symptoms (iron: P < 0.001; DHA/EPA: P = 0.039).

Iron increased the number of days with gastric illness at school (P = 0.006), but this rise was attenuated when DHA/EPA was provided with iron (P = 0.048). Furthermore, iron increased the odds for having gastric symptoms at school (P = 0.036).

TABLE 3

Effects of intervention with iron and DHA/EPA, alone and in combination, on illness and school absenteeism

	Group				Estimated intervention effect		
	Iron + Placebo	DHA/EPA + Placebo	Iron + DHA/EPA	Placebo + Placebo	Iron	DHA/EPA	Iron x DHA/EPA *P*-value
Illness (days *absent combined with ill days at school*)							
Days ill with[1,2]							
All symptoms considered[3]	7.28 ± 0.32[a,4]	2.70 ± 0.19[b]	2.83 ± 0.19[b]	3.05 ± 0.20[b]	0.87 (0.71, 1.03)[5]	–0.12 (–0.31, 0.07)	<0.001
Respiratory symptoms[6]	4.55 ± 0.25[a]	0.81 ± 0.10[b]	1.28 ± 0.13[b]	1.07 ± 0.12[b]	1.45 (1.21, 1.70)	–0.27 (–0.60, 0.06)	<0.001
Gastric symptoms[7]	0.82 ± 0.11	0.77 ± 0.10	0.81 ± 0.10	0.49 ± 0.08	0.50 (0.09, 0.92)	0.45 (0.04, 0.86)	0.104
Duration of illness (per episode) with[8,9]							
All symptoms considered[10]	1.67 (1.48)[11]	1.00 (0.55)	1.00 (0.55)	1.55 (1.00)	0.50 (0.01, 0.99)[12]	–0.18 (–0.68, 0.33)	0.072
Respiratory symptoms[13]	1.50 (1.50)	1.00 (0.64)	1.00 (0.28)	1.25 (0.98)	0.59 (–0.10, 1.30)	–0.21 (–1.07, 0.66)	0.153
Gastric symptoms[14]	1.00 (0.55)	1.00 (0.55)	1.00 (0.00)	1.00 (0.38)	0.06 (–0.49, 0.61)	0.31 (–0.25, 0.87)	0.577
Number of episodes of illness with[1,15]							
All symptoms considered[10]	3.25 ± 0.24[4]	2.86 ± 0.24	3.08 ± 0.25	2.52 ± 0.24	0.25 (0.02, 0.49)	0.12 (–0.13, 0.37)	0.291
Respiratory symptoms[13]	2.81 ± 0.24	2.28 ± 0.36	2.36 ± 0.27	2.08 ± 0.30	0.30 (–0.03, 0.62)	0.09 (–0.32, 0.50)	0.304
Gastric symptoms[14]	1.83 ± 0.27	1.63 ± 0.26	1.74 ± 0.25	1.67 ± 0.30	0.10 (–0.37, 0.56)	–0.03 (–0.50, 0.45)	0.934
Absenteeism							
Days absent due to[1,2]							
Any reason[16]	5.21 ± 0.27[4]	3.87 ± 0.23	5.11 ± 0.26	4.67 ± 0.25	0.11 (– 0.04, 0.26)[5]	–0.19 (–0.34, –0.03)	0.120
All symptoms considered	2.41 ± 0.18[a]	2.19 ± 0.17[b]	1.95 ± 0.16[b]	1.69 ± 0.15[b]	0.33 (0.12, 0.58)	0.26 (0.02, 0.49)	0.004
Headache	0.18 ± 0.05[b]	0.69 ± 0.10[a]	0.13 ± 0.04[b]	0.31 ± 0.06[b]	–0.52 (–1.20, 0.77)	0.82 (0.33, 1.31)	0.021
Fever	0.17 ± 0.05	0.33 ± 0.07	0.40 ± 0.07	0.09 ± 0.04	0.60 (–0.34, 1.53)	1.27 (0.44, 2.11)	0.452
Respiratory symptoms	0.96 ± 0.12	0.39 ± 0.07	0.52 ± 0.08	0.45 ± 0.08	0.75 (0.34, 1.16)	–0.16 (–0.65, 0.34)	0.162
Runny nose	0.13 ± 0.04	0.03 ± 0.02	0.01 ± 0.01	0.04 ± 0.02	1.15 (–0.15, 2.46)	–0.41 (–2.20, 1.38)	0.185
Cough	0.34 ± 0.07	0.21 ± 0.05	0.29 ± 0.06	0.20 ± 0.05	0.53 (–0.12, 1.17)	0.07, (–0.64, 0.80)	0.657
Gastric symptoms	0.59 ± 0.09	0.73 ± 0.10	0.76 ± 0.10	0.48 ± 0.08	0.21 (–0.24, 0.65)	0.42 (0.004, 0.84)	0.558
Stomach pain	0.28 ± 0.06[b]	0.52 ± 0.08[a]	0.25 ± 0.06[b]	0.15 ± 0.04[b]	0.65 (–0.08, 1.39)	1.27 (0.60, 1.93)	0.003
Duration of absenteeism (per episode) with[8,17]							
All symptoms considered[18]	1.00 (0.25)[11]	1.00 (0.33)	1.00 (0.46)	1.00 (0.50)	–0.13 (–0.34, 0.09)[12]	0.02 (–0.19, 0.23)	0.305
Respiratory symptoms[19]	1.00 (0.25)	1.00 (0.00	1.00 (0.13)	1.00 (1.00)	–0.26 (–0.52, 0.002)	–0.27 (–0.57, 0.03)	0.184
Gastric symptoms[20]	1.00 (0.33)	1.00 (0.38)	1.00 (0.00)	1.00 (0.42)	–0.001 (–0.31, 0.31)	0.11 (–0.20, 0.42)	0.522
Days at school with symptoms							
Days ill at school with[1,2]							
Any of the symptoms considered	4.87 ± 0.26[a,4]	0.52 ± 0.08[b]	0.88 ± 0.11[b]	1.36 ± 1.12[b]	1.28 (1.06, 1.50)[5]	–0.96 (–1.33, –0.59)	0.001
Respiratory symptoms	3.59 ± 0.23[a]	0.43 ± 0.08[c]	0.76 ± 0.10[b]	0.61 ± 0.09[b,c]	1.77 (1.45, 2.08)	–0.36 (–0.81, 0.09)	<0.001

Runny nose	3.03 ± 0.21[a]	0.36 ± 0.07[b]	0.49 ± 0.08[b]	0.60 ± 0.09[b]	1.62 (1.30, 1.94)	–0.51 (–0.99, –0.03)	<0.001
Cough	0.34 ± 0.07	0.07 ± 0.03	0.23 ± 0.06	0.08 ± 0.03	1.44 (0.58, 2.34)	–0.18 (–1.37, 1.00)	0.751
Gastric symptoms	0.23 ± 0.06[a]	0.04 ± 0.02[b]	0.05 ± 0.03[b]	0.01 ± 0.01[b]	2.83 (0.81, 4.85)	1.10 (–1.17, 3.36)	0.048

[1]Intervention effects were estimated by Poisson regression using a log linear link adjusted for gender, age, school and grade. Differences between groups were analyzed with Poisson regression using a log linear link with intervention group as between-subject factor and pairwise comparison using Bonferroni adjustment and including gender, age, school and grade as covariates. Means in a row without a common letter differ significantly, P<0.05.

[2]N = 71, 75, 75 and 75 (total study population) in the iron plus placebo, DHA/EPA plus placebo, iron plus DHA/EPA and placebo plus placebo groups, respectively.

[3]Chest pain, cough, diarrhea, ear pain, eye infection, fever, headache, muscle ache, runny nose, shortness of breath, sneezing, sores in the mouth and nose, sore throat, stomach pain and vomiting.

[4]Mean ± SE (all such values).

[5]B value; 95% CI in parenthesis (all such values).

[6]Chest pain, cough, ear pain, runny nose, shortness of breath and sneezing.

[7]Diarrhea, stomach pain and vomiting.

[8]Intervention effects estimated by two-way ANCOVA adjusted for gender, age, school and grade.

[9]Mean per group of [(consecutive days ill (absent and at school) with either all, respiratory or gastric symptoms) divided by number of episodes of that child].

[10]N = 56, 49, 49 and 44 (children who were ill) in the iron plus placebo, DHA/EPA plus placebo, iron plus DHA/EPA and placebo plus placebo groups, respectively.

[11]Median; interquartile range in parenthesis (all such values).

[12]β Value; 95% CI in parenthesis (all such values).

[13]N = 47, 18, 33 and 24 (children who had respiratory illness) in the iron plus placebo, DHA/EPA plus placebo, iron plus DHA/EPA and placebo plus placebo groups, respectively.

[14]N = 24, 24, 27 and 18 (children who had gastric illness) in the iron plus placebo, DHA/EPA plus placebo, iron plus DHA/EPA and placebo plus placebo groups, respectively.

[15]Mean per group of (number of episodes ill (absent and at school) with either all, respiratory or gastric symptoms).

[16]Illness, visits to the clinic, family affairs and no reason given.

[17]Mean per group of [(consecutive days absent due to either all/respiratory/gastric symptoms) divided by number of episodes of that child].

[18]N = 46, 49, 44 and 34 (children who were absent with illness) in the iron plus placebo, DHA/EPA plus placebo, iron plus DHA/EPA and placebo plus placebo groups, respectively.

[19]N = 28, 16, 21 and 16 (children who were absent with respiratory illness) in the iron plus placebo, DHA/EPA plus placebo, iron plus DHA/EPA and placebo plus placebo groups, respectively.

[20]N = 22, 24, 26 and 17(children who were absent with gastric illness) in the iron plus placebo, DHA/EPA plus placebo, iron plus DHA/EPA and placebo plus placebo groups, respectively.

TABLE 4

Odds ratio for the effects of intervention with iron and DHA/EPA, alone and in combination, on school absenteeism and illness[1]

	Group				Odds ratio (95% CI)		
	Iron + Placebo (n = 71)	DHA/EPA + Placebo (n = 75)	Iron + DHA/EPA (n = 75)	Placebo + Placebo (n = 75)	Iron	DHA/EPA	Iron x DHA/EPA
Children absent due to							
Any reason	87[2]	84	89	79	1.93 (0.77, 4.81)[3]	1.35 (0.58, 3.24)	0.80 (0.21, 3.08)
All symptoms considered[4]	65	65	59	45	2.41 (1.19, 4.89)	2.31 (1.15, 4.64)	0.30 (0.11, 0.82)
Headache	15	40	12	20	0.66 (0.27, 1.59)	2.52 (1.17, 5.45)	0.29 (0.08, 1.01)
Fever	8	16	16	4	2.13 (0.51, 9.00)	4.78 (1.27, 18.02)	0.52 (0.10, 2.86)
Respiratory symptoms[5]	39	21	28	21	2.61 (1.21, 5.66)	0.95 (0.42, 2.15)	0.57 (0.19, 1.72)
Runny nose	11	3	1	3	4.76 (0.92, 24.63)	0.96 (0.13, 7.32)	0.11 (0.01, 2.01)
Cough	23	15	19	13	1.91 (0.75, 4.85)	1.07 (0.41, 2.85)	0.65 (0.17, 2.42)
Gastric symptoms[6]	31	32	35	23	1.52 (0.70, 3.23)	1.55 (0.73, 3.32)	0.74 (0.26, 2.12)
Stomach pain	21	24	16	13	1.61 (0.63, 4.12)	2.17 (0.88, 5.35)	0.36 (0.10, 1.29)
Children ill at school with							
All symptoms considered[4]	59	16	29	39	3.20 (1.49, 6.86)	0.22 (0.10, 0.54)	0.82 (0.26, 2.57)
Respiratory symptoms[5]	48	8	24	19	6.14 (2.59, 14.58)	0.32 (0.11, 0.94)	0.66 (0.19, 2.86)
Runny nose	42	5	12	13	7.81 (3.01, 20.27)	0.31 (0.09, 1.13)	0.33 (0.07, 1.61)
Cough	14	3	16	5	3.06 (0.84, 11.10)	0.49 (0.08, 2.91)	4.12 (1.57, 10.85)
Gastric symptoms[6]	11	4	4	1	9.86 (1.16, 83.57)	2.91 (0.29, 29.34)	0.11 (0.01, 1.63)
Stomach pain	11	3	3	1	8.92 (0.74, 106.98)	3.35 (0.23, 49.64)	0.79 (0.01, 2.45)

[1]To examine whether iron or DHA/EPA supplementation increased or decreased the odds ratio (OR) for being absent or ill at school, we used binary logistic regression analyses, adjusting for gender, age, school and grade and including a DHA/EPA x iron interaction term. If there was a significant interaction, groups were compared using chi-square tests with Bonferroni correction.

[2]Percentage of total children per group (all such values).

[3]Exp B value; 95% CI (all such values).

[4]Chest pain, cough, diarrhea, ear pain, eye infection, fever, headache, muscle ache, runny nose, shortness of breath, sneezing, sores in the mouth and nose, sore throat, stomach pain and vomiting.

[5]Chest pain, cough, ear pain, runny nose, shortness of breath and sneezing.

[6]Diarrhea, stomach pain and vomiting.

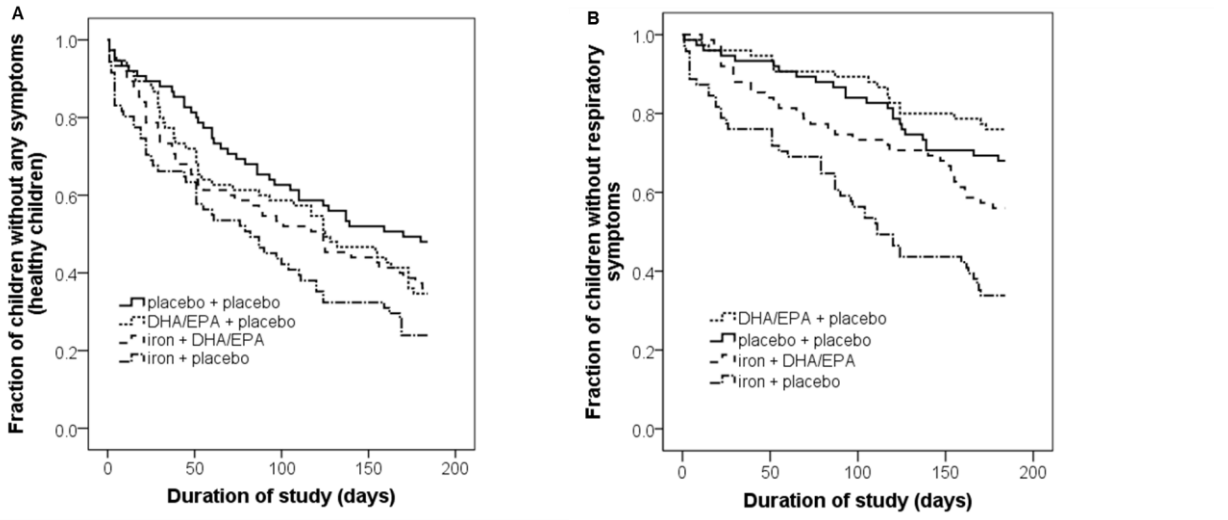

FIGURE 2: Kaplan-Meier survival charts depicting the fraction of children falling ill at least once during the study with (A) all symptoms and (B) only respiratory symptoms. With all symptoms considered the fraction of children ill once receiving iron was significantly increased (*P* < 0.001) and there were significant iron x DHA/EPA interactions (*P* = 0.009), indicating that this increase, caused by iron, was attenuated by DHA/EPA. Furthermore, the fraction of ill children in the iron plus placebo group was significantly higher (*P* = 0.001) than in the placebo plus placebo group at the end of the study, while it tended to be higher (*P* = 0.054) in the DHA/EPA plus placebo group. When only respiratory illness was considered, the fraction of children ill once receiving iron was significantly increased (*P* = 0.001) but the iron x DHA/EPA interaction was not significant (*P* = 0.157). The fraction of ill children in the iron plus placebo group was significantly higher than in all the other groups (all *P* < 0.004) and the iron plus DHA/EPA group also differed significantly from the DHA/EPA plus placebo group (*P* = 0.010). Intervention effects were estimated by Cox regression, using gender, age, school and grade as covariates and the log rank test was used to determine differences between groups. *P*-values < 0.05 were considered significant. DHA/EPA, mixture of docosahexaenoic acid and eicosapentaenoic acid.

DISCUSSION

The most interesting finding of this study in apparently healthy ID school children living in a malaria-free rural area was that iron supplementation increased morbidity, mostly respiratory, but when given in combination with DHA/EPA, this increase in morbidity was prevented.

Our results suggest that the combination of DHA/EPA and iron attenuated the increase in respiratory morbidity due to iron supplementation, leading to the iron plus DHA/EPA group having a similar prevalence of respiratory morbidity to the two groups that did not receive iron. These results were not entirely unexpected, since DHA/EPA supplementation was previously

shown to reduce the likelihood and severity of respiratory illness (14-18). We speculate that DHA/EPA incorporated into macrophage phospholipid bilayers could have improved phagocytosis, thereby improving uptake of iron from senescent red blood cells and thus leaving less unbound extracellular iron needed for pathogen growth. Another possibility is that DHA- and EPA-mediated protection against iron-induced oxidative stress, and improved resolution of inflammation, could have reduced immune cell damage and therefore improved immune response (30, 31).

DHA/EPA on its own reduced the number of days ill at school by 62% and due to respiratory illness by 30%. Furthermore, it significantly reduced the odds for being ill at school, particularly with respiratory symptoms. Others have also reported improved respiratory health with n–3 LCPUFA supplementation in school children and infants (14-18). Thienprasert et al. found that milk fortified with fish oil (providing 200 mg EPA and 1000 mg DHA per day on 5 days per week for 6 months) significantly reduced the number of ill children, as well as ill days per subject (15). Dalton reported a reduced number of children and days absent due to illness in general and respiratory illness in particular after treatment with a bread spread containing ~80 mg EPA and ~190 mg DHA per day on 5 days per week for 6 months (14).

However, in contrast to the positive effect on respiratory symptoms, DHA/EPA increased the number of days absent and the likelihood for being absent through illness when all symptoms were considered – with headache, fever and stomach pain standing out as the main symptoms. Although it is not clear why this happened, headache and gastric upset with LCPUFA supplementation have been noted previously as side effects by others (32). The finding of increased headache is, however, also in contrast with recent studies showing that increased dietary DHA and EPA, as well as fish oil supplementation, reduced chronic headache (33, 34).

Our study population was relatively small for the purpose of monitoring single symptoms. However, we still include these data, since such detailed symptomatic results give further clarity on the unexpected result that general illness-related absenteeism was increased by supplementation individually with either iron or DHA/EPA. A limitation of the statistical analysis is that, since multiple secondary outcomes were tested, the type II error rate could have been inflated. The results should thus be viewed with caution. However, it is clear that DHA/EPA tended to reduce respiratory symptoms, whereas iron tended to attenuate symptoms such as headache. Furthermore, the per-protocol statistical approach followed for the morbidity data could have led to bias. Nevertheless, there was no difference in dropout rates between groups.

A further limitation is that we could not collect data in the holidays or during a teachers' strike, which represented 34% of the total study time.

Contrary to our expectations, we found that iron supplementation increased general morbidity by 139% and respiratory morbidity by 325% (relative to the placebo plus placebo group). There are several possible reasons for these findings which relate to a relatively large, non-physiological bolus of highly absorbable iron entering the gut and target tissues. First and most likely, a transient excess of systemic or topical iron, due to the relatively high dose of 50 mg/d, could have encouraged pathogen growth, which is often seen in areas with a high burden of infectious morbidity, particularly malaria, HIV and TB (9, 11, 12). We specifically chose a malaria-free area, however, and excluded children from the study with self- or parental-admitted chronic diseases. Indeed, one child in the iron group (iron plus placebo) could not finish the study because of being diagnosed with pulmonary TB, which indicates that we cannot exclude TB as an underlying cause of the increased iron-related respiratory morbidity in our study.

