The immune modulatory effects of omega-3 polyunsaturated fatty acids and iron as applied in an animal pulmonary tuberculosis model

A Nienaber

orcid.org / 0000-0002-1013-6740

Thesis accepted for the degree Doctor Philosophy in Dietetics at the Potchefstroom Campus of the North-West University

Promoter: Dr L Malan
Co-promoter: Dr RC Dolman
Co-promoter: Prof R Blaauw

Graduation: May 2020
Student number: 20268866

PREFACE

Every good and perfect gift is from above, coming down from the Father, who never changes (James 1:17).

I firstly want to thank God for the opportunity that He gave me to complete my Ph.D. and how He continued to give me the strength, insight, and perseverance that I needed until the end.

I want to thank my husband, for supporting me. Thank you for taking over most of my responsibilities at home when I had to work, loving and motiving me, and praying for me. I would not have been able to do this without your support. Also, thank you to my two boys, Eckerd and Nikolas for sharing your mommy with her Ph.D. and reminding me that life is not only about work. I also want to thank my family, especially my mom, and all my friends that supported me through this journey.

A special thank you to my supervisor Dr. Linda Malan. Thank you for your unconditional support, patience, understanding and the tireless effort you put into guiding me. You have done so much more than what is expected from a study leader and I've learned many valuable things from you for my career and personal life.

To my two co-supervisors, Dr. Robin Dolman and Prof Renee Blaauw. A big thank you for all the time and effort that you spent in helping me to produce the best thesis that I can. I'm also grateful for all the support and motivation that you provided along the way.

Thank you to our director Prof Marius Smuts for all the effort you put into reviewing my manuscripts and enlightening me with your knowledge. Thank you for your support and motivation and that you believed in my abilities, it meant so much.

Thank you to Dr. Jeannine Baumgartner and Dr. Lizelle Zandberg for the time and effort that you put into helping me. I've learned so much from both of you.

A big thank you to Dr. Suraj Parihar and Dr. Mumin Ozturk from the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, for your hard work in the execution of the study and the laboratory analyses. I also want to thank you for all your guidance and valuable inputs into the manuscripts, for always being available and helping me to understand concepts that were foreign to me. Also, thank you to Kobus Venter for all your inputs into the design of the study.

I want to thank Cecile Cooke and Adriaan Jacobs for all the laboratory work, your roles in this project are irreplaceable.

Also thanks to the M.Sc. students Simone King and Melinda Britz for all your hard work in the execution of the study and analyses. It was a privilege to have worked with you two. Thank you also to Frank Hayford for your contributions to the manuscripts and the study.

I want to thank Prof Du Toit Loots for your valuable inputs into the design of the study and the writing of the first manuscript.

Special thanks to Mary Hoffman and Petra Gainsforth for the language and technical editing of this thesis.

I also want to acknowledge all the personnel of the Centre of Excellence for Nutrition and Nutrition department at the North-West University for their support and cheering me on until the end.

The financial support for this project that was provided by the South African Medical Research Council and the Nutricia Research Foundation is gratefully acknowledged.

ABSTRACT

Background

Non-resolving inflammation is characteristic of tuberculosis (TB). This leads to lung tissue damage and anaemia of infection, which is associated with poor clinical outcomes. Iron supplementation may have limited efficacy and may favour bacterial growth. Therefore, anti-inflammatory and pro-resolving host-directed therapy (HDT) have been suggested. Omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs) including eicosapentaenoic and docosahexaenoic acid (EPA/DHA) may provide such a nutritional approach for HDT.

Aim

The aim of this thesis was to determine the effects of EPA/DHA and iron supplementation, alone and in combination, on inflammatory and clinical outcomes in *Mycobacterium tuberculosis* (*Mtb*)-infected mice and whether these effects were dependent on n-3 polyunsaturated (n-3 PUFA) status prior to infection.

Methods

Male C3HeB/FeJ mice were conditioned on n-3 PUFA sufficient or deficient diets for six weeks prior to infection. One week post *Mtb* infection, n-3 PUFA sufficient mice were randomised to 1) continue on the n-3 PUFA sufficient diet, or were switched to an 2) EPA/DHA-supplemented, 3) iron-supplemented, or 4) EPA/DHA and iron-supplemented diet. Mice conditioned on an n-3 PUFA deficient diet were randomised to 1) continue on the n-3 PUFA-deficient diet, or switched to the 2) n-3 PUFA sufficient, or 3) EPA/DHA-supplemented diets. The mice received these diets for three weeks until euthanasia.

Results

The phospholipid fatty acid composition of cell membranes of mice reflected the dietary fatty acid content. Pro-resolving lung lipid mediator profiles were found in the EPA/DHA-supplemented groups in both n-3 PUFA sufficient and low-status arms, and also when combined with iron. Additionally, EPA/DHA supplementation resulted in lower bacterial loads and lung pathology in the sufficient, but not in the low-status group. Iron and EPA/DHA supplementation both individually lowered systemic and lung cytokines, together with improved anaemia of infection markers. However, whilst EPA/DHA supplementation lowered lung T cells, iron resulted in higher lung immune cell counts. Iron also had no effect on lung pathology or bacterial load but lowered body weight gain. There were iron x EPA/DHA interactions to attenuate the lowering effects of

iron or EPA/DHA on anaemia of infection, and of iron for higher immune cell counts. Furthermore, compared with the n-3 PUFA sufficient diet, EPA/DHA supplementation provided superior benefits in body weight gain, bacterial load, and lung inflammation in low-status mice.

Conclusions

The findings of this thesis showed that EPA/DHA supplementation, after the initial inflammatory response, has antibacterial and inflammation-resolving benefits and improves markers of anaemia of infection in TB, depending on n-3 PUFA status. On the other hand, iron promotes anti-inflammatory effects and improves markers of anaemia, but enhances immune cell recruitment and lowers body weight gain. Providing combination iron and EPA/DHA treatment attenuates their individual beneficial effects. Lastly, in low-status mice, EPA/DHA provides superior effects compared with an n-3 PUFA sufficient diet. Considering this, iron may not be detrimental concerning worsening the bacterial burden in TB, but moreover, n-3 LCPUFA therapy may be a promising approach as HDT in TB.

Keywords: anaemia of infection, inflammation, iron, omega-3 long-chain polyunsaturated fatty acids, tuberculosis

ABBREVIATIONS

AA	arachidonic acid
AD	alzheimer's disease
ALA	alpha-linolenic acid
AMP	adenosine monophosphate
ANOVA	analysis of variance
BSA	bovine serum acid
CCL3	chemokine ligand 3
CEN	Centre of Excellence for Nutrition
CFU	colony-forming units
CHD	coronary heart disease
CIDRI	Centre of Infectious Diseases Research in Africa
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
CRP	C-reactive protein
CVD	cardiovascular disease
CYT P450	cytochrome P450
DC	dendritic cells
DHA	docosahexaenoic acid
DMT-1	divalent metal transporter-1
DNA	deoxyribonucleic acid
DPA	docosapentaenoic acid
EFA	essential fatty acid
EPA	eicosapentaenoic acid
FA	fatty acid
FAME	fatty acid methyl esters
FAO	Food and Agriculture Organisation of the United Nations
GCMS	gas chromatography-mass spectrometry
GMCSF	granulocyte-macrophage colony-stimulating factor
GPR	G-protein coupled receptor

HDHA	hydroxydocosahexaenoic acid
HDT	host-directed therapy
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
HIV	human immunodeficiency virus
HOTrE	hydroxyoctadecatrienoic acid
HPDHA	hydroxyperoxydocosahexaenoic acid
HPEPE	hydroperoxyeicosapentaenoic acid
HPETE	hydroxyperoxyeicosatetraenoic acid
ICGEB	International Centre of Genetic Engeneering and Biotechnology;
IDM	Institute of Infectious Diseases and Molecular Medicine
IL	interleukin
IFN-α	interferon-alpha
IFN -γ	interferon-gamma
IGRA	interferon-gamma release assay
iPLA	calcium-independent phospholipase A
IRIS	immune reconstitution inflammatory syndrome
ISSFAL	International Society for the Study of Fatty Acids and Lipids
LA	linoleic acid
LCPUFA	long-chain polyunsaturated fatty acids
LM	lipid mediator
LOX	lipoxygenase
LT	leukotriene
LTA_4H	leukotriene–A_4 hydrolase
LX	lipoxin
MaR	maresins
MCP-1	monocyte chemoattractant protein-1
MCTR	maresin conjugate in tissue regeneration
MDR-TB	multidrug-resistant tuberculosis
Mo DCs	monocyte-derived dentric cells
MMP	matrix metalloproteinase
Mtb	*Mycobacterium tuberculosis*

NAAT	nucleic acid amplification test
NICE	National Institute for Health and Care Excellence
NK	natural killer
NF-$_k$B	nuclear factor kappa B
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
n-3	omega-3
n-3+	omega-3 long-chain polyunsaturated fatty acid supplemented diet
n-6	omega-6
n-9	omega-9
n-3FAD	omega-3 fatty acid-deficient diet
n-3FAS	omega-3 fatty acid sufficient diet
(n-3)eFAS	omega-3 essential fatty acid sufficient diet
NWU	North-West University
PBMC	peripheral blood mononuclear cell
PCTR	protectin conjugate in tissue regeneration
PE	phosphatidylethanolamine
PG	prostaglandin
PLA_2	phospholipase A_2
PMNL	polymorphonuclear leukocyte
PMN SWAMS	polymorphonuclear leukocyte infiltration
PPAR	peroxisome proliferator-activated receptor
PTB	pulmonary tuberculosis
PUFA	polyunsaturated fatty acids
QOL	quality of life
RA	rheumatoid arthritis
RBC	red blood cell
RCTR	resolvin conjugate in tissue regeneration
RNS	reactive nitrogen species
ROS	reactive oxygen species
RvD	D-series resolvins
RvE	E-series resolvins
SA	South Africa
SAM	severe acute malnutrition

SEM	standard error of the mean
SPSS	Statistical Programme for Social Sciences
SIRS	systemic inflammatory response syndrome
SPM	specialised pro-resolving mediators
TB	tuberculosis
TE	total energy
TfR	transferrin receptor
Th	T helper
TLC	thin-layer chromatography
TNF-α	tumour necrosis factor-alpha
TST	tuberculin skin test
TX	thromboxane
UCT	University of Cape Town
Vit	Vitamin
WHO	World Health Organization
XDR-TB	extensively drug-resistant tuberculosis
Zn	Zinc

LIST OF SYMBOLS AND UNITS

α	alpha
β	beta
CFU	colony-forming units
dL	decilitre
°C	degrees Celsius
IU	international units
g	gram
µL	microliter
mg	milligram
mL	millilitre
n	number
pg	picogram
%	percentage
>	greater than/ above
<	less/ lower than

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

1.1 General background

1.1.1 The burden of tuberculosis globally and locally

Tuberculosis (TB) remains one of the leading causes of death globally (WHO, 2019). In 2018, there were 10 million incident cases worldwide, with the African continent contributing 24% of these cases (WHO, 2019). South Africa (SA) comprises 3% of the total global TB cases. Multidrug-resistant TB (MDR-TB) is another growing concern, accounting for 3.4% of new and 18% of previously treated cases worldwide in 2018 (WHO, 2019). Additionally, MDR-TB and extensively drug-resistant TB (XDR-TB) patients are burdened by lengthy and costly treatment times and poor cure rates, with the resultant side effects, drug toxicity and poor compliance (Dheda *et al.*, 2017). Efficient TB treatment remains lifesaving, as statistics have shown that 48 million deaths have been averted by TB treatment in human immunodeficiency virus (HIV)-negative TB patients between 2000 and 2018 (WHO, 2019). Nevertheless, there is definitely room for improvement in TB management locally and globally, not only to reduce mortality rates and medical costs but also to improve the quality of life in those surviving this disease (De La Mora *et al.*, 2015).

1.1.2 The inflammatory and immune response in tuberculosis and its consequences

The highly infective *Mycobacterium tuberculosis* (*Mtb*) is transferred from an individual with active TB *via* the airborne route and is the most common cause of TB (Jeong & Lee, 2008). The lungs are the primary entry site for *Mtb*-infected droplets and disease manifestation, resulting in 80% of TB patients having pulmonary TB (PTB) (Kaufmann & Dorhoi, 2013; Kumar, 2016). This thesis will, therefore, focus on PTB resulting from *Mtb* infection. An inflammatory and immune response is elicited upon host detection of *Mtb* in order to protect the host from active TB infection. However, *Mtb* manipulates the host response to benefit itself, replicate and spread from cell to cell (Davis & Ramakrishnan, 2009; Ernst, 2012). It is well known that inflammation is an important part of host defense, but TB elicits a hyperactive response and is a disease characterised by chronic non-resolving inflammation (Kumar, 2016; Kumar *et al.*, 2019). Unfortunately, the host's attempt to protect itself eventually contributes to lung tissue destruction and TB transmission (Cooper & Torrado, 2012; Kumar, 2016).

One of the key pathological features of TB is that it recruits immune cells to the pulmonary spaces, followed by the development of lung granuloma and alterations in lung tissue (lesion formation) (Ernst, 2012; Kaplan *et al.*, 2003; Robinson *et al.*, 2015). Granuloma formation is initiated when local alveolar macrophages recognise and phagocytose the bacteria, which leads to the influx of

more macrophages, dendritic cells (DCs) and neutrophils, mediated *via* the secretion of cytokines and chemokines that induce the innate immune responses (Hawn *et al.*, 2013; Marzo *et al.*, 2014). Lymphocytes are then recruited to the granuloma site and surround the core cells (Dartois, 2014; Ndlovu & Marakalala, 2016). The formation of granulomas is intended to separate the *Mtb*-infected macrophages from surrounding healthy tissues, but also to keep them in close contact with T cells (Dorhoi & Kaufmann, 2016; Egen *et al.*, 2008). However, granuloma formation also provides a safe place for bacteria to persist and replicate (Dorhoi & Kaufmann, 2016; Egen *et al.*, 2008; Peters & Ernst, 2003).

Cavity formation from liquefied granuloma is the most destructive form of TB and correlates with high transmission rates (Dorhoi & Kaufmann, 2016). Cavitation results partly from the host's exacerbated inflammatory response. Therefore, it can be reasoned that the host immune response, under the direction of the pathogen, actually facilitates lung tissue damage (Dorhoi & Kaufmann, 2016; Kumar *et al.*, 2019). Unfortunately, in 14 to 100% of patients, cavities, scarring (fibrosis), and pleural adhesions persist, contributing to persistent abnormal lung function even after TB cure and the resultant lower quality of life (De La Mora *et al.*, 2015; Manji *et al.*, 2016; Meghji *et al.*, 2016; Nihues *et al.*, 2015; Theegarten *et al.*, 2006). It is clear that the lowering of the heightened inflammatory response in TB may be of utmost importance.

1.1.3 Iron deficiency and anaemia of infection in tuberculosis

In addition to lung tissue damage, iron-deficiency anaemia and anaemia of infection are also common complications among TB patients. These anaemias affect 30 to 94% of TB patients, with anaemia of infection being the most prevalent (in up to 71.7% of TB patients) (Devi *et al.*, 2003; Hella *et al.*, 2018; Isanaka *et al.*, 2011; Karyadi *et al.*, 2000; Lee *et al.*, 2006; Sahiratmadja *et al.*, 2007; Van Lettow *et al.*, 2005). The causes of iron-deficiency anaemia can be multi-factorial and either TB-related or from other causes (Kant *et al.*, 2015; Karyadi *et al.*, 2000). On the other hand, anaemia of infection occurs as a protective strategy by the host's innate immune system to reduce iron availability for the bacteria, as bacteria require iron from the host for growth and persistence (Agoro & Mura, 2019; Ganz, 2019; Mishra *et al.*, 2018). Anaemia of infection is partly caused by the up-regulation of hepcidin mediated by the host inflammatory response. Hepcidin causes the sequestration of iron into macrophages, shifting available iron (bound to transferrin), to stored iron (in the ferritin form), together with reduced iron absorption (Kurthkoti *et al.*, 2017; Mishra *et al.*, 2018; Schmidt, 2015). Chronic inflammation in TB patients causes continuous up-regulation of hepcidin and low iron availability for the physiological functions of the host, such as erythropoiesis (Nemeth *et al.*, 2004; Nicolas *et al.*, 2002). Low iron levels can have detrimental consequences in TB patients, including symptoms such as impaired motor activity as well as compromised immune function (Brock, 2018; Ekiz *et al.*, 2005; Ifeanyi, 2018; Mishra *et al.*, 2018; Oppenheimer, 2001). Therefore, anaemia is a risk factor for poor TB outcomes and has been linked to TB

recurrence, higher mortality rates and delayed sputum conversion (Isanaka *et al.*, 2011; Isanaka *et al.*, 2012; Nagu *et al.*, 2014; Shimazaki *et al.*, 2013).

1.2 Rationale for this study

As mentioned, TB treatment is still not ideal, therefore, the authors of recent reviews recommend that the host's response in TB should be addressed by future treatment strategies (Hawn *et al.*, 2013; Kim & Yang, 2017; Palucci & Delogu, 2018; Stek *et al.*, 2018; Zumla *et al.*, 2013). This is where host-directed therapies (HDTs) have emerged as a promising field in TB treatment, serving as support for current treatment regimes. In HDT, therapy is directed at enhancing the host's protection and bacterial killing, rather than being aimed directly at the pathogen (Baindara, 2019; Hawn *et al.*, 2013; Kim & Yang, 2017; Palucci & Delogu, 2018; Stek *et al.*, 2018). Therefore, these HDTs are not subject to bacterial drug resistance. Various HDT options have been explored, one of which includes anti-inflammatory drugs (Kim & Yang, 2017; Mayer-Barber *et al.*, 2014; Mayer-Barber & Sher, 2015; Palucci & Delogu, 2018; Stek *et al.*, 2018). Anti-inflammatory drugs reduce inflammation, thereby attenuating lung pathology, including the size and number of lung lesions; reducing bacillary loads; and improving mortality and survival rates in TB (Byrne *et al.*, 2006; Byrne *et al.*, 2007; Critchley *et al.*, 2013; Kroesen *et al.*, 2017; Kroesen *et al.*, 2018; Marzo *et al.*, 2014; Vilaplana *et al.*, 2013). However, apart from beneficial effects, they also carry well-known side effects, such as the risk of gastrointestinal ulcers, bleeding and cardiovascular risks (Ivanyi & Zumla, 2013). A nutritional approach to enhance anti-inflammatory and pro-resolving pathways may be a promising way to deliver the same benefits, but with fewer side effects.

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) may be the answer as they facilitate pro-resolving and anti-inflammatory pathways by altering the membrane phospholipid fatty acid composition of blood and tissue cells that are important in immune and inflammatory responses (Browning *et al.*, 2012; Calder, 2017; Jakiela *et al.*, 2013). They firstly partially replace arachidonic acid (AA) in membranes as the substrate for pro-inflammatory lipid mediators (LMs) (Browning *et al.*, 2012; Calder, 2015; Healy *et al.*, 2000). Furthermore, they also serve as precursors for the specialised pro-resolving mediators (SPMs), which are synthesised from the n-3 long-chain PUFAs (LCPUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These SPMs, including resolvins (Rv), protectins and maresins, promote anti-inflammatory pathways and actively contribute to inflammation resolution and the restoration of tissue functioning (Barden *et al.*, 2016; Jaudszus *et al.*, 2013; Mas *et al.*, 2016; Morris *et al.*, 2009; Serhan, 2017a). They also alter immune cell recruitment by halting neutrophil infiltration and lowering T cell proliferation (Calder, 2015b; Fielding *et al.*, 2019; Serhan *et al.*, 2017b). In addition, SPMs have direct effects on the stimulation of monocytes to migrate and differentiate into macrophages for phagocytic activity, and to enhance bacterial phagocytosis and killing (Chiang *et al.*, 2012; Codagnone *et al.*, 2018; Lee & Zeldin, 2015; Spite *et al.*, 2009).

Promising results have been found with n-3 PUFA supplementation as an adjunct treatment in various conditions with inflammation as one of the main contributors to disease pathology (Calder, 2015b). One may then predict that n-3 LCPUFAs could also be beneficial in TB with its exacerbated inflammatory response and resultant lung damage. According to Fullerton *et al.* (2014), the manipulation of LMs can be useful as part of immunomodulatory therapy in TB and work synergistically or additively with other standard treatments. Furthermore, there has recently also been shown interest in the importance of pro-resolving LM profiles in TB outcomes (Colas *et al.*, 2019). Omega-3 LCPUFAs may be beneficial in TB by enhancing inflammation resolution and contributing to limiting bacterial growth in order to protect lung tissue (Dietzold *et al.*, 2015; Lenaerts *et al.*, 2015). However, studies on the application of n-3 LCPUFAs in TB are very limited. The few available studies have portrayed mixed results with some showing benefit with regard to bacterial killing and pulmonary inflammation (Bonilla *et al.*, 2010b; Jordao *et al.*, 2008), whilst others reported harm, with higher bacterial loads in n-3 PUFA supplemented groups (Bonilla *et al.*, 2010b; Bonilla *et al.*, 2010a; Mayatepek *et al.*, 1994; McFarland *et al.*, 2008; Paul *et al.*, 1997). These experiments, when examined critically, have a number of limitations that have led to heterogeneous results. Moreover, in the only two clinical trials that have been conducted on n-3 LCPUFA therapy in TB patients, a positive effect was found on sputum smear conversion, body weight gain, inflammation resolution, and $CD4^+$ T cell count. In these trials, however, n-3 LCPUFA supplementation was combined with other micronutrients, and, therefore, the effects found cannot be attributed to n-3 LCPUFAs alone (Durry *et al.*, 2018; Nenni *et al.*, 2013).

More research is clearly needed in order to determine whether the beneficial results that have been found with the provision of anti-inflammatory drug treatment can be replicated with this nutritional approach. This is where the current research project will contribute. C3HeB/FeJ mice were used as TB model, which have been extensively utilised in TB experiments and may provide more accurate predictions of treatment effects than other animal models, thereby avoiding premature, long and costly phase three trials (Driver *et al.*, 2012; Gupta *et al.*, 2013; Irwin *et al.*, 2015; Lanoix *et al.*, 2015; Lenaerts *et al.*, 2015).

In addition, TB patients may not have ideal n-3 PUFA intake and the resultant status, as it has been found that in most individuals consuming Western diets (including those in SA), n-3 PUFA intake is low with an unbalanced dietary n-6:n-3 PUFA ratios and the resultant low n-3 PUFA status (Baker *et al.*, 2016; Baumgartner *et al.*, 2012; Blasbalg *et al.*, 2011; Bolton-Smith *et al.*, 1997; Childs *et al.*, 2008; Ford *et al.*, 2016; Meyer *et al.*, 2003; Rahmawaty *et al.*, 2013; Schloss *et al.*, 1997; Stark *et al.*, 2016). Therefore, our model will further mimic a group of TB patients with sufficient versus low n-3 PUFA status at TB infection, to investigate how this may influence n-3 LCPUFA treatment outcomes.

As mentioned earlier, iron-deficiency anaemia and anaemia of infection is a high burden in TB patients. As anaemia of infection is related to the inflammatory response, iron supplementation may not be beneficial in such patients (Ganz, 2019; Kerkhoff *et al.*, 2015; Weiss *et al.*, 2019). The immunomodulatory effects of n-3 LCPUFAs as an adjunct to TB treatment may contribute to inflammation resolution and the restoration of iron status. Nevertheless, iron-deficiency anaemia may remain unresolved even with effective TB treatment and inflammation resolution, thereby requiring iron supplementation (Hella *et al.*, 2018; Isanaka *et al.*, 2011; Minchella *et al.*, 2014). The supplementation of iron in TB is complicated as iron provision may also increase iron availability for the pathogen, and iron absorption may be compromised (Ifeanyi, 2018; Mishra *et al.*, 2018). In this regard, Malan *et al.* (2016) found that supplementing SA iron-deficient schoolchildren with iron alone resulted in a pro-inflammatory LM profile, but that providing iron in combination with a mixture of n-3 LCPUFAs maintained the anti-inflammatory LM profile resulting from n-3 LCPUFA supplementation. Additionally, the increased respiratory morbidity resulting from iron supplementation was prevented by combined supplementation of iron with n-3 LCPUFAs (Malan *et al.*, 2014; Malan *et al.*, 2016). Therefore, combining iron supplementation with n-3 LCPUFAs may be a promising approach to delivering iron more safely in TB patients as well, and will also be investigated in this research.

This being said, in low-and middle-income countries with a high TB burden and where TB drug treatment already comprises a large portion of the national public health budget (Massyn *et al.*, 2017; WHO, 2019), EPA and DHA supplementation as HDT may seem to add to this financial burden. Although somewhat controversial, the essential n-3 LCPUFA precursor alpha-linolenic acid (ALA) has been found to have a dose-dependent inflammation-resolving effect in animal diabetes, cancer, aortic banding and asthma-induced lung inflammation models (Duda *et al.*, 2008; Jangale *et al.*, 2016; Kaveh *et al.*, 2019; Moura-Assis *et al.*, 2018; Schiessel *et al.*, 2016). Moreover, there is some evidence in animals that the effects of supplementary concentrations of ALA itself on inflammation are similar to supplementing preformed EPA and DHA (Jangale *et al.*, 2016; Schiessel *et al.*, 2016). This is mostly related to the conversion of ALA to EPA and DHA which is more efficient in rodents than in humans (Brenna *et al.*, 2009). Conversion rates are higher in a stimulated immune response (Sibbons *et al.*, 2018) and in low n-3 PUFA status (Igarashi *et al.*, 2007). Furthermore, when lowering the linoleic acid (LA):ALA ratio, ALA conversion have also been shown to be higher, as the conversion of ALA to EPA and DHA utilises the same desaturase enzymes for metabolism as does LA for conversion to AA (Gibson *et al.*, 2011; Gibson *et al.*, 2013; Goyens *et al.*, 2006). As mentioned above, the general population's essential n-6:n-3 PUFA intake ratio is high (Baker *et al.*, 2016; Baumgartner *et al.*, 2012; Ford *et al.*, 2016; Richter *et al.*, 2014; Stark *et al.*, 2016). It was, therefore, further hypothesised that altering dietary intake to favour providing sufficient amounts of ALA and reducing the high n-6:n-3 PUFA ratios may provide similar beneficial effects as EPA and DHA supplementation. Therefore,

in the public health context, providing dietary education to provide sufficient n-3 PUFA intake and improve n-6:n-3 PUFA ratios, may possibly, avoid the financial burden of supplementing with preformed EPA and DHA in TB patients. The conceptual framework of this research project is presented in Figure 1.1.

1.3 The conceptual framework for this research

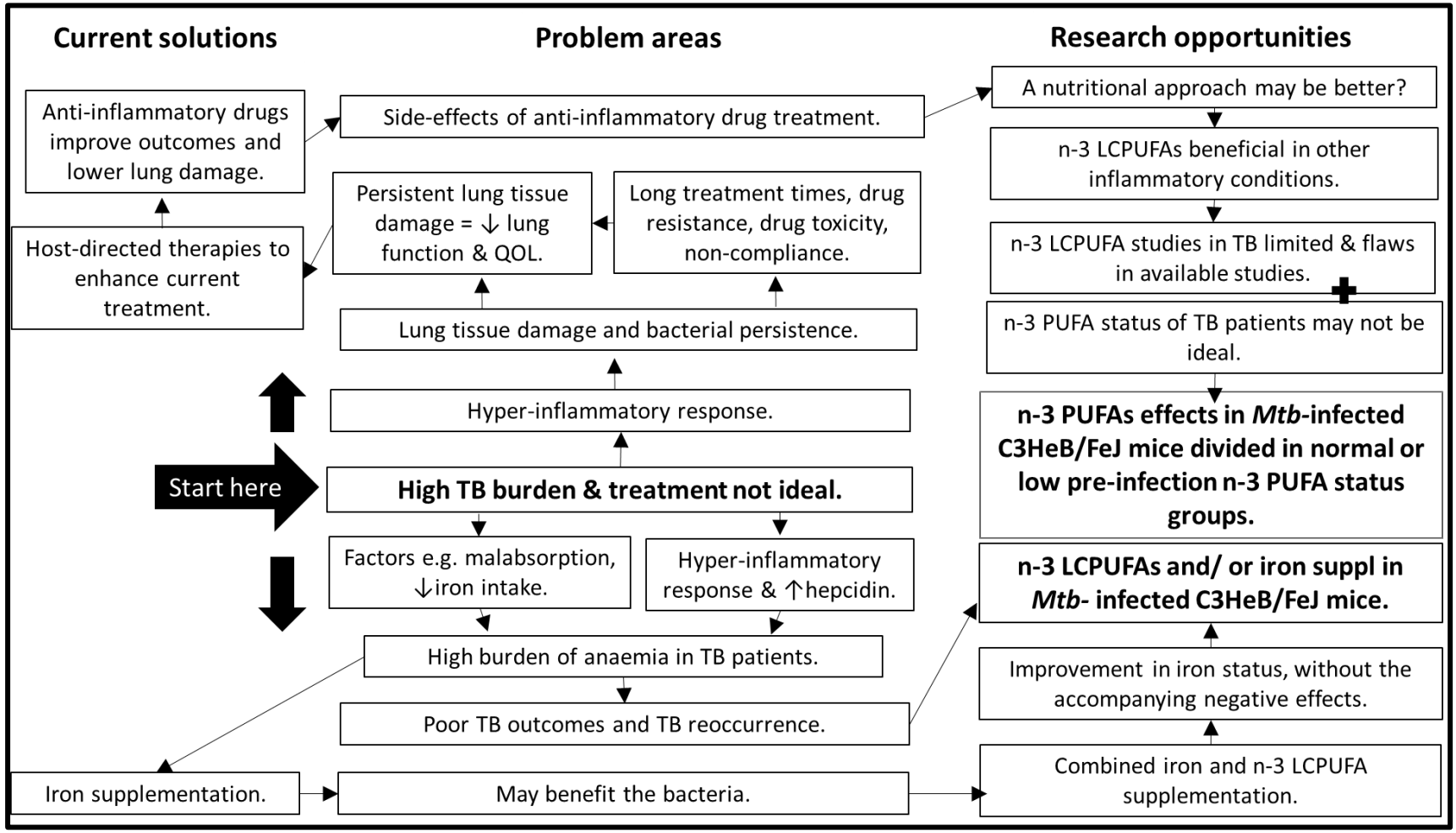


Figure 1-1 The conceptual framework for this thesis

n-3 LCPUFA: omega-3 long-chain polyunsaturated fatty acid; n-3 PUFA: omega-3 polyunsaturated fatty acid; TB: tuberculosis; QOL: quality of life.

1.4 Study aim and objectives

The aim of this thesis is to determine the effects of n-3 LCPUFA and iron supplementation, alone and in combination, on the inflammatory response and clinical outcomes in *Mtb*-infected mice and whether these effects are dependent on n-3 PUFA status prior to infection.

The objectives of this thesis are to:

1. Determine the effects of n-3 LCPUFA supplementation in *Mtb*-infected mice with an n-3 PUFA sufficient or low status, respectively, prior to infection and supplementation on:

(a) Clinical outcome measures, including lung- and spleen-weight indexes, body weight change, bacillary load (lung), and lung pathology (free alveolar space);

(b) Markers of the immune and inflammatory response, including lung LMs, cytokines, and immune cell phenotypes; and

(c) Long-chain PUFA status (total phospholipid fatty acid composition in red blood cells (RBCs), crude lung tissue homogenates, and peripheral blood mononuclear cells (PBMCs));

2. Investigate the effect of n-3 LCPUFA and iron supplementation, alone or in combination in *Mtb*-infected mice, on:

(a) Clinical outcome measures, including lung- and spleen-weight indexes, body weight change, bacillary load (lung), and lung pathology (free alveolar space);

(b) Markers of the immune and inflammatory response, including lung LMs, lung and plasma cytokines, and lung immune cell phenotypes;

(c) Long-chain PUFA status (total phospholipid fatty acid composition in RBCs, crude lung tissue homogenates, and PBMCs); and

(d) Indices of iron status and anaemia of infection, including haemoglobin, ferritin, soluble transferrin receptor (sTfR), and hepcidin; and

3. Compare the effects of a diet sufficient in the n-3 essential fatty acid, ALA to an n-3 LCPUFA supplemented diet in *Mtb*-infected mice with a low n-3 PUFA status on:

(a) Clinical outcome measures, including lung- and spleen-weight indexes, body weight change, bacillary load (lung), and lung histology (free alveolar space);

(b) Markers of the inflammatory response, including lung LMs and cytokines; and

(c) Polyunsaturated fatty acid status (total phospholipid fatty acid composition in RBCs, crude lung tissue homogenates, and PBMCs).

1.5 Ethical approval

This study was approved by the AnimCare Animal Research Ethics Committee of the North-West University (ethics number: NWU-00260-16-A5) (Annexure A), as well as the Animal Research Ethics Committee of the University of Cape Town (AEC 015/040) (Annexure B).

1.6 Thesis outline

Chapter one provides an introduction to this thesis. Chapter two consists of a literature review focussing on the role of n-3 PUFA and iron in the immune and inflammatory response as applied in TB. The first part of the literature review provides an overview of TB as an infectious disease, including statistics, disease pathology, presentation, diagnosis, and current treatment. The second part of the literature review focusses on n-3 PUFAs and its functions in the immune and inflammatory response, as well as its application in TB. The subsequent sections elaborate on iron metabolism in inflammation, as well as the contextualisation of iron in the TB setting and on iron supplementation in this disease. Thereafter, the literature on the interaction between iron and n-3 PUFAs is reviewed. The last section of this literature review focuses on the use of animal models in TB, thereby concluding Chapter two.

Chapter three is a manuscript entitled “Omega-3 long-chain fatty acids promote antibacterial and inflammation-resolving effects in *Mycobacterium tuberculosis*-infected C3HeB/FeJ mice, dependent on fatty acid status”. This manuscript has been submitted to *Mucosal Immunology* and the chapter was, therefore, prepared according to the instructions to authors and style of the journal (Annexure C).

Chapter four presents the manuscript entitled “Both post-infection n-3 fatty acid and iron supplementation alone, but not in combination, lower inflammation and anemia of infection in *Mycobacterium tuberculosis*-infected mice” that will be submitted to *The Journal of Nutrition* and prepared according to the instructions of the journal (Annexure D).

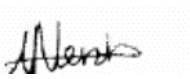
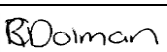
The last manuscript entitled “Switching low (n-3) fatty acid status mice to a sufficient diet does not provide the antibacterial and inflammation-resolving benefits of (n-3) long-chain fatty acid supplementation in tuberculosis” will be submitted to *The Journal of Nutrition*, is provided in Chapter five and was prepared according to the instructions of the journal (Annexure D).

Chapter six concludes this thesis. This chapter provides a discussion of the results, conclusions, indicates how objectives were met, describes the implications of the findings and, lastly, gives recommendations for future research.

1.7 Contributions of the authors to the articles presented in this thesis

The members of the research team and their contributions are described in Table 1.1.

Table 1-1 Research team and contributions

Team member*	Role and responsibilities	Institution	Signature
Mrs A Nienaber	Ph.D. student. Responsible for the conceptualisation and planning of the research project. Involved in the management and execution of the study. The student also performed the statistical analyses and the writing of manuscripts and thesis.	CEN, NWU	
Dr L Malan	Promoter: Principal investigator of research project. Played a role in the conceptualisation, planning, and execution of the research project and managed laboratory analyses. Co-authored the three manuscripts (Chapter 3, 4 and 5).	CEN, NWU	
Dr RC Dolman	Co-promoter: Experienced in therapeutic nutrition. Involved in the conceptualisation and planning of this research project. Co-authored the three manuscripts (Chapter 3, 4 and 5).	CEN, NWU	
Prof. R Blaauw	Co-promoter: Experienced in therapeutic nutrition. Involved in the conceptualisation and planning of this research project. Co-authored the three manuscripts (Chapter 3, 4 and 5).	Division of Human Nutrition, Stellenbosch University	
Prof. CM Smuts	Expert in fatty acids. Involved in conceptualisation of the study and co-authored all three manuscripts (Chapter 3, 4 and 5).	CEN, NWU	
Dr SP Parihar	Expert in TB mice model studies, immunology, and laboratory analyses. Played an advisory role and assisted with the planning and execution of the research project. Co-authored the three manuscripts (Chapter 3, 4 and 5).	CIDRI-Africa and IDM, University of Cape Town, Cape Town	
Dr M Ozturk	Expert in TB mice model studies, immunology, and laboratory analyses. Assisted with the execution of the research project. Co-authored the three manuscripts (Chapter 3, 4 and 5).	ICGEB, IDM, Division of Immunology, University of Cape	

Team member*	Role and responsibilities	Institution	Signature
		Town, Cape Town	
Prof. F Brombacher	Expert in experimental models of infectious diseases and immunology. Head of the host laboratory where the experiments were conducted. Co-authored the three manuscripts (Chapter 3, 4 and 5).	ICGEB-IDM, Division of Immunology, University of Cape Town, Cape Town	
Prof. D Loots	Expert in tuberculosis. Advisor in the planning of the research project. Co-authored the first manuscript (Chapter 3).	Laboratory of Infectious Disease Metabolomics, Centre for Human Metabolomics, NWU	
Dr L Zandberg	Expert in fatty acid metabolism. Co-authored the three manuscripts (Chapter 3, 4 and 5).	CEN, NWU	
Dr J Baumgartner	Expert in iron, fatty acids and animal studies. Advisor in the planning of the research project. Co-authored the second manuscript (Chapter 4).	ETH, Zurich CEN, NWU	
Mr FEA Hayford	Assisted with the execution of the research project and co-authored all three manuscripts (Chapter 3, 4 and 5).	CEN NWU University of Ghana	

*No conflicts of interest. CIDRI: Centre of Infectious Diseases Research in Africa; CEN: Centre of Excellence for Nutrition; ICGEB: International Centre of Genetic Engeneering and Biotechnology; IDM: Institute of Infectious Diseases and Molecular Medicine; NWU: North-West University; TB: tuberculosis.