Second, there is some evidence that iron supplementation can increase infectious morbidity to a greater extent in ID children than in those with ID anemia (IDA) (5-7). Only 20.6% of the children studied here were anemic. In our study, there might have been less of an increase in the duration and number of episodes of respiratory illness in the IDA children than in those with ID alone, but this is difficult to conclude given our limited population size (data not shown). Third, iron overload could have adversely influenced immune function (35). Even so, the children included in our study were all ID and the dose, which corresponds to a mean intake of 28 mg/d for 64 days and 36 mg/d for the final 40 d, was within the 30–60 mg/d recommended by the WHO to prevent ID anemia (36). Even though the supplementation lasted for 8.5 mo, the actual treatment time was 105 d, just exceeding the recommended 3 mo treatment period (36). Furthermore, none of the children had an endpoint ferritin value over 200 μg/L, which would have indicated possible iron overload (37).

In a comparable study, De Silva et al. supplemented 5–10-year-old Sri Lankan children with ID anemia with 60 mg iron as ferrous sulphate per day and found reduced numbers of episodes and total sick days due to upper respiratory tract infections (5). The Sri Lankan study lasted only 8 wk, however, and ours was 8.5 mo (5). Moreover, fish intake in our study area was notably small, as confirmed by the low n–3 LCPUFA levels in PBMCs and erythrocytes, whereas fish intake contributed to more than 50% of the total animal protein intake in Sri Lanka (38). It seems therefore that the poor n–3 LCPUFA status of our children could have affected the outcome.

We also investigated the n–3 LCPUFAs in total phospholipid of PBMC membranes and found an increase of 240% for EPA and 78 % for DHA, but still not to such an extent as to reach the 1% EPA and 2.5% DHA seen in individuals who follow a typical Western diet (39). Arachidonic acid levels of 19.6% in our study compared closely with the 20% seen in Western diet areas and did not change significantly during the study. Such a poor n–3 LCPUFA status was expected because a dietary assessment in our study population found that the ratio of intakes of n–6:n–3 PUFAs was about 60:1 (28). Our dose of EPA (80 mg, 4 times a week; with a total of 7.8 g over the total study period) was probably too low to improve the EPA status to the typical Western diet level as in a report by Covar et al., for example, where EPA was increased from ~0.5% to ~1.4% within 12 weeks in 6–14-y-old children by supplementing with 3 g EPA per day (40).

In conclusion, we demonstrated in this study that iron supplementation increased morbidity, mostly respiratory, but that this adverse effect of iron was attenuated completely when provided in combination with a dose of 420 mg DHA and 80 mg EPA for four days per week for a period of 8.5 mo. Although DHA/EPA on its own reduced respiratory morbidity when the children were present at school, surprisingly it increased the likelihood of being absent with headache and fever. We also confirmed that supplementation with DHA/EPA improved the n–3 LCPUFA composition of PBMCs in ID South African school children. These results indicate that an iron-induced increase in respiratory morbidity could be mitigated by the simultaneous supplementation of DHA/EPA, especially in areas with low fish intake. Iron supplementation is in many cases a necessity to prevent or treat ID and we therefore recommend that this strategy be further researched in order to identify a potentially safer means of administration.

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Authors' contributions

The authors' responsibilities were as follows: CMS, MZ, JB and LM: designed the study; CMS, MZ, JB, and LM conducted the study; LM and JB analyzed the biochemical indicators and performed the statistical analyses; LM, JB, and CMS wrote the first draft of the manuscript; PCC

and MZ helped to finalize the manuscript. All authors were involved in the interpretation of results and had full access to all the data. LM had the responsibility for the final content of the paper and the decision to submit for publication.

Conflict of interest

PCC, JB and CMS have received speaking honoraria from Unilever. LM and MBZ have no conflict of interest.

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Chapter 4 Manuscript 2

Iron and a mixture of docosahexaenoic and eicosapentaenoic acid supplementation, alone and in combination, affect bioactive lipid signalling and morbidity of iron deficient South African school children in a two-by-two randomised controlled trial

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[4]*Abbreviations: ALOX-15*, arachidonate lipoxygenase-15; *COX-2*, cyclooxygenase-2; DHA, docosahexaenoic acid; *DMT1,* divalent metal transporter; EPA, eicosapentaenoic acid; *GPx-4*, glutathione peroxidase-4; HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid LCPUFA, long-chain polyunsaturated fatty acids; *MT1E,*

metallothionein 1E; *NRAMP2,* natural resistance to infection with intracellular pathogens; PBMC, peripheral blood mononuclear cell; PG, prostaglandin; *PGC-1α,* transcriptional co-activator peroxisome proliferator-activated receptor-γ coactivator-1 α; *PLA2G4A,* cytosolic phospholipase 2 group IVA; *PTGS2,* prostaglandin-endoperoxide synthase 2; *SLC11A2 IRE+,* solute carrier family 11A2 with iron response element, *SLC11A2 IRE–,* solute carrier family 11A2 without iron response element; *SOD2,* superoxide dismutase 2.

ABSTRACT

Both iron and n-3 polyunsaturated fatty acids (PUFA) act through multiple mechanisms to influence immune cell responses. We recently reported that iron supplementation increased respiratory morbidity in iron deficient South African children. This increase, however, was attenuated when iron was provided in combination with a mixture of docosahexaenoic and eicosapentaenoic acid (DHA/EPA).

To explore potential underlying mechanisms, we examined the effects of iron and DHA/EPA, alone and in combination, on plasma lipid-derived immune modulator concentrations and related gene expression in peripheral blood mononuclear cells (PBMC). Lipid-derived immune modulators were analysed with liquid chromatography tandem mass spectrometry and gene expression with quantitative real time PCR.

DHA/EPA decreased inflammatory 12-hydroxyeicosatetraenoic acid and tended to increase anti-inflammatory and pro-resolving 17-hydroxydocosahexaenoic acid (17-HDHA), while iron decreased 17-HDHA. However, in combination with iron, the anti-inflammatory effect of DHA/EPA was maintained. These results were supported by directionally altered targeted PBMC gene expression results and predicted functional pathways. Thus, the biochemical findings presented here may explain the prevention of iron-induced respiratory morbidity which we observed when iron was supplemented in combination with DHA/EPA during the 8.5 mo randomised controlled trial. Also, this novel finding might lead to a safer approach of delivering iron supplementation.

Keywords: Omega-3 fatty acids; lipid-derived immune modulators; gene expression; inflammation; oxidative stress.

1. Introduction

Both iron and long-chain polyunsaturated fatty acids (LCPUFA) affect immune function [1, 2]. They act through multiple mechanisms to influence immune cell responses [1-4]. N-6 and n-3 LCPUFA are the precursors of inflammatory and anti-inflammatory (pro-resolving) immune modulators, respectively. This appears to be a key mechanism by which LCPUFA affect immune responses and inflammatory processes [2, 5] On the other hand, iron deficiency (ID) mostly impairs cell-mediated immunity [1, 3], by being structurally part of enzymes or involved in enzyme activation needed for cellular immune responses, such as myeloperoxidase [6] and phospholipase C [7]. As such, ID can increase susceptibility to infections, which might be overcome by iron supplementation [8]. In turn, it is known that iron supplementation can favour the growth of certain microorganisms [9] and this could be harmful in some settings [10]. We found, in a 8.5 mo randomised controlled trial (RCT), that supplementation of 50 mg iron per day increased respiratory morbidity in 6–11-y-old rural ID South African children. However, the combination of iron supplementation with 420 mg DHA and 80 mg EPA per day prevented this increase in respiratory morbidity [11]. This suggests a biologically important interaction between iron and n-3 LCPUFA.

There may be positive and negative interactions between iron and LCPUFA of relevance to immune function. The most obvious of these is that iron is a co-factor for the desaturase enzymes involved in LCPUFA synthesis [12], as well as of the catalytic sites of the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, which are involved in the production of lipid-derived immune modulators [13, 14]. Thus, ID may

impair both LCPUFA synthesis and synthesis of modulatory lipid mediators. On the other hand, membrane n-3 LCPUFA composition influences membrane order (“fluidity”) and may affect cellular uptake of iron and the handling of intracellular iron [15-17]. Thus, n-3 LCPUFA deficiency may limit availablity of iron to immune cells. Through these interactions iron and n-3 LCPUFA may influence one another’s effects and combined deficiency may have additive effects on functional outcomes, as was described recently for cognitive and infectious morbidity outcomes [11, 18, 19].

Therefore, we examined the effects of iron and a mixture of the two bioactive n-3 LCPUFA, DHA and EPA, alone and in combination, on plasma lipid-derived immune modulator concentrations and expression of genes involved in their synthesis, as well as on antioxidative and iron regulatory gene expression in peripheral blood mononuclear cells (PBMC).

2. Methods

2.1. Study design and subjects

This study was part of an 8.5 mo randomized, placebo-controlled, double-blind, two-by-two factorial intervention which investigated the effects of 50 mg iron, and a mixture of 420 mg DHA and 80 mg EPA per day, alone and in combination, on cognitive function and infectious morbidity in 6–11-y-old rural ID South African children [11, 19]. The present study aims to determine the effects of the supplementations on selected cellular parameters fundamental to immune function. The study was conducted at four

primary schools in a malaria-free area in KwaZulu-Natal and 321 children participated. Children were allocated randomly (stratified by school and by grade) to one of four groups, to receive: 1) iron and placebo (*n* = 81), 2) DHA/EPA and placebo (*n* = 81), 3) iron and DHA/EPA (*n* = 79), or 4) placebo and placebo (*n* = 80). The mean total supplement intake during the trial was 4.8 g iron in the groups that received iron tablets; and 41.2 g DHA and 7.8 g EPA in the groups that received DHA/EPA capsules. The following inclusion criteria were used: 1) age 6–11 y; 2) hemoglobin (Hb) > 8 g/dL; 3) ID, defined as either serum ferritin (SF) < 20 µg/L, serum transferrin receptor (TfR) > 8.3 mg/L, or zinc protoporphyrin (ZnPP) > 70 µmol/mol haem in washed RBC; 4) apparently healthy, with no chronic illness; and 5) no consumption of iron or n-3 PUFA-containing supplements. Plasma lipid-derived immune modulators (n = 137) and PBMC gene expressions (n = 29) were measured in randomly selected subgroups. Parents or guardians gave written informed consent and children gave verbal assent before the study started. The ethical committees of North-West University, Potchefstroom, South Africa and ETH Zürich, Switzerland, approved the study protocol.

2.2 Blood preparation

Venous blood was collected at baseline and endpoint for the preparation of peripheral blood mononuclear cells (PBMC) and plasma. It was collected in 4 ml cell preparation tubes from Becton Dickenson (Franklin Lakes, United States of America) and kept in the dark immediately after collection. PBMC and plasma were separated and PBMC

prepared according to the manufacturer's instructions, concealed from direct light. PBMC and plasma were frozen in dry ice and stored until analysis at −80ºC.

2.3. Lipid-derived immune modulator analysis

Plasma concentrations of lipid-derived immune modulators were analysed in a subsample (n = 137), randomly selected with n = 38 in the iron plus placebo; n = 33 in the DHA/EPA plus placebo; n = 37 in the iron plus DHA/EPA and n = 29 in the placebo plus placebo groups, respectively (Table 2). The subsample size was based on a previous study showing intervention effects on circulating plasma lipid mediators with ~1120 mg DHA and ~1590 mg EPA per day for 10 wk in mildly hypertriacylglycerolemic subjects (n = 18 per group) [20]. Baseline and endpoint samples for each subject were prepared and analysed with liquid chromatography tandem mass spectrometry (LCMSMS) together in the same batch. Plasma 17-hydroxydocosahexaenoic acid (17-HDHA); 5- and 12-hydroxyeicosapentaenoic acid (HEPE); 5-, 8-, 11-, and 12-hydroxyeicosatetraenoic acid (HETE) and prostaglandin (PG) E_2 and D_2 were measured. As outlined in Fig. 1, 17-HDHA is produced from DHA and is the precursor of the D-series resolvins and protectins, which are inflammation-resolving immune modulators [2, 21]. PGE_2, PGD_2 and HETE are mainly pro-inflammatory and are derived from AA, whereas HEPE and its products have anti-inflammatory functions and are derived from EPA [2, 21].

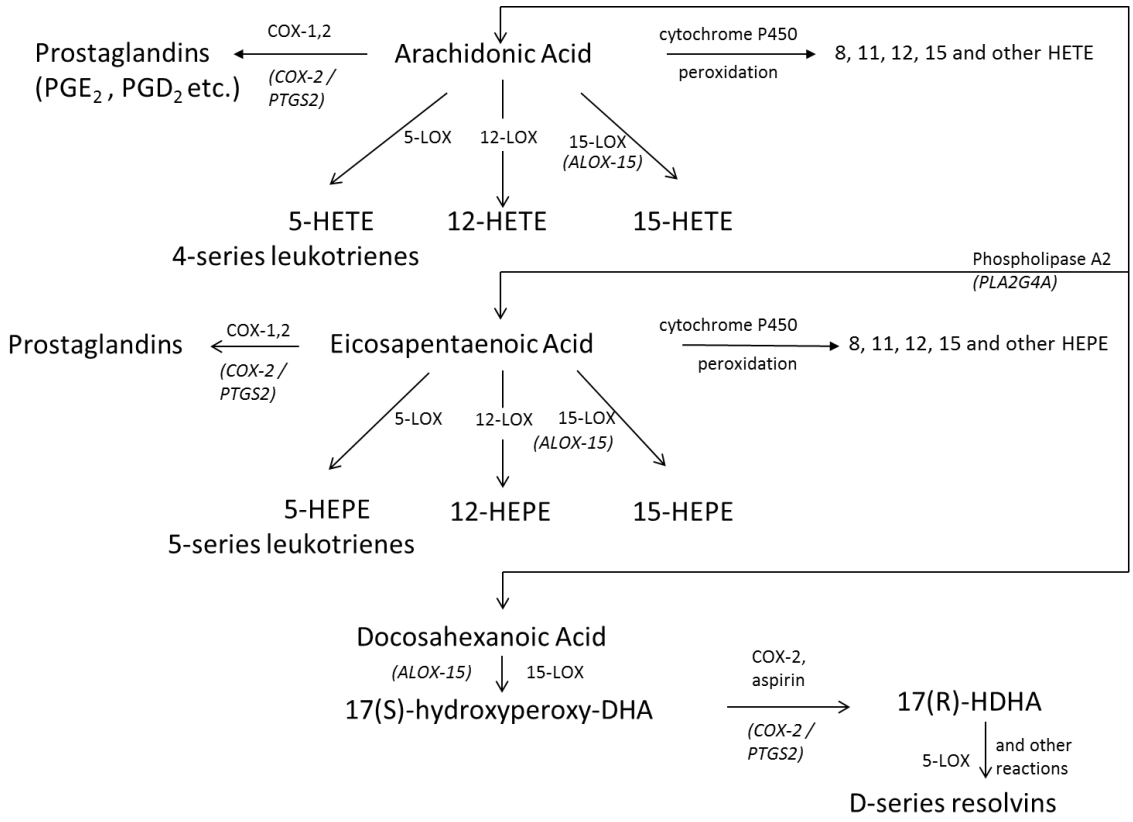


Fig. 1. Schematic overview of the enzymatic and non-enzymatic reactions mediating the production of lipid-derived modulators from AA, EPA and DHA. AA can be metabolized by COX enzymes to prostaglandins; through 5-LOX and 12/15-LOX enzymes into HETE and the 4-series leukotrienes, and by cytochrome P450 enzymes and peroxidation to HETE. EPA is metabolized similarly to HEPE compounds. DHA can be metabolized to 17-HDHA and subsequently to the D-series resolvins. Related genes measured in the study are indicated in parenthesis.

Lipid-derived immune modulators were extracted from plasma with solid phase extraction (SPE) using Strata-X (Phenomenex, Torrance, United States of America). The method was modified for Strata-X SPE columns from a previously described method [22]. Briefly, plasma samples (1 ml) were diluted with water and adjusted to 15% methanol (v/v), to a final volume of 4.6 ml. Internal standards, PGB_2-d4 and 12-HETE-d8 (500 pg of each), were added to each sample. The samples were incubated on ice for 30 min and then centrifuged at 3000 rpm for 8 min to remove any precipitated

proteins. The resulting clear supernatants were acidified with 1N hydrochloric acid to pH 3.0 and immediately applied to SPE cartridges that had been preconditioned with 2 ml methanol followed by 4 ml water. The cartridges were then washed with 10 ml 15% (v/v) methanol, 10 ml water, and 4 ml hexane in succession. Lastly, the lipid-derived immune modulators were eluted with 6 ml methyl formate. A vacuum manifold (Phenomenex) was used to perform the SPE and the vacuum was regulated that single drops could be seen from each cartridge. The methyl formate was evaporated under a fine stream of nitrogen and the residue was dissolved in 20 µl acetonitrile, flushed with nitrogen and stored, at the most, for four days at –20°C before LCMSMS analysis.

Samples were analysed with an Agilent Technologies 6410 MSMS, coupled to an Infinity 1260 HPLC pump (Santa Clara, United States of America). The instrument was operated in negative electrospray ionisation mode. At least two transitions were monitored for each compound, using a dynamic multiple reaction monitoring (MRM) method. The collision and fragmentor voltages were optimized with flow injection analysis. The gas temperature and flow was set at 350°C and 12 l/min respectively, and the capillary voltage at 4000 V. Chromatographic analysis was performed on a C18 column (Poroshell, 2.7µ, 100 x 2.1 mm; Agilent Technologies) with a flow rate of 0.4 ml/min and column temperature of 50°C. Sample injections were performed with an Agilent G1367B autosampler. The sample chamber temperature was set at 5°C and the injection volume was 15 µl, subsequent to mixing 5 µl of sample (in acetonitrile) with 10 µl water in the autosampler, just before injection. The analysis was performed using an acetonitrile-based system with a flow program, by mixing two solvents (A and B) with

programmed ratio changes for optimal separation of compounds. Solvent A was water/glacial acetic acid, 99:1 (v/v) and solvent B was 100% acetonitrile.

Data were quantified with Masshunter B05 02, using external calibration for each compound and two internal standards to correct for losses and matrix effects during sample preparation and analysis.

2.4. Assessment of targeted gene expression effects

PBMC gene expression analyses were planned in a randomly selected subgroup (n = 60), but due to low RNA yield in 29 samples and low qPCR quality of two samples, either in the baseline or endpoint sample, 31 subjects' samples had to be excluded from the analysis. Finally, quantitative real-time PCR (qPCR) was performed on samples from 29 children, with n = 3 in the iron plus placebo; n = 5 in the DHA/EPA plus placebo; n = 13 in the iron plus DHA/EPA and n = 8 in the placebo plus placebo groups, respectively (Table 3). The sample sizes per group were relatively small, especially for the iron plus placebo group, but when analysed in a two-by-two design (iron verum vs iron placebo and DHA/EPA verum vs DHA/EPA placebo), the sample size compared well to the sample sizes of similar intervention studies (16 vs 13 and 18 vs 11, respectively). For example, Bakker *et al*. found an effect of an anti-inflammatory dietary mix intervention with a sample size of 16 per group in PBMC with a nutrigenomics approach (lipidomics and transcriptomics) [23].