1.8 References

Agoro, R. & Mura, C. 2019. Iron Supplementation Therapy, A Friend and Foe of Mycobacterial Infections? *Pharmaceuticals*, 12:75-103.

Baindara, P. 2019. Host-directed therapies to combat tuberculosis and associated non-communicable diseases. *Microbial Pathogenesis*, 130:156-168.

Baker, E.J., Miles, E.A., Burdge, G.C., Yaqoob, P. & Calder, P.C. 2016. Metabolism and functional effects of plant-derived omega-3 fatty acids in humans. *Progress in Lipid Research*, 64:30-56.

Barden, A.E., Mas, E. & Mori, T.A. 2016. n-3 Fatty acid supplementation and proresolving mediators of inflammation. *Current opinion in lipidology*, 27(1):26-32.

Baumgartner, J., Smuts, C.M., Malan, L., Kvalsvig, J., van Stuijvenberg, M.E., Hurrell, R.F. & Zimmermann, M.B. 2012. Effects of iron and n-3 fatty acid supplementation, alone and in combination, on cognition in school children: a randomized, double-blind, placebo-controlled intervention in South Africa. *The American journal of clinical nutrition*, 96(6):1327-1338.

Blasbalg, T.L., Hibbeln, J.R., Ramsden, C.E., Majchrzak, S.F. & Rawlings, R.R. 2011. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. *The American journal of clinical nutrition*, 93(5):950-962.

Bolton-Smith, C., Woodward, M. & Tavendale, R. 1997. Evidence for age-related differences in the fatty acid composition of human adipose tissue, independent of diet. *European journal of clinical nutrition*, 51(9):619.

Bonilla, D.L., Fan, Y.Y., Chapkin, R.S. & McMurray, D.N. 2010b. Transgenic mice enriched in omega-3 fatty acids are more susceptible to pulmonary tuberculosis: impaired resistance to tuberculosis in fat-1 mice. *The Journal of infectious diseases*, 201(3):399-408.

Bonilla, D.L., Ly, L.H., Fan, Y.Y., Chapkin, R.S. & McMurray, D.N. 2010a. Incorporation of a dietary omega-3 fatty acids impairs murine macrophage responses to Mycobacterium tuberculosis. 5(5):e10878.

Brenna, J.T., Salem Jr, N., Sinclair, A.J. & Cunnane, S.C. 2009. α-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. *Prostaglandins, leukotrienes and essential fatty acids*, 80(2-3):85-91.

Brock, J.H. 2018. Iron and the immune system. (*In* Lauffer, R.B. *ed.* Iron and human disease. CRC Press. p. 161-178).

Browning, L.M., Walker, C.G., Mander, A.P., West, A.L., Madden, J., Gambell, J.M., Young, S., Wang, L., Jebb, S.A. & Calder, P.C. 2012. Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish. *The American journal of clinical nutrition*, 96(4):748-758.

Byrne, S.T., Denkin, S.M. & Zhang, Y. 2006. Aspirin and ibuprofen enhance pyrazinamide treatment of murine tuberculosis. *Journal of antimicrobial chemotherapy*, 59(2):313-316.

Byrne, S.T., Denkin, S.M. & Zhang, Y. 2007. Aspirin Antagonism in Isoniazid Treatment. *Antimicrobial agents in chemotherapy*, 51(2):794.

Calder, P.C. 2015a. Functional roles of fatty acids and their effects on human health. *Journal of Parenteral and Enteral Nutrition*, 39(S1):18S-32S.

Calder, P.C. 2015b. Marine omega-3 fatty acids and inflammatory processes: effects, mechanisms and clinical relevance. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1851(4):469-484.

Calder, P.C. 2017. Omega-3 fatty acids and inflammatory processes: from molecules to man. *Biochemical Society Transactions*, 45(5):1105-1115.

Chiang, N., Fredman, G., Bäckhed, F., Oh, S.F., Vickery, T., Schmidt, B.A. & Serhan, C.N. 2012. Infection regulates pro-resolving mediators that lower antibiotic requirements. *Nature*, 484(7395):524-528.

Childs, C.E., Romeu-Nadal, M., Burdge, G.C. & Calder, P.C. 2008. Gender differences in the n-3 fatty acid content of tissues. *Proceedings of the Nutrition Society*, 67(1):19-27.

Codagnone, M., Cianci, E., Lamolinara, A., Mari, V., Nespoli, A., Isopi, E., Mattoscio, D., Arita, M., Bragonzi, A. & Iezzi, M. 2018. Resolvin D1 enhances the resolution of lung inflammation caused by long-term Pseudomonas aeruginosa infection. *Mucosal immunology*, 11(1):35-45.

Colas, R.A., Nhat, L.T.H., Thuong, N.T.T., Gómez, E.A., Ly, L., Thanh, H.H., Mai, N.T.H., Phu, N.H., Thwaites, G.E. & Dalli, J. 2019. Proresolving mediator profiles in cerebrospinal fluid are linked with disease severity and outcome in adults with tuberculous meningitis. *The FASEB Journal*, 33(11):13028-13039.

Cooper, A.M. & Torrado, E. 2012. Protection versus pathology in tuberculosis: recent insights. *Current Opinion in Immunology*, 24(4):431-437.

Critchley, J.A., Young, F., Orton, L. & Garner, P. 2013. Corticosteroids for prevention of mortality in people with tuberculosis: a systematic review and meta-analysis. *The Lancet infectious diseases*, 13(3):223-237.

Dartois, V. 2014. The path of anti-tuberculosis drugs: from blood to lesions to Mycobacterial cells. *Nature Reviews Microbiology*, 12(3):159-167.

Davis, J.M. & Ramakrishnan, L. 2009. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell*, 136(1):37-49.

De La Mora, I.L., Martinez-Oceguera, D. & Laniado-Laborin, R. 2015. Chronic airway obstruction after successful treatment of tuberculosis and its impact on quality of life. *The International Journal of Tuberculosis and Lung Disease*, 19(7):808-810.

Devi, U., Rao, C.M., Srivastava, V.K., Rath, P.K. & Das, B.S. 2003. Effect of iron supplementation on mild to moderate anaemia in pulmonary tuberculosis. *British Journal of Nutrition*, 90(3):541-550.

Dheda, K., Gumbo, T., Maartens, G., Dooley, K.E., McNerney, R., Murray, M., Furin, J., Nardell, E.A., London, L. & Lessem, E. 2017. The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. *The lancet Respiratory medicine*, 5(4):291-360.

Dietzold, J., Gopalakrishnan, A. & Salgame, P. 2015. Duality of lipid mediators in host response against Mycobacterium tuberculosis: good cop, bad cop. *F1000Prime Reports*, 7:29.

Dorhoi, A. & Kaufmann, S. 2016. Pathology and immune reactivity: understanding multidimensionality in pulmonary tuberculosis. *Seminars in Immunopathology*, 38(2):153-166.

Driver, E.R., Ryan, G.J., Hoff, D.R., Irwin, S.M., Basaraba, R.J., Kramnik, I. & Lenaerts, A.J. 2012. Evaluation of a mouse model of necrotic granuloma formation using C3HeB/FeJ mice for testing of drugs against Mycobacterium tuberculosis. *Antimicrobial agents and chemotherapy*, 56(6):3181-3195.

Duda, M.K., O'Shea, K.M., Tintinu, A., Xu, W., Khairallah, R.J., Barrows, B.R., Chess, D.J., Azimzadeh, A.M., Harris, W.S. & Sharov, V.G. 2008. Fish oil, but not flaxseed oil, decreases inflammation and prevents pressure overload-induced cardiac dysfunction. *Cardiovascular research*, 81(2):319-327.

Durry, F.D., Wirjatmadi, B. & Adriani, M. 2018. The role of zinc sulphate and omega 3 on the improvement of weight and speed conversion conversion on tuberculosis parent patients at Surabaya Park Hospital, 2015. *Jurnal Ilmiah Kedokteran Wijaya Kusuma*, 7(1):62-74.

Egen, J.G., Rothfuchs, A.G., Feng, C.G., Winter, N., Sher, A. & Germain, R.N. 2008. Macrophage and T cell dynamics during the development and disintegration of Mycobacterial granulomas. *Immunity*: 28:271-284.

Ekiz, C., Agaoglu, L., Karakas, Z., Gurel, N. & Yalcin, I. 2005. The effect of iron deficiency anemia opresn the function of the immune system. *The Hematology Journal*, 5(7):579-583.

Ernst, J.D. 2012. The immunological life cycle of tuberculosis. *Nature Reviews Immunology*, 12(8):581-591.

Fielding, B.A., Calder, P.C., Irvine, N.A., Miles, E.A., Lillycrop, K.A., von Gerichten, J. & Burdge, G.C. 2019. How does polyunsaturated fatty acid biosynthesis regulate T-lymphocyte function? *Nutrition Bulletin*, doi.org/10.111/nbu.12404.

Ford, R., Faber, M., Kunneke, E. & Smuts, C.M. 2016. Dietary fat intake and red blood cell fatty acid composition of children and women from three different geographical areas in South Africa. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 109:13-21.

Fullerton, J.N., O'Brien, A.J. & Gilroy, D.W. 2014. Lipid mediators in immune dysfunction after severe inflammation. *Trends in immunology*, 35(1):12-21.

Ganz, T. 2019. Anemia of inflammation. *New England Journal of Medicine*, 381(12):1148-1157.

Gibson, R.A., Muhlhausler, B. & Makrides, M. 2011. Conversion of linoleic acid and alpha-linolenic acid to long-chain polyunsaturated fatty acids (LCPUFAs), with a focus on pregnancy, lactation and the first 2 years of life. *Maternal & child nutrition*, 7:17-26.

Gibson, R.A., Neumann, M.A., Lien, E.L., Boyd, K.A. & Tu, W.C. 2013. Docosahexaenoic acid synthesis from alpha-linolenic acid is inhibited by diets high in polyunsaturated fatty acids. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 88(1):139-146.

Goyens, P.L., Spilker, M.E., Zock, P.L., Katan, M.B. & Mensink, R.P. 2006. Conversion of α-linolenic acid in humans is influenced by the absolute amounts of α-linolenic acid and linoleic acid in the diet and not by their ratio. *The American journal of clinical nutrition*, 84(1):44-53.

Gupta, S., Tyagi, S., Almeida, D.V., Maiga, M.C., Ammerman, N.C. & Bishai, W.R. 2013. Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. *American journal of respiratory and critical care medicine*, 188(5):600-607.

Hawn, T.R., Matheson, A.I., Maley, S.N. & Vandal, O. 2013. Host-directed therapeutics for tuberculosis: can we harness the host? *Microbiol. Mol. Biol. Rev.*, 77(4):608-627.

Healy, D., Wallace, F., Miles, E., Calder, P. & Newsholme, P. 2000. Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. *Lipids*, 35(7):763-768.

Hella, J., Cercamondi, C.I., Mhimbira, F., Sasamalo, M., Stoffel, N., Zwahlen, M., Bodmer, T., Gagneux, S., Reither, K. & Zimmermann, M.B. 2018. Anemia in tuberculosis cases and household controls from Tanzania: contribution of disease, coinfections, and the role of hepcidin. *PloS one*, 13(4):e0195985.

Ifeanyi, O.E. 2018. A review on iron homeostasis and anaemia in pulmonary tuberculosis. *International Journal of Healthcare and Medical Sciences*, 4(5):84-89.

Igarashi, M., DeMar, J.C., Ma, K., Chang, L., Bell, J.M. & Rapoport, S.I. 2007. Upregulated liver conversion of α-linolenic acid to docosahexaenoic acid in rats on a 15 week n-3 PUFA-deficient diet. *Journal of lipid research*, 48(1):152-164.

Irwin, S.M., Driver, E., Lyon, E., Schrupp, C., Ryan, G., Gonzalez-Juarrero, M., Basaraba, R.J., Nuermberger, E.L. & Lenaerts, A.J. 2015. Presence of multiple lesion types with vastly different microenvironments in C3HeB/FeJ mice following aerosol infection with Mycobacterium tuberculosis. *Disease models & mechanisms*, 8(6):591-602.

Isanaka, S., Aboud, S., Mugusi, F., Bosch, R.J., Willett, W.C., Spiegelman, D., Duggan, C. & Fawzi, W.W. 2012. Iron status predicts treatment failure and mortality in tuberculosis patients: a prospective cohort study from Dar es Salaam, Tanzania. *PloS one*, 7(5):e37350.

Isanaka, S., Mugusi, F., Urassa, W., Willett, W.C., Bosch, R.J., Villamor, E., Spiegelman, D., Duggan, C. & Fawzi, W.W. 2011. Iron deficiency and anemia predict mortality in patients with tuberculosis. *The Journal of nutrition*, 142(2):350-357.

Ivanyi, J. & Zumla, A. 2013. Nonsteroidal antiinflammatory drugs for adjunctive tuberculosis treatment. *Journal od Infectious Diseases*, 208(2):185-188.

Jakiela, B., Gielicz, A., Plutecka, H., Hubalewska, M., Mastalerz, L., Bochenek, G., Soja, J., Januszek, R., Musial, J. & Sanak, M. 2013. Eicosanoid biosynthesis during mucociliary and mucous metaplastic differentiation of bronchial epithelial cells. *Prostaglandins & other lipid mediators*, 106:116-123.

Jangale, N.M., Devarshi, P.P., Bansode, S.B., Kulkarni, M.J. & Harsulkar, A.M. 2016. Dietary flaxseed oil and fish oil ameliorates renal oxidative stress, protein glycation, and inflammation in streptozotocin–nicotinamide-induced diabetic rats. *Journal of physiology and biochemistry*, 72(2):327-336.

Jaudszus, A., Gruen, M., Watzl, B., Ness, C., Roth, A., Lochner, A., Barz, D., Gabriel, H., Rothe, M. & Jahreis, G. 2013. Evaluation of suppressive and pro-resolving effects of EPA and DHA in human primary monocytes and T-helper cells. *Journal of lipid research*, 54(4):923-935.

Jeong, Y.J. & Lee, K.S. 2008. Pulmonary tuberculosis: up-to-date imaging and management. *American Journal of Roentgenology*, 191(3):834-844.

Jordao, L., Lengeling, A., Bordat, Y., Boudou, F., Gicquel, B., Neyrolles, O., Becker, P.D., Guzman, C.A., Griffiths, G. & Anes, E. 2008. Effects of omega-3 and-6 fatty acids on Mycobacterium tuberculosis in macrophages and in mice. *Microbes and Infection*, 10(12):1379-1386.

Kant, S., Gupta, H. & Ahluwalia, S. 2015. Significance of nutrition in pulmonary tuberculosis. *Critical Reviews in Food Science & Nutrition*, 55(7):955-963.

Kaplan, G., Post, F.A., Moreira, A.L., Wainwright, H., Kreiswirth, B.N., Tanverdi, M., Mathema, B., Ramaswamy, S.V., Walther, G. & Steyn, L.M. 2003. Mycobacterium tuberculosis growth at the cavity surface: a microenvironment with failed immunity. *Infection and immunity*, 71(12):7099-7108.

Karyadi, E., Schultink, W., Nelwan, R.H., Gross, R., Amin, Z., Dolmans, W.M., van der Meer, J.W., Hautvast, J.G.J. & West, C.E. 2000. Poor micronutrient status of active pulmonary tuberculosis patients in Indonesia. *The Journal of nutrition*, 130(12):2953-2958.

Kaufmann, S.H.E. & Dorhoi, A. 2013. Inflammation in tuberculosis: interactions, imbalances and interventions. *Current Opinion in Immunology*, 25(4):441-449.

Kaveh, M., Eftekhar, N. & Boskabady, M.H. 2019. The effect of alpha linolenic acid on tracheal responsiveness, lung inflammation, and immune markers in sensitized rats. *Iranian journal of basic medical sciences*, 22(3):255.

Kerkhoff, A.D., Meintjes, G., Burton, R., Vogt, M., Wood, R. & Lawn, S.D. 2015. Relationship between blood concentrations of hepcidin and anemia severity, Mycobacterial burden, and mortality among patients with HIV-associated tuberculosis. *The Journal of infectious diseases*, 213(1):61-70.

Kim, Y.R. & Yang, C.S. 2017. Host-directed therapeutics as a novel approach for tuberculosis treatment. *Journal of microbiology and biotechnology*, 27(9):1549-1558.

Kroesen, V.M., Gröschel, M.I., Martinson, N., Zumla, A., Maeurer, M., van der Werf, T.S. & Vilaplana, C. 2017. Non-steroidal anti-inflammatory drugs as host-directed therapy for tuberculosis: a systematic review. *Frontiers in immunology*, 8:772.

Kroesen, V.M., Rodríguez-Martínez, P., García, E., Rosales, Y., Díaz, J., Martín-Céspedes, M., Tapia, G., Sarrias, M.R., Cardona, P.J. & Vilaplana, C. 2018. A beneficial effect of low-dose aspirin in a Murine Model of active tuberculosis. *Frontiers in immunology*, 9:798.

Kumar, N.P., Moideen, K., Banurekha, V.V., Nair, D. & Babu, S. 2019. Plasma pro-inflammatory cytokines are markers of disease severity and bacterial burden in pulmonary tuberculosis. *Open forum infectious diseases,* DOI:10.1093/ofid/ofz257.

Kumar, P. 2016. Adult pulmonary tuberculosis as a pathological manifestation of hyperactive antimycobacterial immune response. *Clinical and Translational Medicine*, 5(1):38.

Kurthkoti, K., Amin, H., Marakalala, M.J., Ghanny, S., Subbian, S., Sakatos, A., Livny, J., Fortune, S.M., Berney, M. & Rodriguez, G.M. 2017. The capacity of Mycobacterium tuberculosis to survive iron starvation might enable it to persist in iron-deprived microenvironments of human granulomas. *MBio*, 8(4):e01092-01017.

Lanoix, J.P., Lenaerts, A.J. & Nuermberger, E.L. 2015. Heterogeneous disease progression and treatment response in a C3HeB/FeJ mouse model of tuberculosis. *Disease models & mechanisms*, 8(6):603-610.

Lee, C.R. & Zeldin, D.C. 2015. Resolvin infectious inflammation by targeting the host response. *New England Journal of Medicine*, 373(22):2183-2185.

Lee, S.W., Kang, Y., Yoon, Y.S., Um, S.-W., Lee, S.M., Yoo, C.G., Kim, Y.W., Han, S.K., Shim, Y.S. & Yim, J.J. 2006. The prevalence and evolution of anemia associated with tuberculosis. *Journal of Korean medical science*, 21(6):1028-1032.

Lenaerts, A., Barry III, C.E. & Dartois, V. 2015. Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunological reviews*, 264(1):288-307.

Malan, L., Baumgartner, J., Calder, P.C., Zimmermann, M.B. & Smuts, C.M. 2014. n–3 Long-chain PUFAs reduce respiratory morbidity caused by iron supplementation in iron-deficient South African schoolchildren: a randomized, double-blind, placebo-controlled intervention. *The American journal of clinical nutrition*, 101(3):668-679.

Malan, L., Baumgartner, J., Zandberg, L., Calder, P. & Smuts, C. 2016. Iron and a mixture of dha and epa 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. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)*, 105:15-25.

Manji, M., Shayo, G., Mamuya, S., Mpembeni, R., Jusabani, A. & Mugusi, F. 2016. Lung functions among patients with pulmonary tuberculosis in Dar es Salaam–a cross-sectional study. *BMC pulmonary medicine*, 16(1):58.

Marzo, E., Vilaplana, C., Tapia, G., Diaz, J., Garcia, V. & Cardona, P.J. 2014. Damaging role of neutrophilic infiltration in a mouse model of progressive tuberculosis. *Tuberculosis*, 94(1):55-64.

Mas, E., Barden, A., Burke, V., Beilin, L.J., Watts, G.F., Huang, R.C., Puddey, I.B., Irish, A.B. & Mori, T.A. 2016. A randomized controlled trial of the effects of n-3 fatty acids on resolvins in chronic kidney disease. *Clinical Nutrition*, 35(2):331-336.

Massyn, N., Padarath, A. & Peer, N. 2017. District health barometer 2016/17. Durban: Health Systems Trust.

Mayatepek, E., Paul, K., Leichsenring, M., Pfisterer, M., Wagner, D., Domann, M., Bremer, H. & Sonntag, H. 1994. Influence of dietary (n-3)-polyunsaturated fatty acids on leukotriene B 4 and prostaglandin E 2 synthesis and course of experimental tuberculosis in guinea pigs. *Infection*, 22(2):106-112.

Mayer-Barber, K.D., Andrade, B.B., Oland, S.D., Amaral, E.P., Barber, D.L., Gonzales, J., Derrick, S.C., Shi, R., Kumar, N.P. & Wei, W. 2014. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature*, 511(7507):99.

Mayer-Barber, K.D. & Sher, A. 2015. Cytokine and lipid mediator networks in tuberculosis. *Immunological Reviews*, 264(1):264-275.

McFarland, C.T., Fan, Y.Y., Chapkin, R.S., Weeks, B.R. & McMurray, D.N. 2008. Dietary polyunsaturated fatty acids modulate resistance to Mycobacterium tuberculosis in guinea pigs. *The Journal of nutrition*, 138(11):2123-2128.

Meghji, J., Simpson, H., Squire, S.B. & Mortimer, K. 2016. A systematic review of the prevalence and pattern of imaging defined post-TB lung disease. *PloS one*, 11(8):e0161176.

Meyer, B.J., Mann, N.J., Lewis, J.L., Milligan, G.C., Sinclair, A.J. & Howe, P.R. 2003. Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. *Lipids*, 38(4):391-398.

Minchella, P.A., Donkor, S., Owolabi, O., Sutherland, J.S. & McDermid, J.M. 2014. Complex anemia in tuberculosis: the need to consider causes and timing when designing interventions. *Clinical Infectious Diseases*, 60(5):764-772.

Mishra, S., Taparia, M.P., Yadav, D. & Koolwal, S. 2018. Study of Iron Metabolism in Pulmonary Tuberculosis Patients. *International journal of health sciences and research,* 8(3):70-77.

Morris, T., Stables, M., Hobbs, A., de Souza, P., Colville-Nash, P., Warner, T., Newson, J., Bellingan, G. & Gilroy, D.W. 2009. Effects of low-dose aspirin on acute inflammatory responses in humans. *The Journal of Immunology*, 183(3):2089-2096.

Moura-Assis, A., Afonso, M.S., de Oliveira, V., Morari, J., dos Santos, G.A., Koike, M., Lottenberg, A.M., Catharino, R.R., Velloso, L.A. & da Silva, A.S.R. 2018. Flaxseed oil rich in omega-3 protects aorta against inflammation and endoplasmic reticulum stress partially mediated by GPR120 receptor in obese, diabetic and dyslipidemic mice models. *The Journal of nutritional biochemistry*, 53:9-19.

Nagu, T.J., Spiegelman, D., Hertzmark, E., Aboud, S., Makani, J., Matee, M.I., Fawzi, W. & Mugusi, F. 2014. Anemia at the initiation of tuberculosis therapy is associated with delayed sputum conversion among pulmonary tuberculosis patients in Dar-es-Salaam, Tanzania. *PloS one*, 9(3):e91229.

Ndlovu, H. & Marakalala, M.J. 2016. Granulomas and inflammation: host-directed therapies for tuberculosis. *Frontiers in Immunology*, 7(434).

Nemeth, E., Rivera, S., Gabayan, V., Keller, C., Taudorf, S., Pedersen, B.K. & Ganz, T. 2004. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *The Journal of clinical investigation*, 113(9):1271-1276.

Nenni, V., Nataprawira, H.M. & Yuniati, T. 2013. Role of combined zinc, vitamin A, and fish oil supplementation in childhood tuberculosis. *Southeast Asian J. Trop. Med. Public Health*, 44(5):854-861.

Nicolas, G., Chauvet, C., Viatte, L., Danan, J.L., Bigard, X., Devaux, I., Beaumont, C., Kahn, A. & Vaulont, S. 2002. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *The Journal of clinical investigation*, 110(7):1037-1044.

Nihues, S.D.S.E., Mancuzo, E.V., Sulmonetti, N., Sacchi, F.P.C., Viana, V.d.S., Martins Netto, E., Miranda, S.S. & Croda, J. 2015. Chronic symptoms and pulmonary dysfunction in post-tuberculosis Brazilian patients. *Brazilian Journal of Infectious Diseases*, 19(5):492-497.

Oppenheimer, S.J. 2001. Iron and its relation to immunity and infectious disease. *The Journal of nutrition*, 131(2):616S-635S.

Palucci, I. & Delogu, G. 2018. Host directed therapies for tuberculosis: futures strategies for an ancient disease. *Chemotherapy*, 63(3):172-180.

Paul, K.P., Leichsenring, M., Pfisterer, M., Mayatepek, E., Wagner, D., Domann, M., Sonntag, H.G. & Bremer, H.J. 1997. Influence of n-6 and n-3 polyunsaturated fatty acids on the resistance to experimental tuberculosis. *Metabolism*, 46(6):619-624.

Peters, W. & Ernst, J.D. 2003. Mechanisms of cell recruitment in the immune response to Mycobacterium tuberculosis. *Microbal Infections,* 5(2):151-158.

Rahmawaty, S., Charlton, K., Lyons-Wall, P. & Meyer, B.J. 2013. Dietary intake and food sources of EPA, DPA and DHA in Australian children. *Lipids*, 48(9):869-877.

Richter, M., Baumgartner, J., Wentzel-Viljoen, E. & Smuts, C.M. 2014. Different dietary fatty acids are associated with blood lipids in healthy South African men and women: the PURE study. *International journal of cardiology*, 172(2):368-374.

Robinson, R.T., Orme, I.M. & Cooper, A.M. 2015. The onset of adaptive immunity in the mouse model of tuberculosis and the factors that compromise its expression. *Immunological reviews*, 264(1):46-59.

Sahiratmadja, E., Wieringa, F.T., van Crevel, R., de Visser, A.W., Adnan, I., Alisjahbana, B., Slagboom, E., Marzuki, S., Ottenhoff, T.H. & van de Vosse, E. 2007. Iron deficiency and NRAMP1 polymorphisms (INT4, D543N and 3′ UTR) do not contribute to severity of anaemia in tuberculosis in the Indonesian population. *British Journal of Nutrition*, 98(4):684-690.

Schiessel, D.L., Yamazaki, R.K., Kryczyk, M., Coelho de Castro, I., Yamaguchi, A.A., Pequito, D.C., Brito, G.A., Borghetti, G., Aikawa, J. & Nunes, E.A. 2016. Does oil rich in alpha-linolenic fatty acid cause the same immune modulation as fish oil in walker 256 tumor-bearing rats? *Nutrition and cancer*, 68(8):1369-1380.

Schloss, I., Kidd, M., Tichelaar, H.Y., Young, G.O. & O'Keefe, S.J. 1997. Dietary factors associated with a low risk of colon cancer in coloured west coast fishermen. *South African medical journal,* 87(2):152-158.

Schmidt, P.J. 2015. Regulation of iron metabolism by hepcidin under conditions of inflammation. *Journal of Biological Chemistry*, 290(31):18975-18983.

Serhan, C.N. 2017a. Treating inflammation and infection in the 21st century: new hints from decoding resolution mediators and mechanisms. *The FASEB Journal*, 31(4):1273-1288.

Serhan, C.N., Chiang, N. & Dalli, J. 2017b. New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration. *Molecular aspects of medicine*, http://dx.doi.org/10.1016/j.mam.2017.08.002.

Shimazaki, T., Marte, S., Saludar, N., Dimaano, E., Salva, E., Ariyoshi, K., Villarama, J. & Suzuki, M. 2013. Risk factors for death among hospitalised tuberculosis patients in poor urban areas in Manila, The Philippines. *The International Journal of Tuberculosis and Lung Disease*, 17(11):1420-1426.

Sibbons, C.M., Irvine, N.A., Pérez-Mojica, J.E., Calder, P.C., Lillycrop, K.A., Fielding, B.A. & Burdge, G.C. 2018. Polyunsaturated fatty acid biosynthesis involving Δ8 desaturation and differential DNA methylation of FADS2 regulates proliferation of human peripheral blood mononuclear cells. *Frontiers in immunology*, 9:432.

Spite, M., Norling, L.V., Summers, L., Yang, R., Cooper, D., Petasis, N.A., Flower, R.J., Perretti, M. & Serhan, C.N. 2009. Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature*, 461:1287.

Stark, K.D., Van Elswyk, M.E., Higgins, M.R., Weatherford, C.A. & Salem Jr, N. 2016. Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults. *Progress in lipid research*, 63:132-152.

Stek, C., Allwood, B., Walker, N.F., Wilkinson, R.J., Lynen, L. & Meintjes, G. 2018. The immune mechanisms of lung parenchymal damage in tuberculosis and the role of host-directed therapy. *Frontiers in microbiology*, 9:2603.

Theegarten, D., Kahl, B. & Ebsen, M. 2006. Frequency and morphology of tuberculosis in autopsies: increase of active forms. *Deutsche medizinische Wochenschrift (1946)*, 131(24):1371-1376.

Van Lettow, M., West, C., Van der Meer, J., Wieringa, F. & Semba, R. 2005. Low plasma selenium concentrations, high plasma human immunodeficiency virus load and high interleukine-6 concentrations are risk factors associated with anemia in adults presenting with pulmonary tuberculosis in Zomba district, Malawi. *European journal of clinical nutrition*, 59:526-532.

Vilaplana, C., Marzo, E., Tapia, G., Diaz, J., Garcia, V. & Cardona, P.J. 2013. Ibuprofen therapy resulted in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. *Journal of infecious diseases,* 208:199-202.

Weiss, G., Ganz, T. & Goodnough, L.T. 2019. Anemia of inflammation. *Blood, The Journal of the American Society of Hematology*, 133(1):40-50.

World Health Organization. 2019. Global Tuberculosis Report. Gineva.

Zumla, A., Nahid, P. & Cole, S.T. 2013. Advances in the development of new tuberculosis drugs and treatment regimens. *Nature reviews Drug discovery*, 12(5):388-404.

CHAPTER 2 LITERATURE REVIEW

2.1 Tuberculosis: a heavy burden globally and in South Africa

Pulmonary tuberculosis (PTB) was in earlier years referred to as the wasting disease or white plague (Basil & Levy, 2016; Dubos & Dubos, 1952). Today, tuberculosis (TB) is still a global health problem with an estimated 10 million incident cases worldwide in 2018, irrespective of the average fall of 2% between 2017 and 2018 (WHO, 2019). The African region accounted for 24% of these cases. What is more disturbing, however, is that Africa has the most severe burden relative to its population size, with an average 2450 incident cases per 1060 000 people (more than double the global average). Moving to more local frontiers, South Africa (SA) is ranked as one of the eight countries accounting for two-thirds of the global cases (3% of the global total) (Figure 2.1) (WHO, 2019).

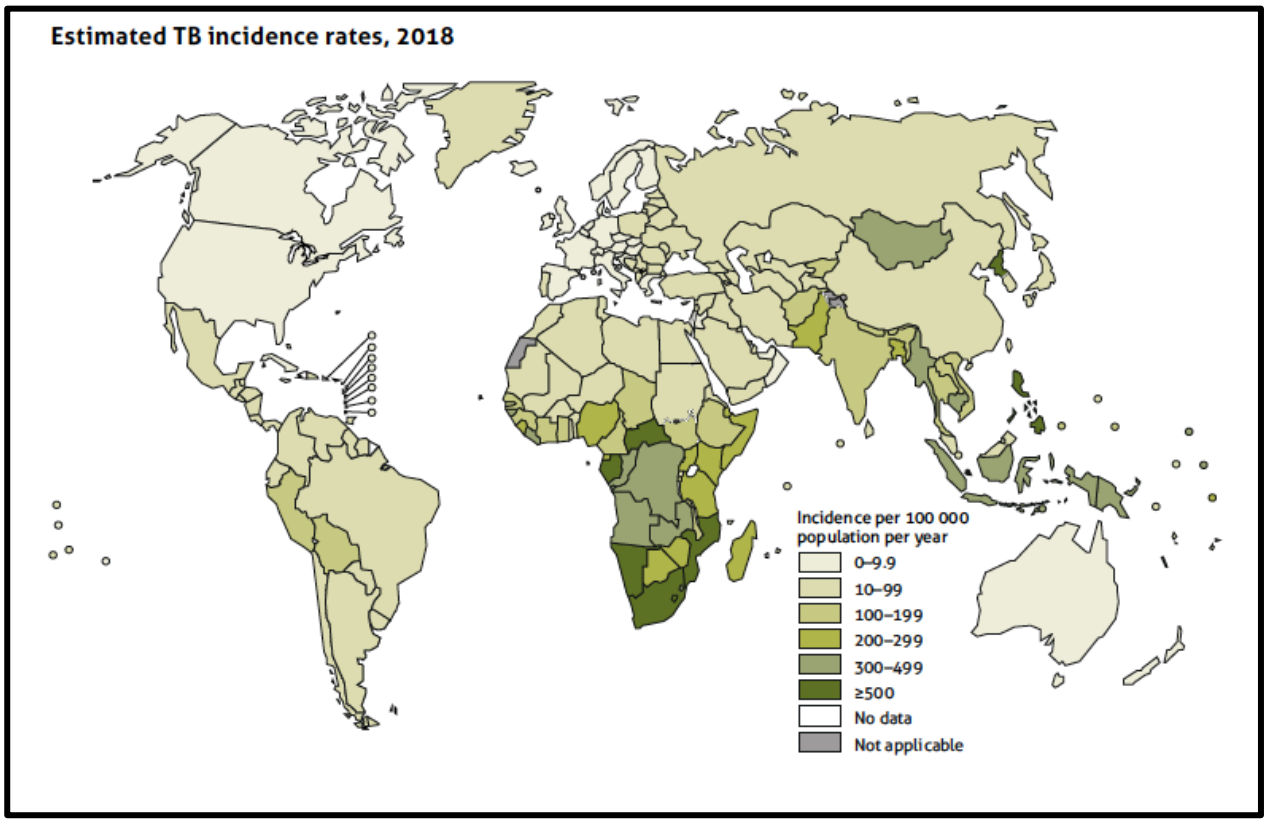


Figure 2-1 Estimated tuberculosis incidence rates in 2018 (WHO, 2019)

TB: tuberculosis

Human immunodeficiency virus (HIV) and its accompanying secondary infections are currently a heavy and escalating burden. Immune dysfunction, including HIV, has a serious impact on TB incidence, countering the gains in the control of TB. Owing to the suppressed immune system of

these patients, it is estimated that 50% to 60% of HIV-positive patients infected with TB go on to develop active TB and that they have a 20 to 30 times higher chance of progressing from latent to active TB (Department of Health, 2014). High TB and HIV co-infection rates have been reported globally, but also locally, exceeding 59% of TB cases in SA. Furthermore, multidrug-resistant TB (MDR-TB) is now a growing concern, which is also partly responsible for poor TB cure rates. In 2018, 3.4% of new cases and 18% of previously treated cases presented with MDR-TB worldwide (WHO, 2019).

Tuberculosis cure rates are also not ideal, as the most recent World Health Organization (WHO) report revealed that 1.24 million deaths resulted from TB in 2018, even though mortality rates fell by 11% from 2015 to 2018. Tuberculosis is not only the 10th leading cause of death globally but also the leader as an infectious agent (WHO, 2019). Even though the high mortality rates in TB persist, 48 million lives of HIV-negative and 10 million lives of HIV-positive people were saved by TB treatment between 2000 and 2018 (WHO, 2019). With this in mind, the most efficient treatment of patients with TB is of utmost importance to aid in reducing mortality rates and medical costs and to improve the quality of life of TB survivors. To understand the targets of TB treatment, it is necessary to first review the disease pathology of TB.