In the current study, PBMC were used to examine the effect of the supplementation regime on genes involved in, 1) lipid mediator synthesis and inflammatory response;

arachidonate lipoxygenase 15 *(ALOX-15)*, cyclooxygenase-2 *(COX-2 / PTGS2)*, glutathione peroxidase 4 *(GPx-4)*, and cytosolic phospholipase A2 group IVA *(PLA2G4A),* 2) oxidative stress response; glutathione peroxidase 4 *(GPx-4)* – also involved in lipid-mediator synthesis, superoxide dismutase 2 *(SOD2)*, metallothionein 1E *(MT1E)* and transcriptional co-activator peroxisome proliferator-activated receptor-γ coactivator-1 α *(PGC-1α)* and 3) iron regulation; the ubiquitously expressed solute carrier family 11A2 *(SLC11A2)*, also termed natural resistance to infection with intracellular pathogens *(NRAMP2)*; coding for divalent metal transporter 1 (DMT1), both isoforms – with and without the iron response element (IRE+ and IRE–).

Total RNA was isolated with the RNeasy® mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol and additional on-column DNase digestion was performed. The yield, quality and integrity of the total RNA preparations were assessed according to the integrity of 28 S rRNA and 18 S rRNA, as well as the RNA integrity number (RIN) values, using a NanoDrop 1000 Spectrophotometer and Agilent Bioanalyser 2100. cDNA was prepared with the QuantiTect® Whole Transcriptome kit from Qiagen (Gibco, Gaithersburg, USA) according to manufacturer's instructions.

QPCR analyses were performed in triplicate for each sample to analyse the change in gene expression over the 8.5 mo intervention. The following TaqMan® (Thermo Fisher, Waltham, United States of America) hydrolysis probe chemistry based gene expression assays were selected: *ALOX-15*, Hs00609608_m1; *COX-2,* Hs00153133_m1*; PLA2G4A*, Hs00233352_m1; *GPx-4,* Hs00157812_m1*; SOD2*, Hs00167309_m1; *MT1E,* Hs01938284_g1 and *PGC-1α,* Hs01016719_m1. The assays for *SLC11A2 IRE+* (forward primer, 5' catcagagccagtgtgtttctatgg '3; reverse primer, 5'

ccaaccaacggttgagtcataaac ’3, probe, 5’gtatgtgccgttgcacagactgt’3) and *SLC11A2* IRE– (forward primer, 5’ gggaagggtgttcaaaactgac ’3, reverse primer, 5’gccaatcgtttaactctgggag’3, probe, 5’acccacccacctcataacagtcat’3) were custom designed, spanning exon junctions, to avoid amplification of potential traces of genomic DNA. Quantification cycle (Cq) values were determined for each sample using the comparative Cq method and relative gene expression ratios were normalised to the arrhythmic mean of the reference genes, large ribosomal protein (*RPLPO*, Hs_99999902_m1) and ß-actin (endogenous control assay, non-primer limited). Relative gene expression changes between baseline and endpoint were calculated according to the $2^{-\Delta\Delta Ct}$ method [24, 25].

2.5. Molecular and functional network analysis

Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com, build version 321501M, content version 21249400), was used to investigate the molecular relationships and functional network interactions associated with the biochemical parameters measured in the study. Twenty eight of the total of 34 molecules uploaded for functional analysis were found in the IPA knowledgebase: Eight immune modulators (17-HDHA, 5-, 8-, 11-, 12- and 15-HETE, PGE_2, PGD_2 [Table 2]), the nine genes investigated (Table 3) , as well as PBMC DHA, EPA and AA [11]; the following iron parameters, namely haemoglobin (Hb), transferrin receptor, ferritin (the light and heavy chain), zinc protoporphyrin (ZnPP) and total body iron (two ID’s, but analysed as one unit); and c-reactive protein [19]. It is important to note that six

molecules, 5- and 12-HEPE, n-3 PUFA, n-6 PUFA, n-3 LCPUFA and n-6 LCPUFA were not recognised in the IPA knowledgebase and therefore not included in the functional and prediction analyses. Also noteworthy is that SLC11A2/IRE+ and SLC11A2/IRE- could not be distinguished by the knowledge base. The analysis were set to include mapped IPA molecules and endogenous chemicals experimentally observed to have direct as well as indirect knowledge based relationships in *H. sapiens*. Additionally, a targeted approach was followed and a combined interaction network for AA, DHA, EPA, iron and reactive oxygen species (ROS) was compiled to further inspect and characterise the intervention effects. The interaction network was modified to include those molecules that showed direct as well as indirect downstream effects with iron and DHA/EPA intervention, alone or in combination. The modified interaction network was separately overlaid with the intervention effects (B values) of iron and DHA/EPA, alone or in combination, without taking significance into consideration. The predicted activation and inhibition character of the overlaid interaction network were independently determined for each intervention.

2.6. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics (version 21; IBM Corporation). Results are expressed as means ± SD when data were distributed parametrically and as medians and range, for non-parametric data. Data and residuals were examined for normality with P-P plots and the Shapiro-Wilk test.

Differences in baseline characteristics between the subgroup for PBMC gene expression analysis, the subgroup in which plasma lipid-derived mediator analysis was performed, and the total group were examined with independent *t*-tests for continuous variables and with the chi-square test for categorical variables. Furthermore, between-intervention group differences were examined within the total group and each subgroup by using ANOVA for continuous variables and with the chi-square test with Bonferroni correction for categorical variables. A per-protocol approach was followed for analysing intervention effects.

Estimated intervention effects of iron and of DHA/EPA and their interaction on endpoint lipid-derived immune modulators and relative gene expression (Log2 ratio of each sample to its own group of the relative difference in expression between baseline and endpoint (ΔΔCT), normalised to the arithmetic mean ΔCT of the two housekeeping genes), were analyzed with two-way ANCOVA. Baseline concentrations (for lipid-derived immune modulators), sex, school and grade were used as individual level covariates. Non-parametric lipid-derived immune modulator data were transformed before performing the ANCOVA. Between-group differences for lipid-derived immune modulators were analysed with ANCOVA and the least significant difference post hoc test, adjusted for respective baseline values, sex, school and grade. The level of significance was set at a p-value less than 0.05.

3. Results

There were no significant differences in baseline characteristics between the total group (n = 296), and the subgroups analysed for PBMC gene expression (n = 29) and plasma lipid-derived immune modulator concentrations (n = 137) (Table 1). However, baseline Hb was higher in the iron plus DHA/EPA group than in the placebo plus placebo group within the lipid-derived immune modulator subgroup (P = 0.024). Furthermore, the prevalence of ID based on ZnPP was higher (P = 0.020) in the iron plus placebo group than the other three treatment groups within the gene expression subgroup.

DHA/EPA supplementation significantly decreased plasma 12-HETE (P = 0.033) and tended to increase 17-HDHA (P = 0.054) (Table 2). Iron supplementation tended to decrease plasma 17-HDHA (P = 0.062) and 5-HEPE (P = 0.063). Furthermore, plasma 17-HDHA in the DHA/EPA plus placebo (P < 0.001) and DHA plus iron (P = 0.022) groups was significantly higher than in the iron plus placebo group at endpoint. Likewise, 12-HEPE was significantly higher in the DHA/EPA plus placebo group than in the iron plus placebo group (P = 0.027) at endpoint. The pro-inflammatory 5- (P = 0.026) and 15-HETE (P = 0.047) were significantly lower in the iron plus DHA/EPA than in the placebo plus placebo group at endpoint. Furthermore, 12- HETE was significantly lower in the DHA/EPA plus placebo (P = 0.033) group than in the placebo plus placebo group.

Table 1: Baseline characteristics of all children and of the subgroups studied for PBMC gene expression and plasma lipid-derived immune modulator analyses[1]

	All children (*n* = 236–322)	Gene expression (PBMC) (n = 21–29)	Lipid-derived immune modulators (plasma) (n = 99–137)
Age (y)	8.9 ± 1.3[a]	9.0 ± 1.3	9.0 ± 1.4
Ratio male:female (%)	51:49	45:55	48:52
Height (m)	1.28 ± 0.09	1.29 ± 0.09	1.29 ± 0.09
Weight (kg)	27.8 (17.9–48.1)[b]	28.3 (18.9–48.1)	28.0 (18.9–48.1)
Anthropometric indices [*n* (%)]			
Stunting (HAZ < –2SD)	18 (5.8)	2 (7.1)	10 (7.3)
Mildly stunted (HAZ < –1SD ≥ –2SD)	99 (32.1)	8 (28.6)	45 (32.9)
Underweight (WAZ < –2SD)	7 (2.9)	0 (0.0)	3 (3.0)
Overweight (BAZ > 1SD < 2SD)	64 (21.1)	8 (28.6)	29 (21.5)
Obese (BAZ ≥ 2SD)	21 (6.9)	3 (10.7)	8 (5.9)
Blood haemoglobin (g/dL)[c]	12.1 ± 0.8	12.0 ± 0.8	12.1 ± 0.8
Serum ferritin (µg/L)[d]	19.1 (3.1–73.1)	19.8 (5.2–62.5)	19 (3.8–73.1)
Serum transferrin receptor (mg/L)	5.7 (2.4–11.8)	5.2 (3.4–9.4)	5.6 (2.5–11.8)
Body iron stores (mg/kg)	3.0 ± 2.5	3.4 ± 2.6	3.0 ± 2.5
Zinc protoporphyrin (µmol/mol haem)	75.0 (33.0–215.0)	75.0 (47.0–162.0)	74 (33–171)
C-reactive protein (mg/L)	0.4 (0.0–17.8)	0.5 (0.0–6.4)	0.4 (0.0–17.8)
Serum zinc (µg/L)	73.3 (45.8–106.2)	68.7 (51.3–102.4)	76.6 (54.3–106.2)
Deficiencies [*n* (%)]			
Anemia (Hb < 11.5 g/dL)	65 (20.6)	6 (21.4)	25 (18.2)
Iron deficiency based on SF (<15.0 µg/L)	78 (28.1)	6 (26.1)	31 (27.0)
Iron deficiency based on TfR (>8.3 mg/L)	36 (11.3)	3 (10.7)	18 (13.2)
Iron deficiency based on ZnPP (>70 µmol/mol haem)[e]	197 (62.7)	17 (58.6)	81 (60)
Shortage of body iron (negative values)[f]	33 (12.0)	3 (13.6)	16 (14.0)
Iron deficiency anemia (Hb < 11.5 g/dL and SF < 15 µg/L)	25 (9.2)	3 (14.3)	8 (7.0)
Zinc deficiency (SZn < 65 µg/L)	70 (24.8)	11 (40.7)	26 (22.2)
Acute-phase protein [*n* (%)]			
C-reactive protein (>5 mg/L)	22 (7.2)	1 (4.2)	11 (8.5)

Abbreviations: HAZ, height-for-age ƺ score; WAZ, weight-for-age ƺ score; BAZ, BMI-for-age ƺ score; Hb, haemoglobin; SF, serum ferritin; TfR, transferrin receptor; ZnPP, Zinc protoporphyrin.

[1]Differences between all children and the gene expression and lipid-derived immune modulator subgroups were examined by using independent *t* tests for continuous variables and by using the chi-square test for categorical variables ($P<0.05$).

[a] Mean ± SD (all such values).

[b] Median; minimum to maximum in parentheses (all such values).

[c] Hb differed between the iron plus DHA/EPA and the placebo plus placebo groups within the lipid-derived immune modulator group ($P = 0.024$).

[d] Serum ferritin values of all children with CRP > 5 mg/L were excluded.

[e] The prevalence of ID based on ZnPP was higher ($P = 0.020$) in the iron-supplemented children within the gene expression group.

[f] Total body iron was calculated on the basis of Cook et al. [47].

Table 2

Effects of intervention with iron and DHA/EPA, alone and in combination, on plasma lipid-derived immune modulators over 8.5 months.

	Group				Estimated intervention effect[1]		
	Iron + Placebo (*n* = 38)	DHA/EPA + Placebo (*n* = 33)	Iron + DHA/EPA (*n* =37)	Placebo + Placebo (*n* = 29)	Iron	DHA/EPA	Iron x DHA/EPA
17-HDHA (pg/µl)							
Baseline	1.11 (0.00–7.62)[2]	1.14 (0.03–4.68)	1.24 (0.00–7.64)	1.42 (0.00–6.94)	–0.113 (–0.231, 0.006)[3]	0.120 (–0.002, 0.243)	–0.011 (–0.158, 0.181)
Endpoint	0.52 (0.00–23.85)[b]	2.73 (0.00–13.01)[a]	1.63 (0.05–17.44)[a]	1.51 (0.06–19.30)[a,b]			
5-HEPE (pg/µl)							
Baseline	0.67 (0.20–2.34)	0.79 (0.11–6.21)	0.77 (0.25–2.81)	0.62 (0.15–2.36)	–0.158 (–0.325, 0.009)	0.062 (–0.111, 0.235)	0.035 (–0.202, 0.272)
Endpoint	0.63 (0.19–3.32)	1.12 (0.27–12.40)	1.02 (0.12–8.53)	1.03 (0.32–17.92)			
12-HEPE (pg/µl)							
Baseline	0.22 (0.00–1.89)	0.16 (0.00–5.07)	0.33 (0.00–11.91)	0.28 (0.00–2.29)	–0.084 (–0.406, 0.237)	0.265 (–0.064, 0.594)	–0.006 (–0.450, 0.437)
Endpoint	0.17 (0.00–5.39)[b]	0.52 (0.00–8.65)[a]	0.56 (0.00–8.68)[a,b]	0.50 (0.04–6.06)[a,b]			
5-HETE (pg/µl)							
Baseline	3.95 (1.61–13.46)	4.57 (1.53–17.30)	4.86 (2.04–11.11)	4.50 (1.53–10.85)	–0.039 (–0.144, 0.065)	–0.076 (–0.184, 0.031)	–0.004 (–0.151, 0.143)
Endpoint	3.70 (1.71–14.84)[a,b]	3.60 (1.16–13.67)[a,b]	3.66 (1.40–26.21)[b]	4.94 (1.35–29.75)[a]			
8-HETE (pg/µl)							
Baseline	1.00 (0.24–3.44)	0.93 (0.29–3.60)	0.87 (0.43–4.25)	1.06 (0.36–3.72)	–0.046 (–0.159, 0.067)	–0.053 (–0.170, 0.064)	0.026 (–0.135, 0.186)
Endpoint	0.76 (0.28–5.60)	0.74 (0.26–3.17)	0.76 (0.33–5.64)	1.13 (0.30–5.21)			
11-HETE (pg/µl)							
Baseline	1.43 (0.83–3.36)	1.41 (0.84–3.78)	1.32 (0.93–3.38)	1.43 (0.86–3.21)	–0.043 (–0.118, 0.031)	–0.045 (–0.122, 0.032)	0.031 (–0.075, 0.138)
Endpoint	1.23 (0.83–6.38)	1.33 (0.84–3.47)	1.37 (0.79–4.61)	1.60 (0.86–4.52)			
12-HETE (pg/µl)							
Baseline	14.17 (1.17–102.49)	12.91 (1.92–78.76)	14.20 (1.93–101.93)	12.80 (1.72–89.14)	–0.105 (–0.285, 0.075)	–0.209 (–0.400,–0.017)	0.213 (–0.047, 0.474)
Endpoint	13.07 (2.12–145.06)[a,b]	8.58 (2.10–74.21)[b]	13.88 (2.99–95.13)[a,b]	22.34 (3.54–73.06)[a]			
15-HETE							
Baseline	5.13 (0.80, 19.26)	4.67 (0.95, 17.77)	4.20 (1.37, 12.95)	5.61 (0.90, 22.57)	–0.092, (–0.238, 0.053)	–0.126, –0.276, 0.025	0.070 (–0.136, 0.275)
Endpoint	3.57 (0.90, 39.21)[a,b]	3.39 (0.68, 17.03)[a,b]	3.66 (0.75, 25.08)[b]	7.04 (0.90, 26.50)[a]			
PGE_2 (pg/µl)							
Baseline	1.85 (0.52–14.58)	2.98 (0.68–20.96)	1.85 (0.32–18.54)	2.94 (0.55–30.71)	–0.038 (–0.186, 0.109)	–0.008 (–0.160, 0.144)	–0.005 (–0.213, 0.204)
Endpoint	2.09 (0.73–25.20)	2.54 (0.56–20.04)	2.22 (0.45–33.98)	2.34 (0.71–11.17)			
PGD_2 (pg/µl)							
Baseline	0.74 (0.00–7.36)	1.22 (0.21–8.32)	0.68 (0.18–5.17)	0.71 (0.07–9.64)	–0.008 (–0.155, 0.139)	–0.053 (–0.204, 0.099)	0.039 (–0.172, 0.249)
Endpoint	0.96 (0.26–6.11)	1.08 (0.14–8.42)	0.82 (0.14–9.36)	0.91 (0.19–5.28)			

Abbreviations: DHA/EPA; mixture of docosahexaenoic and eicosapentaenoic acid; HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxytetraenoic acid; PG, prostaglandin. [1] Intervention effects estimated by two-way ANCOVA adjusted for respective baseline values, sex, school and grade. All compounds were log transformed before performing the two-way ANCOVA. Between-group differences were analysed with one-way ANCOVA and least significant difference post hoc test adjusted for respective baseline values, sex, school and grade. Values in one row with different letters differ significantly. [2] Median; minimum to maximum in parentheses (all such values). [3] B value; 95% CI in parenthesis (all such values).

There were no main effects or significant interactions on the expression of the targeted genes (Table 3). However, *SOD2* expression tended to be lower ($P = 0.079$) in the children who received iron (iron verum) when compared to the children not receiving iron (iron placebo). In addition, *COX-2* (*PTGS2*) and *ALOX-15* were both non-significantly down regulated by the individual supplementations, iron ($P = 0.461$ and $P = 0.171$, respectively) and DHA/EPA ($P = 0.363$ and $P = 0.692$, respectively), but were non-significantly up regulated by the combination of iron and DHA/EPA ($P = 0.132$ and $P = 0.161$).

Knowledge based untargeted functional analysis of 28 molecules (genes and endogenous chemicals) suggested a significant association ($P = 1 \times 10^{-39}$) with a predicted functional network of free radical scavenging, lipid metabolism and small molecule biochemistry (Table 4). Eighteen molecules were associated with free radical scavenging ($P = 8.31 \times 10^{-19} – 2.56 \times 10^{-4}$), 17 with lipid metabolism ($P = 1.23 \times 10^{-12} – 1.16 \times 10^{-3}$) and 20 with the small molecule biochemistry network ($P = 1.23 \times 10^{-12} – 1.42 \times 10^{-3}$).