2.2 Clinical representation and diagnosis of tuberculosis

Tuberculosis is a microbial infection caused by *Mycobacteria tuberculosis* (*Mtb*), *Mycobacteria bovus*, *Mycobacteria microti,* and *Mycobacteria canetti*. However, *Mtb* infection is the most common cause of TB and is transferred *via* the airborne route (Jeong & Lee, 2008). Droplets carrying *Mtb*, produced by patients with active TB while coughing, are highly infective and can be kept airborne in an indoor dark space for up to four hours, but direct sunlight kills the bacilli. As the lungs are the primary site of entry and disease manifestation, 80% of infected patients present with PTB (Kaufmann & Dorhoi, 2013; Kumar, 2016). Nonetheless, TB can also infect other parts of the human body and is then termed extra-pulmonary TB, TB meningitis, or disseminated TB. These are found more frequently in immunocompromised individuals, as disease in these patients is characterised as more progressive and severe (Kumar, 2016).

As referred to earlier, not all humans infected with *Mtb* develop clinical signs, and the immune response of individuals toward active TB infection differs greatly. Following the exposure to *Mtb,* the exposed individuals can be divided into three groups, which also characterise the stages of TB pathology: 1) individuals who show no symptoms of *Mtb* infection, because of the failure of the bacteria to establish such an infection (eradication stage); 2) infected individuals, who develop strong cell-mediated immunity without developing the disease (latent TB infection stage); 3) and

last, a smaller group of individuals (5 – 10%) who develop the active disease (progressing primary TB or reactivation TB) (Apt & Kramnik, 2009; Robinson *et al.*, 2015).

The progression from latent TB to the active form and the response of the host are calculated processes. Latently infected individuals contain the pathogen, but fail to eliminate it. During this phase, the pathogen is controlled by the immune system through inflammation, which minimises damage to the host (Ernst, 2012). When the balance between these individuals' immune function and *Mtb* becomes uneven, the *Mtb* becomes metabolically active (Jeong & Lee, 2008; Kaufmann & Dorhoi, 2013). Disturbances in this balance are largely affected by the cell type the *Mtb* encounters upon first entry into the lung, the innate immune response of the individual and the individual's capacity to activate T cells in response to *Mtb* infection (Robinson *et al.*, 2015). The patients with the highest risk of developing active TB are those that are malnourished, very young (younger than five years) or very old, drug or alcohol abuse patients, cancer patients, those receiving chemotherapy, chronic or end-stage renal disease patients, patients who have had an organ transplant, diabetic or HIV-positive patients, and those on immunosuppressive therapy (Hoppe *et al.*, 2016; Jeong & Lee, 2008).

Patients with active TB may present with the following symptoms: coughing (sometimes blood-laced), chest pain, fever, night sweats, weight loss and anorexia (Mahan & Raymond, 2017). Definitive TB diagnosis can exclusively be made by culturing organisms from patient specimens (Jeong & Lee, 2008). According to the National Institute for Health and Care Excellence (NICE) guidelines, active PTB in adults is diagnosed with a positive respiratory (sputum smear) sample (three samples to be taken) and routine tests, including microscopy, culture and histology (Hoppe *et al.*, 2016). Additionally, a complete TB evaluation could include the medical history of the patient, a physical examination, a tuberculin skin test (TST), a serology test and imaging techniques, including a chest X-ray and computed tomography scan of the thorax (Hoppe *et al.*, 2016; Jeong & Lee, 2008). A nucleic acid amplification test (NAAT) should be requested if there is a clinical suspicion of active TB, the patient is HIV-positive, rapid information will alter the patient care, or there is a need for a large contact-tracing initiative (Hoppe *et al.*, 2016). The detection and treatment of latent TB is also an important public health effort to combat the disease. Latent TB can be detected by a TST (specifically, the Mantoux test); however, it is recommended that the whole-blood interferon-gamma (IFN-γ) release assay (IGRA) be conducted for its higher accuracy in latent TB detection (Hoppe *et al.*, 2016; Jeong & Lee, 2008).

Tuberculosis also affects the patient's nutritional status. As early as 1968, Scrimshaw *et al.* described the link between malnutrition and infections. It is well known that undernutrition increases the incidence, severity and mortality rate of various infections (such as TB), whilst on

the other hand, infections negatively affect nutrition status, possibly leading to undernutrition (Kant *et al.*, 2015; Scrimshaw *et al.*, 1968). Clinical features of undernutrition, such as underweight and wasting, are frequently evident in TB patients (Karyadi *et al.*, 2002; Zachariah *et al.*, 2002). This is because TB increases the energy requirements of patients as a result of their heightened metabolic rate. Furthermore, a greater percentage of ingested protein is oxidised for energy production, which reduces protein availability for endogenous protein synthesis (termed the anabolic block) (MacAllan *et al.*, 1998). This response, coupled with a possible lower nutrient intake (due to a lack of appetite) and nutrient malabsorption, regularly found in TB patients, will ultimately lead to undernutrition (Kant *et al.*, 2015; Karyadi *et al.*, 2002).

Nutritional status is clinically important in TB patients as nutrition plays an important part in immunity and, therefore, malnutrition has a significant effect on TB outcomes (Kant *et al.*, 2015). Malnutrition (including micronutrient deficiencies) can lead to an altered immune response by affecting the actions of specific immune cells and pathways, which include impaired cytokine production and response, and a blunted acute-phase response (Alpert, 2017; Cunningham-Rundles *et al.*, 2005; Dülger *et al.*, 2002; Kant *et al.*, 2015; Manary *et al.*, 2004). As a result, evidence suggests that malnutrition, or more specifically, underweight, wasting and micronutrient deficiencies, are associated with higher TB mortality rates (Cunningham-Rundles *et al.*, 2005; Kant *et al.*, 2015; Yen *et al.*, 2016; Zachariah *et al.*, 2002). This highlights the importance of nutrition intervention in TB patients. For the purpose of this research, a more in-depth discussion of the pathophysiology and immune response to *Mtb* infection is required.

2.3 The pathophysiology and immune response in tuberculosis

Inflammation is an important part of host defence. On the other hand, the resolution of inflammation is an equally important coordinated process and under tight control from the onset of inflammation. Excessive inflammation is known to contribute to disease pathology and unwanted cell and tissue damage (Serhan, 2017a). *Mycobacterium* TB is highly inflammatory and, following infection, a hyperactive immune response and non-resolving inflammation are characteristic features of TB. This bacterium takes advantage of the inflammatory process and manipulates the host immune response by initiating the recruitment of immune cells to the usually sterile pulmonary space to create an appropriate environment for multiplication (Kaufmann & Dorhoi, 2013; Robinson *et al.*, 2015). This serves as a vital pathogenic feature of TB and leads to host lung tissue destruction, thereby enabling TB transmission (Cooper & Torrado, 2012; Kaufmann & Dorhoi, 2013; Kumar, 2016). This pathogenesis and immune response in TB can broadly be categorised into four stages, including the innate immune response, immunological equilibrium (latent TB), reactivation, and finally, transmission (Figure 2.2) (Ernst, 2012). These

four stages will now be briefly discussed followed by a section describing the expected lung histopathology and lung cytokine response in *Mtb* infection.

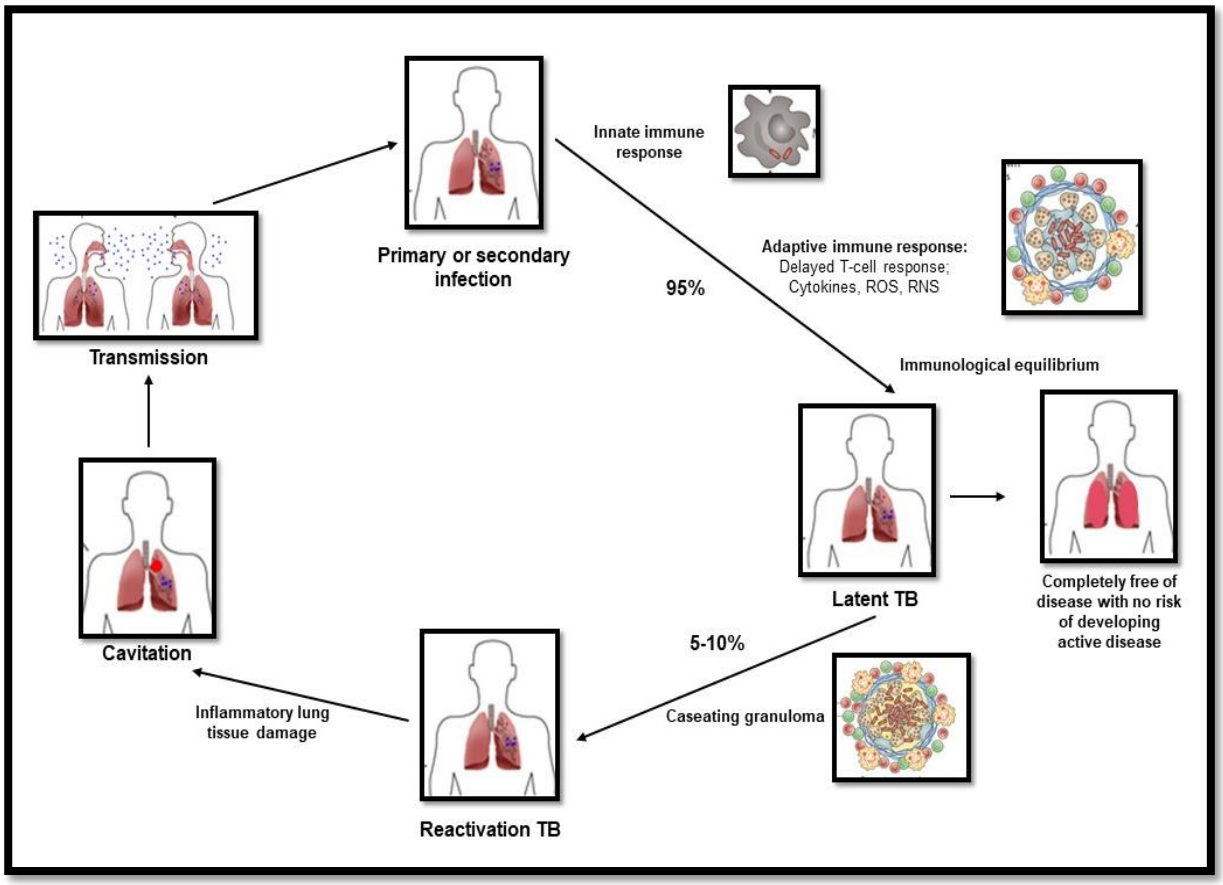


Figure 2-2 **The immunological stages in the life cycle of *Mycobacterium tuberculosis* and tuberculosis progression** (Adapted from Dorhoi & Kaufmann, 2016; Ernst, 2012; Kumar *et al.*, 2011; Kumar, 2016; Lenaerts *et al.*, 2015; Nunes-Alves *et al.*, 2014)

RNS: reactive nitrogen species; ROS: reactive oxygen species; TB: tuberculosis.

2.3.1 The innate immune response in *Mycobacterium tuberculosis* infection

The pathology of TB is initiated when the *Mtb* droplet nuclei, produced by an active TB patient, moves down the trachea and into the bronchial tree where they are deposited in bronchioles or alveoli (Figure 2.2) (Kumar, 2016). Here the early innate immune response is initiated, leading to the accumulation of phagocytic cells, mostly macrophages. Local alveolar macrophages recognise and phagocytose the *Mtb,* forming granulomas at the site of bacterial replication, where the mycobacteria reside in the macrophage phagosome (Davis & Ramakrishnan, 2009; Ernst, 2012; Kaufmann & Dorhoi, 2013; Kumar, 2016). This is where the *Mtb* spends an important part

of its life cycle and infection now depends on the anti-microbial ability of the macrophages, as well as the bacterial virulence of the *Mtb* (Ernst, 2012; Kaufmann & Dorhoi, 2013).

Mycobacterium TB uses various mechanisms to manipulate the host's immune response to their own advantage, spreading bacteria from cell to cell (Davis & Ramakrishnan, 2009; Ernst, 2012). Usually, phagocytes will confine and sometimes eliminate pathogens, but this is not the case in mycobacterial infection, where, instead, they provide a platform for bacterial expansion (Davis & Ramakrishnan, 2009). Within the phagolysosome of macrophages, the *Mtb* is exposed to reactive oxygen species (ROS) and reactive nitrogen species (RNS). However, the *Mtb* can resist the oxidative and nitrosative stress, and arrest phagosome maturation in order to replicate and facilitate the formation of granulomas (Arbues *et al.*, 2014; Cambier *et al.*, 2014; Simeone *et al.*, 2012; Vergne *et al.*, 2005). It further causes phagosomal rupturing and then leaves the cytosol to enable bacterial replication and host tissue necrosis (Simeone *et al.*, 2012; Simeone *et al.*, 2015). Alveolar macrophages serve mostly as a site for bacterial replication, but activated macrophages serve as effectors against *Mtb* (Dorhoi & Kaufmann, 2016). They produce pro-inflammatory (e.g. tumour necrosis factor-alpha (TNF-α)) and regulatory cytokines (e.g. interleukin (IL) -10) and chemokines, ROS and antimicrobial peptides. This leads to an influx of leukocytes into the bronchioalveolar space (Dorhoi & Kaufmann, 2016; Ernst, 2012). More monocytes move towards infected tissue and differentiate into macrophages and dendritic cells (DCs), mediated by the local cytokines, as well as growth factors (Krutzik *et al.*, 2005). Inflammatory mediators, e.g. TNF-α and IL-12, further cause the migration of infected DCs to the draining lymph nodes which, in turn, leads to the initiation of the adaptive immune response (Dorhoi & Kaufmann, 2016; Keeton *et al.*, 2014).

Early control of *Mtb* infection is thought to be mediated by innate lymphocytes (e.g. natural killer cells (NK) and T cells) and granulocyte-colony stimulating factor (GCSF) (Meraviglia *et al.*, 2011). If the bacillus can survive this initial defence, it can grow and replicate the number of organisms in the alveolar macrophages (Jordao *et al.*, 2008; Kaufmann, 2003; Kaufmann & Dorhoi, 2013). For more or less a week *Mtb* replicates within the alveolar macrophages, whereafter neutrophils are recruited (Bru & Cardona, 2010; Dorhoi & Kaufmann, 2016). Neutrophils have a protective role early in the immune response against TB concerning cell death and inflammation and are found in large amounts in the lung alveoli following infection (Dorhoi & Kaufmann, 2016; Ernst, 2012). However, neutrophils have also been found to have different phenotypic subsets, where some subsets contribute to pulmonary immunopathology (Lenaerts *et al.*, 2015; Tsuda *et al.*, 2004). From the above literature, it is clear that the innate immune response to TB infection is dynamic and that the bacteria usually dominate this stage, whereas there is little immediate

antibacterial effect. This stage, however, serves as a platform for the initiation of the T cell response to follow.

2.3.2 The adaptive immune response in *Mycobacterium tuberculosis* infection and the immunological equilibrium

The acquired immune response against TB infection is delayed as, in humans, the initiation of the adaptive immune responses may occur only about 42 days after initial *Mtb* infection (including the time it took for the initial recognition and phagocytosis of bacteria and the recruitment of innate immune cells) (Ernst, 2012). The main contributing factor to the delayed initiation is the fact that *Mtb* inhibits DC migration and it takes seven to nine days, following the initial recruitment of innate immune cells, for the delivery of bacteria (*via* infected and antigen-loaded DCs) to the draining lymph nodes (Blomgran & Ernst, 2011; Cooper & Torrado, 2012). Nevertheless, by the time that the T cells arrive at the site of inflammation in the lung, phagocytes dominate the lung lesion and the granulomatous environment limits T cell function and survival (Cooper & Torrado, 2012; Pearl *et al.*, 2012). Macrophages are activated by T cells, through IFN-γ and TNF-α. These activated macrophages, as mentioned earlier, restrict *Mtb* growth and kill intracellular bacilli (Blomgran & Ernst, 2011; Ernst, 2012; Kumar, 2016).

As the adaptive immune responses arrest bacterial growth, this phase results in temporary disease symptoms, which may include fever and erythema nodosum. Generally, following the onset of this phase, most individuals become asymptomatic (Ernst, 2012). This is then termed latent TB infection and bacteria will mostly not be shed during this time. It is, however, important to note that the adaptive immune responses may be efficient in preventing *Mtb* growth, but the ability to eliminate the bacterium is limited (Mogues *et al.*, 2001). Research has found that, of the latently infected patients, some will completely clear the infection and escape any risk of the development of active disease in the future, while others will house bacteria that are replicating in the absence of symptoms and these individuals are still at risk of active disease (Lawn *et al.*, 2010; Lenaerts *et al.*, 2015; Young *et al.*, 2009). In this latent TB stage, the bacterial population size will remain constant; however, a subpopulation of bacteria continues to reproduce (Gill *et al.*, 2009).

Together, the innate and adaptive immune responses protect healthy lung tissue from *Mtb*, and through the formation of granulomas and a strong adaptive immune response bacterial replication and host pathology can be limited (Lenaerts *et al.*, 2015). Therefore, the majority (90-95%) of latently infected individuals do not go on to develop active TB and will continue to be healthy life long (Figure 2.2) (Egen *et al.*, 2008; Peters & Ernst, 2003). Granuloma formation and composition is further described in Section 2.3.5.

2.3.3 Reactivation tuberculosis

Following the latent TB phase, there is a phase termed reactivation TB (evident in 5-10% of individuals) (Figure 2.2). In this phase the disease is now active with several symptoms, as well as shedding of bacteria by means of respiratory secretions (Ernst, 2012). There are two subtypes of active TB, including primary TB, occurring rapidly following exposure and mostly in the lower lung zones, and the more often evident post-primary TB, that occurs years or even decades after exposure, mostly in the lung apices, and thought to be caused by alterations in immune status (Lenaerts *et al.*, 2015). There is a lack of literature confirming the exact host response that leads to active TB (Hunter, 2016). Traditionally it was believed that active TB is driven by a weakened immune system. However, more and more researchers are questioning this view. In a review, Kumar (2016) reported that most adult PTB patients are not immunodeficient. There are only two immunodeficiency conditions that have been confirmed to lead to active TB and it is suggested that they account only for a small percentage of active TB cases. One such condition is the quantitative and qualitative defects in the $CD4^+$ T cells in HIV-infected individuals as evidence suggests that $CD4^+$ T cell deficiency causes reactivation TB (Blomgran & Ernst, 2011; Geldmacher *et al.*, 2010; Herzmann *et al.*, 2012). The other mechanism that may be responsible for reactivation TB, by means of a weakened immune response, is the use of therapeutic TNF-blocking agents to treat various inflammatory conditions, such as rheumatoid arthritis (RA) (Harris & Keane, 2010; Miossec, 2018). Tuberculosis in immunocompromised individuals also presents in extra-pulmonary sites and is regularly found in the most severe form (Kumar, 2016).

The body of evidence that indicates that it is a hyperactive antimicrobial immune response, including excessive $CD4^+$ T cell activity and IFN-γ levels, that most likely leads to active TB, even in immunocompetent individuals, is growing (Barber *et al.*, 2011; Kumar, 2016). Other mechanisms are investigated for their contribution to reactivation TB. Some of these mechanisms include altered antigen expression, altered T cell trafficking, and the bacteria as primary drivers (Barber *et al.*, 2011; Kumar, 2016). Certain medical conditions have also been linked to a higher susceptibility for the development of reactivation TB, accounting, however, for the minority of cases. These conditions include diabetes mellitus, glucocorticoid treatment, silicosis, advanced age, uraemia, gastrectomy, cancer chemotherapy, and haematological malignancies (Hayashi & Chandramohan, 2018; Harries *et al.*, 2011; Jick, 2006). Malnourished individuals and those with a thin body habitus have also been found to be at higher risk of progressing from latent to active TB (Palmee *et al.*, 1957).

2.3.4 Transmission

As TB is an infectious disease, the transmission stage in the course of the disease is inevitable. This ensues *via* the airborne route through coughing and inhalation by the new host (Figure 2.2). Some individuals will be far more infectious than others, e.g. cavitary TB patients, with large amounts of lung tissue destruction and macroscopic cavities connected to large airways (further alluded to in Section 2.3.5) (Kaplan *et al.*, 2003). In this regard, it can be reasoned that the host immune response facilitates transmission because T cell responses contribute to inflammatory lung tissue damage and the development of cavities. This also explains why HIV-infected individuals have less efficient TB transmission, owing to their less efficient T cell response and, therefore, possibly less tissue destruction (Ernst, 2012; Kaplan *et al.*, 2003).

2.3.5 Histopathology and immune cell phenotypes of lung granulomas in tuberculosis infection

One of the key pathological features of PTB is the alterations in lung tissue. These are referred to as granulomas (lesions), mentioned previously, which contribute to the isolation, but also the growth of the *Mtb* (Kaufmann, 2005). The reason that TB lesions usually dominate the lungs and not other body parts is that the bacteria and T cells are delivered *via* the circulation to the rest of the body at more or less the same time, providing protection to other tissues by means of a healthy immune response (Robinson et al., 2015). Changes in the immune environment of lungs occur within two to three weeks following infection and the development of primary TB lesions in two to eight weeks following exposure (Cooper, 2009; Dorhoi & Kaufmann, 2016; Krutzik *et al.*, 2005; Kumar, 2016; Pancholi *et al.*, 1993; Wallgren, 1948). During the course of the disease, *Mtb* creates various lesions in the lungs where it resides, but early lesions can mostly be found in the upper lung lobes (Geng *et al.*, 2005; Medlar, 1955). In order to study lesion development in TB, the field of histology is applied, where the anatomy of lung tissues and their granulomas are microscopically investigated in lung sections. Through these histological evaluations, lung pathology can be examined visually and quantitatively expressed by means of free alveolar space (Kroesen *et al.*, 2018; Lenaerts *et al.*, 2015). Histological investigations of this project will be reported in later chapters of this thesis.

2.3.5.1 The cellular composition of granulomas

Although there are various types of granulomas, the classic granuloma structure is briefly described. The structure of granulomas and the classic cellular composition of necrotic granulomas are illustrated in Figure 2.3. In the early stages of infection during the innate immune response, following the establishment of *Mtb* in the alveoli, the lung pathology that occurs includes thickening of the alveolar wall and inflammatory foci in the parenchyma and surrounding

bronchioles, as well as blood vessels (Dorhoi & Kaufmann, 2016). As alluded to earlier, firstly macrophages and DCs, are recruited and accumulate in the lungs. The recruited cells compress the surrounding tissues and transit into the alveolar sacs, thereby creating granulomas (Dorhoi & Kaufmann, 2016). Macrophages comprise the largest part of cells, hosting the *Mtb,* and further differentiate into giant cells, epithelioid cells and foamy macrophages. Foam cells are loaded with lipid bodies, which serve as a nutrient source of fatty acids, mostly triacylglycerol, for the bacteria, which is essential for bacterial survival within granulomas (Hunter, 2016; Meena & Sharma, 2016). In its core, the granuloma contains these infected macrophages, epithelioid cells, foamy macrophages as well as giant cells, neutrophils, and DCs (Figure 2.3). These and other myeloid regulatory cells are also scattered throughout the granulomas. A fibrous cuff separates the granuloma centre from lymphocytes and adjunctive tissues (Dorhoi & Kaufmann, 2016). The core cells are surrounded by $CD4^+$ and $CD8^+$ T cells, and B cells (later fibroblasts) around the edge of granulomas (Figure 2.3) (Dorhoi & Kaufmann, 2016).

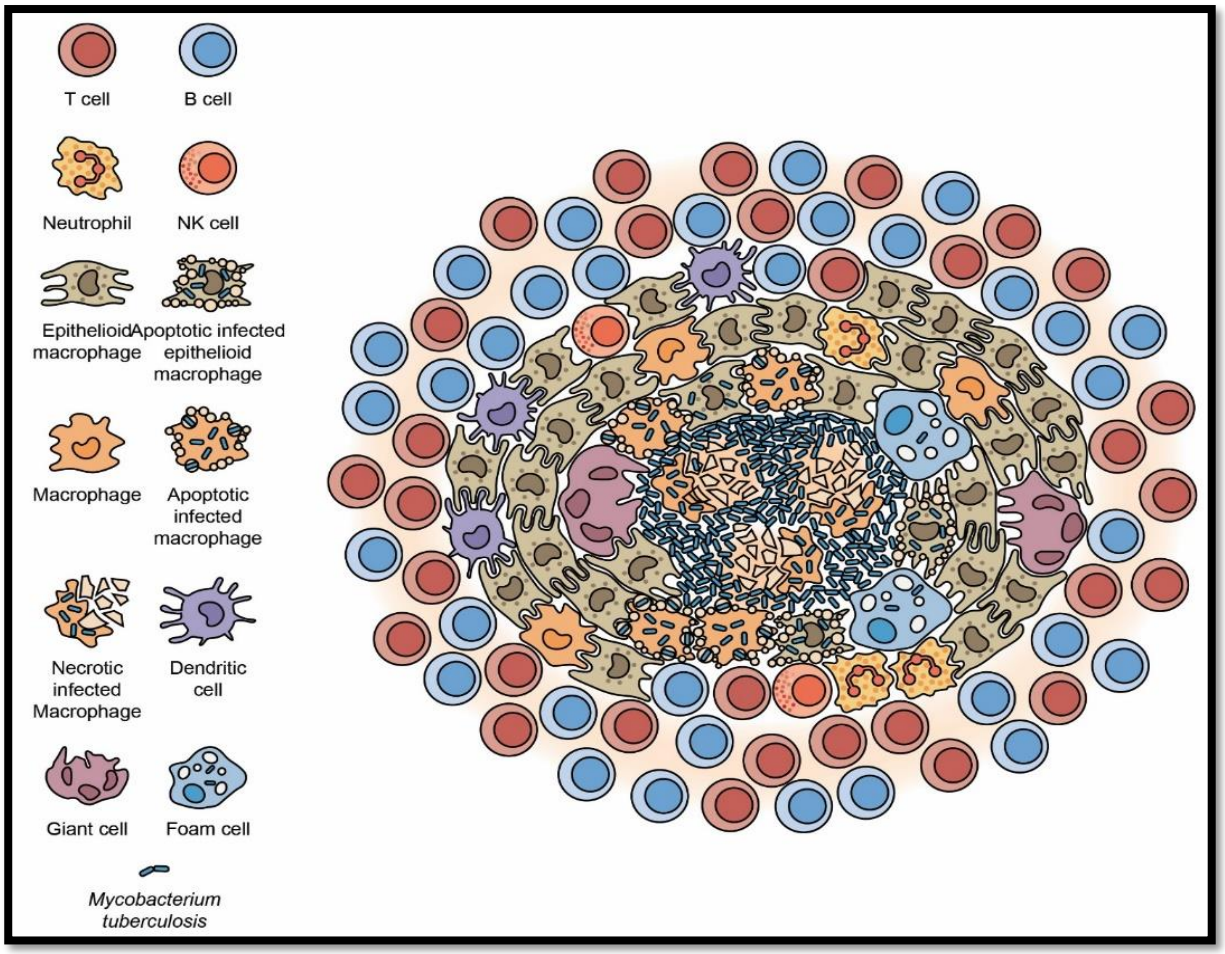


Figure 2-3 **Structure and immune cell types in tuberculosis granuloma** (Adapted from Dartois, 2014; Ndlovu & Marakalala, 2016)

NK: natural killer

The role of T cells in TB is mainly the regulation of the inflammatory environment, more specifically, regulating the innate immune response. $CD4^+$ T lymphocytes are responsible for the protective immunity by limiting pathological damage and the activation of phagocytes (macrophages) for bacterial killing, through pro-inflammatory cytokines such as IFN-γ and TNF-α. T cells are also responsible for forming an outside protective barrier in granulomas (Cooper & Torrado, 2012; O'Garra *et al.*, 2013; Torrado *et al.*, 2011). The formation of granulomas separates infected macrophages from healthy tissues but keeps them in close contact with T cells. However, granulomas also provide a safe place for bacteria to replicate (Agoro & Mura, 2019; Dorhoi & Kaufmann, 2016; Egen *et al.*, 2008; Peters & Ernst, 2003). Immune cell phenotyping can be conducted by means of flow cytometry examinations in order to sort and count cells. This provides quantitative interpretations of immune cells that are recruited in TB infection. Flow cytometry examinations were also applied in this project and reported in later chapters.

It is important to note that *Mtb* modulates the macrophage cell death pathway and evades the innate immune response *via* mediating macrophage necrosis (the premature death of cells, by mitochondrial damage) rather than apoptosis (the normal process of cell death) (Figure 2.4). Apoptosis is described as host protective as it favours an early immune response, limits bacterial growth, aids in antigen presentation by DCs, stimulates the type 1 T helper (Th1) response, and minimises immunopathology. This is in contrast with the harmful necrosis that causes a delayed immune response, poor infection control, and worse lung pathology and transmission. Necrosis of macrophages is one of the mechanisms that *Mtb* uses to protect bacterial growth in the infected host and is TNF-dependent (Chen *et al.*, 2006; Divangahi *et al.*, 2013; Fratazzi *et al.*, 1997; Park *et al.*, 2006; Winau *et al.*, 2006).

Furthermore, *Mtb* inhibits autophagy (Gupta *et al.*, 2016). Autophagy is the process of self-digestion, where an autophagosome engulfs cytosolic material. Thereafter, the material is delivered to lysosomes where it is degraded and the degraded products are then released back to the cytosol (Gupta *et al.*, 2016; Stek *et al.*, 2018). Autophagy is induced by IFN-γ and TNF-α (Songane *et al.*, 2012). In TB, autophagy is important in the defence against *Mtb* as it removes intracellular pathogens (Gutierrez *et al.*, 2004). Furthermore, it down-regulates IL-1β production (Rathinam *et al.*, 2012). Therefore, the consequence of the inhibition of autophagy by *Mtb* is that it may lead to higher IL-1β concentrations and has been shown to be associated with more severe lung damage, whilst the enhancement of autophagy may lessen lung damage and serve as a therapeutic target (Songane *et al.*, 2012; Stek *et al.*, 2018).

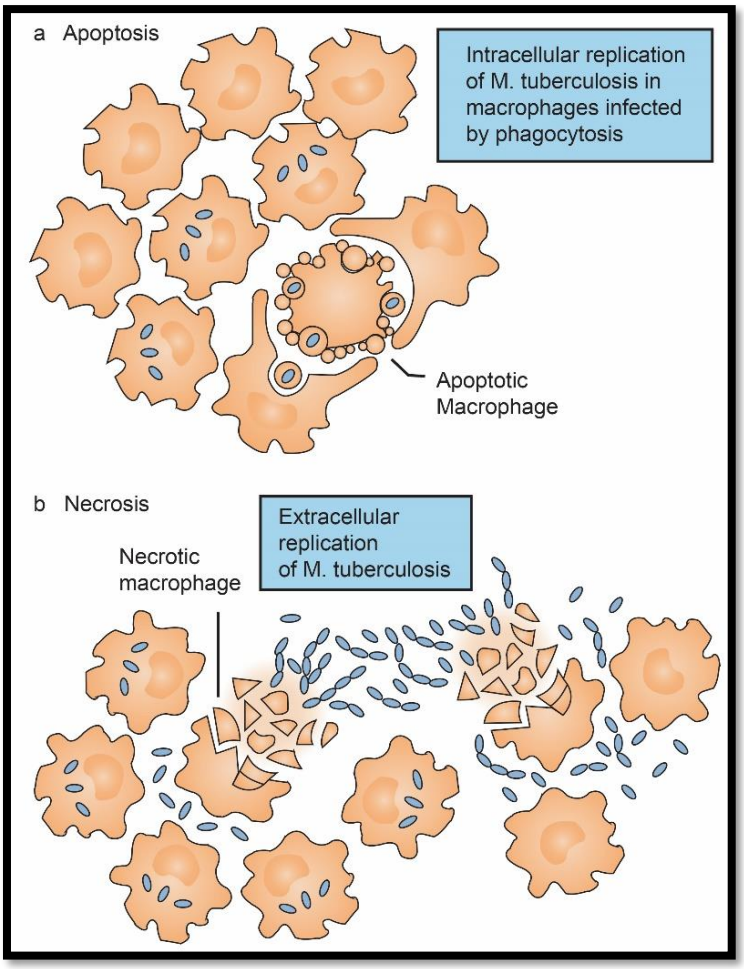


Figure 2-4 **Necrosis versus apoptosis of macrophages in tuberculosis** (Adapted from Dorhoi & Kaufmann, 2016)

(a) The bacteria from infected apoptotic macrophages are distributed to other macrophages by phagocytosis.

(b) Bacteria are released from necrotic macrophages into the extracellular environment, which is highly permissive for mycobacterial growth.

2.3.5.2 Types of granulomas

There are various types of granulomas present in TB and they are also not static, reflecting the course of TB disease (Figure 2.5). These have been defined as either cellular, suppurative, fibrotic or caseous (Cannetti, 1955). Even within a single individual, there are differences in size and inflammatory activity between granulomas (Lenaerts *et al.*, 2015). Granulomas in latent TB individuals contain fewer cells, fibrous encapsulation, are less inflammatory, with prominent sclerosis and often calcification, leading to lung lesions even after granuloma has healed (Cannetti, 1955; Lenaerts *et al.*, 2015). In some granulomas, calcification or fibrosis and

mineralisation of solid and necrotic granulomas hamper replication and spreading (Geng *et al.*, 2005) (Figure 2.5). Mostly, these granulomas will resolve or regress spontaneously and not become active. However, some (5%) of the lesions undergo necrosis and become caseous, which leads to active TB (Figure 2.2 and 2.5) (Hunter, 2016). Solid caseous granulomas, ranging from 1 mm to greater than 2 cm in size, have a cheese-like acellular necrotic area in the middle formed from the degradation of tissue (high in lipids) and are surrounded by inflammatory cells including epithelioid macrophages and lymphocytes (T and B cells) as an outside cuff (Figure 2.3) (Flynn *et al.*, 2011).

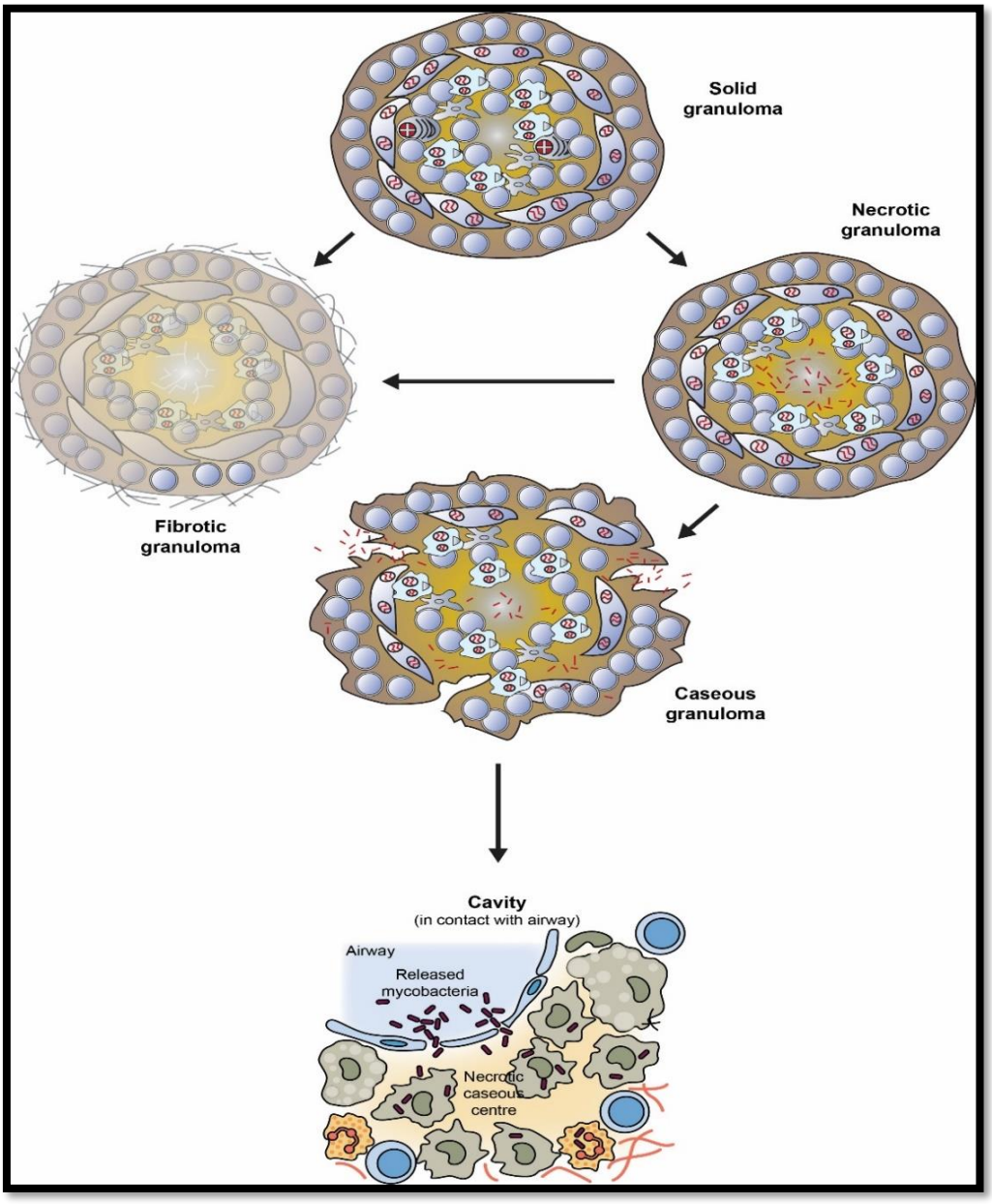


Figure 2-5 **Granuloma progression in tuberculosis** (Adapted from Dorhoi & Kaufmann, 2016; Ndlovu & Marakalala, 2016)

In these granulomas, the solid caseum can sometimes liquefy by means of hydrolysis. Caseous and liquefied granulomas cause the spreading of *Mtb* to adjunct tissue and promote lung tissue injury (Dorhoi & Kaufmann, 2016; Geng *et al.*, 2005; Lenaerts *et al.*, 2015). This liquefied caseum is then emptied into an airway or pleural space and forms a cavity (Figure 2.5). Unlike granulomas, which are known to be protective but also pathological in TB, cavity lesions signify TB progression and transmission. In cavities T cells are almost entirely lacking, thereby permitting bacterial replication (Kaplan *et al.*, 2003). These lesions present a thin caseous-necrotic layer, which is comprised mainly of neutrophils and macrophages, but also of epithelioid and foamy cells, and contains ample bacteria in the cavity wall (Barry *et al.*, 2009; Dorhoi & Kaufmann, 2016). It is from here that bacteria are expectorated for transmission to other individuals, by means of leakage of lesion content into the airways, exposing it to the outside environment (Hunter, 2016).

As in *Mtb*-infected animals, lipid-rich necrotic material builds up in humans as well. However, a second component of cavity formation in humans has been proposed and confirmed by gene expression in that the bacteria cause an autoimmune inflammatory host response (Clayton *et al.*, 2017). Matrix metalloproteinases (MMPs) are found in TB cavities and aid in the breakdown of extracellular material (collagen and elastin). Neutrophils, which have been found to secrete MMPs, have also been reported in high concentrations in cavities and have been implicated in cavity formation (Ong *et al.*, 2015; Ong *et al.*, 2014). Cavitary TB is the most destructive form of TB, and the lung tissue damage is irreversible even with proper treatment. It is difficult to treat, with higher rates of relapse and transitioning to drug-resistant TB, and associates positively with transmission. Cavities are attributed to excessive inflammation, bacillary persistence, and necrosis (Dorhoi & Kaufmann, 2016). Tuberculosis lesions usually heal, but cavities, fibrosis, and pleural adhesions persist in 14% to 100% of patients (Meghji *et al.*, 2016; Theegarten *et al.*, 2006). These persisting pulmonary impairments have also been shown to lead to abnormal lung function in the majority of patients with a TB history (De La Mora *et al.*, 2015; Manji *et al.*, 2016; Nihues *et al.*, 2015). In Figure 2.6, the effects that different immune factors may have on lung damage are illustrated. These factors are also further described in the following section.

2.3.5.3 Factors determining granuloma outcomes

The factors controlling the outcomes of different granulomas in a single individual are not well understood. However, it seems that the local rather than the systemic immune response determines lesion dynamics, resulting in heterogeneous lesions (Lin *et al.*, 2014). Nevertheless, factors that are thought to determine granuloma outcome and disease progression in TB have been identified in the literature. Firstly, apart from the host, the *Mtb* strain strongly influences the host response (Lenaerts *et al.*, 2015; Palanisamy *et al.*, 2009). Secondly, the outcome of granulomas is tightly controlled by local immune factors and inflammatory markers, which may

include lipid mediators (LMs). Specifically, the balance between prostaglandins (PGs), lipoxins (LXs) and leukotrienes (LTs) (especially LT-A4 hydrolase (LTA4H)) has been found to be important in granuloma and TB outcomes (Figure 2.6). These affect tissue damage, macrophage necrosis, and damage and bacterial growth (further discussed in Section 2.6) (Behar *et al.*, 2010; Dorhoi & Kaufmann, 2016; Kumar *et al.*, 2019a; Mayer-Barber *et al.*, 2014; Tobin *et al.*, 2012). In addition to LMs, activated immune cells and cytokines play an important role in granuloma outcomes (Dorhoi & Kaufmann, 2016).

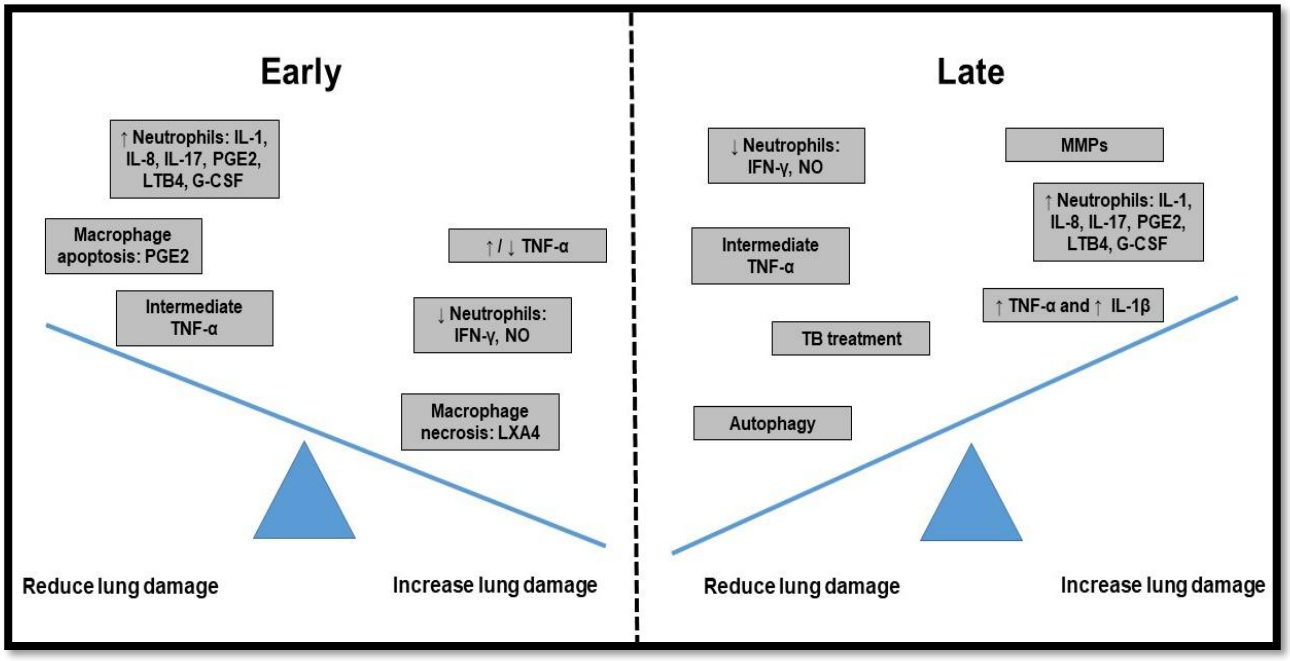


Figure 2-6 Potential effect of immune mediators on lung damage development at different stages of tuberculosis (Adapted from Stek *et al.*, 2018)

GCSF: granulocyte-colony stimulating factor; IL: interleukin; IFN-γ: interferon-gamma; LTB_4: leukotriene B_4; LXA_4: : lipoxinA_4; MMP: matrix metalloproteinase; NO: nitric oxide; PGE_2: prostaglandin E_2; TB: tuberculosis; TNF-α: tumour necrosis factor-alpha.

With regard to immune cells, neutrophils have been found to play a protective role against TB infection (Martineau *et al.*, 2007). However, high neutrophil concentrations later during TB disease are associated with poor clinical outcomes such as lung damage, cavitary TB, slower sputum conversion, disease severity and high mortality rates (Figure 2.6) (Condos *et al.*, 1998; Lowe *et al.*, 2013). In granulomas where neutrophils dominate, high bacterial concentrations have also been noted (Eum *et al.*, 2010; Lowe *et al.*, 2012). These outcomes are attributed to the neutrophil-dependent disease pathology, including neutrophil macrophage interaction, and neutrophil facilitation of TB replication (Lowe *et al.*, 2012). Macrophage-facilitated removal of apoptotic neutrophils supports *Mtb* killing, whilst necrotic neutrophil removal causes *Mtb* survival and

replication in macrophages (Dallenga *et al.*, 2017). As alluded to earlier, *Mtb* induces cell necrosis and this is also true for neutrophils. Neutrophils further store MMPs, which, considering their role in lesion pathology, may contribute to worse outcomes as a result of cavity formation, as discussed earlier (Lowe *et al.*, 2012; Ong *et al.*, 2015; Stek *et al.*, 2018).

Pro-inflammatory cytokines that play an important role in granuloma events are TNF-α and IL-1β (Figure 2.6) (further discussed in Section 2.3.6). These two cytokines have been associated with chest X-ray abnormalities as well as cavity formation (Chowdhury *et al.*, 2014; Sigal *et al.*, 2017; Walker *et al.*, 2012). They control autophagy and antimicrobial peptide release to limit bacteria replication and modulate macrophage cell death within granulomas. Interleukin-17 is also important in granuloma formation, but this cytokine can also cause excessive neutrophil recruitment and tissue damage when overproduced (Figure 2.6) (Cooper & Khader, 2008; Khader *et al.*, 2006). Other cytokines that elicit important functions in granuloma formation include IL-12 and IFN-γ (Clay *et al.*, 2008; Lin *et al.*, 2010; Lin *et al.*, 2007; Romero-Adrian *et al.*, 2015; Wilkinson *et al.*, 2009). In addition, the cytokines IL1β, IL-17, TNF-α and IFN-γ have all been implicated in the expression of MMPs and, therefore, cavity formation and lung damage (Aguilo *et al.*, 2013; Davis & Ramakrishnan, 2009; Keane *et al.*, 2001; Mayer-Barber *et al.*, 2014; Tobin *et al.*, 2012). Cytokines that play important roles in TB will be discussed in further detail in the following section.

2.3.6 Cytokines in tuberculosis

Immune cells communicate *via* signalling either by receptor-receptor contact or secreting molecules. Cytokines are such signalling molecules and are synthesised and released by immune cells in reaction to mycobacterial infection (Cooper *et al.*, 2011). Cytokines refer to protein molecules, including the TNF family, ILs as well as chemokines, and can either have pro- or anti-inflammatory functions (Domingo-Gonzalez *et al.*, 2016). Various cytokines play pivotal roles in the defence against *Mtb* infection. However, if these cytokines are unregulated, they may rather contribute to disease pathology in the chronic setting by causing delayed inflammation resolution, poor tissue repair and tissue damage (Mayer-Barber & Sher, 2015; Stek *et al.*, 2018). The functions of individual cytokines that have been established in animal studies are also well supported by human research. This agreement between the functions of cytokines in human and rodent experiments is one of the reasons why mice studies are justified when exploring the inflammatory response in TB (Mayer-Barber & Sher, 2015). None of these cytokines can be described as good or harmful in TB; most promote defensive, but also pathological outcomes. The positive and negative roles of the different cytokines that will be referred to in this thesis are presented in Table 2.1 (Domingo-Gonzalez *et al.*, 2016).

Table 2-1 The positive and negative roles of specific cytokines in tuberculosis infection*

Cytokine	Positive role in TB	Negative role in TB	Examples of human TB studies which tested the specific cytokines	Examples of TB studies which tested the specific cytokines in mouse models
Tumour necrosis factor-alpha (TNF-α)	• Essential for survival following *Mtb* infection • Initiation of innate cytokine and chemokine response and phagocyte activation	• Mediator of tissue damage	Fallahi-Sichani *et al.,* 2012 Gleeson *et al.,* 2016 Gonzalez *et al.,* 2018 Keane *et al.,* 2000 Zeng *et al.,* 2011	Hölscher *et al.,* 2005 Hölscher *et al*., 2008 Juffermans *et al.,* 2000 Lockhart *et al.,* 2006 Maiga *et al.,* 2015 McNab *et al.,* 2014 Ordway *et al.,* 2007 Wilson *et al.,* 2010
Interferon-gamma (IFN-γ)	• Essential for survival following TB infection • Coordinates and maintains mononuclear inflammation • Expressed by antigen-specific T cells	• Potentially pathogenic	Abu-Taleb *et al.,* 2011 Diel *et al.,* 2011 Dong & Yang, 2015 Gonzalez *et al.,* 2018 Matthews *et al.,* 2012 Özbek *et al.,* 2005 Stefan *et al.,* 2010 Vankayalapati *et al.,* 2000 Wilkinson *et al.,* 1999 Zeng *et al.,* 2011	Behrends *et al.,* 2013 Dhiman *et al.,* 2012 Green *et al.,* 2012 Hölscher *et al.,* 2005 Juffermans *et al.,* 2000 Lockhart *et al.,* 2006 Manca *et al.,* 2001 Moguche *et al.,* 2015 Monin *et al.,* 2015 Ordway *et al.,* 2007 Sakai *et al.,* 2014 Schneider *et al.,* 2010 Wilson *et al.,* 2010
Interleukin-6 (IL-6)	• Potentiates early immunity – nonessential unless a high dose infection	-	George *et al.,* 2015 Nolan *et al.,* 2013 Oh *et al.,* 2018	Lockhart *et al.,* 2006 Manca *et al.,* 2001 Ordway *et al.,* 2007

Cytokine	Positive role in TB	Negative role in TB	Examples of human TB studies which tested the specific cytokines	Examples of TB studies which tested the specific cytokines in mouse models
				Sodenkamp *et al.,* 2012
Interleukin-1α (IL-1α)/ Interleukin-1β (IL-1β)	• Essential for survival following TB infection • Induction of IL-17 • Promotes PGE_2 to limit IFN-α	-	Gleeson *et al.,* 2016 Gonzalez *et al.,* 2018 Wilkinson *et al.,* 1999	Guler *et al.,* 2011 Juffermans *et al.,* 2000 Lockhart *et al.,* 2006 Maiga *et al.,* 2015 McNab *et al.,* 2014
Interleukin-12 (IL-12)	• IL-12p40 and IL-12p35 essential for survival following TB infection. • Mediate early T cell activation, polarisation, and survival	• Overexpression of IL-12p70 is toxic during TB infection	Altare *et al.,* 2001 George *et al.,* 2015 Özbek *et al.,* 2005 Vankayalapati *et al.,* 2000	Behrends *et al.,* 2013 Hölscher *et al.,* 2005 Hölscher *et al*., 2008 Lockhart *et al.,* 2006 Manca *et al.,* 2001 McNab *et al.,* 2014 Monin *et al.,* 2015 Ordway *et al.,* 2007
Interleukin-17 (IL-17)	• Essential for survival following infection with some strains of TB • Induction and maintenance of chemokine gradients for T cell migration	• Drives pathology *via* S100A8/A9 and neutrophils	Bandaru *et al.,* 2014 Dong & Yang, 2015 George *et al.,* 2015 Matthews *et al.,* 2012 Singh *et al.,* 2018	Behrends *et al.,* 2013 Khader *et al.,* 2007 Lockhart *et al.,* 2006 Wilson *et al.,* 2010

*Adapted from Domingo-Gonzalez *et al.* (2016).

IFN-α: interferon-alpha; IFN-γ: interferon-gamma IL: interleukin; *Mtb*: *Mycobacterium tuberculosis*; PGE_2: prostaglandin E_2; TB: tuberculosis.

Pro-inflammatory cytokines that play major roles in TB include type 1 cytokines (IFN-γ and TNF-α), type 17 cytokines (IL-17A and IL-17F), IL-6, IL-12 and IL-1 (α and β) (Domingo-Gonzalez *et al.*, 2016; Kumar *et al.*, 2019b). These pro-inflammatory cytokines exert various important functions in the up-regulation of the immune response, such as macrophage activation, apoptosis, and immune cell recruitment (Kumar *et al.*, 2019b). Deficiency in pro-inflammatory cytokines has been shown to increase TB susceptibility (Casanova & Abel, 2002; O'Garra *et al.*, 2013).

The activity of Th1 cells is of utmost importance in the defence against TB and the cytokines that are associated with this response include IL-12, IFN-γ, and TNF-α, each providing a distinct role (Romero-Adrian *et al.*, 2015). Interleukin-12 (a pro-inflammatory cytokine) is synthesised and released by antigen-presenting cells early in *Mtb* infection (Cooper, 2009; Cooper *et al.*, 2011; O'Garra *et al.*, 2013). Its release causes the migration of DCs from the lung to the lymph nodes (Khader *et al.*, 2006). In addition, it stimulates the production of IFN-γ by NK cells and causes $CD4^+$ T cells to differentiate into Th1 cells that also release IFN-γ (Cooper, 2009; Cooper *et al.*, 1997). Interferon-γ lowers disease susceptibility and is important in protecting against *Mtb* (Casanova & Abel, 2002; Cooper, 2009; O'Garra *et al.*, 2013). In turn, IFN-γ also increases IL-12 release, completing the cycle (Mayer-Barber *et al.*, 2011). The IL-12 / IFN-γ axis links the innate to the adaptive immune response. Macrophages are activated and release TNF in response to IFN-γ (Cooper *et al.*, 2011; Davis & Ramakrishnan, 2009; O'Garra *et al.*, 2013). All of these actions are aimed at mycobacterial containment and killing and this is why the IL-12 / IFN-γ axis is an attractive target for host-directed therapy (HDT). However, researchers have found that Th1 cells that produce IFNs can also be pathogenic and that they can suppress IL-1β and IL-12 production by macrophages, as well as IFN-γ-mediated bacterial killing (Barber *et al.*, 2011; Juffermans *et al.*, 2000; Mayer-Barber *et al.*, 2011). In this regard, Type I IFNs (specifically IFN-α / β) have been implicated in supporting *Mtb* growth and exacerbating the disease (Manca *et al.*, 2005). Therefore, when using the IL-12 / IFN-γ axis as a therapeutic target, the outcomes can be as unpredictable as they can be valuable and more research is needed in this area of TB treatment (Mayer-Barber & Sher, 2015).

The important roles of TNF-α in resistance against TB have been well established (Cooper *et al.*, 2011; Lin *et al.*, 2007). This cytokine restricts mycobacterial growth in macrophages by leading to the production of ROS and RNS that elicit intracellular killing. In addition, TNF-α plays an irreplaceable role in granuloma maintenance, protects against reactivation TB and aids in bacterial containment (Cooper, 2009; Flynn *et al.*, 2011; Lin *et al.*, 2010; O'Garra *et al.*, 2013; Ramakrishnan, 2012). However, some studies have deemed TNF-α as pro-bacterial, because the bacteria use granuloma as protection, and macrophage necrosis is caused by ROS production (Clay *et al.*, 2008; Roca & Ramakrishnan, 2013). Tumour necrosis factor-alpha is also being investigated as a possible target for HDT. Some studies and case reports found that the inhibition

of TNF-α by blocking agents, improves treatment response, especially in complicated TB (Clay *et al.*, 2008; Jorge *et al.*, 2012; Lee *et al.*, 2012). Others have shown an increased susceptibility to TB with a lack of important functions of TNF-α in treated individuals (Hsu *et al.*, 2015; Lin *et al.*, 2010; Lin *et al.*, 2007; Wallis *et al.*, 2004). Nevertheless, the combination of valuable and damaging functions of TNF-α during TB infection has called for more research in this area.

The IL-1 cytokine family and IL-6 also play an important protective role in *Mtb* infection, with significant functions in the innate immune response, thereby influencing *Mtb* susceptibility and host resistance (Juffermans *et al.*, 2000; Mayer-Barber *et al.*, 2011; Romero-Adrian *et al.*, 2015; Verbon *et al.*, 1999b; Yamada *et al.*, 2000). Interleukin-6 acts in cellular apoptosis, differentiation and proliferation and plays a protective role in early immunity by causing IFN-γ expression. Although its effects have been described as minimal, it is an important cytokine in high TB exposure and in immunocompromised individuals (Domingo-Gonzalez *et al.*, 2016). Interleukins-1α and -1β are both strong inflammatory cytokines and modulators of the immune response against *Mtb.* Interleukin-1α is described as one of the main mediators of the innate immune response and is necessary to protect against pulmonary tissue damage and the resultant morbidity and mortality (Guler *et al.*, 2011). In addition, interleukin-1β specifically acts on macrophages to kill *Mtb,* promoting resistance against TB (Romero-Adrian *et al.*, 2015:16; Verbon *et al.*, 1999a:110). Both Type 1 IFNs inhibit IL-1α and IL-1β production. This action of IFN, which is also accompanied by increased IL-10 production, will reduce the host's resistance against TB that is mediated by IL-1 (Mayer-Barber *et al.*, 2011; Novikov *et al.*, 2011). Counter-regulatory IL-1 suppresses Type 1 IFN expression to ensure bacterial control (Mayer-Barber *et al.*, 2014). On the other hand, the IL-1 family has strong inflammatory effects, and if directed by high bacterial loads, it can lead to host tissue damage (Figure 2.6). Interleukin-1β affects the secretion of MMPs, fibroblast activation and neutrophil recruitment, causing lung damage, and it has been found that high IL-1β concentrations correlate with active TB development, disease severity and poor clinical outcomes (Borthwick, 2016; Kumar *et al.*, 2019b; Lowe *et al.*, 2013; Zhang *et al.*, 2014). Therefore, this cytokine is tightly controlled by the host response (Dinarello, 2009).

The measurement of these inflammatory markers in blood and tissue can provide valuable information about the inflammatory and immune response in TB. Kumar *et al.* (2019b) found significantly higher levels of IFN-γ, TNF-α, IL-17A, IL-17, IL-6, IL-12 and IL-1β in the plasma of individuals with active TB when compared with those with latent TB or with their healthy counterparts. In addition, higher levels of IFN-γ, TNF-α, IL-17, and IL-1β could be found in those patients suffering from bilateral or cavitary TB (Kumar *et al.*, 2019b). Disease progression, measured by bacterial load, was further positively correlated with IFN-γ, TNF-α, IL-17, and those patients with delayed culture conversion (longer time from the start of TB treatment to a smear-negative result) also presented with significantly higher levels of these cytokines and IL-1β. This

portrays cytokines as valuable markers of disease progression and severity (Kumar *et al.*, 2019b). On the other hand, Nolan *et al.* (2013) found that IL-6 concentrations were significantly lower in pulmonary fluids of cavitary TB patients, perhaps demonstrating that IL-6 is rather a marker of controlled TB (Nolan *et al.*, 2013). In addition, Wu *et al.* (2017b) reported higher levels of IL-2, IL-10, IFN-γ, and TNF-α in a latent TB patient group, compared with a group with active TB. The authors further suggested that IL-2 and IL-10 may possibly be important markers to differentiate latent from active TB, concluding that cytokines are highly important in bacterial control (Wu *et al.*, 2017b). The importance of inflammatory balance indicates why HDT could be a valuable approach at the correct stage of the disease.

2.4 Tuberculosis management

2.4.1 Medical treatment

Tuberculosis treatment is aimed at curing TB, reducing its transmission, and the prevention of the development of acquired drug resistance, relapse, further complications and death (Department of Health, 2014). According to the NICE, as well as the SA Department of Health guidelines (2014), the updated pharmacological treatment regime for PTB patients, without central nervous system involvement, includes the administration of isoniazid (with pyridoxine), rifampicin, pyrazinamide, and ethambutol for two months followed by isoniazid (with pyridoxine) and rifampicin for a further four months (Department of Health, 2014; Hoppe *et al.*, 2016; Kim & Yang, 2017). The intensive phase (first two months) is intended to kill the bacilli and to ensure that the patient becomes less infectious within 10 to 14 days, whilst the continuation phase (following four months) eliminates the bacilli and prevents relapse (Department of Health, 2014; Kim & Yang, 2017). One of the problems associated with successful TB treatment is poor compliance, which may, in turn, lead to MDR-TB (Zumla *et al.*, 2013). For the treatment of MDR-TB and extensively drug-resistant TB (XDR-TB) the WHO recommends four TB drugs for at least 20 months, resulting in extremely long and costly treatment periods, together with unwanted side effects and the resultant poor compliance (Dheda *et al.*, 2017). To reduce the TB treatment time, the number of drugs required, and improve the resultant toxicity, lung function, drug resistance, and costs, would be of major benefit from a physiological and economic perspective (Gomez *et al.*, 2016; Palucci & Delogu, 2018). This is where HDT fits in as adjunct treatment to current TB treatment regimens.

An HDT is a treatment that supports the host response, including immune defence or/and inflammation, but does not target the pathogen directly (Ivanyi & Zumla, 2013; Stek *et al.*, 2018). These treatments are aimed at providing antimicrobial or other beneficial effects by 1) altering the host mechanisms that the pathogen uses to grow and replicate; 2) enhancing host defence mechanisms; and 3) targeting pathological pathways e.g. hyperinflammation (Kaufmann *et al.*, 2018). More and more research is being poured into this field in order to find the best supportive

treatment. Several HDTs have been found to lower the inflammatory lung damage at different stages of TB disease. Anti-tuberculous treatment itself has been implicated as an HDT that has been shown to reduce systemic and pulmonary inflammation, as well as MMP release, and to enhance autophagy (Kim *et al.*, 2012; Manca *et al.*, 2013; Singh *et al.*, 2014; Yuhas *et al.*, 2007). Other HDTs can be categorised into those targeting either 1) autophagy (e.g. Metformin, Rapamycin, Vitamin D_3, and anti-convulsants e.g. Valproic acid and Lithium, and Statins); 2) the immune and inflammatory response (e.g. non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, IFN-γ, Statins, Zileuton); or 3) granuloma structure (e.g. TNF-α blocking agents, Bevacizumab, MMP-inhibitors, mesenchymal stromal cells) (Carrillo-Alvarado *et al.*, 2019; Kim & Yang, 2017; Mayer-Barber *et al.*, 2014; Mayer-Barber & Sher, 2015; Palucci & Delogu, 2018; Stek *et al.*, 2018). Table 2.2 provides an overview of the current host-directed strategies that are being investigated (Kim & Yang, 2017; Palucci & Delogu, 2018; Stek *et al.*, 2018).

2.4.2 Anti-inflammatory treatment as host-directed therapy in tuberculosis

As mentioned previously, a hyperactive immune response is a prominent pathological feature of TB. Anti-inflammatory drugs such as corticosteroids (e.g. prednisone) and NSAIDs (e.g. ibuprofen) have been investigated as treatment options in this regard. The reasoning behind the use of anti-inflammatory treatment in TB is to aid in the reduction of inflammation-causing lung damage, specifically targeting granulomatous lung lesions that are caused by an inflow and crowding of immune cells (Ivanyi & Zumla, 2013; Kroesen *et al.*, 2017; Stek *et al.*, 2018).

In the past, corticosteroids have been frequently used in TB patients (Critchley *et al.*, 2013). A systematic review and meta-analysis on the effects of the administration of corticosteroids in TB patients revealed that it reduced mortality by 17% (Critchley *et al.*, 2013). However, when including studies conducted only in PTB patients, no significant effects could be found. The authors concluded that more research in PTB patients is needed (Critchley *et al.*, 2013). In a more recent review, Schutz *et al.* (2018) reported that adjunctive corticosteroid treatment supported the prevention of HIV-TB immune reconstitution inflammatory syndrome (IRIS). They further reported that corticosteroid administration benefited meningeal and pericardial TB in HIV-uninfected patients. It was concluded that more high-quality clinical research is needed in this area (Schutz *et al.*, 2018).

Non-steroidal anti-inflammatory drugs, which are the most frequently used analgesic and anti-inflammatory medication, inhibit cyclooxygenase (COX)-1 and COX-2. This reduces the conversion of arachidonic acid (AA) to LMs such as PGE_2, prostacyclin, and thromboxane A_2 (TXA_2), thereby reducing pain, inflammation, fever, platelet aggregation and vasoconstriction. However, the major effects of NSAIDs in TB are mostly ascribed to a reduction in PGE_2 production (Ivanyi & Zumla, 2013; Kroesen *et al.*, 2017; Vilaplana *et al.*, 2013).

Table 2-2 Host-directed therapies potentially inhibiting lung damage and/or promoting lung repair*

Host directed therapy	Potential mechanism
Corticosteroids	↓ IFN-γ, TNF-α, IL-1β ↓ IL-6, IL-10, IL12p40 and MMP-7 in TB-IRIS
Doxycycline	↓ MMP-1, -3 and -9
Vitamin D_3	Innate immune response activator ↓ MMP-7 and -9, IFN-γ, TNF-α, IL-6, IL-10 ↑ Autophagy
Rapamycin, Everolimus	↓ MMP-1 and -3 ↑ Autophagy
NSAIDs (Ibuprofen, Acetylsalicylic acid, Diclofenac)	COX inhibitors ↓ PGE_2 and ↑ LXA_4
Zileuton	↓ 5-LOX
Phosphodiesterase	↓ TNF-α ↓ Neutrophil recruitment
Metformin	Targets AMP-activated protein kinase activator ↓ TNF-α ↑ Autophagy Induces mitochondrial ROS and phagolysosome fusion
Statins	↑ Autophagy Promotes phagosomal maturation and phagolysosome fusion
TNF-α blockers (Adalimumab, Enbrel)	↓TNF-α
PGE_2	↑ PGE_2
IFN-γ	↑ IFN-γ
IL-2	↑ IL-2
GMCSF	Cytokine modulation
Mesenchymal stromal cells	Controls inflammation and immediate tissue repair

*Adapted from Kim & Yang (2017), Palucci & Delogu (2018) and Stek *et al.* (2018).

AMP: adenosine monophosphate; COX: cyclooxygenase; GMCSF: granulocyte-macrophage colony-stimulating factor; IFN-γ: Interferon-gamma; LOX: lipoxygenase; LXA_4: lipoxin A_4; IL: interleukin; MMP: matrix metalloproteinase; NSAIDs: non-steroidal anti-inflammatory drugs; PGE_2: prostaglandin E2; ROS: reactive oxygen species; TB-IRIS: tuberculosis immune reconstitution inflammatory syndrome; TNF-α: tumour necrosis factor-alpha.

Various animal studies have been conducted on the use of ibuprofen and acetylsalicylic acid (aspirin), specifically, in TB (Kroesen *et al.*, 2017). Byrne *et al.* (2006 and 2007) conducted two experiments in which they investigated the effect of ibuprofen and aspirin, in combination or alone, as adjunct therapy with pyrazinamide or isoniazid treatment in TB-infected mice. The authors reported that ibuprofen (10, 20, and 40 mg/kg body weight /day) and aspirin (10, 20, and 40 mg/kg body weight/day) enhanced the effect of isoniazid and pyrazinamide by reducing bacterial count (Byrne *et al.*, 2006; Byrne *et al.*, 2007). Ibuprofen was further reported to be a better adjunct

therapy than aspirin (Byrne *et al.*, 2007). Vilaplana *et al.* (2013) studied the effect of ibuprofen (80 mg/kg body weight /day) in *Mtb*-infected mice and found statistically significant improvements in lung histopathology, survival and bacillary load (Vilaplana *et al.*, 2013). Soon thereafter, Marzo *et al.* (2014) also reported that aspirin increased survival and improved bacillary load control with aspirin administration (3 mg/kg body weight/day) (Marzo *et al.*, 2014). Recently Kroesen *et al.* (2018), in a murine model, found that low-dose aspirin (3 mg/kg/day) reduced lung pathology and bacillary load, whilst increasing survival and favouring anti-inflammatory responses. This was ascribed to its anti-inflammatory effects at systemic and local lung tissue level. Aspirin produced a more anti-inflammatory profile on local tissue level in the lungs, with less neutrophil recruitment. The authors concluded that NSAID administration starting later on in active TB may help reduce excess inflammation and enhance Th1-cell responses (Kroesen *et al.*, 2018).

Human studies in this area are scarce. An older study in 1964 administered low-dose short-term aspirin as an adjunct therapy in TB patients and showed that it reduced some of the side effects of pyrazinamide treatment (Petty & Dalrymple, 1964). In addition, Horsefall *et al.* (1979) reported a reduction in TB treatment side effects when aspirin was administered as co-treatment. Aspirin has also been investigated as supportive treatment in TB meningitis paediatric patients, where 150 mg aspirin daily resulted in fewer strokes and lower three-month mortality rates (Misra *et al.*, 2010).

On the other hand, NSAID use has also been recently correlated with an increased risk of active TB. It is, however, unclear whether this association is causal or rather related to the fact that TB patients are known to have increased NSAID use (Wu *et al.*, 2017a). Supporting this, Rangel Moreno *et al.* (2002) found that in TB-infected mice, lung pathology and inflammation were increased with the administration of ibuprofen. This was linked to PGE_2 inhibition early in the onset of the disease, whereas inhibition later in the disease reduced neutrophil inflow and the resultant lung pathology (Rangel Moreno *et al.*, 2002). It seems that the time of administration of anti-inflammatory treatment is important. Other NSAIDs, including indomethacin and diclofenac have also been investigated for their use in TB. It has been found that indomethacin reduces granuloma size, helps to correct T cell imbalances in granulomas and enhances immunisation response (Hernandez-Pando *et al.*, 1995; Shroff *et al.*, 1990). Similarly, studies on diclofenac showed promising results with regard to the reduction in lesions and bacillary load and increased survival (Dutta *et al.*, 2004; Dutta *et al.*, 2007). In conclusion, NSAIDs and steroids can be beneficial when used as supportive TB treatment owing to their anti-inflammatory properties, but the timing of administration will be crucial. These anti-inflammatory drugs do, however, also carry various side effects such as the risk of gastro-intestinal ulcers, bleeding and cardiovascular risks, including hypotension and myocardial infarctions (Ivanyi & Zumla, 2013). The effect of nutrients that also contribute to anti-inflammatory and pro-resolving responses is, therefore, worthy of

further investigation. These nutrients may provide benefits as anti-inflammatory medications do, but with the advantage of fewer side effects. One such group of nutrients is omega-3 polyunsaturated fatty acids (n-3 PUFAs), which will be discussed in greater detail in Section 2.5.

2.4.3 Nutritional Treatment

2.4.4 The evidence on nutrition interventions in tuberculosis patients

Nutrition and TB are strongly linked as malnutrition causes reduced immune competence, whilst infection can, in turn, lead to malnutrition (Mishra *et al.*, 2018). Nutritional intervention is seen as an adjunct to TB treatment. According to Bhargava (2016), nutrition in TB patients can no longer be neglected as this is incompatible with the ultimate goal of TB control (Bhargava, 2016). Nutrition support in TB patients is aimed at the restoration of body weight, lean body mass, physical functioning and better quality of life (Kant *et al.*, 2015).

With regard to macronutrient provision, in a recent study conducted by Frediani *et al.* (2016), it was found that an increased macronutrient intake over time in TB patients increased body mass index, but did not alter body composition (Frediani *et al.*, 2016). Supporting this, the authors of a 2016 systematic review concluded that macronutrient supplementation will most likely produce modest weight gain, but also mentioned that this was not the case in all the studies included in the systematic review (Grobler *et al.*, 2016). Furthermore, it was found that the available trials were too small to demonstrate whether the provision of sufficient macronutrients *via* food or supplementation plays an important role in TB-specific outcomes (Grobler *et al.*, 2016).

Specific micronutrients have also been investigated for their importance in TB patients. Lower levels of micronutrients, such as Vitamin A, Vitamin D, Vitamin E, Iron, Vitamin C, Zinc (Zn), Selenium, and a higher Coper/Zn ratio have been reported in TB patients. These may have significant clinical implications for host defence and TB outcomes (Devi *et al.*, 2003; Rao, 2009; Vijayamalini & Manoharan, 2004; WHO, 2013). The supplementation of various single micronutrients has consistently been shown to increase these low plasma levels, but clinically important benefits resulting from these increased levels have not been proven (Grobler *et al.*, 2016; Sinclair *et al.*, 2011; WHO, 2013).

One micronutrient that is recognised as an HDT in TB, is Vitamin D. It is well-known that low Vitamin D levels have been associated with a higher risk of TB and that Vitamin D therapy has various positive effects on TB outcomes (Kumar, 2016). Vitamin D supplementation as co-treatment has been found to improve sputum conversion and show radiological improvements in TB patients (Kota *et al.*, 2011; Nursyam *et al.*, 2006). Salahuddin e*t al.* (2013) supported these findings, reporting faster radiographic improvements and immune activation when supplementing Vitamin D-deficient TB patients with 60000 IU of Vitamin D_3 intramuscularly in two dosages

(Salahuddin *et al.*, 2013). In addition, Mily *et al.* (2015) supplemented TB patients with 5000 IU of Vitamin D_3 and found higher odds of sputum culture being negative (2.2 times higher) and a higher percentage of patients with negative sputum cultures at week four when supplementing standard treatment with Vitamin D (Mily *et al.*, 2015). The beneficial therapeutic effects of Vitamin D therapy are partly ascribed to its role in the activation of the innate immune response, the resolution of inflammation (inhibiting inflammatory cytokines such as IL-6, IFN-γ and TNF-α), antimicrobial activity, the inhibition of MMP-7 and MMP-9 and the fact that it induces autophagy (Anand & Selvaraj, 2009; Campbell & Spector, 2012; Coussens *et al.*, 2012; Harishankar *et al.*, 2014; Vidyarani *et al.*, 2007; Yuk *et al.*, 2009). However, others found that Vitamin D_3 supplementation had no effects on sputum conversion and clinical outcomes at dosages of either twice 50 000 IU (Ralph *et al.*, 2013) or once with 2.5 mg Vitamin D_3, with the exception of patients with certain genotypes (Martineau *et al.*, 2011). Nevertheless, Vitamin D_3 may be worth considering as a supplement in TB patient treatment.