Table 3

Effects of intervention with iron and DHA/EPA, alone and in combination, on gene expression of peripheral blood mononuclear cells over 8.5 months

	Group				Estimated intervention effect[1]		
	Iron + placebo (*n* = 1–3)	DHA/EPA + Placebo (*n* = 2–5)	Iron + DHA/EPA (*n* = 8–13)	Placebo + Placebo (*n* = 7–8)	Iron	DHA/EPA	Iron x DHA/EPA
COX-2 (PTGES2)	–8.01 (4.02)[2]	–3.98 (1.25)	–2.05 (0.70)	–4.56 (2.43)	–2.051 (–7.956, 3.855)[3]	–2.744 (–9.109, 3.621)	5.991 (–2.120, 14.101)
ALOX-15	–2.83 (1.36)	–2.30 (0.57)	–0.38 (3.06)	1.48 (2.33)	–6.068 (–15.151, 3.016)	–1.446 (–9.206, 6.314)	8.843 (–4.041, 21.726)
GPx-4	2.3	–0.66 (0.74)	–2.23 (0.63)	–0.43 (1.56)	–0.331 (–7.247, 6.585)	–0.456 (–4.500, 3.587)	–2.310 (–10.312, 5.691)
PLA2G4A	–1.45 (1.85)	–2.77 (2.00)	–3.08 (1.56)	–2.91 (1.22)	2.048 (–5.733, 9.830)	–0.236 (–6.785, 6.313)	–2.160 (–12.100, 7.780)
MT1E	–0.72 (1.17)	–1.95 (1.50)	1.25 (1.20)	2.35 (2.87)	–3.055 (–11.287, 5.177)	–4.554 (–11.616, 2.508)	5.072 (–5.888, 16.031)
SOD2	–3.21 (3.26)	1.02 (1.17)	–3.17 (1.79)	–0.32 (1.44)	–1.249 (–8.268, 5.771)	0.217 (–6.215, 6.648)	–2.197 (–11.292, 6.898)
PGC-1α	–2.72 (3.30)	–4.73 (4.36)	–1.04 (2.26)	–3.64 (2.57)	–0.425 (–10.510, 9.661)	–1.086 (–9.577, 7.405)	2.286 (–10.634, 15.207)
SLC2A11/IRE+	–4.10 (2.56)	3.71 (3.20)	–0.88 (1.31)	–1.48 (0.90)	–0.198 (–8.283, 4.446)	3.998 (–2.603, 10.599)	–2.516 (–12.938, 7.906)
SLC2A11/IRE–	–1.41 (2.80)	0.13 (2.11)	–2.48 (1.66)	–3.03 (1.88)	2.089 (–6.208, 10.386)	1.186 (–6.881, 9.253)	–3.009 (–13.877, 7.859)

Abbreviations: EPA/DHA a mixture of eicosapentaenoic and docosahexaenoic acid; *ALOX-15*, arachidonate lipoxygenase-15; *COX-2*, cyclooxygenase-2; *GPx-4*, glutathione peroxidase-4; *MT1E,* metallothionein 1E; *PGC-1α,* transcriptional co-activator peroxisome proliferator-activated receptor-γ coactivator-1 α; *PLA2G4A,* cytosolic phospholipase 2 group IVA; *PTGS2* prostaglandin-endoperoxide synthase 2*, SLC11A2 IRE+,* solute carrier family 11A2 with iron response element, *SLC11A2 IRE–,* solute carrier family 11A2 without iron response element and *SOD2,* superoxide dismutase 2.

[1] Intervention effects estimated by two-way ANCOVA adjusted for respective baseline values, sex, school and grade. Housekeeping gene-adjusted data were analysed using the ΔΔCT method [25], using the Log2 ratio of each sample ΔΔCT to the mean ΔΔCT of each group of the relative endpoint to baseline expression (ΔΔCT) for ANCOVA, and expressed as fold change (baseline to endpoint) values.

[2] Mean (SEM) (all such values). [3] B value; 95% CI in parenthesis (all such values).

Table 4
Associated functional networks

Functional networks	p-Value		Molecules	Number of molecules
Free radical scavenging	8.31E-19	2.56E-04	12-hydroxyeicosatetraenoic acid,15(S)-HETE,5-hydroxyeicosatetraenoic acid,arachidonic acid,CRP,docosahexaenoic acid,eicosapentenoic acid,FTH1,FTL,GPX4,HBA1/HBA2,iron,PLA2G4A,PPARGC1A,prostaglandin E2,PTGS2,SOD2,TFRC	18
Inflammatory response	1.39E-07	1.38E-03	arachidonic acid,prostaglandin E2,12-hydroxyeicosatetraenoic acid,ALOX15,docosahexaenoic acid,CRP,5-hydroxyeicosatetraenoic acid,prostaglandin D2,PLA2G4A,eicosapentenoic acid,SOD2,PPARGC1A,GPX4,PTGS2	14
Lipid metabolism	1.23E-12	1.16E-03	12-hydroxyeicosatetraenoic acid,15(S)-HETE,ALOX15,arachidonic acid,docosahexaenoic acid,PLA2G4A,eicosapentenoic acid,prostaglandin D2,GPX4,PTGS2,iron,PPARGC1A,prostaglandin E2,zinc protoporphyrin IX,SOD2,CRP,FTL	17
Small molecule biochemistry	1.23E-12	1.42E-03	15(S)-HETE,12-hydroxyeicosatetraenoic acid,arachidonic acid,ALOX15,PLA2G4A,GPX4,eicosapentenoic acid,prostaglandin D2,docosahexaenoic acid,FTH1,iron,FTL,PTGS2,prostaglandin E2,zinc protoporphyrin IX,TFRC,PPARGC1A,HBA1/HBA2,CRP,SOD2	20

The top five predicted canonical pathways (Table 5) were identified as eicosanoid signalling (8/81, P = 9.95 x 10^{-15}), prostanoid biosynthesis (4/15, P = 1.17 x 10^{-9}), macrophage migration inhibitory factor (MIF)-mediated glucocorticoid regulation (4/35, P = 4.43 x 10^{-8}), MIF-regulation of innate immunity (4/44, P = 1.14 x 10^{-7}) and the role of mitogen-activated protein kinase (MAPK) signalling in the pathogenesis of influenza (4/71, P = 8.03 x 10^{-7}). Furthermore, 14 of the 22 molecules were involved in the inflammatory response (14/28, P = 1.39 x 10^{-7}– 1.38 x 10^{-3}).

Table 5
Canonical pathways associated with the 34 parameters investigated in the study.

Symbol	Entrez Gene Name	B-values	p-value	Human Entrez Gene ID for
Eicosanoid signalling				
12-hydroxyeicosatetraenoic acid	--	-0.054	2.49E-01	
15(S)-HETE	--	-0.092	2.12E-01	
ALOX15	arachidonate 15-lipoxygenase	-6.068	1.71E-01	246
arachidonic acid	--	-0.24	7.89E-01	
PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium-dependent)	2.048	5.86E-01	5321
prostaglandin D2	--	-0.008	9.18E-01	
prostaglandin E2	--	-0.038	6.08E-01	
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-2.051	4.61E-01	5743
Prostanoid biosynthesis				
arachidonic acid	--	-0.24	7.89E-01	
prostaglandin D2	--	-0.008	9.18E-01	
prostaglandin E2	--	-0.038	6.08E-01	
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-2.051	4.61E-01	5743
MIF-mediated glucocorticoid regulation				
arachidonic acid	--	-0.24	7.89E-01	
PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium-dependent)	2.048	5.86E-01	5321
prostaglandin E2	--	-0.038	6.08E-01	
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-2.051	4.61E-01	5743
MIF-mediated innate immunity				
arachidonic acid	--	-0.24	7.89E-01	
PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium-dependent)	2.048	5.86E-01	5321
prostaglandin E2	--	-0.038	6.08E-01	
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-2.051	4.61E-01	5743
Role of MAPK signalling in the pathogenesis of influenza				
arachidonic acid	--	-0.24	7.89E-01	
PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium-dependent)	2.048	5.86E-01	5321
prostaglandin E2	--	-0.038	6.08E-01	
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-2.051	4.61E-01	5743

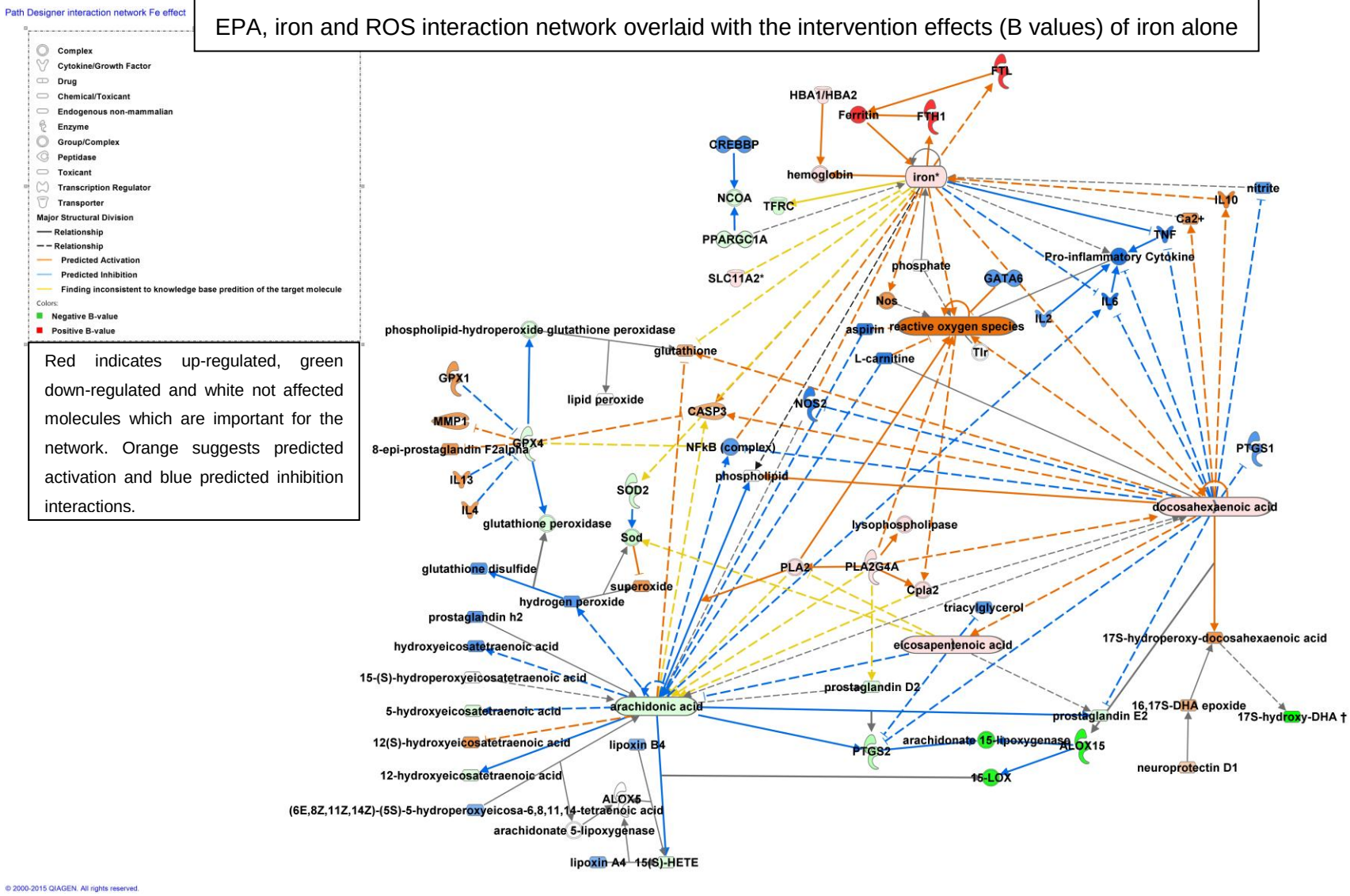
The proposed interaction network for DHA, EPA, AA, iron and ROS, overlaid with the intervention effects (B-values) for iron, reflected an increase of total body iron, an increase in PBMC DHA and a decrease in PBMC AA (Supplementary fig. 2A). These represent real values uploaded into IPA, but were all non-significant changes [11, 19].

Notably, an activation response for ROS production and Caspase 3 (CASP3) were predicted.

The DHA/EPA intervention effect overlay character (Supplementary fig. 2B) was generally similar to iron's, as also seen in Table 2. It must however be taken into consideration that the anti-inflammatory 5- and 12-HEPE (Table 2) were not recognised in the IPA knowledge base and could therefore not be included in the analysis. Nevertheless, the prediction analyses still showed induction of *CASP3* irrespective of a weaker activation for ROS production.

The combined intervention effects of iron and DHA/EPA evident in supplementary figure 2C, are the down regulated *PLA2GA* expression, decreased PBMC DHA and decreased total body iron (actual, but non-significant B-values) [11, 19]. Furthermore, *ALOX-15* and *COX-2* (*PTGS2)* were up regulated (both were down-regulated by iron and DHA/EPA alone), as also apparent in Table 3 (actual, but non-significant B-values).

Fig. 2A Graphical representation and functional relationship predictions of the compiled AA, DHA, EPA, iron and ROS interaction network overlaid with the intervention effects (B values) of iron alone



Red indicates up-regulated, green down-regulated and white not affected molecules which are important for the network. Orange suggests predicted activation and blue predicted inhibition interactions.

Fig. 2B Graphical representation and functional relationship predictions of the compiled AA, DHA, EPA, iron and ROS interaction network overlaid with the intervention effects (B values) of DHA/EPA alone

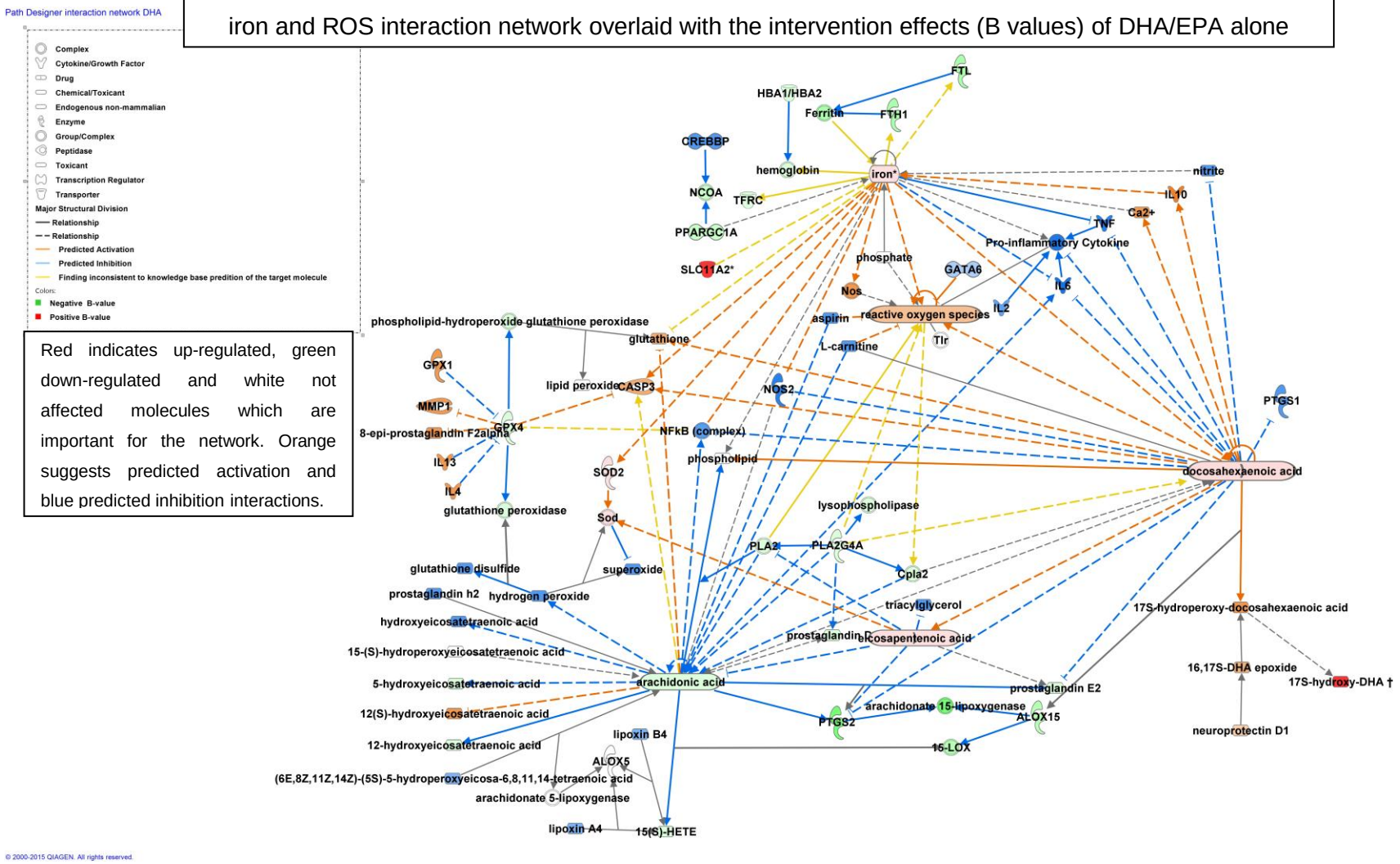
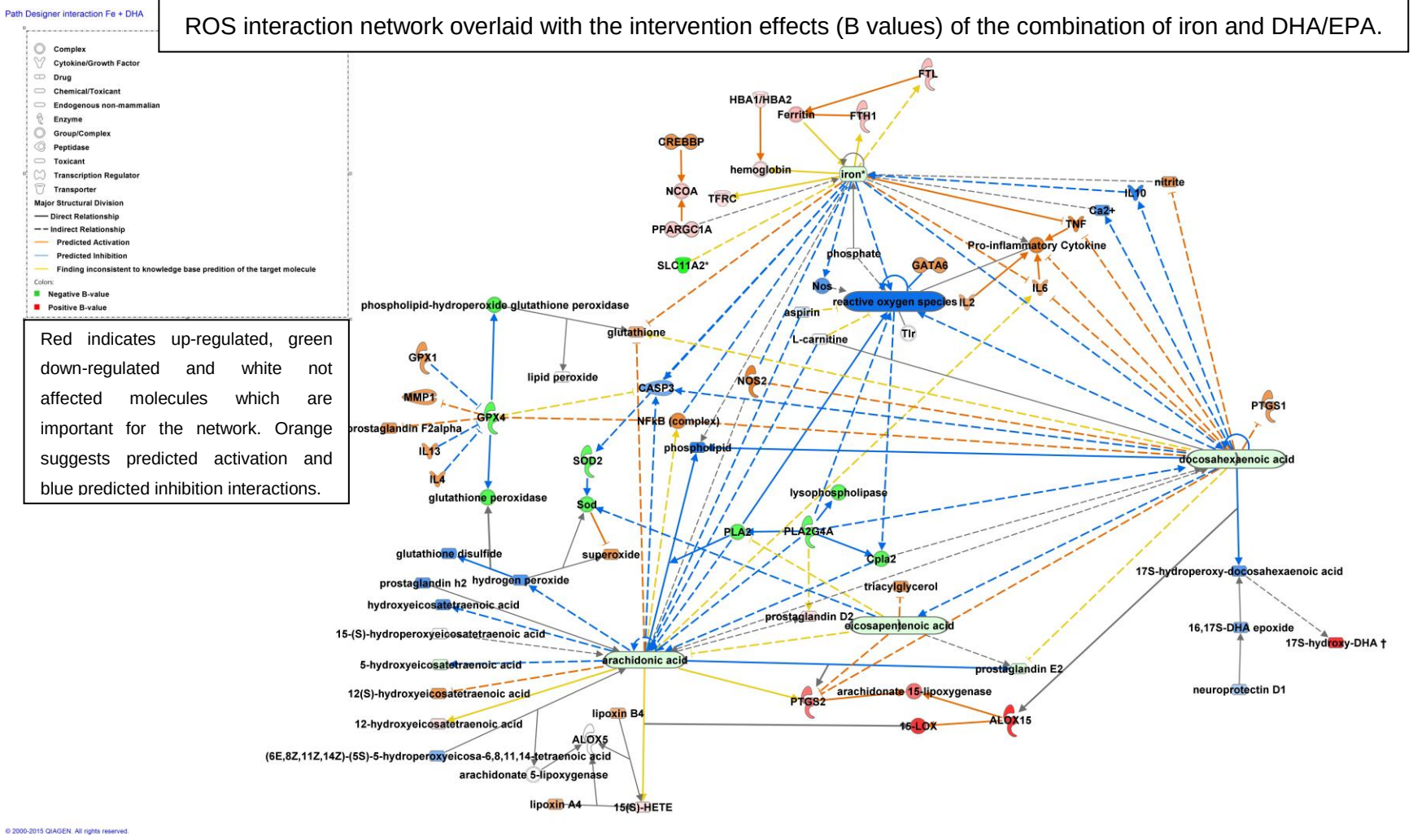


Fig. 2C Graphical representation and functional relationship predictions of the compiled AA, DHA, EPA, iron and ROS interaction network overlaid with the intervention effects (B values) of the combination of iron and DHA/EPA.