General multi-micronutrient supplementation of 50-150% of the recommended daily allowance of micronutrients is thought to improve the immune response to TB and reduce TB recurrence (Kant *et al.*, 2015; Karyadi *et al.*, 2002; Mahan & Raymond, 2017). This is supported by Villamore *et al.* (2008), reporting the benefit of micronutrient supplementation on TB recurrence, immune response, the incidence of peripheral neuropathy and mortality rates (Villamor *et al.*, 2008). However, reviews of the available evidence suggest that multi-micronutrient supplementation studies seem to show little or no benefit with regard to weight gain during treatment and provide inadequate evidence to confirm whether it affects TB-specific clinical outcome measures, such as TB cure rate (Grobler *et al.*, 2016; WHO, 2013). Therefore, even though lower plasma levels of certain micronutrients may be evident in TB patients, the supplementation above general recommended values is not routinely advised (Grobler *et al.*, 2016). An in-depth discussion of the effects of single micronutrients on TB patients falls beyond the scope of this literature review, but the role of iron supplementation will be discussed in detail in later sections (Section 2.7). From the above literature, it is clear that one cannot conclude that dietary intervention or micronutrient supplementation in TB patients will clinically improve treatment outcomes, with the exception of Vitamin D, which shows promising anti-inflammatory effects and improved clinical outcomes.

2.4.4.1 Current practice recommendations on the nutritional treatment of tuberculosis patients

In 2013, the WHO published practice guidelines on the nutritional treatment of TB patients (WHO, 2013). They formulated five guiding principles, including the following: 1) TB care should be provided according to WHO guidelines; 2) adequate essential macronutrients and micronutrients should be provided; 3) thorough nutrition screening, assessment and management should form an integral part of TB care; 4) wider socio-economic issues, including poverty and food insecurity,

should be addressed; and 5) other comorbidities that frequently accompany TB, such as HIV and diabetes, should be considered during nutrition screening, assessment and counselling (WHO, 2013). The WHO further recommends that TB patients (WHO, 2013):

- on diagnosis and throughout treatment should receive a thorough nutritional assessment followed by appropriate counselling on their nutritional status;
- with severe acute malnutrition (SAM), should be treated according to the WHO recommendations on SAM;
- with moderate undernutrition should receive nutrition counselling and be provided with locally available nutrient-rich or fortified supplementary foods; and
- should receive a daily micronutrient supplement providing the recommended nutrient intake in the case where fortified and supplementary food should have been provided but was not available.

It is difficult to calculate quantitatively what the energy requirements of TB patients are, but based on low-quality evidence it should be calculated at around 14% higher than the basal metabolic rate (Kant *et al.*, 2015; WHO, 2013). Mahan and Raymond (2017) suggest a total energy intake of 35 – 45 kcal per kilogram ideal body weight per day of which 15% (or 1.2 to 1.5 g per kilogram per day) should be provided from protein (Mahan & Raymond, 2017).

In practice, pyridoxine (Vitamin B6) is recommended at a dosage of 25 mg per day for all adults started on TB treatment in order to prevent peripheral neuropathy, which is most commonly caused by isoniazid. It is further recommended that this dosage be increased to 50 – 75 mg per day if a TB patient develops peripheral neuropathy (Department of Health, 2014; Mahan & Raymond, 2017). Further dietary intervention should be aimed at managing the symptoms of the disease and treatment and the management of malnutrition, if present (Mahan & Raymond, 2017; WHO, 2013). This includes dietary modifications for the management of anorexia, nausea and abdominal pain, among others. Dietary adjustments should also be aimed at addressing gastrointestinal intolerance of TB medication (most commonly rifampicin) e.g. nausea, vomiting, loss of appetite, mild abdominal pain and diarrhoea. The patient's diet should also be modified with regard to the timing of a meal in relation to medication intake, e.g. taking medication prior to a meal to prevent drug–nutrient interactions (Department of Health, 2014; Kant *et al.*, 2015; WHO, 2013). The role of n-3 PUFAs and iron in TB will be discussed in greater detail in the following sections.

2.5 Omega-3 polyunsaturated fatty acids

2.5.1 Terminology, dietary sources, and synthesis of omega-3 polyunsaturated fatty acids

Fatty acids are classified according to their number of carbon atoms, as well as double bonds. Polyunsaturated fatty acids contain two or more double bonds and can be divided into either the n-3, omega-6 (n-6) or omega-9 (n-9) series, according to the position of their first double bond from the methyl end (Table 2.3) (Calder, 2010; Vanek *et al.*, 2012). Fatty acid nomenclature includes systematic or trivial names and shorthand notations (Table 2.3). The systematic name indicates the number of carbon atoms and double bonds, as well as the double bond positions from the carboxyl end (e.g. 5,8,11,14,17-eicosapentaenoic). The trivial names are descriptive of the number of carbon atoms or historic name e.g. eicosapentaenoic or osbond acid, and the shorthand notation indicates the number of carbon atoms and double bonds, as well as the place of the first double bond from the methyl end (e.g. 20:5n-3) (Burdge & Calder, 2015; Fielding *et al.*, 2019; Sala-Vila *et al.*, 2008). The various fatty acids that will be referred to in this thesis are listed in Table 2.3.

Linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) are essential fatty acids and need to be consumed, as humans cannot add a double bond at the n-3 or n-6 positions of the fatty acid carbon chain. Alpha-linolenic acid is produced in plants and can be found in different seeds, nuts, and oils. Green leaves, canola, soybean (~ 10% of fatty acids), and flaxseed (or linseed) (> 50% of total fatty acids) and their oils are the main dietary sources (Baker *et al.*, 2016; Burdge & Calder, 2015; Nettleton, 1991). Consumed ALA can have various metabolic fates. Firstly, it can be incorporated into adipose tissue as triglycerides where ALA is the most abundant n-3 PUFA. Furthermore, it can be subjected to beta-oxidation to be converted to acetyl-coenzyme A for energy and carbon dioxide or for subsequent saturated and monounsaturated fatty acid production (Andersen *et al.*, 1999; Burdge *et al.*, 2003; Geerling *et al.*, 1999; Nettleton, 1991). Dietary ALA can also be elongated and desaturated to form the long-chain PUFAs (LCPUFAs). A series of enzymatic reactions, occurring mainly in the liver, bring about the elongation of both ALA and LA by the addition of two-carbon units, as well as desaturation, introducing additional double bonds (Burdge & Calder, 2015; Nakamura & Nara, 2004; Sprecher *et al.*, 1995). Alpha-linolenic acid is converted to the longer, more unsaturated eicosapentaenoic acid (EPA, 20:5n-3) and then docosahexaenoic acid (DHA, 22:6n-3), whilst AA is synthesised from LA (Figure 2.7) (Burdge & Calder, 2015).

Table 2-3 Fatty acid nomenclature

Systematic name	Trivial name	Abbreviation	Shorthand notation
Omega-6 PUFAs			
cis-9,cis12-octadecadienoic	Linoleic acid	LA	18:2n-6
All cis-6,9,12-octadecatrienoic	Gamma-Linolenic acid	GLA	18:3n-6
All cis-11,14 eicosadienoic acid	Eicosadienoic acid	-	20:2n-6
All cis-8,11,14-eicosatrienoic	Dihomo-gamma-Linolenic acid	DGLA	20:3n-6
All cis-5,8,11,14-eicosatetraenoic	Arachidonic acid	AA	20:4n-6
All cis-7,10,13,16-docosatetraenoic acid	Adrenic acid	-	22:4n-6
All cis-4,7,10,13,16-docosapentaenoic	n-6 docosapentaenoic / Osbond acid	n-6 DPA	22:5n-6
Omega-3 PUFAs			
All cis-9,12,15-octadecatrienoic	Alpha-linolenic acid	ALA	18:3n-3
All cis-6,9,12,15-octadecatetraenoic acid	Stearidonic acid	-	18:4n-3
All cis-11,14,17-eicosatrienoic acid	Eicosatrienoic acid	-	20:3n-3
All cis-5,8,11,14,17-eicosapentaenoic	Eicosapentaenoic acid	EPA	20:5n-3
All cis-7,10,13,16,19-docosapentaenoic	Docosapentaenoic acid	n-3 DPA	22:5n-3
All cis-4,7,10,13,16,19-docosahexaenoic	Docosahexaenoic acid	DHA	22:6n-3

PUFAs: polyunsaturated fatty acids

Diets with high ALA content have been found to result in increased EPA and docosapentaenoic acid (DPA) content in blood and tissues but with relatively minor effects on DHA composition (Abedin *et al.*, 1999; Arterburn *et al.*, 2006; Barceló-Coblijn & Murphy, 2009; Barceló-Coblijn *et al.*, 2005; Bowen & Clandinin, 2000; Burdge & Calder, 2005a; Dittrich *et al.*, 2015). These LCPUFAs are also incorporated into phospholipids which are the building blocks of cell membranes. It should, however, be noted that the extent to which the n-3 LCPUFAs can be synthesised from the intake of ALA is generally limited in humans and rodents (Abedin *et al.*, 1999; Brenna *et al.*, 2009a; Micha *et al.*, 2014). As little as 6% of ALA is elongated and desaturated to DHA in rodents, whilst additionally, only 16% is incorporated in mainly adipose tissue (Lin & Salem, 2007). This is even lower in humans, where between 8 and 12% is converted to EPA, whilst only 1% of ALA is converted to DHA in infants, with even lower percentages in adults (Arterburn *et al.*, 2006; Brenna *et al.*, 2009b; Burdge, 2004; Goyens *et al.*, 2006).

Furthermore, Baker *et al*. (2016) have reported that for every 1 g of ALA consumed there is a 10% relative increase in plasma phospholipid EPA content. These low conversion rates are a result of the fact that ALA is mainly beta-oxidized (Brenna, 2002; Cunnane & Anderson, 1997; Cunnane *et al.*, 2003).

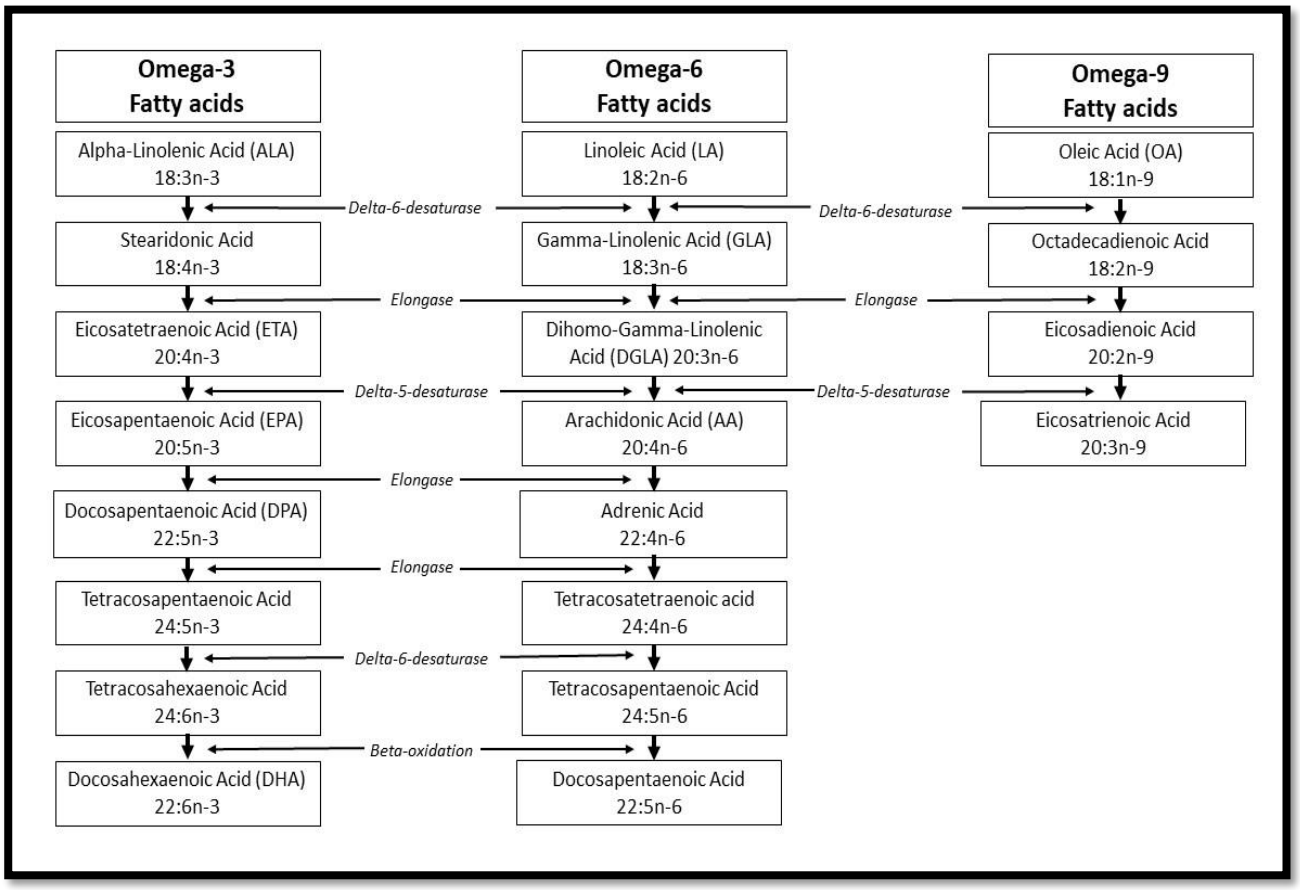


Figure 2-7 **Biosynthesis pathways of omega-3 and omega-6 fatty acids** (Adapted from Calder, 2015b; Edwards & O'Flaherty, 2008; Le *et al.*, 2009)

The conversion of ALA to its longer-chain metabolites is dependent on various factors. In humans, gender influences conversion rates, with higher conversion to DHA in women (Burdge *et al.*, 2002; Burdge & Wootton, 2002). Additionally, genetic and epigenetic variations, as well as age, also influence ALA conversion in humans (Baker *et al.*, 2016; Burdge & Calder, 2005b; Childs *et al.*, 2008). The effects of ALA intake on membrane phospholipid fatty acid composition are also tissue-specific and affected by current PUFA status, as n-3 PUFA deficiency in animal models has been found to enhance liver desaturase activity (Barceló-Coblijn *et al.*, 2005; Domenichiello *et al.*, 2014; Igarashi *et al.*, 2007).

The absolute amount of ALA, EPA, DHA, LA, and total PUFAs consumed influences ALA conversion (Baker *et al.*, 2016; Gibson *et al.*, 2011; Gibson *et al.*, 2013; Goyens *et al.*, 2006). The

main determinant of the synthesis of n-3 LCPUFAs from ALA is the absolute concentration of ALA available as substrate (Cho *et al.*, 1999a; Cho *et al.*, 1999b; Tu *et al.*, 2010). The concentration of ALA that is consumed has been shown to influence ALA phospholipid composition and the resultant n-3 LCPUFA composition in blood and tissue in a dose-response relationship in animals (Arterburn *et al.*, 2006; Burdge & Calder, 2006; Gibson *et al.*, 2013; Goyens *et al.*, 2006; Tu *et al.*, 2010). However, in rodents, it has been reported that increments in DHA synthesis from ALA plateau at a certain concentration of ALA intake and actually decline thereafter (Gibson *et al.*, 2013; Sprecher *et al.*, 1995).

Furthermore, as depicted in Figure 2.7, the synthesis of n-3 series LCPUFAs (EPA and DHA) from ALA uses the same enzymes for metabolism as those of the n-6 PUFA series for the production of AA from LA, which leads to competition between ALA and LA conversion pathways (Brenna *et al.*, 2009a; Burdge & Calder, 2006; Chan *et al.*, 1993; Lands *et al.*, 1990; Sprecher *et al.*, 1995). Consequently, the amount of LA that is consumed and its ratio to ALA influences its conversion and determines the PUFA membrane composition of tissues and blood in rodents and humans (Bourre *et al.*, 1993; Gibson *et al.*, 2013; Mohrhauer & Holman, 1963; Riediger *et al.*, 2008). However, delta-6 desaturase, which is the rate-limiting step in the conversion pathway, has been found to have a higher affinity for ALA compared with LA. Therefore, a higher intake of ALA will favour the production of EPA and DHA compared with that of AA (Hrelia *et al.*, 1990). Consequently, the substitution of LA with ALA (lowering the LA:ALA ratio) has been reported to increase the conversion rates of ALA to n-3 LCPUFA in rodents and humans (Chan *et al.*, 1993; Gibson *et al.*, 2013; Lands *et al.*, 1990; Riediger *et al.*, 2008; Wood *et al.*, 2014; Wood *et al.*, 2015). At LA:ALA intake ratios of above 7:1 (7:1 is considered normal for rodents), the incorporation of ALA into phospholipids is substantially inhibited, and additionally, the conversion to n-3 LCPUFAs is suppressed (Gibson *et al.*, 2013). In humans, a systematic review further concluded that LA needs to be lowered substantially to less than 2.5 % of total energy intake for ALA to enable increased DHA levels (Wood *et al.*, 2015). Recent evidence in cultured human endothelial cells, however, did not find increases in either EPA nor DHA when lowering the LA:ALA ratio (Bork *et al.*, 2019). These authors and others suggested that the absolute amount of ALA intake, rather than the LA:ALA ratio, is the main determinant of conversion rates (Bork *et al.*, 2019; Goyens *et al.*, 2006).

Eicosapentaenoic acid and DHA can also be consumed from dietary sources, of which cold-water fatty fish, such as mackerel, salmon, trout, sardines and their oils are the main dietary sources. These foods vary in EPA and DHA content (Calder, 2014). Essential fatty acid, as well as EPA and DHA intake, differ greatly between countries. Alpha-linolenic acid is generally consumed in higher amounts than EPA and DHA, but its relative contribution to cell membrane fatty acid composition is lower in both tissue and blood, except for adipose tissue. Although essential fatty

acid deficiencies are very rare, in most Western diets the intake of n-6 PUFA is generally higher and n-3 PUFA lower, leading to high n-6:n-3 PUFA ratios (Baker *et al.*, 2016; Blasbalg *et al.*, 2011; Bolton-Smith *et al.*, 1997; Childs *et al.*, 2008; Meyer *et al.*, 2003; Rahmawaty *et al.*, 2013; Simopoulos, 2008). This is also the case in SA, where n-3 PUFA intakes have been found not to be ideal, with n-6:n-3 PUFA ratios as high as 60/1 (Baumgartner *et al.*, 2012; Ford *et al.*, 2016; Richter *et al.*, 2014).

It is recommended that sources rich in DHA and EPA be consumed as part of a healthy balanced diet; however, no dietary reference intakes for n-3 LCPUFAs exist. Current recommendations by expert groups and international bodies recommend 0.7% of total energy from ALA; 250 mg – 500 mg of DHA plus EPA; 0.5 - 2% DHA and EPA of total energy intake; or two to three servings of oily fish or oily fish consumption once or twice per week for healthy males and females. In addition, a minimum of 500 mg/day or up to ≥ 1000 mg per day is recommended for protection against cardiovascular disease (CVD) (Elagizi *et al.*, 2018; FAO, 2010; Flock *et al.*, 2013a; ISSFAL, 2004; Nestel *et al.*, 2015). There are no recommendations for DHA or EPA alone, owing to insufficient evidence for formulating such recommendations (FAO, 2010). Higher dosages of 3 to 4 g of EPA and DHA have also been recommended for specific inflammatory conditions without adverse effects (Flock *et al.*, 2013a; Rangel-Huerta *et al.*, 2012). Nevertheless, it seems that less than 20% of the global population consumes the target of 250 mg EPA and DHA per day (Micha *et al.*, 2014).

2.5.2 Polyunsaturated fatty acids as structural and functional components of cell membranes

Polyunsaturated fatty acids form important functional and structural components of the phospholipid bilayer of plasma membranes throughout the body. In membranes, phospholipids are the most abundant lipid class. Phospholipids are constituted of carbon atoms (glycerol backbone), fatty acids and a polar head (Masi *et al.*, 2013). In Figure 2.8, the structure of the lipid bilayer is illustrated. This lipid bilayer comprises all cell membranes and also some organelle membranes; however, the fatty acid composition between membranes differs according to cell type and function and the phospholipid classes present in the membranes (Calder, 2015a; Masi *et al.*, 2013). The EPA and DHA composition of cell membranes influences cell signalling pathways and gene expression and alters the physical properties of cell membranes (Georgiadi *et al.*, 2012; Ma *et al.*, 2004). The fatty acid composition of the membrane determines the membrane fluidity (by the saturation of fatty acids), influencing the membrane protein function and the movement of membrane proteins (Calder, 2015a; Murphy, 1990; Stubbs & Smith, 1984). Fatty acids can also be released from the membrane or taken up in cells to serve as specific functional, metabolic or signalling molecules. They act as precursors for signalling molecules e.g. LMs and endocannabinoids (Calder, 2014; Calder, 2015a). Polyunsaturated fatty acids exert

important functions in the brain, eyes, and inflammatory processes, amongst others, but the specific function that will be discussed in greater detail in this literature review is its role in the immune and inflammatory response (Section 2.5.4) (Calder, 2015a).

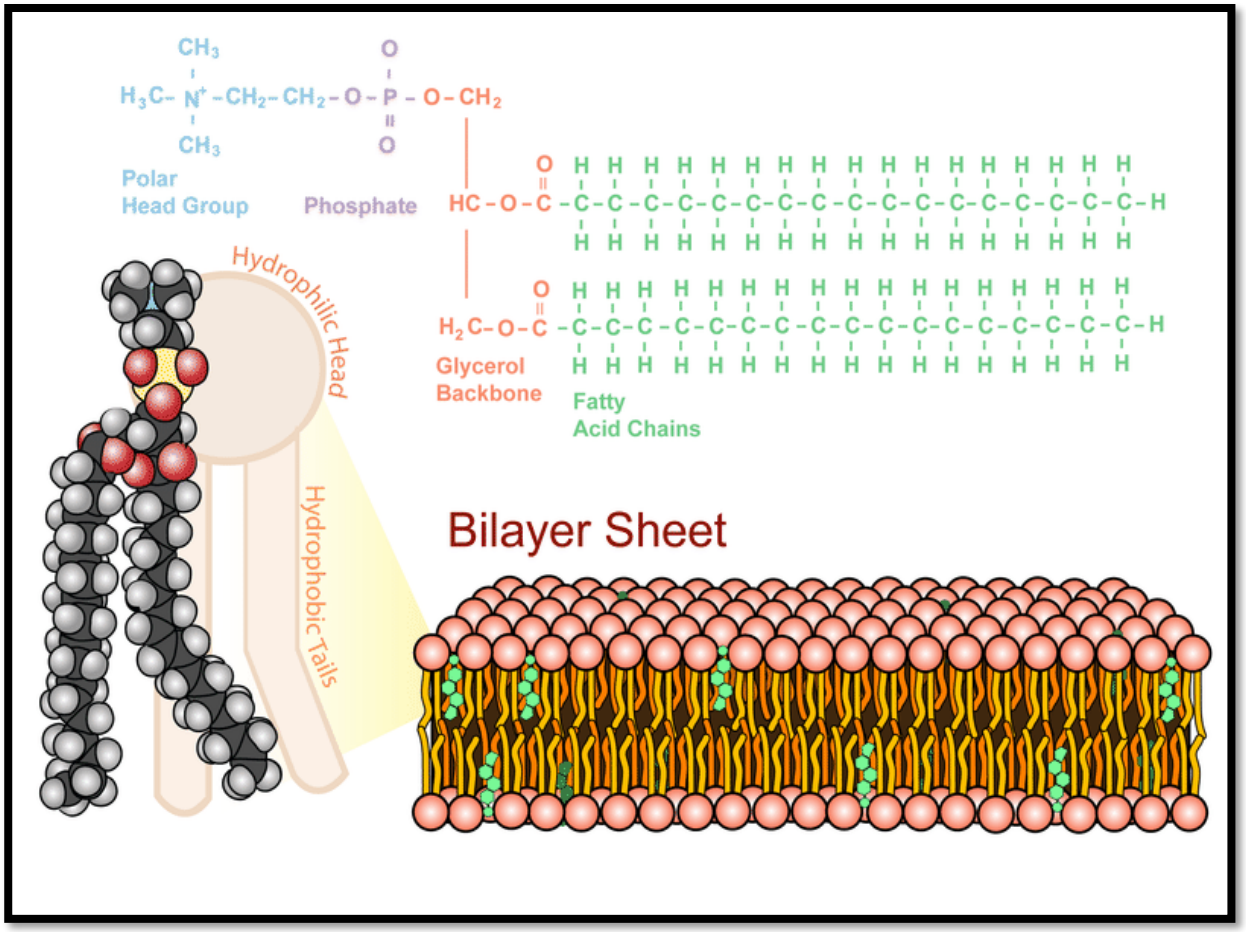


Figure 2-8 **Lipid bilayer sheet of the plasma membrane** (Adapted from https://www.ck12.org/biology/phospholipid-bilayer/lesson/Phospholipid-Bilayers-BIO/)

2.5.3 Fatty acid status

2.5.3.1 Fatty acid incorporation into blood cells and tissues

An individual's oral intake of n-3 LCPUFAs will be reflected in cell membranes of blood cells and tissues at different amounts in different pools. Therefore, when n-3 LCPUFA intake increases, n-3 LCPUFA content will also be higher, but EPA and DHA composition will then reduce and return to baseline levels when intake returns to normal (Browning *et al.*, 2012; Katan *et al.*, 1997; Metherel *et al.*, 2009; Von Schacky *et al.*, 1985; Yaqoob *et al.*, 2000). Eicosapentaenoic acid is incorporated more quickly into membranes than DHA but is also lost more quickly when intake is reduced (Yaqoob *et al.*, 2000). Although the most influential, diet is not the only factor that affects

the EPA and DHA composition of blood pools. Other factors include sex, lifestyle (e.g. smoking and physical activity levels) and age (Block *et al.*, 2008; Flock *et al.*, 2013b). The incorporation of fatty acids into different pools also depends on the turnover of the pool, with rapid incorporation into pools with higher turnover (Calder, 2014). When examining fatty acid status, the fatty acid pool chosen for measurement should be based on the outcomes that are being investigated. Unfortunately, there is no gold standard for the measurement and reporting of fatty acid status (Stark *et al*., 2016), but best practices have recently been suggested (Brenna *et al.*, 2018).

In human studies, the LCPUFA content in various blood pools is usually measured, because of the difficulty of sampling tissues. There are three blood pools that are mainly sampled in humans and they include red blood cells (RBCs) (erythrocytes), plasma phospholipids and plasma total lipids (Stark *et al.*, 2016). In addition, whole blood total lipids are also measured from dried blood spots. This method of sampling and measurement has become increasingly popular as a result of its easy collection and the fact that no processing or preparation is required. However, it provides a more comprehensive idea of the fatty acids of all blood components and is influenced by triglycerides (strongly dependent on recent dietary intake), white blood cells (composition will change in infection), haematocrit, and lipoproteins (also influenced by recent diet) (Brenna *et al.*, 2018). In this thesis, the fatty acid composition of RBCs, peripheral blood mononuclear cells (PBMCs) and lung tissue homogenates were analysed.

Determining the RBC fatty acid status has advantages as it is described as a more stable, long-term marker of fatty acid intake. In RBCs, phospholipids dominate over triglycerides. Red blood cell phospholipid fatty acid changes are usually minimal when compared with plasma lipid pools (Metherel *et al*., 2009). It is also the main blood pool comprising mostly of a lipid bilayer membrane, which may be more reflective of cell membrane content in tissues (Brenna *et al.*, 2018; Fekete *et al.*, 2009; Hodson *et al.*, 2008). With oral n-3 LCPUFA consumption in humans, EPA is incorporated into RBCs, changing their content within one to four weeks. Docosahexaenoic acid, however, may take longer and changes may be subtler (Browning *et al*., 2012, Metherel *et al*., 2009). A global shift towards the measurement of fatty acids in RBCs has not yet ensued owing to the logistical challenges of sample collection, preparation, and storage (Harris & Von Schacky, 2004; Metherel & Stark, 2015; Stark *et al.*, 2016). Data on typical DHA and EPA blood levels are scarce in SA. This is most likely due to the challenges associated with fatty acid analyses. In an older study, it was reported that individuals in areas that are close to the sea, e.g. St Helena Bay region, have high levels of EPA and DHA (9.49%) (Schloss *et al.*, 1997). Literature that is more recent conveyed that RBC phospholipid DHA levels ranged from 3.5% to 4.6% in SA, with comparable values for inland and coastal areas (4.6% vs 4.2%) (Ford *et al.*, 2016).

The fatty acid composition of white blood cells (leukocytes), including lymphocytes, monocytes, neutrophils, etc., can also be measured. White blood cell n-3 PUFA composition is important as it influences immune functions such as phagocytosis, neutrophil activity and inflammatory responses (Calder, 2015b; Calder, 2017). In this research project, the phospholipid fatty acid composition of PBMCs was measured, which represents a fraction of circulating white blood cells and includes mainly lymphocytes (85%, mostly T cells) and monocytes (15%) (Calder, 2018; Fielding *et al.*, 2019). Docosahexaenoic acid and EPA concentrations measured in PMBCs are generally not good biomarkers of overall n-3 PUFA status (Fekete *et al.*, 2009). This is due to the fact that the incorporation of fatty acids in immune cells is also regulated by the need of these fatty acids for inflammatory and anti-inflammatory responses. In humans following a typical Westernised diet, AA represents the largest part of leukocyte fatty acid membrane composition (15 - 20%), whilst EPA and DHA are found in lower concentrations (about 0.5 -1% and 2 - 3%, respectively) (Covar *et al.*, 2010; Damsgaard *et al.*, 2008; Yaqoob *et al.*, 2000).

Dietary n-3 PUFA intake has been found to impact the fatty acid membrane composition of immune cells of humans in a dose- and time-dependent manner (Browning *et al.*, 2012; Damsgaard *et al.*, 2008; Faber *et al.*, 2011; Healy *et al.*, 2000; Katan *et al.*, 1997; Kew *et al.*, 2003; Kew *et al.*, 2004; Rees *et al.*, 2006b; Yaqoob *et al.*, 2000). Long-chain PUFAs is incorporated even faster into leukocytes compared with erythrocytes, especially in an inflammatory or infectious milieu where there is rapid cell turnover (Browning *et al.*, 2012; Healy *et al.*, 2000; Katan *et al.*, 1997; Rees *et al.*, 2006b; Von Schacky *et al.*, 1985; Yaqoob *et al.*, 2000). The contribution of EPA and DHA to the total fatty acids in the membrane phospholipids of immune cells increases when EPA and DHA are consumed in higher concentrations, reaching maximum composition after a few weeks of supplementation in humans (Browning *et al.*, 2012; Faber *et al.*, 2011; Healy *et al.*, 2000; Kew *et al.*, 2003; Miles *et al.*, 2004; Rees *et al.*, 2006a; Yaqoob *et al.*, 2000).

Omega-3 PUFA status can also be measured in tissues, with the rate of incorporation differing between different tissues. For example, DHA is abundant in the cell membranes of the brain, eyes, and testes and can also be found in substantial concentrations in breast milk of humans (Lands, 2005). Dietary n-3 PUFA intake has also been found to reflect in muscle, heart, gut and adipose tissue of humans (Harris *et al.*, 2004; Hillier *et al.*, 1991; Katan *et al.*, 1997; McGlory *et al.*, 2014; Sorensen *et al.*, 2014). Similarly in rodents, Adkins *et al.* (2019) found that mice that were fed DHA-enriched diets (at either 1, 2 or 4%) for four weeks showed significantly higher DHA and EPA concentrations in tissues, including the liver, adipose tissue, heart, and eye, when compared with a control group. Additionally, high DHA diets significantly lowered n-6 PUFA composition in these tissues (Adkins *et al.*, 2019). This was also reported in mammary glands of mice fed diets with differing n-3 and n-6 LCPUFA content. Mice fed n-3 LCPUFA-enriched diets

for 10 weeks reflected significantly higher concentrations of n-3 LCPUFAs in mammary fat pads, coupled with lower numbers of macrophages, inflammatory mediator expression (PGE_2, IL-6, TNF-α, and IFN-γ) and the resultant risk of tumour formation (Khadge *et al.*, 2018). As research has proven that fatty acid consumption alters fatty acid membrane composition of lung tissue and that lung epithelium biosynthesises LMs, crude lung homogenate fatty acid status was also measured in our study (Jakiela *et al.*, 2013; Kiss *et al.*, 2010; Sanak, 2016). Crude lung homogenates represent both lung tissue and the recruited immune cells.

2.5.3.2 Reporting of fatty acid status

Fatty acids can be expressed in relative or absolute formats. The absolute format is the reporting of the actual concentration of the fatty acid in fluids or tissues, but this may be subject to greater variability between individuals. Furthermore, concentrations are difficult to interpret e.g., high n-3 PUFA levels can be due to general lipaemia (Brenna *et al.*, 2018). A global survey reported that µg of fatty acid/mL of blood was the most common method of expression when reporting absolute fatty acid concentrations (Stark *et al.*, 2016). However, fatty acid status is more frequently reported as a proportion relative to other fatty acids. This is termed the relative format or fatty acid profiling and is mostly expressed as a weight percentage of total fatty acids (Stark *et al.*, 2016). The benefit of reporting in this manner is its lower variability and normal distribution, which limits statistical analyses less than the absolute format does (Brenna *et al.*, 2018). In, addition it provides a clearer picture of the quality of fat. To understand the change in profile, it is better to provide the composition of all the fatty acids (Stark *et al.*, 2016).

In the past, owing to a lack of standardised reporting globally, the n-3 index was proposed (Harris & Von Schacky, 2004). This measurement provides a marker for the total amount of EPA and DHA in erythrocytes and may be a useful predictor of an individual's risk of CVD. When interpreting the n-3 index, 4% is considered high risk, whilst above 8% is considered low risk (Harris & Von Schacky, 2004). Internationally, EPA and DHA blood levels vary, but tend to be mostly low (≤ 4-6%) or very low (≤ 4%) when expressed as a percentage of total plasma lipids (Stark et al., 2016). In this thesis, individual fatty acids will be expressed as a percentage of total fatty acids in phospholipids. The next sections will focus on the functions of n-3 LCPUFAs in the inflammatory and immune response.

2.5.4 Omega-3 polyunsaturated fatty acids and the immune and inflammatory response

There are different ways in which n-3 LCPUFAs affect immune function and inflammation. Table 2.4 provides an overview of the anti-inflammatory and pro-resolving activity of n-3 LCPUFAs (Calder, 2013b; Calder, 2015b).

Table 2-4 The anti-inflammatory and pro-resolving activity of omega-3 long-chain polyunsaturated fatty acids and mechanisms involved*

Effect	Likely mechanism involved
Decreased leukocyte chemotaxis.	Decreased production of some chemoattractants (e.g. LTB_4); and down-regulated expression of receptors for chemoattractants.
Decreased adhesion molecule expression and decreased leukocyte–endothelium interaction.	Down-regulated expression of adhesion molecule genes (*via* NF-κB, PPAR-γ, GPR120 etc.).
Decreased production of eicosanoids from AA.	Lowered membrane content of AA; Inhibition of COX; and down-regulated expression of *COX-2* gene (*via* NF-κB, PPAR-γ, GPR120 etc.).
Decreased production of arachidonic acid-containing endocannabinoids.	Lowered membrane content of AA.
Increased production of less inflammatory eicosanoids from EPA.	Increased membrane content of EPA.
Increased production of anti-inflammatory EPA and DHA-containing Endocannabinoids.	Increased membrane content of EPA and DHA.
Increased production of pro-resolution resolvins, protectins, and maresins.	Increased membrane content of EPA and DHA.
Decreased production of inflammatory cytokines.	Down-regulated expression of inflammatory cytokine genes (*via* NF-κB, PPAR-γ, GPR120 etc.).
Modified T cell reactivity.	Disruption of membrane rafts and intracellular signalling (*via* increased content of EPA and DHA in specific membrane regions).

*Adapted from Calder,(2013b) and Calder (2015b)

AA: arachidonic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; GPR120: G-protein coupled receptor 120; LTB_4: leukotriene B_4; NF-κB: nuclear factor kappa B; PPAR-γ: peroxisome proliferator-activated receptor γ.