4. Discussion

The main finding of this study was that the lipid-derived immune modulator profile was altered to being more anti-inflammatory or pro-resolving by DHA/EPA and to being more pro-inflammatory by iron supplementation (Table 2). Furthermore, when combining DHA/EPA and iron supplementation, the pro-resolving effect of DHA/EPA was maintained (Table 2). These results, together with supportive gene expression and functional pathway analyses (Table 3 and supplementary fig. 2), may well explain the prevention of iron-induced respiratory morbidity that we observed with combined iron and DHA/EPA intervention during the 8.5 mo RCT.

In the present study, in agreement with others [20], but for the first time shown in young children, it was demonstrated that DHA/EPA supplementation altered the profile of lipid-derived immune modulators to being more anti-inflammatory or pro-resolving (Table 2) by decreasing the pro-inflammatory 12-HETE and tending to increase the pro-resolving 17-HDHA. 12-HETE has been shown to be involved in inflammatory processes, oxidative stress, and to be associated with cardiovascular disease, diabetes and hypertension [26]. In contrast, the anti-inflammatory D-series resolvins are produced from 17-HDHA, and have been demonstrated to exert several mechanisms to resolve localised inflammation [27]. On the other hand, iron tended to alter the profile to being more pro-inflammatory, by tending to decrease the concentrations of two anti-inflammatory mediators, 17-HDHA and 5-HEPE. The latter is the precursor molecule for the 5-series leukotrienes, known for their anti-inflammatory properties (Fig. 1) [2]. This novel finding contributes to our knowledge about the pro-inflammatory nature of iron,

implicating that even with this relatively moderate oral iron dose, there is an effect on circulating functional lipid-derived molecules, particularly only on the anti-inflammatory n-3 LCPUFA derived modulators. Notably though, iron alone did not increase the AA-derived pro-inflammatory HETE levels.

Remarkably, the sustaining effect of DHA/EPA towards 17-HDHA was maintained in the combined intervention group, despite the addition of iron (Table 2). In addition to DHA/EPA's recognised anti-inflammatory and anti-oxidative properties [28-30], probably curbing the opposite effects of excess iron [31], this finding might be further explained by directional changes in expression of certain genes, caused by interaction of the combined supplements. Both *AlOX-15* and *COX-2* were down regulated with each supplement alone, and up regulated by the combined intervention (Table 3). These enzymes are both involved in the synthesis of 17-HDHA (Fig.1). Thus, increased substrate (PBMC DHA) in the DHA/EPA plus placebo group likely led to increased 17-HDHA. Furthermore, although not significant, a directional change in expression was caused by the interaction between iron and DHA/EPA, producing an intervention effect change of *AlOX-15* and *COX-2* from negative to positive. This, even though PBMC DHA composition was not significantly increased by the combined intervention [11], likely contributed to the maintenance of the higher 17-HDHA in the DHA/EPA plus iron group.

The changes in the plasma lipid-derived immune modulator levels took place over an 8.5 mo time course, concurrent with increased respiratory morbidity by iron and the attenuation thereof by an interaction between iron and DHA/EPA [11]. Previous studies with DHA/EPA intervention in children have found improved respiratory morbidity [32-

35], whereas iron supplementation has had mixed results [9-11, 36]. We suspect that we did not find an anticipated improvement in overall infectious morbidity with DHA/EPA supplementation alone, as found by others, maybe because of the confounding effect of ID [11]. This highlights the importance of the potential implications if multiple nutrient deficiencies are not addressed together. The radically different predicted functional network results for the three different situations, for example the predicted differences in ROS and *CASP3* activation (Supplementary figure 2A, B and C), further emphasize the possible diverse effects of single and combined supplementation with these specific nutrients on molecular level.

Very recent studies have demonstrated improved morbidity outcomes in patients who received parenteral feeding which contained anti-inflammatory n-3 PUFA, as well as in critically ill patients who regularly used anti-inflammatory drugs [37-41]. Thus it is proposed that the failure of lipid class switching to producing more inflammation-resolving mediators towards the end of the response to infection, negatively affects immune competence of these patients [37, 39]. An extreme example is the case of traumatic insult, which is associated with hyper-inflammation (including oxidative stress), subsequently followed by an immunosuppressed state which increases susceptibility to infection [37]. A range of nutrients, including n-3 PUFA are able to curb inflammation (and the associated oxidative stress) and to maintain or improve immune function [37]. Since iron increased inflammation in these children, we therefore propose that it might have been this pro-resolving mechanism of DHA/EPA, which was maintained when combining the two supplements, that improved immune competence and morbidity outcome in the children studied [11]. This proposed mechanism is further

supported by the predicted reduction of ROS as well as *CASP3* in the functional pathway analysis (Fig 2 C) with combined supplementation. Oxidative stress is known to be closely associated with inflammation [30] and *CASP3* that codes for caspase 3, which is central in apoptosis and is, in turn, proposed to be triggered by oxidative stress [42]. Therefore, the reduction of inflammation and ROS may be one of the key mechanisms by which the immune competences in the children studied were improved to the level of preventing iron-induced respiratory morbidity [11].

Another key mechanism underlying the attenuation of iron-induced morbidity by DHA/EPA, also involves the potentially harmful effect of iron supplementation, but in relation to being more freely available for pathogens, resulting in increased infectious morbidity. The safety of iron supplementation has been questioned recently, as it increased morbidity and mortality, specifically in areas where malaria is prevalent [10, 43]. Therefore, the improvement of respiratory morbidity by supplementing DHA/EPA in combination with iron is a novel finding which might become important in future strategies for safer iron supplementation. Whether this effect was solely due to the reduction of inflammation or also related to improved cellular uptake of iron, was another purpose of this mechanistic investigation. Improved iron uptake mechanisms could, however, not be confirmed by the gene expression analysis in our study, probably limited by the low number of samples suitable for gene expression analysis. This lack of evidence for this mechanism investigated as a possible means of increased iron uptake and sequestering of iron by PBMC to decrease pathogen growth, might also have been due to this mechanism being specific to macrophages and the effect too diluted in total PBMC [44].

Functional pathway analysis predicted that iron may have increased ROS, whereas DHA/EPA attenuated this effect (Supplementary fig. 2). However, the combination of the two nutrients seemed to have had an even stronger antioxidant effect than DHA/EPA alone, as indicated by knowledge-based pathway predictions (Supplementary fig. 2). The particular conclusion around oxidative stress would, however, have been stronger if we had measured other oxidative stress markers than the free-radical-mediated oxidative stress products of AA, 8- and 12-HETE, which showed no significant increase with iron supplementation. Particularly F2-isoprostanes and 9-HETE, both also products of free radical oxidation of AA, have been shown to be sensitive markers of lipid peroxidation and the presence of oxidative stress [45].

The plasma levels of 17-HDHA, 5- and 12-HEPE as well as 5-, 8-, 11-, 12- and 15-HETE observed here were comparable to those reported in other similar intervention studies [20, 46], even though different populations were investigated. Dawczynski *et al*. and Mas *et al*. reported on mildly hypertriacylglycerolemic and healthy adults, respectively, whereas our results originate from children of African (Zulu) descent. In comparison with these two studies, our results are generally about 4 times higher, which might be due to differences in analytical sensitivity, age or ethnicity. Nevertheless, our aim was to investigate intervention effects, rather than absolute values.

The study was limited by the number of subjects that could be included in the gene expression analysis and because we could not measure additional important lipid-derived immune modulators, like resolvins, protectins and leukotrienes, which was below our equipment's detection or quantitation limits.

5. Conclusion

We found that iron and DHA/EPA supplementation resulted in an altered lipid-derived immune modulator profile to being more pro- or more anti-inflammatory, respectively. Moreover, the anti-inflammatory profile resulting from DHA/EPA supplementation was maintained when the supplementations were combined. These biochemical changes, together with supporting gene expression and functional pathway analyses, may explain the prevention of iron-induced respiratory morbidity that we observed when iron was supplemented in combination with DHA/EPA during the 8.5 mo RCT. This novel finding might lead to a promising approach to deliver iron supplementation more safely.

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Chapter 5 Manuscript 3

Allergic disease is associated with alterations in long-chain polyunsaturated and *trans*-fatty acid composition of peripheral blood mononuclear cells, red blood cells and plasma in rural South African school children

Running head: Allergy and fatty acid composition

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[4]*Abbreviations:* DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; LCPUFA, long-chain polyunsaturated fatty acids; PBMC, peripheral blood mononuclear cell

ABSTRACT

Background: Allergic disease has been linked to altered fatty acid status, such as low levels of long-chain polyunsaturated fatty acid (LCPUFA), a high n-6 to n-3 PUFA ratio and elevated *trans*-fat status.

Objective: Associations in rural South African children between allergic disease and the fatty acid composition of three lipid pools were investigated.

Methods: In this cross-sectional study of 321 children (6–11-years old), a subsample (n = 111) of children with allergy data were divided into an allergic (n = 28) and non-allergic (n = 75) group. Total phospholipid fatty acids in peripheral blood mononuclear cells (PBMC, n = 45), red blood cells (RBC, n = 103) and plasma (n = 87) were compared. Additionally, associations between total immunoglobulin E and fatty acid composition of these lipid pools were investigated in the total 321 children. The study was registered at clinicaltrials.gov as NCT01092377.

Results: RBC dihomo-gamma-linolenic acid (DGLA) (P=0.003) and the DGLA:LA ratio (p<0.001) were higher and PBMC arachidonic acid (AA) (P=0.009) and plasma DHA (P=0.041) lower in the allergic children. PBMC *trans*-C18:1n-9 tended to be higher (P=0.073) in the allergic group and correlated negatively (P<0.001) with AA.

Conclusion and clinical relevance: Fatty acid composition indicative of impaired delta-5 desaturase (D5D) activity, lower plasma DHA and higher PBMC membrane *trans*-fat are associated with allergy in these children.

Keywords: Allergic disease, omega-3 fatty acids, *trans*-fat, arachidonic acid, PBMC

1. Introduction

Allergic disease, including rhinitis, asthma and eczema, is an increasing health problem worldwide, both in industrialized and developing countries [1, 2]. It is estimated that approximately 500 million people worldwide and over 30 million in Africa have allergic rhinitis and that about 200 million also have asthma as a co-morbidity [3]. In South Africa, the third phase of the International Study on Asthma and Allergy in Childhood (ISAAC III) found a prevalence for allergic rhinoconjuntivitis (rhinitis with itching and watery eyes), asthma and eczema of 18–20% in 13–14-year olds [4].

It has been hypothesized that the rise in allergic disease could be due to changes in diet [5]. One of the leading diet hypotheses relates to the changes in intake of polyunsaturated fatty acids (PUFA) over recent decades. In particular a higher intake of n-6 PUFA [6] and lower relative intake of n-3 PUFA is considered to be an important contributing factor [7, 8]. There is some mechanistic evidence explaining how the n-6:n-3 PUFA ratio could be causally related to the development of allergic disease [9-11]. Some arachidonic acid (AA; 20:4n-6) derived mediators like prostaglandin E_2 affect differentiation of T and B cells to promote a pro-allergic environment [12]. Conversely, the docosahexaenoic acid (DHA; C22:6n-3) derived anti-inflammatory mediator, protectin D1 (PD1), was found to be decreased in exhaled breath condensates of patients with exacerbated asthma compared to healthy subjects [11]. Furthermore, DHA derived 17-hydroxydocosahexaenoic acid and resolvin D1 (RvD1) strongly reduced the production of immunoglobulin E (IgE), which is elevated in allergic disease, in human B

cells [9]. Thus there is evidence supporting opposing actions of long chain (LC) n-6 and LC n-3 PUFA in allergic disease.

In contrast, some studies also report that lower AA status is associated with allergic disease [13], and intervention with a combined DHA- and AA-supplemented formula delayed onset and reduced incidence of allergic disease in children up to three years of age [14]. Furthermore, it is hypothesized that impaired desaturation, therefore reducing both n-6 and n-3 LCPUFA status, might be associated with allergy [15, 16].

In addition to influences of PUFA, epidemiological studies have suggested that the intake of unnatural *trans*-fatty acids, such as elaidic acid (*trans*-18:1n-9), could be involved in allergic disease [17, 18]. *Trans*-fatty acids are produced by partial hydrogenation of unsaturated fatty acids and were used commonly in margarines and baked goods until recently [19]. Most countries, including South Africa, have now put legislation into place to reduce greatly and soon forbid the use of these fats due to evidence of various detrimental health effects [19-21].

The relation between fatty acid status and allergic disease is poorly explored in African populations. Therefore, the aim of this study was to determine the prevalence of allergic disease as well as to identify any differences between PUFA and *trans*-fatty acid composition in peripheral blood mononuclear cells (PBMC), RBC and plasma of allergic and non-allergic rural South African school children. Furthermore, associations between total immunoglobulin E (tIgE) and fatty acids in PBMC, RBC, and plasma were investigated.

2. Methods

2.1. Study design and subjects

This was a cross-sectional study utilizing baseline data from children enrolled in a randomized, placebo-controlled intervention study aiming to determine the effects of iron and n-3 LCPUFA supplementation, alone and in combination, on cognition, and infectious morbidity [22, 23]. The study was conducted at four primary schools in rural KwaZulu-Natal, South Africa and 321 black children participated. The following inclusion criteria were used: 1) age 6–11 y; 2) hemoglobin (Hb) > 8 g/dL; 3) iron deficiency (ID), defined as either serum ferritin (SF) < 20 µg/L, serum transferrin receptor (TfR) > 8.3 mg/L, or zinc protoporphyrin (ZnPP) > 70 µmol/mol haem in washed RBC; 4) apparently healthy, with no chronic illness; and 5) no consumption of iron or n–3 PUFA-containing supplements. Parents or guardians gave written informed consent and children gave verbal assent before the study started. The ethical committees of North-West University (NWU-0061-08-A1), Potchefstroom, South Africa and ETH Zürich (EK 2008-33), Switzerland, approved the study protocol and the study was registered at clinicaltrials.gov as NCT01092377.

2.2. Assessment of allergic disease

The ISAAC questionnaire was used to classify the children (n = 111) as allergic (n = 30) or non-allergic (n = 81), though this was not an official ISAAC study [24, 25]. This

subsample (n = 111) consisted only of the children from whose parents or caretakers ISAAC questionnaires were completed. The questions were formulated to determine if a child had asthma (wheezing), rhinoconjunctivitis, and/or eczema and were addressed to the children's parents or caretakers. The presence of asthma was estimated on the basis of positive answers to the written question: "Has your child had wheezing or whistling in the chest in the past 12 months?" The presence of allergic rhinoconjunctivitis was estimated on the basis of positive answers to both these questions: "In the past 12 months, has your child had a problem with sneezing or a runny or blocked nose when he/she did not have a cold or the flu?" and if yes, "In the past 12 months, has this nose problem been accompanied by itchy watery eyes?" Eczema symptoms were estimated on the basis of positive answers to two questions: "Has your child had this itchy rash at any time in the past 12 months?" and, "Has this itchy rash at any time affected any of the following places: the folds of the elbows; behind the knees; in front of the ankles; under the buttocks; or around the neck, ears, or eyes." These questions were preceded by the question "Has your child ever had a skin rash which was coming and going for at least 6 months?" A child was considered to have allergic disease if one of these conditions were present [25]. The questionnaire was translated to the local language (Zulu) and validated by consultation with the local community and back translation into English by an independent translator.

2.3. Laboratory measurements

Blood for the preparation of RBC, plasma and serum was collected from all the study children (n = 321) in EDTA and serum tubes, and for the preparation of PBMC for a subsample (n = 124) in cell preparation tubes (CPT) from Becton Dickinson (New Jersey, United States of America). Total IgE (n = 321) was measured in serum with the tIgE ELISA kit from Human Diagnostics (Wiesbaden, Germany). Fatty acid composition was determined in PBMC (n = 124), RBC (n = 315) and plasma (n = 263) as previously described [22]. All samples used for fatty acid analysis were stored at −80ºC for less than 6 months before analysis. Briefly, phospholipids were extracted from PBMC, RBC and plasma with chloroform:methanol (containing 0.01% butylated hydroxytoluene) and methylated by using a modification of the method of Folch et al. [26]. External calibration was used for each fatty acid methyl ester (FAME) and corrected with a weighed heptadecanoic acid internal standard. The gas chromatography separation of FAMEs was carried out on a BPX 70 capillary column (60 m x 0.25 mm x 0.25 μm; SGE Analytic Sciences) by using helium as the carrier gas at a flow rate of 1.3 mL/min. FAMEs, including the FAMEs of the C18 trans fatty acids, *trans*-C18:1n-9 (elaidic acid), *trans* C18:1n-7 (trans-vaccenic acid) and *trans* C18:1n-6 (petroselaidic acid) were separated chromatographically The oven temperature was programmed from 130°C to 240°C, rose from 130°C to 200°C at 2°C/min, was held at 200°C for 4 min, and then rose at 5°C/min to 220°C. After the temperature was held isothermal at 220°C for 5 min, it was increased by 10°C/min to 240°C, where it was retained for 5 min. The total analysis time was 53 min. Quantitation was performed with Masshunter mass

spectrometry data software (B.06.00, Agilent Technologies) after the data were transferred from ChemStation mass spectrometry data software (Agilent Technologies, G1701EA version E.02.00.493). In Masshunter, target and qualifier transitions were selected for each FAME from the full scan data obtained, and a target to qualifier ratio between 80% and 120% was accepted. Further quality control measures entailed spiking and extraction of biological samples with fatty acids to obtain exact chromatographic retention times and target to qualifier ratios within the biological matrices. The relative composition (weight %) of fatty acids was reported as previously described [22]. To compare n-6 and n-3 PUFA compositions between groups, single and total n-6 and n-3 PUFA, total LCPUFA, and the ratio between AA and EPA were analyzed. These fatty acids and ratios were also tested for associations with tIgE [27]. Furthermore, the ratios of n-6 DPA:DHA and n-6 DPA:AA were used as a marker for DHA deficiency and to estimate usage of AA to conserve membrane fluidity, respectively [28-30]. Ratios between fatty acids in the desaturation and elongation pathway were used to reflect enzyme activity differences in this pathway between groups (Figure 1). Although gamma-linolenic acid (GLA, C18:3n-6) is the direct product of D6D, it is rapidly elongated to dihomo-gamma-linolenic acid (DGLA, C20:3n-6) [31] resulting in very low GLA concentrations (erythrocyte mean = 0.05%). Therefore, as previously described [32], DGLA:LA and AA:DGLA ratios were used to estimates D6D and D5D activities, respectively.