Most importantly, the phospholipid PUFA composition of immune cells is of functional importance and can be altered through dietary intake (Calder, 2015b). In these cells, significant increases in percentage EPA have been reported within one week of oral supplementation in humans, while also contributing towards changes in immune and inflammatory responses (Faber *et al.*, 2011). Nevertheless, the literature reports that at four weeks of n-3 PUFA administration, near-maximum incorporations of DHA and EPA can be found in leukocyte membranes of humans (Faber *et al.*, 2011; Yaqoob *et al.*, 2000). Browning *et al.* (2012) found that, when supplementing the equivalent of dietary oily fish intake one to four times per week, maximum incorporation into PBMC membranes was found only at six to nine months, thus over a longer time period (Browning *et al.*, 2012). The period of maximum incorporation may differ between populations and may be faster

in an individual with an infectious disease. In addition, when increasing the dietary consumption of EPA and DHA, not only does the EPA and DHA composition increase in immune cells, but the AA composition is reduced in a dose-dependent manner in rodents (Fritsche, 2007) and humans (Browning *et al.*, 2012; Healy *et al.*, 2000; Rees *et al.*, 2006a).

The first reason for the importance of the membrane phospholipid PUFA composition of immune cells is that it affects the biophysical properties of the membrane, including the membrane fluidity and cell responses dependent on membrane proteins. In cell membranes, rafts can be found with particular fatty acid and lipid compositions. These rafts serve as platforms for the action of receptors and the initiation of intracellular signalling (Lorent *et al.*, 2018; Simons, 2018; Simons & Gerl, 2010). The phospholipid EPA and DHA composition of membranes alter raft formation and, in turn, affect intracellular signalling pathways and gene expression by transcription factor activation, e.g. NF-$_k$B. Higher EPA and DHA membrane composition have been found to improve phagocytosis and signalling of immune cells, affecting immune and inflammatory outcomes (Blasbalg *et al.*, 2011; Calder, 2012; Calder, 2013a; Calder, 2014; Calder & Yaqoob, 2007; Jump *et al.*, 2013; Lorent *et al.*, 2018; Novak *et al.*, 2003; Yaqoob, 2009).

There is evidence suggesting that EPA and DHA play a significant role in the enhancement of bacterial phagocytes and killing by the innate immune cells (Chiang *et al.*, 2012; Codagnone *et al.*, 2018; Lee & Zeldin, 2015; Russell & Schwarze, 2014; Spite *et al.*, 2009). As early as 1992, Chapkin and colleagues proved that fish oil enhanced phagocytosis in peritoneal mice macrophages on which an acute inflammatory response was induced by the injection of zymosan (Chapkin *et al.*, 1992). Higher phagocytosis has also been proven in *Mtb*-infected macrophages, specifically (Bonilla *et al.*, 2010a). This is related to, as discussed above, the alterations in the physical nature of cell membranes and receptor expression that are involved in phagocytosis, together with the role of LMs, which will be discussed in Section 2.5.4.3 (Calder *et al.*, 1990; Lorent *et al.*, 2018).

Another reason for the importance of the composition of PUFAs in membranes is that these serve as precursors for the synthesis of signalling molecules (or LMs), which include eicosanoids, octadecanoids, and docosanoids (Calder, 2015b). The types of LMs that are synthesised and released are dependent on the PUFA composition of membranes. Lipid mediators are hormone-like substances that are produced enzymatically by the oxygenation of PUFAs: AA, EPA and DHA *via* lipoxygenase (LOX), COX (COX-1 and COX-2) and cytochrome P450 (CYT P450) pathways in response to pathogens or cytokines, for example. These LMs may also be produced independently of enzymatic activity. Various cell types, such as epithelial cells (that also line bronchi and alveoli), platelets, monocytes, macrophages, neutrophils, fibroblasts and endothelial cells, produce LMs (Basil & Levy, 2016; Jakiela *et al.*, 2013; Kiss *et al.*, 2010; Sanak, 2016; Serhan *et al.*, 2017b).

In response to infection, PUFAs are hydrolysed from membrane phospholipids by phospholipase enzymes, specifically phospholipase A_2 (PLA_2), to release free fatty acids locally at the site of infection or to be transported to the inflammatory site intracellularly and also extracellularly, carried by proteins *via* oedema (Basil & Levy, 2016; Dennis *et al.*, 2011; Kasuga *et al.*, 2008; Serhan *et al.*, 2017b). There are different families of PLA, of which cytosolic PLA_2 ($cPLA_2$) has been reported as being mainly part of pro-inflammatory LM production. This enzyme is calcium-dependent and a calcium influx into the cell is part of the infection signal, causing $cPLA_2$ to excise phospholipid fatty acids from the *sn-2* position, which is mainly AA (Soberman & Christmas, 2003). Calcium-independent PLA (iPLA) excises fatty acids from the *sn-3* position, including the n-3 LCPUFAs (Quach *et al.*, 2014; Ryan *et al.*, 2014; Soberman & Christmas, 2003). The released fatty acids give rise to different LMs in order to mediate the pro-inflammatory and pro-resolving responses (Basil & Levy, 2016; Serhan *et al.*, 2017b). By signalling either the progression or termination of inflammation, balanced LM production and the correct timing is of utmost importance in inflammation control (Kaufmann & Dorhoi, 2013; Robinson *et al.*, 2015). In Figure 2.9 the different LMs and their biosynthesis pathways which will be referred to in this thesis (font in bold red), are illustrated. These LMs and their functions are discussed in more detail in the following sections.

2.5.4.1 Omega-6 polyunsaturated fatty acid-derived lipid mediators

Arachidonic acid is the main substrate for LM synthesis owing to its high concentrations in cell membranes. Shortly following infection or injury, AA is released and free AA serves as a substrate to form pro-inflammatory LMs by CYT P450, COX and LOX enzymes (Calder, 2018). The LMs produced from the metabolism of AA include the LXs, 4-series LTs, 2-series PGs, and TXs. The LOX enzymes that are responsible for the synthesis of pro-inflammatory LMs include 5-LOX, 12-LOX and 15-LOX and these are found in mast cells, monocytes, macrophages, granulocytes and epithelial cells (Figure 2.9) (Calder, 2015b; Tilley *et al.*, 2001). These LOX enzymes lead to the production of LTs and LXs. Additionally, COX enzymes (COX-1 and COX-2) mediate the production of PGs and TXs, whilst hydroxyeicosatetraenoic acids (HETEs) are produced by CYT P450. The LMs derived from AA are mostly pro-inflammatory, except for LXs, which also display anti-inflammatory and pro-resolving effects (Kaufmann & Dorhoi, 2013; Robinson *et al.*, 2015; Serhan & Petasis, 2011). In addition, the n-6 essential fatty acid LA can also directly be metabolised by LOX to LMs such as the hydroxyoctadecadienoic acids (HODEs) and oxo-HODEs, and can, therefore, also directly affect inflammation (Innes & Calder, 2018; Vangaveti *et al.*, 2016).

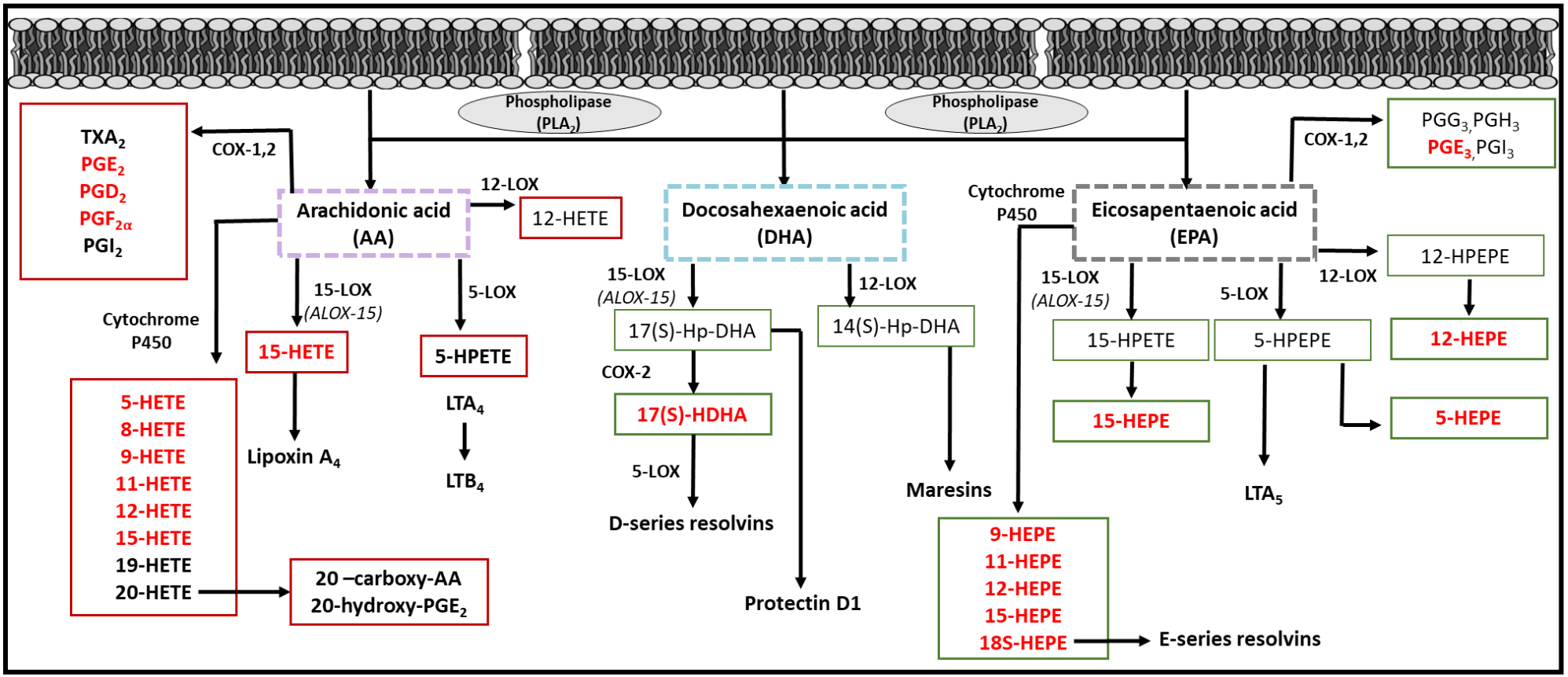


Figure 2-9 **Schematic overview of the biosynthesis pathways of lipid mediators from arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid*** (Figure compiled by L Zandberg and adapted by A Nienaber (Calder, 2015b; Calder, 2018; Serhan *et al.*, 2017b))

*Lipid mediators that were measured in lung tissue homogenates in this thesis are indicated in red bold font.

AA: arachidonic acid; COX: cyclooxygenase; HDHA: hydroxydocosahexaenoic acid; HEPE: hydroxyeicosapentaenoic acid; HETE: hydroxyeicosatetraenoic acid; Hp-DHA: hydroxyperoxydocosahexenoic acid; HPEPE: hydroxyperoxyeicosapentaenoic acid HPETE: hydroperoxyeicosatetraenoic acid; LOX: lipoxygenase; LT: leukotriene; PE: phosphatidylethanolamine; PG prostaglandin; PLA_2: phospholipase A_2; TX: thromboxane.

These LMs bind mostly to G-protein-coupled receptors to result in the expression of enzymes, inflammatory mediators and growth factors aimed at the destruction of the invading pathogens and tissue repair (Calder *et al.*, 2013a; Fielding *et al.*, 2019; Serhan & Petasis, 2011). Each pro-inflammatory LM has a unique role in the inflammatory response. An in-depth discussion of the functions of all the pro-inflammatory LMs is beyond the scope of this thesis; however, several will be mentioned in later sections and, therefore, contextualisation of these is necessary. In this regard 5-, 8-, 11-, 12- and 15-HETE were measured in this research project. Five-hydroxyeicosatetraenoic acid is an intermediate of the LTA_4 and LTB_4 pathway and improves lymphocyte proliferation, and plays an important role in the acute inflammatory response (Colas *et al.*, 2014; Dalli *et al.*, 2015; Serhan, 2014), whilst 12-HETE has been found to benefit inflammatory processes and oxidative stress (Porro *et al.*, 2014). On the other hand, 15-HETE inhibits lymphocyte proliferation (Calder, 2010). Other pro-inflammatory LMs that will also be discussed later in this thesis include PGE_2 and PGD_2. Prostaglandins (PGE_2 and PGD_2) and LTs are pro-inflammatory LMs that cause the recruitment of monocytes and macrophages initially in the inflammatory response. Leukotrienes enhance pro-inflammatory cytokine production such as IL-1, IL-2, IL-6, and TNF-α, whilst LTB_4 aids in neutrophil recruitment. Prostaglandin E_2 and PGD_2 heightens acute inflammation and the signs that this response elicits. In addition, PGE_2 control T and B cell responses, stimulates the production of immunoglobulin E and contribute to blood flow regulation (Buckley *et al.*, 2014; Kalinski, 2012; Kaufmann & Dorhoi, 2013; Robinson *et al.*, 2015; Serhan & Petasis, 2011). On the other hand, PGE_2 and PGD_2 are also important in inflammation resolution, as they stimulate 15-LOX (Buckley *et al.*, 2014; Levy & Serhan, 2014). Other anti-inflammatory effects of PGE_2 that have been reported include the suppression of lymphocyte proliferation, as well as NK cell activity and the inhibition of the production of certain inflammatory markers such as TNF-α, IFN-γ, IL-1, IL-2, and IL-6 (Calder, 2010). A more detailed description of n-6 LCPUFA-derived LMs in the context of TB, will be provided in Section 2.6.

2.5.4.2 Omega-3 long-chain polyunsaturated fatty acid-derived lipid mediators

The n-3 LCPUFAs EPA and DHA also generate LMs by COX and LOX activity. Firstly, as described above, increased intake and availability of n-3 PUFAs alter the lipid membrane composition, partially replacing AA and thereby reducing AA as the substrate for pro-inflammatory LM production (Calder, 2015b; Fullerton *et al.*, 2014; Serhan & Petasis, 2011). Furthermore, the EPA-derived 5-series LTs and 3-series PGs are less potent when compared with AA-derived LMs (Calder, 2018).

Secondly, the LMs that are produced from n-3 LCPUFAs exert pro-resolving effects. As mentioned earlier, inflammation forms an important part of host defence; however, the resolution thereof together with the clearance of phagocytes can be seen as equally important (Serhan *et al.*, 2017b). Contrary to early beliefs, research has shown that there are active biochemical

pathways involved not only in inflammatory responses but also in inflammation resolution (Serhan *et al*., 2007, Serhan, 2014, Serhan *et al*., 2015, Serhan *et al*., 2017b). This is where specialised pro-resolving mediators (SPMs) that are synthesised from both EPA and DHA fit in, actively contributing to inflammation resolution and the restoration of tissue functioning (Barden *et al.*, 2016; Jaudszus *et al.*, 2013; Mas *et al.*, 2016; Morris *et al.*, 2009). The SPMs that are produced from EPA include the E-series resolvins (RvE) (eicosanoids) and those from DHA include the D-series resolvins (RvD), protectins and maresins (docosanoids) (Bannenberg *et al.*, 2005; Hong *et al.*, 2003; Serhan, 2017a). Their biosynthetic pathways are illustrated in Figures 2.9 and 2.10.

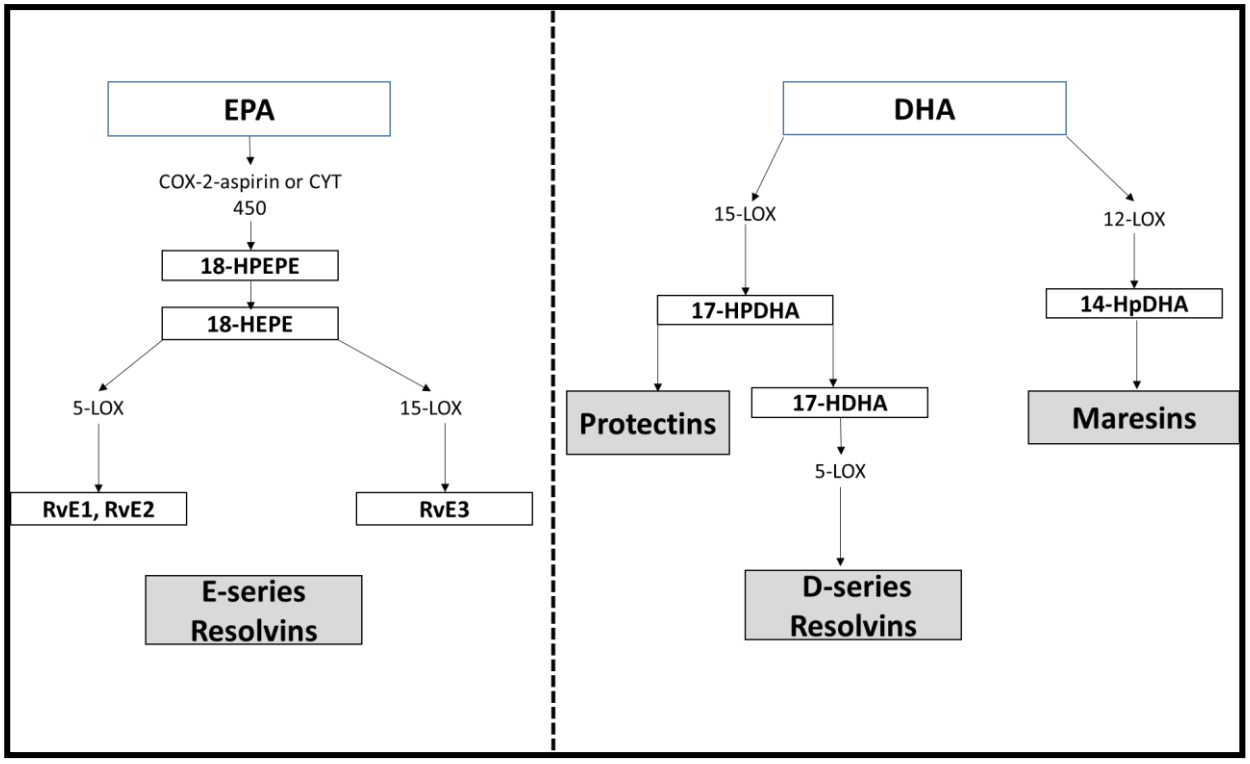


Figure 2-10 Specialised pro-resolving mediator biosynthesis from eicosapentaenoic acid and docosahexaenoic acid (Adapted from Serhan *et al.* (2017b))

COX: cyclooxygenase; CYT P450: cytochrome P450; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; HDHA: hydroxydocosahexaenoic acid; HEPE: hydroxyeicosapentaenoic acid; HPDHA: hydroxyperoxydocosahexaenoic acid; HPEPE: hydroxyperoxyeicosapentaenoic acid; LOX, lipoxygenase; Rv: resolvin.

As mentioned above, consuming diets high in n-3 PUFAs increases the phospholipid n-3 PUFA composition of immune cells (including macrophages, neutrophils that are apoptotic etc.), but also body tissues and, thereby, the production of these LMs (Dalli & Serhan, 2012; Jones *et al.*, 2013; Lundström *et al.*, 2013; Malan *et al.*, 2016). Specialised pro-resolving mediators can be measured in human blood and have been found in breast milk, lymph nodes, lung tissue, synovial fluids and human placenta, suggesting that other tissues produce SPMs in addition to the innate immune system cells and inflammatory exudate (Arnardottir *et al.*, 2016; Colas *et al.*, 2014; Jakiela *et al.*,

2013; Keelan *et al.*, 2014; Kiss *et al.*, 2010; Norling *et al.*, 2016; Sanak, 2016). Additionally, circulating n-3 LCPUFAs and SPMs themselves can be transported by proteins, extracellular vesicles (exosomes) and, *via* oedema, to the area of inflammation (Boilard, 2018; Dalli & Serhan, 2012; Frediani *et al.*, 2014; Kasuga *et al.*, 2008).

Again, phospholipase A_2 releases the n-3 LCPUFAs from phospholipids in membranes. Eicosapentaenoic acid then gives rise to the intermediates, 18-hydroperoxyeicosapentaenoic acid (18HpEPE) and 18-hydroxyeicosapentaenoic acid (18-HEPE) by either COX-2 or CYT P450 enzymes (Oh *et al.*, 2011) (Figure 2.9). Eighteen-hydroxy-eicosapentaenoic acid is used to produce the E-series revolvins (RvEs) RvE1, RvE2, and RvE3 by means of 5-LOX (Arita *et al.*, 2005; Isobe *et al.*, 2012; Oh *et al.*, 2011) (Figure 2.10). In addition, EPA is converted by 5-LOX to 5-HPEPE, which is an intermediate for the anti-inflammatory LTs (Figure 2.9) (Calder, 2015b). Docosahexaenoic acid can also be used to form SPMs, including the D-series Rvs (RvD) (six different species), protectins and maresins (Figure 2.9 and 2.10). The protectins and RvDs are produced from DHA by 15-LOX, and maresins by 12-LOX (Serhan, 2014). One of the LM intermediates that is produced in the DHA pathway by 15-LOX is 17-hydroxyperoxy DHA (17-HDHA), which serves as a marker of the synthesis of the RvDs (Serhan *et al.*, 2002). Nine new LMs have recently been identified, named maresin conjugate in tissue regeneration (MCTR), protectin conjugate in tissue generation (PCTR), and Rv conjugate in tissue regeneration (RCTR) (Serhan *et al.*, 2017b).

2.5.4.3 The functions of specialised pro-resolving mediators in the immune and inflammatory response

The various functions of SPMs are illustrated in Figure 2.11. As the inflammatory process progresses, SPMs step in to keep inflammation within boundaries and to improve the restoration of tissue functioning. The cellular targets of these pro-resolving LMs include neutrophils, macrophages, NK cells, innate lymphoid cells, lymphocytes and mucosal epithelial cells (Basil & Levy, 2016). The roles of SPMs differ and can include the countering of acute inflammation by the down-regulation of the production of pro-inflammatory LMs (LT and PG), cytokines and chemokines; the regulation of COX-2 expression; the formation of platelet-activating factor; NF-$_k$B expression; as well as heightened anti-inflammatory mediator production, such as IL-10 (Serhan, 2017a; Serhan *et al.*, 2017b). They have been found to alter the Th1/Th2 balance mainly by inhibiting the secretion of Th1-type cytokines, including IL-2 and IFN-γ, thereby favouring the Th2 response (Wallace *et al.*, 2001; Zhang *et al.*, 2005). However, n-3 LCPUFAs has also been found to enhance the synthesis of Th2-type cytokines (Petursdottir & Hardardottir, 2008). Specialised pro-resolving mediators further regulate leukocyte trafficking by halting polymorphonuclear leukocyte (PMNL) or neutrophil infiltration (which are proven functions of RvE1, RvE2, RvE3, RvD1, and protectin D1) (Arita *et al.*, 2005; Isobe *et al.*, 2012; Oh *et al.*, 2011; Serhan, 2017a;

Serhan *et al.*, 2017b). However, SPMs still facilitate monocytes to migrate and differentiate into macrophages for phagocytic activity (Serhan, 2017a; Serhan *et al.*, 2015).

Apart from the effects of SPMs on reducing PMNL infiltration and clearance of apoptotic PMNL, as well as their effects on the adaptive immune system, they also actively contribute to the clearance of infections. Specialised pro-resolving mediators have been found to enhance phagocytosis and the activation of immune cells for bacterial killing (Codagnone *et al.*, 2018; Serhan *et al.*, 2017b; Spite *et al.*, 2009). This is a very novel and important function of SPMs as it may contribute to the lowering of the need for pharmacological treatment such as antibiotics (Chiang *et al.*, 2012). It should still be kept in mind that n-3 LCPUFAs cannot serve as a substitute for prescription medication, but should rather be used as adjunctive therapy to help limit bacterial resistance and lower the need for medication by enhancing the host's response to infection and other insults (Serhan, 2017a).

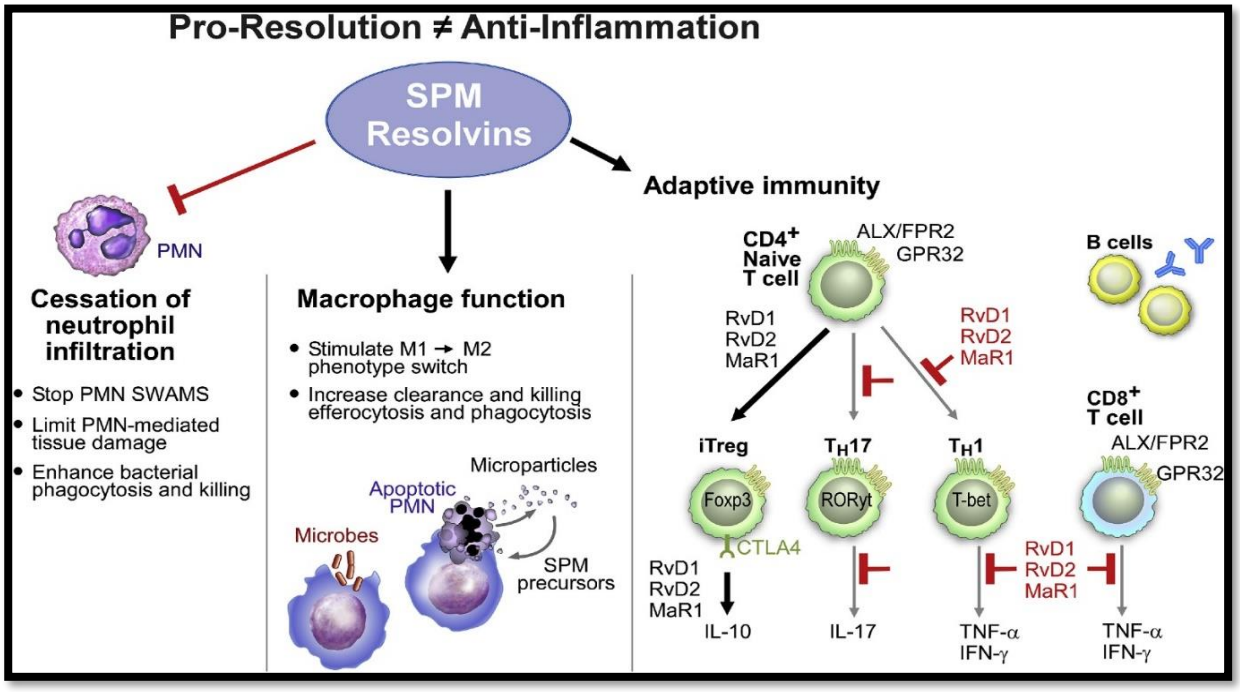


Figure 2-11 **The main actions of SPMs in the immune system** (Used with permission from Serhan *et al*. (2017b))

ALX/FpR2: N-formyl peptide receptor; GPR: G protein-coupled receptor; IFN-γ: interferon-gamma; IL interleukin; MaR: maresin; PMN SWAMS: polymorphonuclear leukocyte infiltration; Rv: resolvin; SPM: specialised pro-resolving mediators; Th: T helper; TNF-α: tumour necrosis factor-alpha.

From the above literature, it is clear that anti-inflammatory functions are not necessarily the same as pro-resolving functions, as pro-resolving encompasses all the actions including phagocytosis of PMNs, bacterial killing and clearance, and enhanced efferocytosis (the removal of apoptotic and necrotic cells by phagocytes) (Serhan, 2017a). In a recent review, Serhan *et al*. (2017b) also

noted that SPMs are not immunosuppressive, as they do not block the inflammatory response. If these SPMs are found in lower concentrations than required, it can add to disease pathology by causing a failed resolution mechanism (Serhan *et al.*, 2007).

2.5.4.4 Omega-3 long-chain polyunsaturated fatty acids and T cell activity

The importance of T lymphocytes in the immune response in TB is well described, as alluded to in earlier sections. These T cells can be divided into sub-classes, including Th1, Th2, Th17 and regulatory T cells with different roles in inflammation and immunity (Liston & Gray, 2014; Noack & Miossec, 2014; Romagnani, 1997). Omega-3 LCPUFAs influence the T cell response, as they have been shown to inhibit T cell proliferation and to alter the production of Th1- and Th2-type cytokines *in vitro* (Calder et al., 1992; Calder et al., 1991; Calder & Newsholme, 1992), in animal models (Jolly *et al.*, 1997; Wallace *et al.*, 2001; Yaqoob *et al.*, 1994a) and in humans (Meydani *et al.*, 1991). The mechanisms underlying the effects of n-3 LCPUFA on T cells have been ascribed to lipid raft disruption (Allen *et al.*, 2014; Kim *et al.*, 2010; Stulnig & Zeyda, 2004), altered LM production (Calder, 2015b) and changes in signalling (Denys *et al.*, 2005; Jolly *et al.*, 1997). Omega-3 PUFAs has been shown to influence lymphocyte function by the up-regulation of peroxisome proliferator-activated receptor alpha (PPAR-α), PPAR-β, and PPAR-γ expression in $CD4^+$ T cells (Unoda *et al.*, 2013). Apart from being incorporated into membranes, it has also been found that PUFAs are oxidised in the metabolism of lymphocytes, which affects the immune cell phenotype differentiation of both macrophages and T cells (Chiaranunt *et al.*, 2015; Fielding *et al.*, 2019; Mills & O'Neill, 2016; Namgaladze & Bruene, 2016; Yaqoob *et al.*, 1994b).

2.5.4.5 The effects of alpha-linolenic acid on inflammatory and immune responses

The essential n-3 PUFA ALA also potentially plays a role in immune and inflammatory responses. These effects are mainly ascribed to the fact that it serves as the precursor for EPA and DHA synthesis. In the immune and inflammatory response, the extrahepatic conversion of ALA to n-3 LCPUFAs is also important (Burdge, 2019). As reviewed by Baker *et al.* (2016), dietary intake of ALA changes membrane fatty acid composition to favour EPA composition of cells involved in inflammation, such as monocytes, neutrophils, and lymphocytes. In humans, higher dietary ALA intake has been found to result in higher PBMC and neutrophil EPA, but not DHA composition (Caughey *et al.*, 1996; Healy *et al.*, 2000; Kew *et al.*, 2003; Zhao *et al.*, 2004). Although delta-6 desaturase activity has been found to be absent in peritoneal macrophages (Chapkin *et al.*, 1988), n-3 LCPUFA can be converted from ALA in lymphocytes which contain delta-6 desaturase, elongase, and delta-5 desaturase activity (Anel *et al.*, 1990b; Calder *et al.*, 1994). Additionally, upon mitogenic stimulation of PBMCs, higher delta-5, delta-6, and delta-9 desaturase activity in lymphocytes has been reported (Calder *et al.*, 1994) and the resultant increased composition of LCPUFAs in PBMCs and lymphocytes (Anel *et al.*, 1990a; Anel *et al.*, 1990b). Consistent with these findings, more recently, Sibbons *et al.* (2018) found that the stimulation of human PBMCs

resulted in higher ALA uptake and conversion to n-3 LCPUFAs (Sibbons *et al.*, 2018). This conversion of ALA to EPA then produces the anti-inflammatory and pro-resolving activity of EPA, as discussed in the previous sections. Furthermore, ALA has been found to directly give rise to its own LMs, such as 13-hydroxyoctadecatrienoic acid (13-HOTrE) catalysed by 15-LOX, which possesses anti-inflammatory functions (Schulze-Tanzil, 2002; Tam, 2013). Alpha-linolenic acid's anti-inflammatory effects are also ascribed to COX inhibition and reducing NO production (Anand & Kaithwas, 2014; Ren & Chung, 2007).

Although not specific to TB and somewhat controversial, especially in humans, ALA supplementation has previously been found to enhance neutrophil phagocytosis capacity; reduce T cell proliferation and activation; and to lower inflammation (Anand & Kaithwas, 2014; Erdinest *et al.*, 2012; Fielding *et al.*, 2019; Ren & Chung, 2007; Schiessel *et al.*, 2016; Sibbons *et al.*, 2018; Tyagi *et al.*, 2012; Zhao *et al.*, 2007; Zhao *et al.*, 2004). Even though there has been no research conducted on ALA in TB, Baker *et al.* (2016) reviewed clinical trials on the effects of ALA supplementation on inflammatory outcomes. Nine out of the 19 studies reviewed reported that higher ALA intake for a minimum of four weeks decreased inflammatory markers such as TNF-α, CRP and IL-1β concentrations, in a dose- and time-dependent manner (Baker *et al.*, 2016). The studies included were mostly conducted in healthy adults, or dyslipedaemic and hypercholesterolaemic patients (Baker *et al.*, 2016). However, a recent meta-analysis on clinical trials investigating the effect of ALA on blood inflammatory markers found no effect of ALA supplementation on TNF-α, IL-6 or CRP (Su *et al.*, 2018). This research was based either on healthy populations or on those with CVD or diabetes, and, therefore, may not be applicable to the infectious disease setting (Su *et al.*, 2018).

In animal studies, the reduction of several pro-inflammatory cytokines has been proven with ALA supplementation in diabetes, cancer and aortic banding models (Duda *et al.*, 2008; Jangale *et al.*, 2016; Moura-Assis *et al.*, 2018; Schiessel *et al.*, 2016). When provided at dosages of 54.5 g $\pm$ 0.8 per 100 g diet, ALA has been found to enhance neutrophil phagocytosis capacity by two-fold, whilst lowering macrophage and lymphocyte TNF-α, and macrophage IL-6 production of tumour-bearing rats (Schiessel *et al.*, 2016). In diabetic mice, flaxseed or fish oil for 35 days exerted similar anti-inflammatory effects, down-regulating IL-6 and NF-kB concentrations (Jangale *et al.*, 2016). Flaxseed oil at 10% of total caloric intake for eight weeks has also been found to reduce inflammation by reducing IL-1β and TNF-α in diabetic mice (Raygan *et al.*, 2019). Alpha-linolenic acid has further been reported to lower lung inflammation in a dose-dependent manner in an animal model, mimicking the effects of asthma-induced lung inflammation (Kaveh *et al.*, 2019).

Additionally, whilst clinical trials have found ALA's effects only to be modest and dependent on its conversion to EPA (Zhao *et al.*, 2007), there is some evidence in animals that the effects of

ALA on inflammation are similar to those of providing preformed EPA and DHA (Jangale *et al.*, 2016; Schiessel *et al.*, 2016). Nevertheless, in a recent clinical trial, 100 mg n-3 PUFA from either fish oil or flaxseed oil administered twice per day for 12 weeks in type 2 diabetic patients exerted similar effects on CRP concentrations (Raygan *et al.*, 2019). The LA:ALA ratio is also an important consideration when discussing the effects of ALA on the immune and inflammatory response. It has previously been reported that n-6:n-3 PUFA ratios of 7:1 and 4:1 are associated with higher IL-6, IL-8 and TNF-$\propto$ concentrations in human alveolar cells. On the other hand, ratios of 1:1 and 2:1 potentiated lower concentrations of these cytokines (Cotogni *et al.*, 2011). This is related to the competition of LA and ALA pathways for n-3 LCPUFA biosynthesis. The next section will elaborate on the clinical relevance of n-3 LCPUFA in some conditions with inflammation as a central component of pathology.

2.5.4.6 Omega-3 long-chain polyunsaturated fatty acids applied as therapy in inflammatory conditions

Previously, the treatment of inflammation has been directed at suppressing, stopping or inhibiting mostly pro-inflammatory mediators by means of pharmacological agents, such as salicylate and other NSAIDs; however, recently, the need for new therapeutic approaches has been highlighted (Serhan, 2017a). Utilising pro-resolving pathways may be a promising approach and has been referred to in recent literature as resolution pharmacology. This approach is aimed at the minimisation and elimination of the immunosuppressive effects of currently utilised anti-inflammatory treatments (Serhan, 2017a). There are various conditions that are associated with, and sometimes caused or exacerbated by, inflammation (Rangel-Huerta *et al.*, 2012). The provision of n-3 LCPUFAs as a therapeutic agent in some of these conditions will now be mentioned, but not discussed in detail.

Rheumatoid Arthritis is an autoimmune disease that is characterised by chronic inflammation. Animal research and clinical trials have proven that n-3 LCPUFA supplementation in RA provides inflammation-resolving and clinical benefits (Lerna *et al.*, 2010; Leslie *et al.*, 1985; Miles & Calder, 2012; Volker *et al.*, 2000). However, Gioxari *et al.* (2018), recently conducted a meta-analysis of the available clinical trials on the effect of n-3 LCPUFA supplementation in RA patients and found that it reduced only LTB_4 among the five markers included in the analyses, but improved early morning stiffness (Gioxari *et al.*, 2018). Omega-3 LCPUFA supplementation has also been investigated in inflammatory bowel disease (Calder, 2009a), including ulcerative colitis (Masoodi *et al.*, 2013) and Crohn's disease (Turner *et al.*, 2009), as well as cystic fibrosis (Oliver & Watson, 2016) and dermatitis (Reese & Werfel, 2015) with promising results.

In patients with inflammatory conditions specifically affecting the lungs, the supplementary benefit of n-3 LCPUFAs seems promising, but controversial. Omega-3 LCPUFAs has been reported as

possibly playing a role in asthma and allergy prevention or treatment (Bilal *et al.*, 2011; Miyata & Arita, 2015). However, more clinical research is required in this regard. In acute respiratory distress syndrome, acute lung injury (Parish *et al.*, 2014; Zhu *et al.*, 2014), chronic obstructive pulmonary disease (Fulton *et al.*, 2015) and respiratory failure, some studies have proven benefit. Others reported no effect with regard to ventilator-free days, oxygenation and lung mechanics indices, depending on the route of n-3 LCPUFA administration and quality of studies (Koekkoek *et al.*, 2019; Preiser *et al.*, 2015). Further research with high-quality research designs is required to confirm earlier findings of benefit with n-3 LCPUFA therapy in these patient groups. Keeping this in mind, one would expect that n-3 LCPUFAs might also benefit TB patients, owing to the disease's inflammatory nature and the lungs as its main target. Section 2.6 will now focus on the application of n-3 LCPUFAs, specifically in PTB.

2.6 Omega-3 long-chain polyunsaturated fatty acids in tuberculosis

2.6.1 Lipid metabolism and tuberculosis

The lipid metabolism of *Mtb* plays a distinct role in the course of TB infection and lipid biosynthesis in these mycobacteria is very complex. It has been found that the cell wall of the mycobacterium has various layers and that the outer membrane (layer) has a lipid bilayer structure. The *Mtb* can also synthesise various lipids, which are then used as a substrate for biosynthetic pathways (Bloch, 1975; Hoffmann *et al.*, 2008; Sani *et al.*, 2010). These lipids play an important role in the entry of *Mtb* into host immune cells. Furthermore, certain lipids influence phagosomal maturation, the exit of *Mtb* from phagosomes, bacterial survival and host cell necrosis (Augenstreich *et al.*, 2017; Axelrod *et al.*, 2008; Passemar *et al.*, 2014; Quigley *et al.*, 2017). Apart from the importance of the bacterium's lipid metabolism, a clear interaction between the host's lipid metabolism and *Mtb* has also been established. Targeting the host's lipid metabolism may be a useful strategy as supportive TB therapy since the availability of host lipids, as well as the types of lipids available, affect TB susceptibility and disease progression (Lovewell *et al.*, 2016).

One mechanism that *Mtb* uses to alter host lipid metabolism is the increasing of the host's PPARs, which leads to lipid body accumulation and the formation of foamy macrophages, which play an important part in granulomas, as discussed earlier (Almeida *et al.*, 2012; Gago *et al.*, 2018; Lovewell *et al.*, 2016). In addition, host lipids are not only important modulators of the immune response in TB, but also serve as a nutrient source for the pathogen (Lovewell *et al.*, 2016). As mentioned earlier, *Mtb* exists, grows and replicates within macrophages, and DCs as well as extracellularly within granuloma, and uses nutrients derived from the host in these environments (Lee *et al.*, 2013). Even though *Mtb* utilises different sources of carbon at different stages of infection, it preferentially uses lipids, specifically cholesterol, as the main fuel source for the metabolism of the bacterium (Gago *et al.*, 2018; Lovewell *et al.*, 2016; VanderVen *et al.*, 2015).

Therefore, the availability of certain fatty acids as a nutrient source may alter the susceptibility of the host to TB infection. It has been found, for example, that hypercholesteraemic mice are more susceptible to TB (Martens *et al.*, 2008). In addition, Parihar *et al.* (2014) found that statins (that reduce cholesterol levels) inhibit the host mevalonate pathway by enhancing phagosome maturation and autophagy, thereby providing protection against TB (Parihar *et al.*, 2014). Furthermore, TB also interacts with fatty acids by the alteration of the production of certain LMs. The manipulation of host LMs, in turn, affects the progression of the disease.

2.6.2 Lipid mediators in tuberculosis

As mentioned earlier, LM signalling forms a very important part of the immune and inflammatory response and this is also true in *Mtb* infection (Basil & Levy, 2016). Tobin and Ramakrishnan (2013) reported that the inflammatory balance in TB is highly dependent on LM production and that this may be an important target for HDT (Tobin & Ramakrishnan, 2013). Research on the role of LM production and its manipulation as HDT in TB has focussed mainly on n-6 PUFA-derived LMs (illustrated in Figure 2.12). In 2005, Bafica *et al.* (2005) proved that 5-LOX-deficient mice (with resulting lower LX levels) presented with higher IL-12 and IFN-γ levels and lower bacterial loads, and therefore, better bacterial control, implicating the negative effects of LXs on the host response. This was one of the first indications of the significant role of LMs in TB and was followed up by numerous studies investigating LM manipulation as HDT.

As alluded to earlier, apoptosis of macrophages is seen as host protective, whilst necrosis improves TB bacilli growth, persistence, and transmission (Dietzold *et al.*, 2015). Lipid mediators play a major role in the determination of the fate of macrophages (either necrosis or apoptosis) (Behar *et al.*, 2010). In this regard, research has suggested that PGE_2, generated from AA by COX-1 and -2 and secreted by activated macrophages, provides resistance against bacteria by regulating cell death outcomes of macrophages that are *Mtb*-infected in an autocrine way. Prostaglandin E_2 protects against necrosis and favours apoptosis by lowering mitochondrial damage and aiding in membrane repair (Behar *et al.*, 2010; Chen *et al.*, 2008; Divangahi *et al.*, 2009; Tobin & Ramakrishnan, 2013). This is also an example of how the functioning of cytokines and LMs are tightly connected, as IL-1β adds to host protection by increasing PGE_2 formation *via* the up-regulation of COX-2 expression (Mayer-Barber *et al.*, 2014; Mayer-Barber & Sher, 2015). The production of PGE_2, in turn, inhibits Type I IFN which helps diminish the reduced bacterial control caused by IFN expression (Mayer-Barber *et al.*, 2014; Mayer-Barber & Sher, 2015). Unfortunately, when macrophages become infected with virulent *Mtb* strains, they suppress PGE_2 production to counter host control of bacteria. Nevertheless, the role of PGE_2 is not fully understood as lower levels of this LM have been found early during TB infection, and higher levels later, suggesting that its role may change during the disease course (Lee & Zeldin, 2015; Mayer-Barber *et al.*, 2014; Rangel Moreno *et al.*, 2002; Stek *et al.*, 2018).

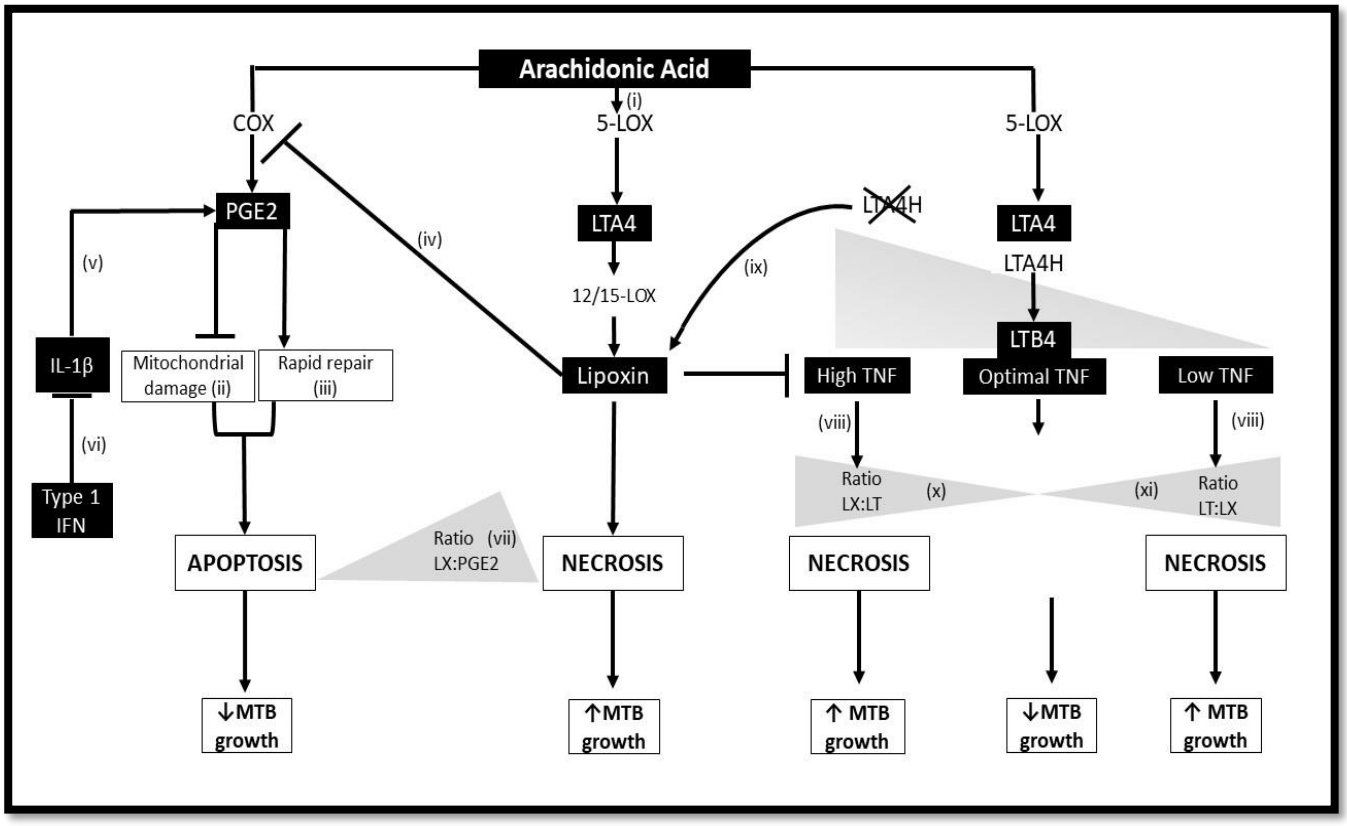


Figure 2-12 Cross-regulation of eicosanoid pathways restricting *Mycobacterium tuberculosis* growth (Adapted from Dietzold *et al.* 2015)

(i) Arachidonic acid metabolism leads to the induction of PGE_2, LXs, and LTs.

(ii) PGE_2 promotes apoptosis and prevents necrosis by inhibiting mitochondrial damage.

(iii) PGE_2 also induces rapid plasma membrane repair.

(iv) LX can inhibit PGE_2 *via* inhibition of COX-2 expression leading to necrosis.

(v) IL-1β enhances PGE_2 expression.

(vi) IL-1β expression is inhibited by type I IFN.

(vii) In the presence of Type I IFN, IL-1β expression is decreased and consequently PGE_2, resulting in an overall increased ratio of LX to PGE_2 thereby promoting *Mtb* growth.

(viii) Low expression of *LTA_4H* leads to reduced TNF-α and the inability to control bacteria and the necrosis of macrophages.

(ix) Removal of *LTA_4H* deviates LM expression towards enhanced LX levels.

(x) An increased ratio of LX to LTs leads to macrophage necrosis.

(xi) High expression of *LTA_4H* is also detrimental since it tips the balance toward high LT expression, excess TNF, macrophage necrosis and enhanced *Mtb* growth.

COX: cyclooxygenase; IFN: Interferon; IL-1β: interleukin-I beta; LOX: lipoxygenase; LTA_4: leukotriene A4; LTA_4H: leukotriene A4 hydrolase; LTB_4: leukotriene B4; LX: lipoxin; MTB: *Mycobacterium tuberculosis*; PGE_2: prostaglandin E2; TNF: tumour necrosis factor.

On the other hand, as mentioned earlier, LXA_4 (anti-inflammatory LM) serves an opposing role and, instead, benefits bacterial growth, and reduces the host's control by initiating necrosis and bacterial transmission. Therefore, excessive amounts of LXA_4 can be detrimental to the host (Figure 2.12) (Bafica *et al.*, 2005; Chen *et al.*, 2008; Divangahi *et al.*, 2009; Kaul *et al.*, 2012; Tobin & Ramakrishnan, 2013). It has been reported that 5-lipoxygenase knock-out mice are more resistant to TB, whereas the provision of exogenous LXs was found to increase susceptibility (Bafica *et al.*, 2005). Research conducted on Zebrafish also reported LXs as the driver with regard to TB susceptibility (Divangahi *et al.*, 2009). The higher susceptibility to TB caused by excess LX production is thought to be a result of the reduction of TNF-α release related to high LX levels, another example of the link between LM production and cytokines. Lower TNF-α release reduces bacterial killing in macrophages. In addition, LXs also limits the adaptive immune responses and causes PGE_2 production to be inhibited by reducing COX-2 expression, thereby mediating necrotic cell death and mitochondrial damage (Figure 2.12) (Chen *et al.*, 2008; Divangahi *et al.*, 2010). In the most recent publication on LMs in TB, Kumar *et al.* (2019) found that LXA_4 positively correlated with IFN-γ, TNF-α, IL1β, and bacterial burden (Kumar *et al.*, 2019a).

Another AA-derived LM, LT have also been implicated in TB resistance. Studies have found that a balanced expression of the *leukotriene–A_4 hydrolase* (*LTA_4H*) gene (a gene responsible for the encoding of the enzyme that converts LTA_4 to LTB_4) promotes the best response to TB, whilst either an excess or a shortage may be detrimental (Tobin *et al.*, 2012). Leukotriene B_4 has been shown to correlate positively with TB severity on chest X-rays (El-Ahmady *et al.*, 1997). Marakalala *et al.* (2016) found high concentrations of *LTA_4H* in granuloma's necrotic centres. This again is mediated mainly through TNF-α production, where the excessive expression of *LTA_4H* leads to high levels of the pro-inflammatory LM, LTB_4, which causes high TNF-α production (Figure 2.12) (Marakalala *et al.*, 2016). As described earlier, TNF-α is host protective; however, excessive TNF-α concentrations leads to necrosis, lysis of infected macrophages, and the release of bacteria (Roca & Ramakrishnan, 2013; Tobin *et al.*, 2012). Additionally, a negative association has been reported recently, between LTB_4 and IFN-γ and IL-1β in TB patients (Kumar *et al.*, 2019a). Once more, this highlights the importance of the close regulation of the inflammatory response in TB *via* the control of cytokine to cytokine and cytokine to LM networks (Mayer-Barber & Sher, 2015).

The literature above clearly indicates the importance of the balanced production of LTs, PGE_2, and LXs to regulate apoptosis and necrosis of macrophages, limit bacterial growth and protect lung tissue (Lenaerts *et al.*, 2015). The importance of LMs in TB regulation, as well as the close connection between cytokine and LM networks in TB control, accentuates the possibilities of LMs

as immunotherapy targets in the management of TB patients (Dietzold *et al.*, 2015; Mayer-Barber & Sher, 2015).

There is a paucity of literature on pro-resolving LMs in TB and their potential as targets to serve as HDT. However, there is a growing interest. The plasma metabolomics of newly diagnosed human PTB patients have been investigated. The results revealed that a pro-resolving plasma LM profile, including higher concentrations of the RvDs, was evident in these patients (Frediani *et al.*, 2014). Very recently, Colas *et al.* (2019) investigated the effects of aspirin on TB meningitis and found that a pro-resolving LM profile (specifically Rvs) positively correlated with 80-day survival, whilst lower levels of SPMs was linked to more severe disease (Colas *et al.*, 2019). More studies are clearly needed in this area.

2.6.3 Omega-3 polyunsaturated fatty acid supplementation in tuberculosis

As mentioned earlier, supportive anti-inflammatory treatment can help reduce the persistent inflammation and coupled inflammatory tissue damage that is characteristic of TB infection. The addition of these host modulators is aimed at shorter treatment times and a reduction in lung tissue damage (Baindara, 2019; Hawn *et al.*, 2013; Palucci & Delogu, 2018). According to Fullerton *et al.* (2014), the manipulation of LMs can be useful as part of immunomodulatory therapy and work synergistically or additively with other standard treatments. Previous studies on the role of n-3 LCPUFAs in *Mtb* are, however, limited (only five studies), having been conducted only in animals and cell cultures (Table 2.5), with the exception of two recent clinical trials. Some of these studies have raised awareness that n-3 LCPUFA supplementation may cause an increased TB susceptibility and reduced ability of the host to control the infection (Bonilla *et al.*, 2010b; Bonilla *et al.*, 2010a; McFarland *et al.*, 2008; Paul *et al.*, 1997). Bonilla *et al.* (2010b) found that *fat-1* mice with higher n-3 PUFA levels had a higher susceptibility to TB and that higher bacterial loads correlated with higher n-3 PUFA composition (Table 2.5). The authors ascribed this to the fact that macrophages of these mice were deficient in various important functions (Bonilla *et al.*, 2010b).

Supporting this, McFarland *et al.* (2008) and Paul *et al.* (1997) also reported that n-3 LCPUFA-fed guinea pigs infected with *Mtb* had a higher bacterial burden when compared with their n-6 PUFA-fed counterparts. However, Paul *et al.* (1997) found more concentrated levels of AA in the animals of the n-3 LCPUFA-supplemented group when compared with the n-6 PUFA-supplemented group, which leaves the interpretation of these results questionable. The group speculated that the right amount of LA might have been provided in the diet administered to the n-3 LCPUFA supplemented group. In addition to these studies, Bazinet *et al.* (2004) reported that n-3 PUFA supplementation (35 g flaxseed oil per kg feed) in piglets increased the levels of antibodies in response to TB immunisation (Bazinet *et al.*, 2004).

Contrasting with these studies, Jordao *et al.* (2008) found n-3 LCPUFA administration to be anti-mycobacterial as lower a bacterial load was evident in the n-3 LCPUFA-supplemented mice group, compared with the n-6 PUFA-supplemented or control groups (Jordao *et al.*, 2008). The reason for the discrepancy in results between this study and the other three may be the fact that, in the other studies, macrophages were used (Bonilla *et al.*, 2010a); the diets with higher n-3 PUFA content were provided as conditioning prior to, but not during, infection as therapy; the fat, EPA, and DHA content of the diets provided in the studies differed; the control diet provided may not have been ideal; the animals used were not the best reflection of TB in humans; and the outcomes measured did not reflect the effect of n-3 PUFAs on inflammation (Bonilla *et al.*, 2010b; McFarland *et al.*, 2008; Paul *et al.*, 1997) (Table 2.5). In this regard, Bonilla *et al.* (2010b) reported a reduction in pulmonary inflammation in *Mtb*-infected mice supplemented with n-3 LCPUFAs.

Only two clinical trials have been conducted on this topic. The first supplemented a combination of fish oil (350 mg n-3 LCPUFA), vitamin A (1500 UI) and Zn (10 mg) with standard TB drug treatment, in paediatric TB patients aged five to 14 years in Indonesia. The group receiving supplementation (n=11) for one month had lower TNF-α levels (and a better decrease in TNF-α levels from baseline) and an improved body mass index, compared with a group that received only standard drug treatment (Nenni *et al.*, 2013). The timing of the initiation of supplementation during the course of the disease and drug treatment was not mentioned. In the second trial, n-3 LCPUFA (300 mg) was supplemented in combination with Zn sulphate (15 mg) once per day for two months in a small number (n=10 per group) of adult Indonesian PTB patients receiving standard TB treatment (Durry *et al.*, 2018). Although the exact timing of the supplementation was not indicated in the article, the authors reported that supplementation had a non-significant, positive impact on sputum smear conversion rates (P = 0.080) and significant improvements in body weight and $CD4^+$ T cell counts compared with the control group (Durry *et al.*, 2018). However, n-3 LCPUFAs were supplemented in combination with other nutrients in these studies and, therefore, beneficial outcomes cannot be ascribed to the n-3 LCPUFAs alone. More basic studies with optimal timing, dosages, and duration of n-3 LCPUFA supplementation, measuring the correct outcomes concerning inflammatory effects and tissue damage in an animal model that reflects the course of the disease in humans, are clearly needed to explain earlier conflicting results and to clearly determine outcomes.

Table 2-5 *In vivo* and *in vitro* studies conducted on the effect of omega-3 long-chain polyunsaturated fatty acids on tuberculosis outcomes

	Reference	TB model	Intervention		Outcomes
			Treatment	Duration	
1)	Bonilla *et al*. (2010a)	Murine macrophage-like cells line J774.A1 (ATCC TIB-67) infected with virulent H37Rv *Mtb*.	Cells conjugated with either: (1) BSA and DHA (DHA:BSA = 3:1) or (2) BSA alone (control).	Seven days	Modified fatty acid composition membranes of macrophages significantly. DHA- treated group had a higher bacterial load compared with the control group ($P < 0.05$) at 3 days post-infection. DHA-treated cells produced fewer pro-inflammatory cytokines at 48 hours post-infection (TNF-α, IL-6, and MCP-1) ($P < 0.01$).
	Bonilla *et al.* (2010b)	8 to 10-week old Wild type (control group) and *fat-1* transgenic mice infected with virulent H37Rv *Mtb* via the aerosol route.	*Fat-1* mice produce n-3 PUFA endogenously. All mice fed AIN -76A rodent diet with 10% safflower oil.	Mice infected for 12 weeks. Sacrificed 5 mice per group at 2, 4, 8, and 12 weeks post-infection.	Higher bacterial loads in the spleens of *fat-1* mice at week 4 and 8 ($P< 0.05$) and week 12 ($P < 0.01$).
		Ex vivo experiment on peritoneal cells of either Wild type or *fat-1 mice*, infected with virulent H37Rv *Mtb*.	N/A	N/A	Higher bacterial loads in *fat-1* mice cells compared with Wild type mice cells at 1 hour, 1, 3, 5 and 7 days post-infection ($P < 0.001$). n-3 PUFA levels positively correlated with CFU. TNF-α ($P < 0.05$), IL-6, ($P < 0.001$), IL-1β ($P < 0.05$), and MCP-1 ($P < 0.05$), were down-regulated in *fat-1* mice.
2)	Jordao *et al.* (2008)	6 to 8-week old female, BALB/c mice infected intranasally with 103 CFU of *Mtb* bacilli.	Fat-free diet supplemented with either: (1) 10% safflower oil (LA content 7.5% of TE) or (2) 10% Ropufa (EPA content 1.5% and DHA 1.1% of TE) or (3) Control diet.	Diets introduced 2 weeks prior to infection. Mice were euthanised at day 21 and 63 post-infection.	n-3 LCPUFA-supplemented diet led to lower bacterial loads (CFU) in lung and spleen at 21 and lungs at 63 days compared with the n-6 PUFA-rich diet and the control diet ($P < 0.05$).

	Reference	TB model	Intervention		Outcomes
			Treatment	Duration	
	Jordao *et al.* (2008)	Mouse macrophage cell line J774A.1 infected with Mtb.	Lipid solution added to the culture medium at a concentration of either 125µM AA or 15µM EPA.	Lipid solution added to culture medium 3 days prior infection and additional lipids daily post-infection.	Higher CFU counts in macrophages treated with EPA and lower in AA-treated cells ($P < 0.05$). EPA reduced TNF-α secretion, whilst AA increased TNF-α secretion ($P < 0.05$).
3)	Mayatepek *et al.* (1994)	H37Rv-infected guinea pig macrophages.	Control: 2.8% LA of TE n-6 rich feed: 15.4% LA of TE. n-3 rich feed: 10.1% LA, 1.4% EPA and DHA of TE.	Diet administered for 13 weeks prior to and during infection period (7 weeks).	PGE_2 and LTB_4 significantly lower in n-3 PUFA rich feed group ($P < 0.05$). Spleen bacterial load significantly higher in n-3 PUFA rich feed group ($P < 0.05$).
4)	McFarland *et al.* (2008)	Weanling male and female outbred Hartley strain guinea pigs infected with virulent *Mtb* H37Rv via the respiratory route for 6 weeks until necropsy.	Diets contained as main lipid source either: (1) corn oil (n-6) (5.8% of TE LA content) or (2) Menhaden fish oil (n-3) (fish oil: corn oil = 3:1 and LA content 1.6% of TE).	Provided corn oil diet for 2 to 3 weeks as washout period and then switched to experimental diets for 3 weeks prior to infection.	Higher bacterial load in lungs of n-3 PUFA-fed group compared with the n-6 PUFA-fed group at 3 weeks ($P = 0.01$). Higher bacterial load in lungs in n-3 PUFA-fed group compared with the n-6 PUFA-fed group at 6 weeks ($P = 0.001$). No difference in histological evaluations between groups.
5)	Paul *et al.* (1997)	Male weanling guinea pigs infected with 180 CFU of H37Rv *Mtb* for 7 weeks until necropsy.	Animals divided into 3 groups: (1) n-6 PUFA group or (2) n-3 LCPUFA group (EPA: 5.24 % and DHA 3.44 % weight of fat mixture) (3) Reference group	Fed experimental diets for 13 weeks after which they were infected; kept on diets during the experiment.	Spleens of the n-3 LCPUFA-fed group contained the highest amounts of mycobacteria compared with the n-6 PUFA-fed and reference groups ($P < 0.05$) at 7 weeks post-infection.

AA: Arachidonic acid; BSA: Bovine Serum Acid; CFU: colony-forming units; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; IL: interleukin; LA: linoleic acid; LCPUFA: long-chain polyunsaturated fatty acids; LTB_4: leukotriene B_4; MCP-1: monocyte chemoattractant protein-1; *Mtb: Mycobacterium tuberculosis*; n-3: omega 3; n-6: omega-6; PGE_2: prostaglandin; PUFA: polyunsaturated; TE: total energy.

2.7 Iron

2.7.1 Iron metabolism

Iron is an important trace element, essential for various life functions. It serves as a co-factor that plays important roles in electron transfer (mitochondrial respiration and energy production), enzyme catalysis (deoxyribonucleic acid (DNA) synthesis and repair, and central metabolism), and gene expression (Brzóska *et al.*, 2006; Pantopoulos *et al.*, 2012; Py & Barras, 2010). It is, therefore, also irreplaceable in immune function. The main function of iron is the synthesis of haemoglobin, the oxygen-carrying pigment in erythrocytes, for which 25 mg of iron is required daily in humans (Schmidt, 2015). On the other hand, the recycling of erythrocytes is also the main iron source in the blood (Ganz, 2019).

The body contains three to five grams of iron, mostly found in haemoglobin, but also present in macrophages and muscle myoglobin, as well as in the liver (Ifeanyi, 2018). Biological iron consists of circulating (Fe^{3+}) and ferrous (Fe^{2+}) iron bound to transferrin and lactoferrin. Additionally, haem iron is the most abundant circulating form, which is mostly incorporated into haemoglobin. As haemoglobin contains 80% of bodily iron, this is the greatest iron reservoir in the body (Lane *et al.*, 2015b; Waldvogel-Abramowski *et al.*, 2014). To meet the requirement of iron in order for it to exert its important physiological functions, 1 to 2 mg is absorbed from dietary sources, replacing iron losses. As excretion has not yet been found to play a role in iron homeostasis, iron absorption from dietary consumption plays a key role in iron balance (Anderson & Frazer, 2017). In addition, the iron pool is maintained mainly by recycled senescent RBCs (RBCs in growth arrest) that are phagocytised by macrophages (Schmidt, 2015). As too much or too little iron is detrimental, dietary iron intake, iron recycling from aged or damaged erythrocytes and, iron release and uptake from cells are tightly controlled in order to prevent anaemia or iron overload that may lead to oxidative stress and tissue damage (Ifeanyi, 2018; Schmidt, 2015).

Dietary iron can be consumed in two forms, either haem or non-haem iron (Carpenter & Mahoney, 1992). Non-haem iron is found in plant and animal sources and its absorption is usually influenced by various factors, such as stomach pH and dietary components, e.g. tannins as it is not tightly bound to a carrier molecule (Dasa & Abera, 2018; Hallberg *et al.*, 1991; Storcksdieck, 2007). On the other hand, haem iron is sequestered in protoporphyrin, protecting it from influential factors and, therefore, haem iron is deemed more bioavailable. This form of iron is mostly found in animal myoglobin and haemoglobin (Anderson & Frazer, 2017; West & Oates, 2008).

The absorption of dietary iron mainly occurs in the duodenum, where iron must cross both the apical and basolateral borders of enterocytes to enter the circulation (Fuqua *et al.*, 2012). Dietary

iron is reduced from the ferric (Fe^{3+}) to the ferrous form (Fe^{2+}) by ferric reductase activity, e.g. by duodenal cytochrome B on the brush border (Lane *et al.*, 2015a). Reduced dietary non-haem iron (Fe^{2+}) is transported over the apical membrane of duodenal enterocytes by divalent metal-ion transporter-1 (DMT-1) (Figure 2.13) (Fuqua *et al.*, 2012). Dietary iron is then either stored as ferritin in enterocytes if the body does not need it or exported from enterocytes into circulation over the basolateral membrane by ferroportin (Figure 2.13). If it is exported to the circulation, iron is then transformed into the ferric state (Fe^{3+}) once again *via* oxidation by either membrane-bound multicopper ferroxidase hephaestin or soluble ferroxidase ceruloplasmin (Donovan *et al.*, 2005; Vulpe *et al.*, 1999). Haem iron absorption is less well understood, but evidence exists that it is transported into enterocytes by endocytosis or haem carrier protein-1/proton-coupled folate transporter. It is then thought to be split from haem by oxygenases and also carried over the basolateral membrane *via* ferroportin (Figure 2.13) (Anderson & Frazer, 2017; West & Oates, 2008).

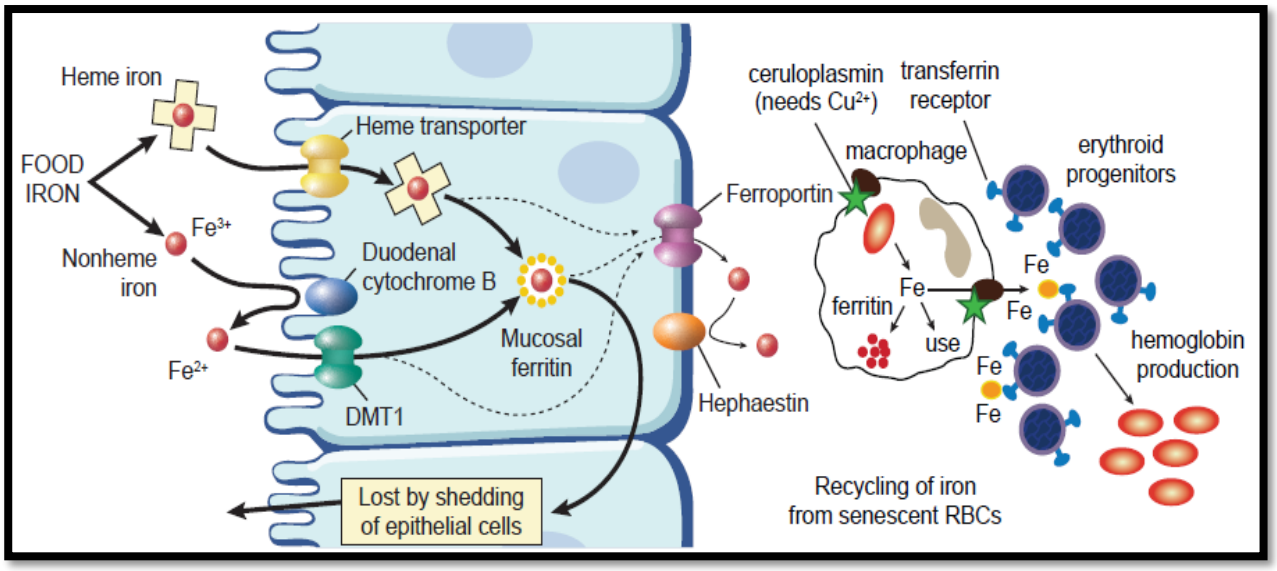


Figure 2-13 Iron absorption and transport*

*Adapted from http://eclinpath.com/wp-content/uploads/Iron-absorption-from-the-diet.jpg and https://greek.doctor/medical-biochemistry/lectures/9-iron-metabolism-and-related-disorders/

DMT-1: divalent metal-ion transporter-1; Fe: iron; RBC: red blood cell.

Once in circulation, the ferric form of iron is taken up by the protein transferrin and diferric transferrin is transported to various tissues (especially the erythroid marrow) to bind to transferrin receptor (TfR) (Gkouvatsos *et al.*, 2012). This receptor can be found on most cell surfaces and when transferrin binds to it, the complex is internalised into the cell *via* endocytosis (Gkouvatsos *et al.*, 2012). Transferrin receptor expression can be measured in serum as the protein can be cleaved at the plasma membrane in its extracellular domain, proportionately complementing the

TfR on cell surfaces (Harms & Kaiser, 2015; Koulaouzidis *et al.*, 2009). Transferrin receptor-1 and -2 detect the level of transferrin in the blood together with sensors for stored liver iron and, thereby, affect the signalling pathway that controls hepcidin synthesis (Wang & Babitt, 2019). Transferrin receptor concentrations will be higher when iron concentrations are lower, making it a valuable marker to identify iron deficiency.

Hereafter, transferrin-bound ferric iron is reduced and released from transferrin to be transported into the cytoplasm over the endosomal membrane by DMT-1 in order to serve its metabolic functions, to be stored in ferritin, or again to be transported out of the cell *via* ferroportin (Anderson & Frazer, 2017). The storage of iron intracellularly with the protein ferritin is very important to provide a pool for impending iron metabolic requirements (Theil, 2013). Ferritin is also secreted by cells into the circulation and the ferritin concentrations in serum correlate with cellular ferritin levels, making it an accurate indicator for measuring iron stores (Theil, 2013). Transferrin-bound iron is not the only manner in which iron can be delivered to and taken up by cells. It can also be delivered as non-transferrin-bound iron or by ferritin (Gelvan *et al.*, 1996; Jenkitkasemwong *et al.*, 2015).

As mentioned earlier, iron balance is very important. Iron regulatory proteins control the concentration of iron at the cellular level. These iron regulatory proteins determine the production of ferritin and TfR expression to regulate storage and uptake of iron into cells (Wilkinson & Pantopoulos, 2014). In addition, hepcidin regulates systemic iron homeostasis, controlling iron absorption and the exportation of iron from cells to plasma, playing pivotal roles in iron regulation in hypoxia and anaemia, as well as, infection and inflammation (Clark *et al.*, 2011). Hepcidin is a peptide derived from the liver, its concentration increasing or decreasing in accordance with iron demands, e.g. hepcidin concentrations increase when iron demands are low (Schmidt, 2015). Any alterations in bodily iron needs are communicated to the liver, leading to hepcidin expression. Hereafter, hepcidin is distributed to the target sites to bind to ferroportin, acting as hepcidin receptor (Clark *et al.*, 2011). The hepcidin-ferroportin complex is then internalised into cells, such as macrophages, enterocytes, and hepatocytes, and degraded, thereby blocking iron release into the circulation (Clark *et al.*, 2011; Guo *et al.*, 2013; Nemeth *et al.*, 2004b). This causes iron sequestration in the reticuloendothelial system coupled with a reduction in iron absorption (Guo *et al.*, 2013). Hepcidin transcription is regulated through the protein-SMAD regulatory pathway, inflammatory cytokines, hypoxia and numerous other elements such as hormones. High iron stores, as well as inflammatory conditions, increase hepcidin expression, but low iron stores and hypoxia reduce hepcidin expression (Anderson & Frazer, 2017; Ganz & Nemeth, 2015; Guo *et al.*, 2013; Hou *et al.*, 2012; Peyssonnaux *et al.*, 2008).

2.7.2 Iron and Tuberculosis

2.7.2.1 Iron metabolism in tuberculosis

An important function of iron is that it affects immune function (both innate and adaptive), whilst the immune response also influences iron status. Concerning infectious microbes, iron is vital in the host immune response and has a significant impact on the course of an infectious disease (Agoro & Mura, 2019). The reason for this is that iron is necessary for the functionality of various enzymes, by acting as co-enzyme. Iron is specifically important for the bactericidal activity of macrophages, as well as the number of T cells and their functioning (Brock, 2018; Cherayil, 2010; Jonker & van Hensbroek, 2014). However, iron is also essential for the survival and replication of mycobacteria as it serves as co-factor for their metabolic enzymes, with its availability correlating with virulence (Cherayil, 2010; Cole *et al.*, 1998; Ganz, 2018). Various microbes rely on iron acquired from the host, as is the case for *Mtb,* utilising iron from the host's stores for growth and virulence (Agoro & Mura, 2019). It is for this reason that it is one of the functions of the innate immune responses to reduce iron availability in order to oppose iron uptake by bacteria (Ifeanyi, 2018; Mishra *et al.*, 2018; Weiss & Goodnough, 2005).

The first mechanism by which iron availability is limited is by means of iron-sequestering proteins found in mucosal fluids. Lactoferrin is such a protein that binds to two ferric iron atoms similarly to transferrin; however, this protein does not release iron even in a highly acidic environment. Therefore, lactoferrin serves as part of the first barrier against iron availability for microbes (Mayeur *et al.*, 2016). Another iron-sequestering protein is lipocalin-2, which isolates bacterial siderophores that bind iron and the protein uses catechol metabolites as chelators of iron to protect it from bacteria (Shields-Cutler *et al.*, 2016; Sia *et al.*, 2013). In TB specifically, it has been found that extracellular sequesters of iron, such as transferrin, haptoglobin, and haemopexin, accumulate in the necrotic centres of caseous granuloma. This is complemented by iron-restricting antimicrobial proteins such as lactoferrin and lipocalin, which sequester siderophores and inhibit mycobacterial growth in granuloma (Kurthkoti *et al.*, 2017). All the iron-restricting proteins found in granulomas deprive the *Mtb* of iron. Phagocytes are also produced at the infection sites to compete with microbes for iron (Ifeanyi, 2018). When macrophages are activated by infection they also reduce the lifecycle of erythrocytes (Ganz, 2019; Libregts *et al.*, 2011; Macdougall & Cooper, 2002).