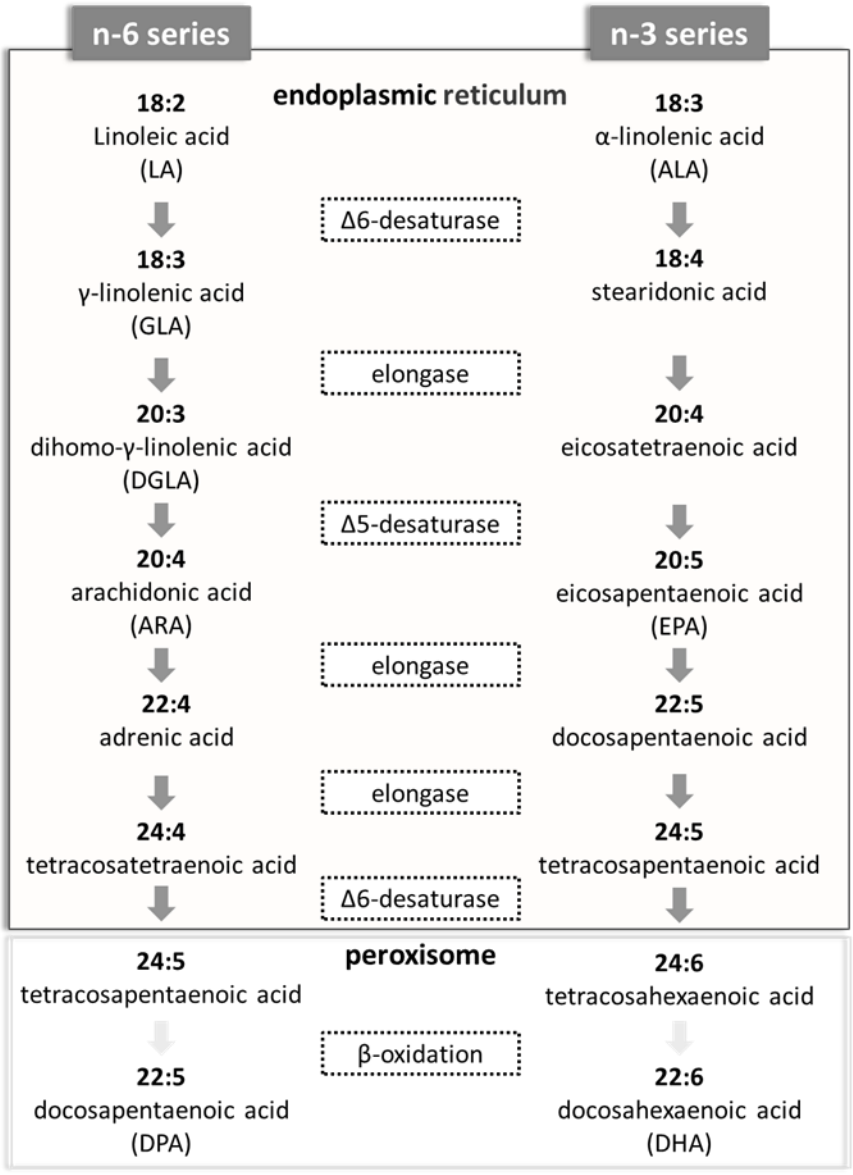

Fig. 1. N-6 and n-3 polyunsaturated fatty acid desaturation and elongation pathways (Reproduced by kind permission of Portland Press from [54])

2.4. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics (version 21; IBM Corporation). All data were checked for the presence of outliers (±3 SD from the mean).

Differences between the allergic and non-allergic group for fatty acids in PBMC, erythrocyte and plasma phospholipids, were examined by using independent *t*-tests for normally distributed data and with Mann-Whitney U tests for non-normally distributed data. If a large effect size occurred (d = 0.7), the RBC sample size of n = 75 and n = 28 for the non-allergic and allergic group, respectively, would give 88% power to detect a difference with an independent t-test with a significance level of 0.05. Furthermore, if a large effect size occurred, (d = 0.7 as for n-6 LCPUFA), the sample PBMC size of n = 31 and n = 14 for the non-allergic and allergic group, respectively, would give 68% power to detect a difference with an independent t-test with a significance level of 0.05. To determine associations between tIgE and specific fatty acids, Pearson's or Spearman's rank correlations were performed, for normally distributed and non-normally distributed data, respectively. Results are expressed as means ± SD when data were distributed normally and as medians and range (min to max) for nonparametric data. The level of significance was set at a p-value less or equal than 0.05.

3. Results

Allergic and non-allergic children did not differ significantly with respect to sex distribution, age, weight and iron status indices, but height and age differed significantly between children with and without ISAAC data (*P* = 0.012 and 0.016, respectively) (Table 1). In total, 296 ISAAC questionnaires were sent to parents and caretakers, of which 111 were completed and returned. Based on the ISAAC questionnaire 27.2% of the study children had allergic disease, consisting of a mixture of rhinoconjuntivitis,

asthma and eczema symptoms. The median tIgE in the study population was 131.7 IU/ml (range, 0.0 – 921 IU/ml) and tIgE did not differ significantly (*P* = 0.390) between allergic and non-allergic children (Table1).

For the purpose of investigating the differences in fatty acid composition between the allergic and non-allergic children with ISAAC data available (111 children), the phospholipid fatty acid composition was available in PBMC for 45 children, in RBC for 103 children and in plasma for 87 children (Table 2). The mean DHA composition was 1.5% in PBMC, 3.7% in RBC and 3.3% in plasma. Correlations between phospholipid fatty acids and tIgE were analyzed in a subsample of PBMC of 124 children; and in the total study sample (n = 321) of RBC of 315 children and plasma of 263 children (Table 3). The median serum zinc concentration (*P* = 0.048) and ID prevalence on the basis of SF (*P* = 0.030) were higher, and zinc deficiency prevalence was lower (P = 0.039) in the group in whom PBMCs were studied (PBMC group) than in the group in whom PBMCs were not studied [23].

Table 1

Baseline characteristics of all children, children with and without ISAAC data as well as allergic and non-allergic children[1]

	All children (*n* = 236–321)	Children with ISAAC data (n = 72–111)	Children without ISAAC data (n = 164–210)	Allergic children (*n* = 18–28)	Non-allergic children (*n* = 54–75)
Age (y)	8.9 ± 1.3[a]	9.2 ± 1.3	8.8 ± 1.3	9.3 ± 1.4[a]	9.1 ± 1.4
Ratio male:female (%)	51:49	45:55	54:45	57:43	41:59
Height (m)	1.28 ± 0.09	1.30 ± 0.09	1.27 ± 0.08	1.31 ± 0.08	1.29 ± 0.09
Weight (kg)	27.8 (17.9–48.1)[b]	29.0 (19.3–48.1)	27.1 (17.9–48.1)	28.9 (20.2–47.2)[b]	29.1 (19.3–48.1)
Anthropometric indices [*n* (%)]					
Stunting (HAZ < –2SD)	18 (5.8)	6 (5.4)	12 (6.0)	1 (3.3)	5 (6.3)
Mildly stunted (HAZ < –1SD ≥ –2SD)	99 (32.1)	37 (33.6)	62 (31.3)	11 (36.7)	26 (32.5)
Underweight (WAZ < –2SD)	7 (2.9)	2 (2.7)	5 (3.0)	0 (0.0)	2 (3.7)
Overweight (BAZ > 1SD < 2SD)	64 (21.1)	22 (20.4)	42 (21.4)	4 (13.3)	18 (23.1)
Obese (BAZ ≥ 2SD)	21 (6.9)	5 (4.6)	16 (8.2)	0 (0.0)	4 (5.1)
Plasma total IgE (IU/ml)	131.7 (0.0–921.0)	135.9 (0.0–921.0)	114.7 (0.0–887.0)	104.6 (0.0 – 740.1)	135.9 (0.0–740.0)
Blood hemoglobin (g/dL)	12.1 ± 0.8	12.1 ± 0.7	12.1 ± 0.8	12.2 ± 0.7	12.1 ± 0.7
Serum ferritin (µg/L)[c]	19.1 (3.1–73.1)	21.1 (5.4–64.6)	18.9 (3.1–73.1)	18.8 (5.4 – 43.8)	20.5 (6.2–64.6)
Serum transferrin receptor (mg/L)	5.7 (2.4–11.8)	5.7 (2.5–9.7)	5.7 (2.4–11.75)	5.3 (2.5 – 9.2)	5.6 (2.5–9.7)
Body iron stores (mg/kg)	3.0 ± 2.5	3.2 ± 2.3	2.9 ± 2.6	2.8 ± 2.3	3.3 ± 2.3
Zinc protoporphyrin (µmol/mol heme)	75.0 (33.0–215.0)	72.0 (40.0–141.0)	75.0 (33.0–171.0)	72.0 (48.0 – 112.0)	74.0 (40.0–161.0)
C-reactive protein (mg/L)	0.4 (0.0–17.8)	0.3 (0.0–3.9)	0.3 (0.0–4.9)	0.4 (0.0–9.9)	0.3 (0.0–17.8)
Serum zinc (µg/L)	73.3 (45.8–106.2)	74.7 (45.8–104.1)	72.6 (46.2–106.2)	70.6 (55.9–102.2)	75.5 (45.8–104.1)
Deficiencies [*n* (%)]					
Anemia (Hb < 11.5 g/dL)	65 (20.6)	18 (16.2)	47 (23.0)	3 (10.0)	15 (18.5)
Iron deficiency based on SF (<15.0 µg/L)	78 (28.1)	24 (25.0)	54 (29.7)	9 (37.5)	15 (20.8)
Iron deficiency based on TfR (>8.3 mg/L)	36 (11.3)	12 (10.9)	24 (11.5)	3 (10.0)	9 (11.3)
Iron deficiency based on ZnPP (>70 µmol/mol heme)	197 (62.7)	64 (58.7)	133 (64.9)	18 (60.0)	46 (58.2)
Shortage of body iron (negative values)[d]	33 (12.0)	11 (11.6)	22 (12.2)	4 (16.7)	7 (9.9)
Iron deficiency anemia (Hb < 11.5 g/dL and SF < 15 µg/L)	25 (9.2)	7 (7.3)	18 (10.2)	2 (8.3)	5 (6.9)
Zinc deficiency (SZn <65 µg/L)	70 (24.8)	22 (22.4)	48 (26.1)	8 (32.0)	14 (19.2)
Acute-phase protein [*n* (%)]					
C-reactive protein (>5 mg/L)	22 (7.2)	7 (6.6)	15 (7.5)	4 (13.8)	3 (3.9)

Abbreviations: HAZ: height-for-age ȥ score; WAZ: weight-for-age ȥ score; BAZ: BMI-for-age ȥ score; Hb: haemoglobin; SF: serum ferritin; SZn: serum Zinc; tIgE: total immunoglobulin E; TfR: transferrin receptor; ZnPP: Zinc protoporphyrin.

[1]Differences between the children with and without ISAAC data, as well as the allergic and non-allergic children were examined by using independent *t* tests for continuous variables and by using the chi-square test for categorical variables ($P<0.05$). Height and age differed significantly between children with and without ISAAC data ($P=0.012$ and 0.016, respectively).

[a] Mean ± SD (all such values).

[b] Median; minimum to maximum in parentheses (all such values).

[c] Serum ferritin values of all children with CRP>5 mg/L were excluded.

[d] Total body iron was calculated on the basis of Cook et al. [53].

In PBMC phospholipids, the major n-6 LCPUFA, AA (P = 0.009), as well as total n-6 PUFA and total n-6 LCPUFA (both P = 0.013), were significantly lower in the allergic children than in the non-allergic children (Table 2). Furthermore, elaidic acid (*trans*-C18:1n-9), tended to be higher (P = 0.073) in the allergic group (Table 2) and correlated negatively with medium strength (r = -0.424, P < 0.001) with AA (Figure 2) in PBMC phospholipids. PBMC phospholipid total n-3 LCPUFA (r = -0.244, P = 0.006) and n-3 docosapentaenoic acid (n-3 DPA; C22:5n-3) correlated negatively (r = -0.295, P = 0.001) and the n-6:n-3 PUFA ratio positively (r = 0.223, P = 0.013) with tIgE (Table 3).

In RBC phospholipids dihomo-gamma-linolenic acid (DGLA; C20:3n-6) (P = 0.003) and the ratio of DGLA:LA (P <0.001) were significantly higher in the allergic children (Table 2). N-6 docosapentaenoic acid (n-6 DPA; C22:5n-6), also called osbond acid, tended to be higher (P = 0.080) in the allergic group (Table 2). Furthermore, tIgE correlated positively with the ratios of n-6 DPA to DHA (r = 0.124) and n-6 DPA to AA (r = 0.135) in RBC phospholipids (Table 3).

Plasma phospholipid DHA was significantly lower (P = 0.041) and the total n-3 LCPUFA tended to be lower (P = 0.067) in the allergic children (Table 2). Furthermore, total n-6 PUFA (P = 0.079) tended to be lower in plasma phospholipids of the allergic group (Table 2). There were no correlations between tIgE and fatty acid composition in plasma.

Table 2

Peripheral blood mononuclear cell (PBMC), red blood cell (RBC) and plasma total phospholipid fatty acid composition of allergic and non-allergic children[1].

	PBMC			**RBC**			**Plasma**		
	Allergic children n = 14	Non-allergic children n = 31		Allergic children n = 28	Non-allergic children n = 75		Allergic children n = 25	Non-allergic children n = 62	
			p-Value			*p*-Value			*p*-Value
Elaidic (*trans*-C18:1n-9)	2.99 (0.57 – 5.56)[a]	2.48 (0.61 - 4.59)	0.073	0.18 (0.03 – 0.48)	0.17 (0.05 – 1.18)	0.625	0.22 (0.11 – 1.28)	0.22 (0.06 – 1.22)	0.694
LA (C18:2n-6)	7.65 ± 2.88[b]	7.23 ± 1.25	0.611	12.91 ± 1.17	13.33 ± 1.03	0.080	23.03 ± 2.35	23.52 ± 2.63	0.457
GLA (C18:3-n6)	Not detected	Not detected	-	0.05 (0.03 – 0.09)	0.04 (0.01 – 0.12)	0.261	0.08 (0.03 – 0.23)	0.07 (0.01 – 0.42)	0.632
DGLA (C20:3n-6)	1.46 (0.94 – 1.90)	1.56 (1.01 – 2.33)	0.377	1.44 (1.03 – 1.84)	1.30 (0.93 – 1.71)	0.003	2.72 (1.54 – 4.54)	2.58 (1.33 – 4.09	0.330
DGLA:LA ratio	0.21 ± 0.06	0.22 ± 0.06	0.419	0.11 ± 0.02	0.10 ± 0.01	<0.001	0.12 (0.07 – 0.24)	0.11 (0.06 – 0.20)	0.302
AA (C20:4n-6)	18.11 (13.33 – 23.12)	21.79 (15.11 – 28.46)	0.009	15.04 ± 1.04	14.88 ± 1.25	0.537	13.48 ± 2.06	14.06 ± 1.90	0.217
AA:DGLA ratio	12.50 (9.08 – 23.69)	13.79 (8.56 – 19.64)	0.078	10.78 ± 2.08	11.60 ± 1.69	0.039	4.86 (3.14 – 10.00)	5.46 (3.07 – 11.38)	0.062
n-6 DPA (C22:5n-6)	0.47 (0.33 – 1.09)	0.53 (0.28 – 1.14)	0.310	1.35 (0.87 – 2.02)	1.24 (0.80 – 2.23)	0.080	0.80 (0.42 – 1.31)	0.77 (0.26 – 2.18)	0.666
Total n-6 PUFA	31.25 (27.64 – 35.05)	34.28 (24.53 – 42.08)	0.013	35.08 ± 1.56	34.94 ± 1.59	0.682	40.94 (32.39–45.66)	42.00 (34.87–48.03)	0.079
Total n-6 LCPUFA	23.72 ± 2.90	26.90 ± 4.01	0.013	22.12 ± 1.45	21.56 ± 1.69	0.127	17.80 ± 2.30	18.38 ± 2.28	0.301
EPA (C20:5n-3)	0.13 (0.05 – 0.41)	0.11 (0.02 – 0.54)	0.211	0.16 (0.08 – 0.38)	0.17 (0.03 – 0.38)	0.896	0.18 (0.09 – 0.40)	0.18 (0.02 – 1.33)	0.694
n-3 DPA (C22:5n-3)	1.30 (0.36 – 2.36)	1.18 (0.48 – 2.00)	0.692	1.67 ± 0.20	1.63 ± 0.30	0.508	0.80 ± 0.14	0.83 ± 0.26	0.476
DHA (C22:6:n-3)	1.36 (0.88 – 2.16)	1.51 (0.96 – 2.91)	0.259	3.76 ± 0.55	3.75 ± 0.74	0.958	3.12 ± 0.58	3.44 ± 0.68	0.041
Total n-3 LCPUFA	2.70 (1.83 – 4.35)	2.97 (1.88 – 4.43)	0.771	5.60 ± 0.69	5.50 ± 0.98	0.562	3.93 (3.08–5.34)	4.30 (2.86–7.13)	0.067
n-6:n-3 PUFA ratio	12.00 (6.68 – 19.11)	12.33 (6.62 – 18.98)	0.653	6.15 (0.70 – 8.71)	6.43 (4.31 – 8.79)	0.514	10.23 (7.27 – 13.67)	9.50 (5.75 – 14.24)	0.330
n-6:n-3 LCPUFA ratio	8.6 (5.15 – 15.15)	9.70 (4.63 – 14.86)	0.315	3.99 (2.93 – 5.59)	4.07 (2.64 – 5.86)	0.901	4.39 (3.01 – 6.51)	4.23 (2.5 – 6.79)	0.355
n-6 DPA:DHA ratio	0.34 (0.17 – 0.78)	0.38 (0.13 – 0.76)	0.787	0.36 (0.21 – 0.62)	0.34 (0.19 – 0.70)	0.213	0.24 (0.11 – 0.44)	0.24 (0.08 – 0.75	0.464
n-6 DPA:AA ratio	0.03 (0.02 – 0.07)	0.03 (0.02 – 0.05)	0.928	0.09 (0.06 – 0.14)	0.08 (0.06 – 0.14)	0.076	0.06 (0.03 – 0.09)	0.06 (0.02 – 0.13)	0.800

Abbreviations: ALA: alpha-linolenic acid; AA: arachidonic acid; DGLA: dihomo-gamma-linolenic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: eicosapentaenoic acid; GLA: gamma-linolenic acid; LA: linoleic acid; LCPUFA: long-chain polyunsaturated fatty acid; PUFA: polyunsaturated fatty acid. [1] Differences between the allergic and non-allergic groups were examined by using independent *t* tests for normally distributed data and with Mann-Whitney U tests for non-normally distributed data. $P < 0.05$ was considered significant. [a] Median; minimum to maximum in parentheses (all such values). [b] Mean ± SD (all such values).

Table 3
Spearman correlation coefficients of total immunoglobulin E (tIgE) with peripheral mononuclear cell (PBMC) and erythrocyte phospholipid fatty acids

PBMC fatty acids **n = 124**			**RBC fatty acids** **n = 315**		
	tIgE			**tIgE**	
	r	*p*-Value		r	*p*-Value
n-3 DPA	-0.295	0.001	n-6 DPA:DHA ratio	0.124	0.048
Total n-3 LCPUFA	-0.244	0.006	n-6 DPA:AA ratio	0.135	0.029
Total n-6:n-3 PUFA ratio	0.223	0.013			

Abbreviations: AA: arachidonic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; LCPUFA: long-chain polyunsaturated fatty acid; PUFA: polyunsaturated fatty acid.

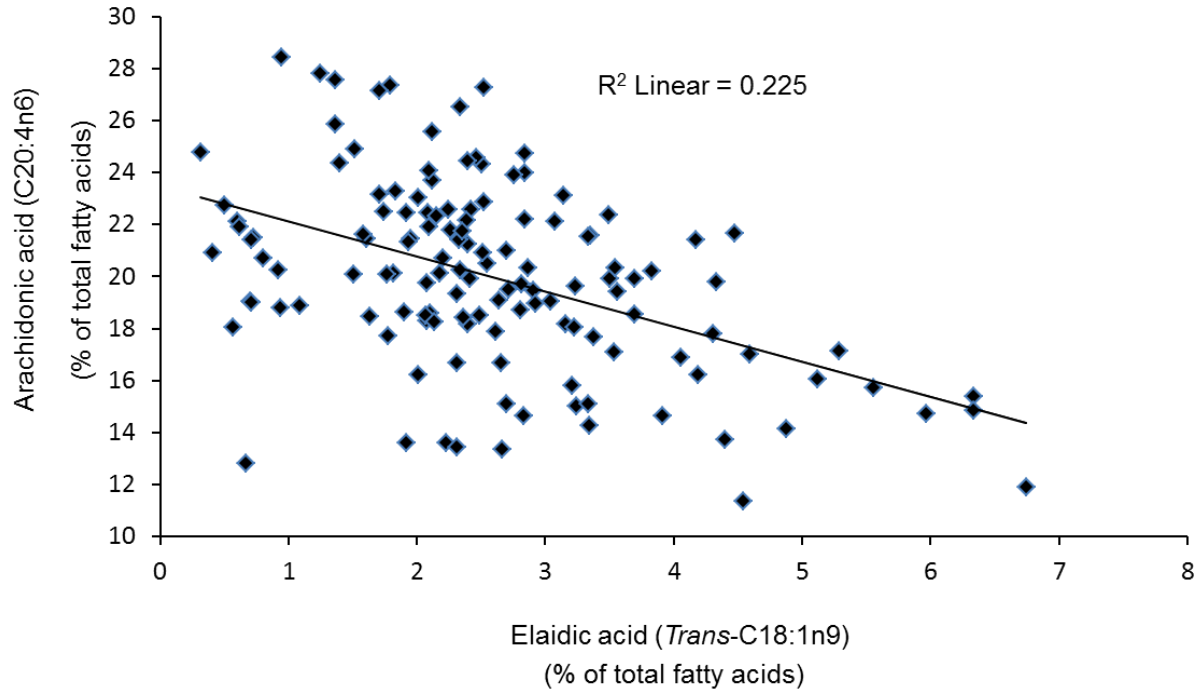

Fig. 2. Scatter plot representing the negative association between arachidonic acid and elaidic acid (*trans*-C18:1n-9) in PBMC. Spearman's rank correlation coefficient r = -0.424 and *P* < 0.001 (n =124).

4. Discussion

The current study identified about 27% of the cohort of school children included to have allergic disease. Even though the study agrees to some extent with the hypothesis that low n-3 LCPUFA status is associated with allergic disease, it also shows that low status of the n-6 LCPUFA, AA, could be linked to allergy. Of the three lipid pools studied, PBMC phospholipids are most closely linked to the cellular responses involved in allergy. Here AA, the main n-6 PUFA present, was lower in the allergic group.

As far as we know this is the first study in black South Africa school children looking at the association of fatty acid composition with allergy. The findings are in general agreement with others who found 47% lower AA and 70% lower DHA in T cell phospholipid membranes of 6 mo to 12-year old children with atopic dermatitis compared to non-atopic controls [8, 33]. In our study AA was 17% and significantly lower and DHA 10% lower in PBMC phospholipids of the allergic children. Even though the current study had a limited number of PBMC samples (n = 45), we still observed significant differences in AA, total n-6 PUFA and n-6 LCPUFA between the allergic (n = 14) and non-allergic (n = 31) children. Therefore, this study adds value to the limited data available about fatty acid composition of PBMC in the context of allergic disease. The overall DHA status of PBMC in the study group (1.5% of fatty acid composition in PBMCs), is very low compared to the ~3% that others have reported [34-36], but the erythrocyte DHA composition (3.7%) is comparable to those reported (3 – 4%) in other studies [35, 37]. According to dietary data obtained by two 24 hour recall dietary questionnaires from 102 children (data not shown), the median fish intake were 0.0 g,

with a range of 0.0 – 83 g and fish intake correlated negatively with plasma AA and n-6 LCPUFA (Spearman r = – 0.219, p<0.5 and r = – 0.240, p <0.05, respectively) and positively with PBMC EPA (Spearman r = 0.281, p <0.05). There was no difference between fish intake of the allergic and non-allergic groups, however, these conclusions are limited by the fact that the number of children with both dietary and allergy data were limited (only to 13 and 31 children in each group).

There are four likely mechanistic explanations for the observation of lowered AA in the allergic children of our study population. First, significantly higher DGLA and lower AA:DGLA ratio in RBC of the allergic children could indicate less effective conversion of DGLA to AA and, therefore, impaired delta-5 desaturase (D5D) activity in this group [38]. Lower AA was seen in PBMC, but not in RBC, perhaps due to additional uses of AA in PBMC, such as for eicosanoid production as part of the ongoing inflammatory process [39]. A reduced ratio of AA:DGLA was also found by Lindskou et al. in mononuclear cells of patients with atopic dermatitis [16]. As reviewed by Lattka *et al*., polymorphisms in the fatty acid desaturases (FADS) gene cluster, specifically FADS1, encoding D5D, are associated with allergic disease [15]. Rhezak *et al*. found an association of D5D polymorphisms with eczema, but could not find any differences in fatty acid composition between eczema and control groups, perhaps because they analyzed plasma only [40]. However, others have also seen differential fatty acid composition in allergic and non-allergic groups in serum [41]. Our data does, however, not support impaired delta-6 desaturase (D6D) activity in the allergic children of the current study, since the ratio of DGLA:LA was higher in the erythrocyte membranes of the allergic children in our study [33, 42].

Second, since AA is significantly lower only in PBMC, it could be that AA was excised in excess from PBMC membranes due to increased activity of AA-specific cytosolic phospholipase 2 (cPLA2) [43]. Indeed, cPLA2 gene promoter polymorphisms in 18–75-year old patients with severe asthma have been associated with induced transcription of cPLA2 with asthma [44]. Furthermore, the allergic children in our study had significantly lower plasma DHA, possibly indicating lower fish intake in this group. DHA has been shown to decrease levels and activity of AA-specific cPLA2 *in vivo* [15, 45]. Lower levels of dietary DHA in this group of children could, therefore, have created a favourable environment for increased excision of AA from phospholipid membranes by cPLA2 [45, 46]. Thus AA may be low in PBMC phospholipids from children with allergic disease because it is being depleted as part of the inflammatory process. Since AA is the precursor for PGE_2, which is known to promote IgE production in B cells [12], such as is found in allergic disease, the scenario of increased release of AA is also supported by the clinical history (allergy symptoms) of these children.

Third, increased n-6 DPA (osbond acid), the n-6 equivalent of DHA, has been described in DHA deficiency [28, 29]. N-6 DPA is incorporated into membranes instead of DHA to conserve polyunsaturation [47, 48]. A tendency towards higher n-6 DPA in the allergic group of our study and the positive correlations of the n-6 DPA:DHA and n-6 DPA:AA ratios with tIgE, might indicate that this mechanism is also present in our study. This mechanism could also have limited the availability of AA by using AA to produce n-6 DPA, meaning less AA was available for incorporation into fast-changing membranes such as PBMCs.

Fourth, AA could have been displaced from PBMC membranes by elaidic acid (*trans*-C18:1-n9), as this *trans*-fatty acid was associated negatively with AA in PBMC. Elaidic acid also correlated negatively with DHA, but this did not reach significance, so it seems that DHA could be less affected, possibly also due to the relatively lower levels of DHA naturally occurring in PBMC membranes [12].

Another novel finding from this study is that the *trans*-fatty acid elaidic acid tended to be higher in PBMC phospholipids of the allergic children. This supports the hypothesis that *trans*-fat might be associated with allergic disease [17, 18]. The median percentage PBMC elaidic acid (2.4%) in the study children overall was very high in comparison with the 0.5% of total *trans*-C18:1 of 6 mo – 12-year old Caucasian allergic children in Italy [33]. The median intake of snacks (unknown brands of chips and cookies) that could possibly contain *trans*-fat was 10.0 g with a range of 0.0 – 47.5 g per day and intake of these snacks are known to be high in this particular area in South Africa (Valley of a Thousand Hills) from a very young age [49, 50]. Furthermore, this finding was specific for PBMC, which are directly involved in immune response and the etiology of allergic disease.

The small sample size, specifically for PBMC, was a limiting factor of this study. It was, however, unavoidable because we could only analyze PBMC phospholipid fatty acids in a subsample. A further limitation of the study was that tIgE did not differ significantly between the allergic and non-allergic children. Even though it is known that there is a significant overlap in tIgE between allergic and non-allergic individuals [51], we were concerned that there could have been a relatively high prevalence of helminthic infections in the children, which could have masked the association of allergy

with tIgE [52]. However, the children were dewormed at baseline of the intervention study, and again half way through the study. Therefore, we investigated the association between baseline and endpoint tIgE, taken 8 mo later. These correlated strongly (Spearman r = 0.847, p <0.001) and thus support the notion that tIgE in this case was an indication of allergic disease and that the associations with the fatty acid status are valid.

5. Conclusion

Our data support the hypothesis that impaired D5D activity might be associated with allergic disease in these rural South African children. Increased release of AA from PBMC phospholipid membranes, therefore, aggravating allergic disease, might also be involved. These findings should be further explored by investigating possible genetic variation of the FADS genes. Furthermore, the findings agree with the hypothesis that a low n-3 LCPUFA intake and, to a lesser extent, high n-6:n-3 PUFA ratio, may be associated with allergic disease. Finally, our results support the notion, shown for the first time in black South African children, that *trans*-fat may also be associated with children's allergic state.

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Conflict of interest

PCC, JB and CMS have received speaking honoraria from Unilever. LM has no conflict of interest.

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Chapter 6 Concluding remarks

The overall aim of this thesis was to explore interactions between iron and n-3 LCPUFA by investigating the effects of combined iron and n-3 LCPUFA supplementation, alone and in combination, on infectious morbidity and immune function. More specifically we aimed to: 1) determine the effect of supplementation with iron and a mixture of DHA/EPA, alone and in combination, on the occurrence and duration of infectious morbidity, in ID children with low n-3 PUFA intake in a 8.5 mo RCT; 2) evaluate the effects of this regime of intervention on fatty acid-derived immune modulators and the expression of certain genes involved in inflammation, iron regulation and oxidative stress; 3) to assess the allergic disease prevalence in the study population and explore the association of the total phospholipid fatty acid composition of three lipid pools, but mainly PBMC, with allergic disease prevalence in the study population.

6.1 Infectious morbidity

In a 8.5 mo RCT, we investigated whether a mixture of 50 mg iron as iron sulphate and 420 mg DHA and 80 mg EPA given four days per week, alone or in combination, affected illness and illness-related absenteeism in ID 6–11 y-old school children with low fish intake. The main finding of this study in apparently healthy school children living in a malaria-free rural area was that iron supplementation increased morbidity by 139% and respiratory morbidity by 325% (relative to the placebo plus placebo group), but when given in combination with DHA/EPA, this increase in morbidity was prevented.

We are not sure whether iron supplementation increased respiratory morbidity specifically due to this population's low n-3 LCPUFA status. Furthermore, no iron intervention studies have, as far as we know, assessed n-3 PUFA status before intervention. Only one near comparable study, with baseline n-3 PUFA status data, has assessed the effect of iron supplementation in children of the same age on infectious morbidity, but at relatively low concentration (18 mg) and within a multiple micronutrient mix (Muthayya *et al.*, 2009; Thomas *et al.*, 2011). In contrast to our findings, the authors found no detrimental effects on morbidity with the micronutrient mix alone, probably due to a lower dose iron and the contribution of the other micronutrients, that supplementation with 100 mg DHA and 900 mg ALA improved respiratory health. Furthermore, combining DHA/EPA supplementation with a micronutrient mix containing 100% of the recommended dietary allowance increased respiratory morbidity (Thomas *et al.*, 2011). However, the baseline RBC total phospholipid DHA composition in the two populations was the same (~3.2% vs. 3.1%). Still, it is difficult to compare these studies due to the different compositions of the interventions. Besides, 60 mg iron as ferrous sulphate per day reduced the

number of episodes and total sick days due to upper respiratory tract in 5–10 y-old IDA Sri Lankan children (de Silva *et al.*, 2003). In this population, n-3 PUFA status may be assumed to be adequate due to high fish intake, but it was not actually measured.

A surprising finding was that DHA/EPA increased the number of days absent and the probability for being absent through illness when all symptoms were considered – with headache, fever and stomach pain standing out as the main symptoms. Although it is not clear why this happened, headache and gastric upset with n-3 LCPUFA supplementation have been noted previously as side effects by others (Wozniak *et al.*, 2007). Our study agreed with that of Thomas et al., finding that combined supplementation interacted to improve gastric upset (Thomas *et al.*, 2011). This is a very interesting finding which also warrants further investigation. The finding of increased headache is, however, also in contrast with recent studies showing that increased dietary DHA and EPA, as well as fish oil supplementation, reduced chronic headache (Ramsden *et al.*, 2013; Van De Ven & Ji, 2013).

Despite limitations, such as a relatively small study population for investigating individual symptoms and reduced data collection due to a strike and holidays, the detailed recording of symptoms gave us insight into our findings. Even though the findings are limited by statistical limitations such as multiple testing (inflating the type II error rate) and a per protocol statistical approach (maybe causing bias) it was clear that iron increased, and DHA/EPA prevented this increase in respiratory illness, whereas iron tended to improve absence with headache, caused by DHA/EPA supplementation.

6.2 Underlying biochemistry of iron and n-3 polyunsaturated fatty acid supplementation

We also aimed to investigate the effects of supplementation with iron and DHA/EPA alone, and in combination, on immune function. Thus, the effects of supplementation on circulating plasma lipid-derived immune modulators and gene expression involved in inflammation, anti-oxidative response and iron regulation, were examined. These biochemical findings helped to explain and illuminate the clinical findings of the 8.5 mo intervention.

We found that DHA/EPA altered the profile of circulating lipid-derived immune modulators to being more anti-inflammatory or pro-resolving. This happened concurrently with improving respiratory morbidity while attending school, but together with an increase of absenteeism through headache and fever. Why this happened, remains to be investigated. We did not find an expected overall improvement in infectious morbidity with DHA/EPA supplementation, as found by others (Minns *et al.*, 2010; Thomas *et al.*, 2012), maybe because of the confounding effect of

ID in the study population. This highlights the importance of not addressing multiple nutrition deficiencies with a single nutrient supplementation.

The pro-inflammatory nature of iron supplementation was emphasised by our results of iron changing the lipid-derived modulator profile to being more pro-inflammatory, but only affecting DHA and EPA-derived mediators and not increasing AA-derived mediators. The pro-inflammatory and oxidative nature of the iron supplementation was also supported by the knowledge-based functional pathway analysis based on our measured gene expressions and metabolites, and predicted according to the knowledge base currently available. This is a novel finding and confirms the notion that a non-physiological relatively high dose of non-haem oral iron – compared to the dietary iron ingested with the basal diet – reaches the circulatory system and influences inflammatory processes in blood. This finding also supports the notion that this excess iron (probably as NTBI) becomes available for pathogens and that is probably why we found that iron increased respiratory infectious morbidity. Lipid-derived immune modulator levels, related gene expression and functional pathway analysis elucidated some possible mechanisms that might have been involved. Furthermore, these data supported previous observations about the anti-inflammatory and pro-resolving nature of DHA/EPA supplementation (Calder, 2014; Dawczynski *et al.*, 2013; Serhan & Petasis, 2011). It suggests that iron enhances the availability and function of DHA/EPA, as well as its incorporation into PBMC which have also been observed previously (Smuts *et al.*, 1995), and support the known mechanism of iron being part of the catalytic centres of the enzymes involved in LCPUFA and fatty acid-derived immune modulators (Gilbert *et al.*, 2011; Hemler & Lands, 1976; Nakamura & Nara, 2004).

The underlying molecular results support the notion that ID confounded DHA/EPA-mediated improvement of response to infectious morbidity, and that addressing ID corrected this confounding affect and brought about the same positive response to infectious morbidity with improved n-3 LCPUFA status, as reported by others (Birch *et al.*, 2002; Dalton, 2006; Thienprasert *et al.*, 2009; Thomas *et al.*, 2012). Part of the underlying mechanism seems to be improved lipid-mediator synthesis and increased DHA/EPA availability which together result in increased pro-resolving DHA-derived immune modulator synthesis. This mechanism likely led to the anti-inflammatory profile being maintained even when iron was supplemented together with DHA/EPA and might be the key mechanism by which the immune competence were improved to the level of preventing iron-induced respiratory morbidity.

The study was limited by the very low concentrations of lipid-derived mediators in circulating blood, e.g. in the order of pg/mL for resolvins and protectins and somewhat higher for their

precursors and PG. Tissues or body fluids where these molecules are more concentrated, such as local inflammatory sites (skin, brain, intestinal cells and sputum) and endpoint products (urine) will be explored in future studies (Bosco *et al.*, 2013; Kendall & Nicolaou, 2013). Blood sampling are also invasive and may also lead to artefactual ex vivo formation of certain eicosanoids (thromboxane B2), thus urine will be a useful sample to measure endpoint metabolites in future (Kumlin, 1996; Sznajer *et al.*, 2004).

6.3 Allergic disease

The association of fatty acid composition with allergic disease was also investigated in this study. In a cross-sectional design, utilizing baseline data from the RCT performed in this thesis, we investigated for the first time in black South African children, the associations between total phospholipid PBMC, RBC and plasma fatty acid composition and allergic disease. We found that 27% of the cohort of school children included had allergic disease. The ISAAC questionnaire addressed to parents or caregivers was used to classify the children as allergic or non-allergic. The prevalence of our study is in agreement with the findings of ISAAC III in South Africa, namely a prevalence for allergic rhinoconjuntivitis (rhinitis with itching and watery eyes), asthma and eczema of 18–20% in 13–14 year olds (Ait-Khaled *et al.*, 2007).

Of the three lipid pools studied, PBMC phospholipids are most closely linked to the cellular responses involved in allergy. Here, AA, the main n-6 PUFA present, was lower in the allergic group. Even though the study agrees to some extent with the hypothesis that low n-3 LCPUFA status is associated with allergic disease, it also shows that low status of particularly the n-6 LCPUFA, AA, could be linked to allergy. First, significantly higher DGLA and lower AA:DGLA ratio in erythrocytes of the allergic children could reflect less effective conversion of DGLA to AA and, therefore, impaired D5D activity in this group (Nakamura & Nara, 2004). Lower AA was seen in PBMC, but not in erythrocytes, perhaps due to additional uses of AA in PBMC, such as for eicosanoid production (Calder, 2013). Another novel finding from this study is that the trans-fatty acid elaidic acid tended to be higher in PBMC phospholipids of the allergic children. This supports the hypothesis that trans-fat might be associated with allergic disease (Nagel & Linseisen, 2005; Weiland *et al.*, 1999). The median percentage PBMC elaidic acid (2.4%) in the study children overall was very high in comparison with the 0.5% of total trans-C18:1 of 6 mo–12 y-old Caucasian allergic children in Italy (Ferreri *et al.*, 2005). Furthermore, this finding was specific for PBMC, which are directly involved in immune response and the aetiology of allergic disease.

6.4 Implications and perspectives

The results of this PhD study contributed to our knowledge base by answering some questions about the functional and molecular effects of single and combined supplementation of iron and DHA/EPA in the background of double-deficiencies of these two nutrients. However, as is the nature of research, new questions have also been raised. Thus we have plans to further investigate this interesting and potentially very valuable field of fatty acid composition in disease and of addressing multiple micronutrient deficiencies and investigating interactions, particularly between iron and n-3 PUFA, on immune function.

The findings of the cross-sectional allergy study results agree to some extent with the hypothesis that low n-3 LCPUFA status is associated with allergic disease, but even more so, supported the hypothesis that impaired delta-5 desaturase could be linked to the allergic state of these children. Another novel finding from this study is that the trans-fatty acid elaidic acid tended to be higher in PBMC phospholipids of the allergic children. Our result therefore also support the theory that trans-fat might be associated with allergic disease. The origin of these results possibly lies in the genetic variance of the delta-5 desaturase enzyme, and it would be valuable to investigate these genetics within the under-explored ethnic context of South-Africa. Furthermore, it should be investigated if trans-fat intake is associated with the increased trans-fat blood levels seen, and whether the legislation limiting the allowed trans-fat content in food in South-Africa (2010), had an impact in reducing trans-fat blood levels. Due to the very detrimental effects associated with trans-fat, also on cardiovascular health, this is a very important question to investigate further.

Future research plans include some animal models, human observational and human intervention studies. In regard to the association of allergic disease with n-3 and n-6 LCPUFA composition, we plan to investigate effects of n-3 PUFA depletion and repletion on allergy and eczema in a rat model, with depleted dams and their offspring. Since the fatty acid status particularly in pregnancy and infants may influence allergic outcomes, it is of importance to work in these populations (pregnancy and infants). In a previous rat model study in our group, skin lesions were seen in n-3 PUFA deficiency, and were rectified with repletion (Baumgartner, unpublished data). We would also like to extend these studies further to other human populations, specifically to pregnancy and infancy, because this is the time that improvement of deficiencies or compromised fatty acid composition may have the most impact.

We would like to elucidate further the underlying biochemistry of the apparent protective effect of DHA/EPA with iron supplementation. Our group is developing a rat model to study iron and n-3 PUFA depletion and repletion, alone and in combination, in dams and their offspring. Within

the study, we plan to investigate response of lipid-derived immune modulators and gene expression in infectious disease, such as malaria and / or TB. This will give us the opportunity to measure morbidity and mortality outcomes together with biochemical immune function in various tissues. Particular mechanisms highlighted by the functional pathway analysis of the study from this thesis will be of interest. One of these includes Caspase-mediated signalling. Caspases are a family of endoproteases that provide critical links in cell regulatory networks controlling inflammation and cell death. It was predicted to be activated by iron and DHA/EPA individually, but was to be inhibited by the combination of the two nutrients. Furthermore, an ex vivo approach with immune response stimuli like LPS in human and animal models, could be a means of improving our understanding of acute immune responses with previous nutrition intervention. Lipid-derived immune modulators, certain cytokines and the expression of iron regulatory, inflammatory and oxidative stress response genes ex vivo in immune cells as well as in certain tissues and body fluids will give valuable insight into the mechanisms involved behind clinical observations.

Infectious morbidity aspects that need to be addressed, are whether this protective effect of co-supplementation of iron with DHA will also be found in n-3 PUFA sufficient populations and if the protective effect extends to serious infectious diseases like malaria, TB and HIV, and their co-morbidities such as Salmonella (with malaria). These infections are known to be influenced by iron status and that ion supplementation must be approached with caution to prevent increased morbidity and mortality (Jonker *et al.*, 2012; Jonker & van Hensbroek, 2014; Roth *et al.*, 2010). Since improving iron status in settings where these diseases are prevalent is problematic (Roth *et al.*, 2010), increasing morbidity even at fortification levels (Soofi *et al.*, 2013), it will be worthwhile to investigate the possibility of safer iron supplementation by means of adding n-3 PUFA or determining n-3 PUFA status before intervention. Even though some suggest that ID might be protective of malaria, the evidence for many benefits of iron sufficiency, also for immune function, are numerous (Jonker & van Hensbroek, 2014; Oppenheimer, 2001).

A possible human observational study, which will likely reveal some mechanistic insight, will be a study set in a critical care hospital setting. Very recent studies have demonstrated improved morbidity outcomes in critically ill patients who regularly used anti-inflammatory drugs, such as aspirin, statins and non-steroidal anti-inflammatory drugs (Fullerton *et al.*, 2014). The failure of lipid class switching to the production of inflammation-resolution mediators is suspected to affect immune competence of these patients negatively. The investigation of lipid mediators together with clinical outcomes would be a valuable addition to gain understanding of this phenomenon.

Evidence about the positive effects of n-3 PUFA and anti-inflammatory drugs on overall immune competence is starting to accumulate (Fullerton *et al.*, 2014; Pradelli *et al.*, 2012).

On the other hand, from our results, it also seems important to take into consideration the iron status when supplementing with DHA/EPA and that ID may compromise positive effects of n-3 PUFA intervention. As such, our finding for increased absenteeism through headache and its attenuation with iron, warrants a closer investigation.

In conclusion, this study contributed to the knowledge base by emphasizing the importance of investigating multiple micronutrient deficiencies, particularly n-3 PUFA status, before intervening with a single nutrient, specifically iron. This approach will prevent unintended harm. Furthermore, our findings support and explain why n-3 PUFA, although they exert anti-inflammatory actions, are not detrimental to immune response if consumed moderately, but part of a process for dealing effectively with infection. This is also reflected in many intervention studies which show enhanced immune response and improved clinical outcomes (infectious morbidity) with moderate n-3 PUFA intake or supplementation. Based on the molecular findings of our study, one of the mechanisms involved seems to be modulation of lipid mediators involving enhanced pro-resolving n-3 PUFA-derived immune modulator levels when sufficient n-3 PUFA was present, and reduced AA as well as AA-specific cPLA2. However, these effects seem to be suboptimal when ID was present, and was activated with iron supplementation.

The biochemical findings therefore compliment the clinical results and support previous observations about DHA/EPA supplementation to reduce inflammation, but add to the knowledge that a relatively high oral dose of non-haem iron also modulates circulating lipid-derived immune modulators and related gene expression. Furthermore, when supplementing with iron and DHA/EPA combined, in this ID population with low fish intake, the anti-inflammatory effect of DHA/EPA was maintained concurrently with attenuation of respiratory morbidity. This finding support the notion that excess iron (probably as NTBI) becomes available for pathogens and this is probably why we found that iron increased respiratory infectious morbidity. The improved clinical outcome with combined supplementation seems to be related to increased lipid-mediator synthesis gene expression and the availability of DHA/EPA, leading to a more pro-resolving profile and enhanced immune competence. Iron deficiency still remains one of the primary challenges globally, and also in South Africa. It is especially a challenge to improve iron status and anaemia in areas where malaria, HIV and TB are prevalent, but this study's results may be the start of an approach to improve the safety of iron supplementation.

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Annexure 1 Ethical approval documents of North-West University and ETH, Zürich.

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Prof CM Smuts

Ethics Committee
Tel +27 18 299 4850
Fax +27 18 293 5329
Email Ethics@nwu.ac.za

Dear Prof Smuts

ETHICS APPROVAL OF PROJECT

21 Oktober 2008

The North-West University Ethics Committee (NWU-EC) hereby approves your project as indicated below. This implies that the NWU-EC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title: The effect of iron and DHA supplementation, alone and in combination, on cognition: a radomized, double-blind, 2x2 intervention trial in SA children

Ethics number: N W U - 0 0 6 1 - 0 8 - A 1
(Institution – Project Number – Year – Status; Status: S = Submission, R = Re-Submission, P = Provisional Authorisation, A = Authorisation)

Approval date: 10 September 2008 **Expiry date**: 09 September 2013

Special conditions of the approval (if any): None

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The project leader (principle investigator) must report in the prescribed format to the NWU-EC:*
  - *annually (or as otherwise requested) on the progress of the project,*
  - *without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.*
- *The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-EC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-EC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-EC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the project;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the project are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the NWU-EC or that information has been false or misrepresented,*
    - *the required annual report and reporting of adverse events was not done timely and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Prof MMJ Lowes
(chair NWU Ethics Committee)

Prof HH Vorster
(Chairman: NWU Ethics Committee: Author)

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ETHICS APPROVAL OF PROJECT

The North-West University Research Ethics Regulatory Committee (NWU-RERC) hereby approves your project as indicated below. This implies that the NWU-RERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title: The effect of iron an DHA supplementation, alone and in combination, on cognition: a randomised, double-blind, 2x2 intervention trial in South African

Project Leader: Prof CM Smuts

Ethics number:

N	W	U	-	0	0	0	6	1	-	0	8	-	A	1
Institution				Project Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Approval date: 2014-11-18 **Expiry date**: 2017-12-31

Special conditions of the approval:

- No new measurements or data will be collected.
- No additional analyses that were not covered by the original application and informed consent will be done.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The project leader (principle investigator) must report in the prescribed format to the NWU-RERC:*
  - *annually (or as otherwise requested) on the progress of the project,*
  - *without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.*
- *The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-RERC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-RERC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-RERC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the project;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the project are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the NWU-RERC or that information has been false or misrepresented,*
    - *the required annual report and reporting of adverse events was not done timely and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Linda du Plessis

Digitally signed by Linda du Plessis
DN: cn=Linda du Plessis, o=NWU, Vaal Triangle Campus, ou=Vice-Rector: Academic, email=linda.duplessis@nwu.ac.za, c=US
Date: 2014.12.04 00:41:57 +02'00'

Prof Linda du Plessis
Chair NWU Research Ethics Regulatory Committee (RERC)

Eidgenössische Technische Hochschule Zürich
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ETH Zurich
PD Dr. Michael Bruce Zimmermann
Institute of Food Science and Nutrition
LFV E 19.2
Schmelzbergstrasse 7
CH-8092 Zurich

Zurich, November 12, 2008
EK 2008-33

Dear Dr. Zimmermann,

Your research project

„The effects of iron and n-3 fatty acid supplementation, alone and in combination, on cognition: a randomized, double blind 2x2 intervention trial in south African children (02.07.2008)"

has been evaluated by the Ethics Committee of ETH Zurich on a correspondence basis:

		Involved in the decision		
			no (reason)	
Name	Institute	yes	absent	withdrawn from the proceeding
Prof. Dr. Lutz Wingert, Präsident	Professorship for Philosophy	X		
Dr. Susanne Boshammer	Ethics Centre, University of Zurich		X	
Prof. Dr. Urs Boutellier	Human Movement Sciences & Sport	X		
Dr. Ines Egli	Food Science & Nutrition			X
Dr. Marino Menozzi	Center for Organizational & Occupational Sciences	X		
Prof. Dr. Alexander Ruch	Professorship for Law		X	
Dr. Alex Stacoff	Biomechanics	X		
Prof. Dr. Robert Steffen	Social- & Preventive Medicine	X		
Prof. Dr. Caspar Wenk	Animal Sciences, Nutrition Biology	X		

Based on the recommendation of the Ethics Committee of ETH Zurich, and your submission of the revised documents by August 26, 2008, the Executive Board of ETH Zurich has made the following decision for your research project:

☒ Full Approval without reservation
☐ Approval with reservation (written notification to the Ethics Committee sufficient)
☐ Rejection, proposal to be revised:
  ☐ Written notification to the Ethics Committee sufficient
  ☐ New evaluation by the Ethics Committee needed
☐ Negative (with justification and comments for new evaluation)
☐ Not evaluated (with justification)

1. General statement

The research project „The effects of iron and n-3 fatty acid supplementation, alone and in combination, on cognition: a randomized, double blind, 2x2 intervention trial in South African children" does not contravene principles and regulations to be followed by research projects involving human beings in Switzerland, namely:

- The scientific validity and relevance of the research project and of the expected results;
- An advantageous benefit–risk ratio;
- The written consent of the research participants;
- The protection of the privacy and confidentiality;
- The compensation in case of an injury;
- The professional qualifications of the Swiss personnel involved in the research project;

However, the Ethics Committee of ETH Zurich was unable to judge the project as being acceptable in respect to ethical aspects, as the local conditions have to be considered and judged by the local Ethics Committee in charge. The approval of the Ethics Committee of ETH Zurich therefore does not include the following aspects:

- The relevance of the research project with regard to the needs of the local health care system;
- The procedure for recruiting the participants, in particular the information sheet and consent form written in the local language;
- The adequateness of the local infrastructure (material, facilities, personnel, etc.) with regard to the optimal protection of the participants;
- The professional qualifications of the Non-Swiss research personnel

⇨ **The above mentioned items need special attention by the Ethics Committee of NWU Potchefstroom.**

2. Final Provisions

We would like to draw your attention to the fact, that you have the duty to inform the Ethics Committee of ETH Zurich in the following cases:

a) Immediately at the occurrence of unexpected events which could affect the security of the participants and/or the continuation of the research project;

b) By making changes at the research protocol or changing the treatment of the participants; or

c) By an abnormal termination of the study.

Last but not least, the comments, reservations and the final approval of the Ethics Committee of NWU Potchefstroom have to be provided to the Ethics Committee of ETH Zurich.

Kind regards,

Prof. P. Chen
Vice-President for Research

Prof. L. Wingert
Chair of the Ethics Committee

cc: Head of the Departement

Annexure 2 Acceptance letter of the American Journal of Clinical Nutrition

RE: AJCN/2013/081208

N–3 long-chain polyunsaturated fatty acids reduce respiratory morbidity caused by iron supplementation in iron-deficient South African school children: a randomized, double-blind, placebo-controlled intervention

Dear Dr. Malan:

Your revised manuscript, listed above, has been re-evaluated by The American Journal of Clinical Nutrition and is approved for publication in The Journal. However, before the manuscript can be forwarded to the Journal's Production Office, several minor items must be corrected. These are shown at the end of this letter. Once we receive a final revision containing the necessary changes, the manuscript will be forwarded directly to The AJCN Production Office.

Please resubmit the manuscript electronically at http://submit.ajcn.org and include in the "rebuttal" part of the online resubmission process your response to the comments below. This should include each comment with your response beneath it. In addition, we require that the most recent changes in your manuscript (but NOT in Supp. Material and Figures) be indicated with red text/ red font, NOT "tracked changes", and please no highlighting and no strike-outs, which make it very difficult to read.

Attached is the text file submitted with the last version of your paper. Please begin with this file when making the requested changes.

We expect to receive your revised manuscript within two weeks from today.

Thank you for your continued interest in The American Journal of Clinical Nutrition.
We look forward to receiving your revised manuscript.

Please direct any questions to ajcnsubmit@nutrition.org.

Sincerely,

Ricardo Uauy
Associate Editor

Dennis M Bier, MD
Editor-in-Chief

The American Journal of Clinical Nutrition
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Annexure 3 ISAAC Questionnaire in Zulu

Table 1: Imobuzo esemqoka mayelana nokucinana kwesifuba okukuke kuhlasele izingane ezineminyaka eyisithupha ukuya kweyisikhombisa

1. Ingane yakho ike yaba nesifuba esicinene noma esikhalayo kwisikhathi esedlule na?
   Yebo[ ]
   Cha[ ]

UMA UPHENDULE NGOKUTHI "CHA" UYACELWA UKUTHI WEQE EMINYE IMIBUZO UYE KUMBUZO WESITHUPHA (6).

2. Ingane yakho ike yaba nesifuba esicinene noma esikhalayo ezinyangeni eziyishumi nambili ezedlule na? **Yebo[ ] Cha[ ]**

UMA UPHENDULE NGOKUTHI "CHA" UYACELWA UKUTHI WEQE EMINYE IMIBUZO UYE KUMBUZO WESITHUPHA (6).

3. Ingane yakho ihlaselwe kangaki yisifuba esikhalayo kulezi zinyanga eziyushumi nambili ezedlule na? **Akazange[ ] 1 ukuya ku 3[ ] 4 ukuya ku 12[ ] Ngaphezu kwe 12 [ ]**
4. Ezinyangeni eziyishumi nambili ezedlule kukangaki lapho ingane yakho ike yaphazamiseka ukalala ngenxa yesifuba esicinene noma esikhalayo na (wheezing)?
   Akakaze avuswe ukucinana[ ] Ngaphansi kobusuku obubodwa ngeviki [ ]
   Kanye noma kabili ngeviki[ ]
5. Ezinyangeni eziyishumi nambili ezedlule, isifuba sike savaleka kangangoba ingane yangakwazi ukukhuluma kahle na, wakwazi ukukhuluma igama elilodwa ukuya kwamabili na? **Yebo[ ] Cha[ ]**
6. Ingane yakho ike yaphathwa isifuba i-**asthma (isifuba esivalekayo kubenzima ukuphefumula)?** **Yebo[ ] Cha[ ]**
7. Ezinyangeni eziyishumi nambili ezedlule, ingane yakho ike yaba nesifuba esikhalayo uma noma emuva kokuzilolonga na? **Yebo[ ] Cha[ ]**
8. Ezinyangeni eziyishumi nambili ezedlule, ingane yakho ike yaba nesifuba esomile uma ikhwehlela ebusuku, ngaphandle kokuphathwa ngumkhuhlane noma ukuba negciwane lesifuba na? **Yebo[ ] Cha[ ]**

Table 2: Imibuzo esemqoka mayelana nesifo samakhala esiki sihlasele izingane ezineminyaka eyisiyithupha ukuya kweyisikhombisa.

1. Ingane yakho ikeyaba nokuthimula, amakhala avuzayo, amakhala avalekile kodwa ingane ibingaphethwe ngumkhuhlane? **Yebo[ ] Cha [ ]**

UMA UPHENDULE NGOKUTHI "CHA" UYACELWA UKUTHI WEQE EMINYE IMIBUZO UYE KUMBUZO WESITHUPHA (6).

2. Ezinyangeni eziyishumi nambili ezedlule, ingane yakho ikeyaba nenkinga yokuthimula, amakhala avuzayo noma amakhala avalekile kodwa ingane ingaphethwe ngumkhuhlane?

Yebo[ ]

Cha[ ]

UMA UPHENDULE NGOKUTHI "CHA" UYACELWA UKUTHI WEQE EMINYE IMIBUZO UYEKUMBUZO WESITHUPHA (6).

3. Ezinyangeni eziyishumi nambili ezedlule, ngabe kuke kwabakhona yini lenkinga yamakhala ehambisana namehlo akhalayo abe eluma futhi na? **Yebo[ ] Cha[ ]**

4. Ezinyangeni eziyishumi nambili ezedlule inkinga yamakhala ike yabakhona na enganeni (Ngizocela ukuthi ukhethe inyanga lapho ingane ibiphethwe yilenkinga)?
   January[ ] February[ ] March[ ] April[ ] May[ ] June[ ]
   July[ ] August [ ] September[ ] October[ ] November[ ] December[ ]

5. Ezinyangeni eziyishumi nambili ezedlule, inkinga yamakhala iyiphazamise kanjani ingane yakho kwisimo esijwayelekile? **Akazange[ ] Kancane[ ] Kahle nje[ ] Kakhulu[ ]**

6. Ingane ike yahlaselwa yimfiva ethimulisayo (**hay fever**)? **Yebo[ ] Cha[ ]**

<u>Table 3: Imibuzo esemqoka mayelana nokuluma okuhambisana namashashazi kwisikhumba sengane eneminyaka eyisithupha ukuya kweyisikhombisa</u>

1. Ingane yakho ike yaphathwa yisikhumba esilumayo futhi esinamashashazi aziphumelayo futhi kuze kwuqhubeke isikhathi esingangezinyanga eziyisithupha na? **Yebo[ ] Cha[ ]**

UMA UPHENDULE NGOKUTHI "CHA" UYACELWA UKUTHI WEQE EMINYE IMIBUZO UYE KUMBUZO WESITHUPHA (6).

2. Ingane yakho ikeyaba nesikhumba esilumayo ezinyangeni eziyishumi nambili ezedlule na?

Yebo[ ]

Cha[ ]

UMA UPHENDULE NGOKUTHI "CHA" UYACELWA UKUTHI WEQE EMINYE IMIBUZO UYE KUMBUZO WESITHUPHA (6).

3. Kungabe lamashashazi alumayo ake avela kulezi zindawo ezilandelayo: ngaphimbili endololwaneni, ngemuva kwamadolo, ngaphambili emaqakaleni, ngaphansi kwezinqe, entanyeni, ezindlebeni noma emehlweni? **Yebo[ ] Cha[ ]**

4. Yayineminyaka emingaki ingane yakho uma amashashazi evela okokuqala?

   Ngaphansi kweminyaka emibili [ ] ,**Iminyaka emibili ukuya kwemine [ ] Iminyaka emihlanu nangaphezulu [ ]**

5. Ake amashashazi anyamalala noma aphela nya esikhathini sezinyanga eziyishumi nambili ezedlule? **Yebo[ ] Cha[ ]**

6. Ezinyangeni eziyishumi nambili ezedlule,kukangaki ,ngendlela eyejwayelekile, kukhona lapho usuku ingane yakho ingazange ilale ngenxa yamashashazi alumayo? **Akakaze kulezinyanga eziyishumi nambili ezedlule[ ] Ngaphansi kobusuku obubodwa ngeviki, Ubusuku noma kaningi evikini[ ]**
7. Ingabe ingane yakho ike yaphathwa yinkinga yokuba nesikhumba esilumayo futhi sibe namashashazi na **(eczema)?** **Yebo[ ] Cha[ ]**