In addition, as part of the acute-phase response, concentrations of certain proteins, named the acute-phase proteins, change in plasma. Transferrin, hepcidin, and ferritin are such proteins that contribute to alterations in iron metabolism during the acute-phase response (Bresnahan *et al.*, 2014; Ifeanyi, 2018). As discussed above, a pro-inflammatory action is elicited upon host *Mtb*

recognition, which causes the up-regulation of hepcidin in the liver by the release of pro-inflammatory cytokines, mainly IL-6, but also IL-1, IL-22, and IFN-α (Ganz, 2018; Lee *et al.*, 2005; Nemeth *et al.*, 2004a; Nicolas *et al.*, 2002; Ryan *et al.*, 2012; Schmidt, 2015; Wrighting & Andrews, 2006). Importantly, hepcidin is not only regulated by inflammation, but also by infection (Armitage *et al.*, 2011; Armitage *et al.*, 2014; Schmidt, 2015). Other factors associated with infection, such as the H_2O_2, that is generated from neutrophils, also affect hepcidin expression (Millonig *et al.*, 2012). Hepcidin synthesis results in the uptake of iron into macrophages, limited gastrointestinal iron absorption, together with a reduced release of iron from body stores. This is seen as the main cause of hypoferraemia in infection and inflammation (Ganz, 2019; Weiss *et al.*, 2019). As alluded to earlier, hepcidin causes the endocytosis and proteolysis of the exporter ferroportin, as well as lower expression of DMT-1, leading to reduced absorption and the sequestration of iron intracellularly into macrophages, hepatocytes and enterocytes as cytoplasmic ferritin (Ganz & Nemeth, 2015; Schmidt, 2015). Additionally, ferroportin expression is also reduced by mechanisms other than hepcidin release (Guida *et al.*, 2015).

In essence, there is a shift from available iron (bound to transferrin) to stored iron (in the ferritin form) in TB (Mishra *et al.*, 2018; Schmidt, 2015; Weiss & Goodnough, 2005). This, however, may bring about the availability of iron for intracellular microbes. *Mycobacterium tuberculosis* is an intracellular pathogen and can use the iron sequestered and stored within macrophages for replication and growth, thereby accelerating disease progression. In the phagosome of host macrophages, *Mtb* has limited access to the host iron stores, but research suggests that siderophores, which are secreted by *Mtb,* cause iron diffusion across phagosomal membranes to provide access to high iron concentrations in the macrophage cytosol (Figure 2.14) (Chao *et al.*, 2019). Extracellular circulating *Mtb* takes iron from circulating transferrin, ferritin, and lactoferrin and lyses RBCs to release haemoglobin. There are also mechanisms by which *Mtb* acquires haem iron from the host and, therefore, in advanced TB infection, high haem levels can be detected in the lungs (Chao *et al.*, 2019). Siderophores are molecules that bind iron better than iron storage proteins and obtain iron from transferrin, lactoferrin, ferritin and haem iron (Chao *et al.*, 2019). In TB, these siderophores include lipophilic, cell-bound mycobactins, as well as free carboxymycobactins (Chao *et al.*, 2019; Madigan *et al.*, 2015; Sritharan, 2016).

Additionally, inflammatory cytokines also play a role in the development of hypoferraemia, in other ways than the upregulation of hepcidin. Firstly, they also favour myeloid cell production, at the expense of erythropoiesis (IFN-γ); TNF-α inhibits erythroid proliferation; IFN-γ activates macrophages causing a shorter erythroid life cycle due to phagocytosis; together with the lower release of iron from macrophages by the effects of IL-6 on hepcidin. Cytokines can also directly

inhibit iron absorption and act on macrophages to favour iron restriction (Ganz, 2019; Weiss *et al.*, 2019).

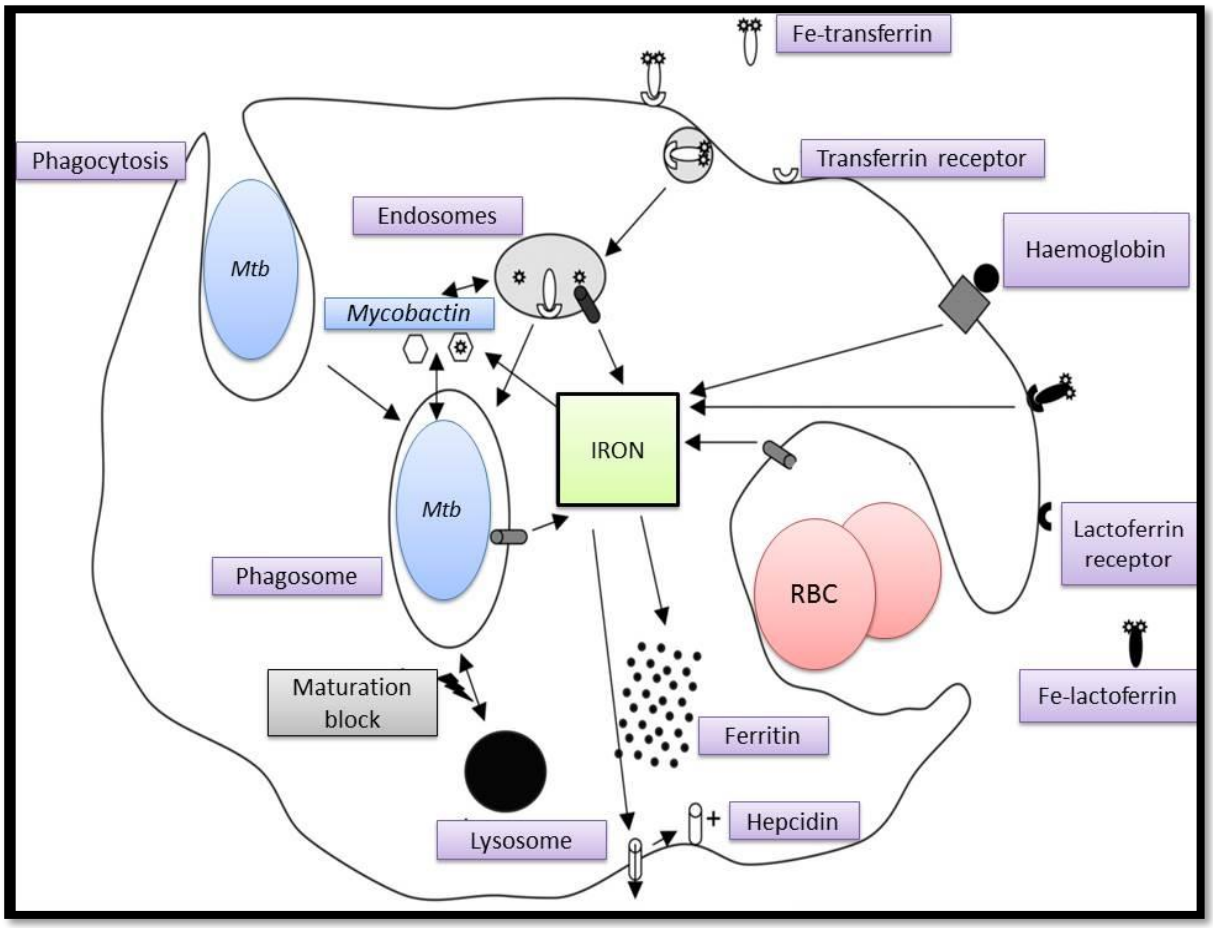


Figure 2-14 Schematic representation of an *Mycobacterium tuberculosis*-infected macrophage and its iron metabolism (Adapted from Boelaert *et al*. (2007))

Fe: Iron; Mtb: Mycobacterium tuberculosis; RBC: red blood cell.

2.7.2.2 Anaemia of infection and iron deficiency anaemia in tuberculosis

All of these mechanisms, described in the previous section, contribute to lower the availability of iron for pathogens (Stefanova *et al.*, 2017); however, they also result in reduced iron absorption and serum iron availability (hypoferraemia) for erythropoiesis (Anderson & Frazer, 2017; Cherayil, 2010; Ganz, 2018). Taken together, the literature suggests three pathways contributing to anaemia of infection including: iron restriction by hepcidin, the inflammatory suppression of erythropoiesis, and a reduced erythrocyte life cycle (Weiss *et al.*, 2019). As mentioned earlier, a large proportion of bodily iron is constricted to the reticuloendothelial system macrophages with resultant low amounts of iron transported by transferrin in circulation. Erythropoiesis is inhibited, which preserves iron for other tissues such as the central nervous system and diverting of bone

marrow progenitors for leukocyte production (Ganz, 2019; Han *et al.*, 2001). As hepcidin continues to be up-regulated by the on-going inflammation in TB, low iron availability for erythropoiesis together with the inhibition of iron absorption from supplements and food eventually results in normocytic normochromic anaemia (Ganz, 2019; Schmidt, 2015). Hypoferraemia, coupled with the higher production of leukocytes, lowers the available erythroid precursors. This, in turn, decreases the body's oxygen-carrying capacity (with lower haemoglobin levels) (Ganz, 2018; Schmidt, 2015). This anaemia is termed anaemia of infection (also known as anaemia of inflammation or anaemia of chronic disease) and, with the exception of critical illness, may take several weeks to develop as erythrocytes have a long lifecycle of 100 to 120 days in adults (Ganz, 2019).

Following iron-deficiency anaemia, anaemia of inflammation is the most common form of anaemia and very common among patients with chronic inflammatory disorders such as RA, inflammatory bowel syndrome, and infections (such as TB) (Ganz, 2019). It should, however, be noted that hepcidin up-regulation and the resultant anaemia of infection are not found in all infections and are pathogen-specific, e.g. hepatitis B and C infection do not induce hepcidin production (Armitage *et al.*, 2014). Over a long serious illness, patients may go on to develop more serious microcytic hypochromic RBCs (Schmidt, 2015). Anaemia of infection and iron deficiency anaemia also often co-exist, especially in developing countries (Shaw & Friedman, 2011). In TB specifically, other factors may also contribute to the development of anaemia (or more specifically, iron-deficiency anaemia). These may include: (Johnson & Wessling-Resnick, 2012; Kant *et al.*, 2015; Karyadi *et al.*, 2000; Lee *et al.*, 2006; Mishra *et al.*, 2018):

- malnutrition as a result of lower food intake and a reduction in appetite;
- malabsorption due to hookworm, inflammation or other secondary infections; and
- haemoptysis, leading to blood loss through sputum.

Iron-deficiency anaemia (microcytic hypochromic anaemia) and the even more prevalent, anaemia of infection, are common complications in TB patients. These types of anaemia affect 30 to 94% of TB patients and can either be TB-related or from other causes, as discussed above (Devi *et al.*, 2003; Hella *et al.*, 2018; Isanaka *et al.*, 2011; Kant *et al.*, 2015; Karyadi *et al.*, 2000; Lee *et al.*, 2006; Mishra *et al.*, 2018; Sahiratmadja *et al.*, 2007; Van Lettow *et al.*, 2005). Research has found normocytic normochromic anaemia to be the main anaemia present in TB patients (up to 71.7%), most likely caused by inflammation (anaemia of infection). This type of anaemia has also been found to improve and resolve with TB treatment in the absence of iron supplementation (Devi *et al.*, 2003; Friis *et al.*, 2009; Hella *et al.*, 2018; Kerkhoff *et al.*, 2016; Lee *et al.*, 2006;

Minchella *et al.*, 2014; Morris *et al.*, 1989; Sahiratmadja *et al.*, 2007). Kerkhoff *et al.* (2016) and Hella *et al.* (2018) reported that iron-deficiency anaemia was present in as few as 7% and 1% of the TB patients in their study populations, whereas 90% and 59.8% presented with anaemia of infection. In this regard, iron supplementation will not really benefit such patients because of the up-regulation of hepcidin, which lowers iron absorption (Kerkhoff *et al.*, 2015). However, Isanaka *et al.* (2011) found that a high burden (53% of their study population) of iron-deficiency anaemia existed in TB patients in Dar es Salaam, Tanzania. The reason for the discrepancy in the proportion of TB patients presenting with iron-deficiency anaemia across different TB populations is probably related to other factors such as co-infections and diet (Isanaka *et al.*, 2012; Kerkhoff *et al.*, 2015). As mentioned above, anaemia of infection will improve with TB treatment and infection resolution, with a reduction of hepcidin levels and the availability of iron in circulation. However, iron-deficiency anaemia will remain unresolved and iron-based interventions for these patients may be required (Hella *et al.*, 2018; Isanaka *et al.*, 2011; Minchella *et al.*, 2014).

2.7.2.3 Biomarkers for the identification of anaemia of infection and iron-deficiency anaemia in tuberculosis

It is difficult to distinguish between iron-deficiency anaemia and anaemia of infection, even though they co-exist. Both of these types of anaemia are characterised by low iron levels (hypoferraemia), however, they differ in that lower ferritin levels are observed in iron-deficiency anaemia compared with higher ferritin and hepcidin levels in anaemia of infection (Table 2.6) (Ganz, 2019). Mishra *et al.* (2018) investigated the effect of TB on iron-related biomarkers. They found that PTB patients had increased total iron-binding capacity, ferritin, and CRP levels when compared with healthy individuals. This is in concordance with earlier and recent research that also reported higher ferritin concentrations in TB patients (Hella *et al.*, 2018; Morris *et al.*, 1989). On a cellular level, Kurthkoti *et al.* (2017) measured ferritin concentrations in the cellular regions of cavitary granulomas and reported high concentrations present (Kurthkoti *et al.*, 2017). Ferritin levels have also been found to change with TB treatment. In TB, ferritin cannot be used as a marker of iron stores but is rather defined as an acute-phase protein (Mishra *et al.*, 2018). There are two mechanisms by which ferritin levels are thought to increase in TB: the first is the fact that ferritin is produced by monocytes and macrophages, and monocytosis is a result of TB infection; and the second is that ferritin is seen as an inflammation marker and is closely correlated with CRP in TB patients, as discussed above (Ganz, 2019; Mishra *et al.*, 2018).

Table 2-6 Biomarker profile expected in anaemia of infection and iron-deficiency anaemia*

Biomarker	Concentrations in iron deficiency anaemia	Concentrations in anaemia of infection
Red blood cells	Low	Low
White blood cells	Low to normal	Normal to high
Serum iron	Decreased	Decreased
Serum Transferrin	High	Low
Transferrin saturation	Low	Low
Serum Transferrin receptor	High	Normal to high
Hepcidin	Decreased	Increased
Haemoglobin	Decreased	Decreased to normal
Ferritin	Low	High
Mean corpuscular volume	Low	Normal
Mean corpuscular haemoglobin	Low	Normal

*Adapted from Oliveira *et al.* (2014), Mishra *et al.* (2018), and Ganz (2019).

Compared with their healthy counterparts, lower iron, haemoglobin, transferrin, and transferrin saturation levels have also been reported in TB patients (Hella *et al.*, 2018; Mishra *et al.*, 2018). These lower transferrin concentrations accord with what is expected in infections as transferrin is seen as a negative acute-phase protein. A negative acute-phase protein indicates that during inflammation there is an increased synthesis of cytokines by the liver and reduced synthesis of other proteins, such as transferrin, which are not essential for host immune function (Mishra *et al.*, 2018). In addition, the shift of iron to iron stores (i.e. ferritin), as discussed above, is also responsible for the lower transferrin levels evident in TB. Lastly, transferrin is not only affected by inflammation, but is also a nutritional status marker, which is influenced by protein and iron intake (Mishra *et al.*, 2018). On the other hand, elevated TfR levels can be expected in inflammatory situations which indicate higher iron demand and erythropoietic stimulus (Rohner *et al.*, 2017). Therefore, TfR concentrations are also not a good indicator of iron-deficiency anaemia as it is influenced by inflammation. Kurthkoti *et al.* (2017) also reported high concentrations of TfR in solid cellular granulomas. These findings are supported by higher circulatory TfR levels found in Tanzanian TB patients (Hella *et al.*, 2018).

The up-regulation of hepcidin concentrations may be a valuable tool to distinguish between iron-deficiency anaemia and anaemia of infection, as lower hepcidin levels are expected in iron-deficiency anaemia, compared with higher levels in anaemia of infection (Girelli *et al.*, 2016).

Indeed, higher hepcidin levels have been reported in TB patients (Harrington-Kandt *et al.*, 2018; Hella *et al.*, 2018; Kerkhoff *et al.*, 2015). Taken together, in anaemia of infection one can expect normocytic normochromic anaemia (indicated by normal mean corpuscular volume and mean corpuscular haemoglobin), together with systemic inflammation, low transferrin saturation, and higher ferritin concentrations (Ganz, 2019). Table 2.6 above reflects the differences in biomarkers expected in a patient with anaemia of infection or iron deficiency anaemia. The parameters highlighted were measured in this research project. The next section will elaborate on the relevance of these iron markers to TB outcomes.

2.7.2.4 Clinical outcomes associated with iron biomarkers in tuberculosis

It has been reported that a reduction in host iron availability or iron uptake by the pathogen restricts *Mtb* growth, whilst an excess enhances growth and can cause damage by ROS catalysation, and impair the macrophages' ability to restrict micro-organism invasion (Boelaert *et al.*, 2007; Ifeanyi, 2018; Madigan *et al.*, 2015; Mishra *et al.*, 2018). However, there are various clinical consequences of iron-deficiency as well, such as lower quality of life, and productivity, and higher mortality and morbidity rates (Nissenson *et al.*, 2003).

Firstly, as mentioned before, the intracellular shift of iron that is elicited upon TB infection may actually benefit the *Mtb,* which is an intracellular pathogen, thereby promoting bacterial growth (Agoro & Mura, 2019). In addition, iron-deficiency also influences host defence, inhibiting cell defence mechanisms and activity of macrophages by a shift from the Th1 to Th2 response (lower IL-1 and IL-6 levels), leading to a reduced ability of macrophages to inflict mycobacterial killing and blocking NO-dependent activity (Cronjé *et al.*, 2005; Gangaidzo *et al.*, 2001; Isanaka *et al.*, 2011; Serafín-López *et al.*, 2004; Weiss *et al.*, 1994). Iron-deficiency also compromises immune function by a reduction in T cell numbers and their unavailability to be structurally a part of, or to activate, enzymes for the immune response (Brock, 2018; Ekiz *et al.*, 2005; Jabara *et al.*, 2016; Kuvibidila *et al.*, 1998; Oppenheimer, 2001). Owing to its effects on immune function, anaemia is also a risk factor for poor TB outcomes (Ifeanyi, 2018). Apart from its effects on the immune response, it also has other detrimental consequences for the host, as it limits iron availability for physiological processes such as erythroid synthesis (Mishra *et al.*, 2018). Therefore, anaemia can lead to various precipitating symptoms, including weakness and impaired motor activity (Mishra *et al.*, 2018).

Various studies have researched the link between host iron status and TB. However, it seems that the relationship is not simple as both iron-deficiency and overload can enhance an individual's TB susceptibility and disease progression (Ifeanyi, 2018; Johnson & Wessling-Resnick, 2012; Jonker & van Hensbroek, 2014; Mishra *et al.*, 2018; Shimazaki *et al.*, 2013). Isanaka *et al.* (2011)

were the first to link poor clinical outcomes and disease recurrence to iron-deficiency and anaemia in TB patients. In this study, anaemia with or without iron-deficiency was associated with a two- to threefold independent increase in mortality risk (Isanaka *et al.*, 2011). In addition, anaemia without iron-deficiency was also associated with TB recurrence (Isanaka *et al.*, 2011). Hereafter, other research supported these findings, indicating that anaemia is associated with delayed sputum conversion and higher mortality rates in TB patients (Nagu *et al.*, 2014; Shimazaki *et al.*, 2013).

Individual biomarkers of iron status were also found to correlate with disease progression and clinical TB outcomes. Circulating ferritin levels have been shown to correlate positively with disease severity and sputum positivity (Mishra *et al.*, 2018). Supporting this, Isanaka *et al.* (2012) found that high plasma ferritin was positively associated with higher mortality rates. On the other hand, the authors also reported that low plasma ferritin levels at TB infection (prior to active TB disease) predicted a higher risk of treatment failure and TB recurrence in HIV patients (Isanaka *et al.*, 2012). Ferritin levels that are measured prior to active disease are different from ferritin levels that are measured during the response to active TB as these levels are affected by inflammation, as discussed above. This is closely associated with hepcidin, which has also been suggested as a marker of TB disease development. Kerkhoff *et al.* (2015) reported that hepcidin concentrations were strongly correlated with mycobacterial burden and predicted mortality in SA TB patients (Kerkhoff *et al.*, 2015). The same was found in Tanzania, where hepcidin correlated with the severity of TB symptoms (Hella *et al.*, 2018). Harrington-Kandt *et al.* (2018) did not agree with this and found that hepcidin levels did not influence *Mtb* growth and that hepcidin concentrations actually decreased following infection. Their findings in an animal TB model also portrayed no association between iron-deficiency and mycobacterial load, therefore, neither benefiting nor slowing disease progression (Harrington-Kandt *et al.*, 2018).

Apart from ferritin and hepcidin, other circulating markers of iron status have also been linked to TB incidence and outcomes. McDermid *et al.* (2013) reported a higher TB incidence and susceptibility with lower markers of circulating iron (transferrin and haemoglobin together with high ferritin) in HIV patients. Studies have also indicated that low transferrin levels are strongly correlated with TB disease severity (Adedapo *et al.*, 2006; Bapat *et al.*, 2015; Mishra *et al.*, 2018). Lastly, in an older study, Morris *et al.* (1989) found that haemoglobin levels were positively correlated with faster sputum conversion rates (Morris *et al.*, 1989). It is clear that iron-deficiency and anaemia can have detrimental consequences in TB patients and that the normalisation of iron status may be a useful way to improve TB outcomes (Isanaka *et al.*, 2012).

2.7.2.5 The supplementation of iron in tuberculosis

The treatment of hypoferraemia in TB is difficult, as the provision of iron to ensure competent immune functioning will also lead to increased iron availability for pathogens (Agoro & Mura, 2019; Murray *et al.*, 1978; Sazawal *et al.*, 2006). Nevertheless, there are still no clear guidelines for the treatment of iron deficiency in infections. More research is clearly needed in this area (Ifeanyi, 2018; Jonker & van Hensbroek, 2014). As mentioned earlier, owing to the up-regulation of the inflammatory response, iron supplementation may not benefit or correct anaemia of infection in TB patients. Other treatment strategies, e.g. anti-TNF therapy, have been suggested, that target the infection or inflammation, which may improve anaemia of infection, together with other TB symptoms (Koutroubakis *et al.*, 2015; Lee *et al.*, 2006). However, studies have also highlighted the fact that iron-deficiency anaemia is present in some patients and that these patients may still benefit from iron supplementation (Hella *et al.*, 2018; Isanaka *et al.*, 2011). Recently, Ganz (2019) suggested intravenous iron therapy, when combined anaemia of inflammation and iron-deficiency anaemia is present in a patient. Nevertheless, iron supplementation studies in TB patients and animal models are scarce.

In studies conducted in cultured human and murine macrophages, a relationship between iron availability and acquisition by the bacteria and enhanced *Mtb* growth has been reported (Rook *et al.*, 1986). Serafin-Lopez *et al.* (2004), reported that iron favoured intracellular growth of the mycobacteria related to the reduced release of TNF-α, which is required to restrict *Mtb* in TB-infected J774 macrophages. Lower TNF-α release further potentiated the lower expression of IL-1 and IL-6 (Serafín-López *et al.*, 2004). Supporting this, in a study conducted in a human mononuclear phagocyte culture system, it was found that iron is necessary to decrease the release of TNF-α and attenuate monocytes' responsiveness to TNF-α. However, the authors argued that this is required for monocytes to differentiate, which is necessary to restrict *Mtb* by limiting cell-to-cell spread (Byrd, 1997).

Furthermore, in animal experiments, results have been inconsistent. In an older experiment, intraperitoneal injections of 100 µg iron twice a day for three weeks benefited *Mtb* growth in TB-infected mice (Kochan, 1973). This was supported by the intraperitoneal injection of 50 mg/kg polymaltose ferric hydroxide three times a day for 42 days in *Mtb*-infected BALB/C mice, where iron-loaded mice presented with higher bacterial loads in lungs and spleens (Lounis *et al.*, 2001). Schaible *et al.* (2002) also found that iron overload exacerbates TB in β-2-microglobulin knockout mice (Schaible *et al.*, 2002). On the other hand, more recent animal experiments found otherwise. In latently TB-infected rabbits, 25 mg iron was injected into animals three times a week for eight weeks. No effects on haemoglobin or haematocrit could be found, but reduced total iron-binding capacity and percentage transferrin saturation in plasma and higher lung iron levels were evident

in iron-supplemented groups (Kolloli *et al.*, 2019). The authors further reported that iron supplementation did not affect disease pathology and bacterial load. Iron supplementation was also found to reduce the systemic expression of host immune response genes, including *TNFA, IL1B, IFNG,* and *IL10,* but up-regulated *IL6* both locally in the lungs and in plasma (Kolloli *et al.*, 2019). Furthermore, dietary iron supplementation of 280 mg iron carbonyl per kg diet was shown to reduce bacterial load in *M. bovis* BCG-infected C57BL/6 mice (Agoro *et al.*, 2017). In this study, the pro-inflammatory cytokines IFN-γ, IL-1β, IL-12p40, and TNF-α were lower in the lungs of iron-supplemented mice. Additionally, immune cell recruitment was enhanced by iron, leading to higher $CD8^+$ T cells (Agoro *et al.*, 2017).

Clinical trials on iron supplementation in TB patients are limited. Early in 1987, Murray *et al.* (1987) investigated the effect of iron supplementation on two iron-deficient TB patients and found that it caused increased disease activity. It was concluded that infection was most likely to occur when iron stores reached near-normal levels of repletion (Murray *et al.*, 1978). Supporting this, dietary iron consumption and high macrophage iron stores were found to be positively associated with active TB and mortality risk, with a 1.3-fold increased hazard ratio of death (Gangaidzo *et al.*, 2001; Johnson & Wessling-Resnick, 2012; Mishra *et al.*, 2018). In a case study, Karakonstantis also indicated that intravenous iron administration led to the activation of TB (Karakonstantis *et al.*, 2019).

Countering the above, in a clinical trial, Devi *et al.* (2003) supplemented PTB patients with 75 mg of ferrous fumarate twice daily and found that supplementation increased blood haemoglobin, total erythrocyte count, packed cell count and mean cell volume at one month from the start of treatment, but not at two months, even with continued supplementation. The initial faster recovery of biochemical values may have slowed because further improvement would be influenced more by balancing the inflammatory process and not by iron supplementation *per se*. This was supported by the fact that the total iron-binding capacity was independent of supplementation and more dependent on inflammation resolution (Devi *et al.*, 2003). They also found that iron supplementation did not influence body mass index or radiological improvements (chest lesion severity). The authors speculated that *Mtb* has a regulatory mechanism to obtain iron from the host, irrespective of host iron status (Devi *et al.*, 2003).

Therefore, limiting the host's continuing inflammatory response induced by TB may make an important contribution towards the repletion of iron stores and the resolution of anaemia. The above literature leaves the recommendation of iron supplementation in TB still unclear. However, very recently Agoro and Mua (2019) reviewed the available literature and suggested that iron supplementation in mycobacterial infections has a biphasic effect. Moderate iron supplementation within the "iron benefit window" may benefit the host response to lower the bacterial load and

inflammation. On the other hand, iron supplementation above the threshold of the “iron benefit window” can have detrimental consequences and promote bacterial growth and virulence, together with poor outcomes (Agoro & Mura, 2019).

2.7.3 The interaction between iron and omega-3 fatty acids

As discussed above, both iron and n-3 LCPUFAs play significant roles in TB progression and outcomes. In addition, it has been suggested that the availability of each of these nutrients may influence the other’s actions. The next three sections will briefly elaborate on this.

2.7.3.1 Iron affects omega-3 fatty acid metabolism

Iron serves as a co-factor for desaturase enzymes that are necessary for LCPUFA synthesis and for catalytic sites of LOX and COX. As iron affects desaturase activity and evidence suggests desaturases have a higher affinity for n-3 PUFAs, the provision of iron leads to n-3 LCPUFAs being incorporated into membranes, preferentially above other fatty acids (Hrelia *et al.*, 1990). Therefore, iron intake has been found to affect n-3 LCPUFA status. This has been confirmed by two independent studies, reporting the effect of iron consumption on n-3 LCPUFA RBC content, and indicating higher incorporation of n-3 LCPUFA into membranes related to a higher iron intake (Del Bò *et al.*, 2009; Smuts *et al.*, 1995).

On the other hand, iron-deficiency may then reduce the activity of these enzymes. Research has found that iron deficiency leads to a shift in cellular membranes, affecting mainly n-3 PUFA membrane composition (Brash, 1999; Gilbert *et al.*, 2011; Kuhn *et al.*, 2005; Nakamura & Nara, 2004; Oloyede *et al.*, 1992; Rao & Larkin, 1984; Shanklin *et al.*, 1994; Smuts *et al.*, 1995; Stangl & Kirchgessner, 1998). Even though not supported by all studies (LeBlanc *et al.*, 2009; Tichelaar *et al.*, 1997), the greater majority of animal and human research has found that iron-deficiency causes impairment in n-3 PUFA metabolism, altered n-3 PUFA incorporation into cell membranes, and reduced n-3 LCPUFA synthesis. Consequently, a reduced n-3 LCPUFA status is associated with iron-deficiency (Cunnane & McAdoo, 1987; Krajčovičová-Kudláčková *et al.*, 2004; Oloyede *et al.*, 1992; Rao & Larkin, 1984; Stangl & Kirchgessner, 1998; Zhou *et al.*, 2011).

Furthermore, Malan *et al.* (2016) found that iron supplementation altered LM profiles in healthy SA children by lowering 17-HDHA and 5-HEPE, which are precursors for RvDs and 5-series LTs. Thus, iron supplementation favoured a pro-inflammatory profile (Malan *et al.*, 2016). However, none of these studies are TB specific and their results cannot be directly extrapolated to an infectious disease setting.

2.7.3.2 Omega-3 long-chain polyunsaturated fatty acids affect iron metabolism

Omega-3 PUFA intake has also been found to influence iron stores. Even though some studies have connected a high n-3 PUFA dietary intake to iron-deficiency (Lukaski *et al.*, 2001; Miret *et al.*, 2003; Rao & Larkin, 1984; Shotton & Droke, 2004; van Dokkum *et al.*, 1983), others have reported that iron status positively correlated with n-3 PUFA status (Clauss *et al.*, 2008; Jamieson *et al.*, 2013; Mutanen *et al.*, 2016). It should be kept in mind that these studies are not specific to TB patents and that the interaction between n-3 PUFA intake and iron metabolism may look completely different where there is a heightened immune response.

Omega-3 PUFA influences iron metabolism by affecting membrane fluidity and, thereby, altering iron uptake and intracellular handling. Consequently, low n-3 LCPUFA levels may limit iron availability for immune cells (Brand *et al.*, 2008; Ober & Hart, 1998; Rodríguez *et al.*, 1996). In *in vitro* research, it has been shown that DHA increases TfR and DMT-1 expression in non-differentiated PC12 pheochromocytoma cells in a dose-dependent manner, increasing intracellular iron uptake (Schonfeld *et al.*, 2007). Supporting this, DHA has been shown to elevate ferritin concentrations i.e. cellular iron content (Brand *et al.*, 2008). Others have argued that higher iron absorption linked to n-3 PUFA intake is rather related to higher lipid peroxidation and the resultant heightened erythroid cell turnover (Miret *et al.*, 2003; Rodríguez *et al.*, 1996).

In TB patients, the known anti-inflammatory and pro-resolving properties of n-3 LCPUFAs may be advantageous. The supplementation of n-3 PUFAs as an adjunct treatment in TB patients may aid in the resolution of inflammation, together with the reduction in pro-inflammatory cytokine release and the resultant hepcidin expression (Serhan *et al.*, 2017b). This, in turn, may lead to the restoration of iron absorption, as well as iron status, and the resolution of anaemia of infection and its unwanted outcomes.

2.7.3.3 The combined treatment effect of iron and omega-3 polyunsaturated fatty acids

Malan *et al.* (2016) found that supplementing SA iron-deficient schoolchildren with 50 mg iron daily resulted in a pro-inflammatory LM profile, but when 50 mg iron was provided in combination with a mixture of DHA (420 mg per day) and EPA (80 mg per day), the anti-inflammatory LM profile resulting from DHA and EPA was preserved (Malan *et al.*, 2016). The authors further noted that, since iron supplementation was found to increase respiratory morbidity, but the combined supplementation of iron with DHA and EPA prevented this, that combination treatment might be a promising approach to administer iron safely (Malan *et al.*, 2014; Malan *et al.*, 2016). Furthermore, Baumgartner *et al.* (2012) found that the combination of n-3 LCPUFAs and iron more effectively improved cognition than providing the nutrients alone (Baumgartner *et al.*, 2012). These studies suggest that iron and n-3 LCPUFAs interact with each other and may assert useful

combined effects. This is also worth investigating in TB patients. There are no studies investigating the effect of combination treatment of iron and n-3 LCPUFAs in TB patients and it is hoped that this thesis will contribute to providing the necessary evidence to be able to assess whether this is worth investigating in human TB patients.

2.8 Animal tuberculosis models and their translation to humans

Various experimental animal TB models have been used and are described in the literature. These animal experiments provide valuable insights into disease pathology and the mechanisms underlying treatment. Although animal models have been scrutinised for their validity, three of them have been found particularly useful for effective TB research, including the mouse, the guinea pig and non-human primates (Apt & Kramnik, 2009; Kaufmann, 2003). Guinea pigs are considered the animal model that most closely reflects human TB pathology, with similar granulomatous lesions and high susceptibility to TB, however, as with non-human primates, they are less economical and practical options (Kaufmann, 2003).

This leaves mice as an attractive option. This model is considered the most cost-effective and refined animal model in which the immune response is well understood and is commonly used in TB research (Kaufmann, 2003). With regard to the inflammatory response, the functions of individual cytokines that have been proven in mice experiments are also well supported by human research. This agreement between the functions of cytokines in humans and rodents is one of the reasons why TB mice studies are justified when exploring the inflammatory response, specifically, in TB (Mayer-Barber & Sher, 2015). Despite this, mouse models have been criticised, as some mouse strains do not develop a similar lung pathology as would be expected in humans. This may lead to concerns with regard to the translation of treatment results to humans (Gupta *et al.*, 2013; Irwin *et al.*, 2015; Lanoix *et al.*, 2015; Lenaerts *et al.*, 2015).

Mouse strains that are widely used, such as the BALB/c and C57BL/6 strains, do not develop necrotic lesions as expected in humans (Lenaerts *et al.*, 2015). However, the C3HeB/FeJ mouse strain develops necrotic heterogeneous granulomas, which is what is expected in TB-infected human lung pathology. This is important because heterogeneous lesion types respond differently to treatment (Driver *et al.*, 2012; Gupta *et al.*, 2013; Irwin *et al.*, 2015; Lanoix *et al.*, 2015; Lenaerts *et al.*, 2015; Rosenthal *et al.*, 2012). C3HeB/FeJ mice, also known as the Kramnik mice, are highly susceptible to TB and have been used extensively as TB mouse model (Driver *et al.*, 2012; Gupta *et al.*, 2013; Irwin *et al.*, 2015; Kramnik & Beamer, 2016; Kroesen *et al.*, 2018; Lanoix *et al.*, 2015; Lenaerts *et al.*, 2015; Marzo *et al.*, 2014; Rosenthal *et al.*, 2012; Vilaplana *et al.*, 2013). Following infection, lesion necrosis can be observed within three to four weeks in C3HeB/FeJ mice, only present in the lungs, and not in the liver or spleen, where the infection is more controlled (Driver

et al., 2012; Kramnik & Beamer, 2016). Lung granulomas in this mouse strain have more prominent liquefactive necrotic areas of degenerating cells and caseation. This leads to the development of various types of lung lesions, which is also the clinical picture expected in human lungs. Cavities can also occur naturally after aerosol infection in C3HeB/FeJ mice but are less likely to develop (Lenaerts *et al.*, 2015).

The lung lesions that are found in C3HeB/FeJ mice can be categorised into either Type I, II or III lesions that progress over time. Type I lesions present with a liquefactive necrotic centre of neutrophil debris, surrounded by functional macrophages in a collagen rim, and a layer of foamy macrophages. Type II lesions show substantial neutrophil recruitment and the resultant large areas of necrosis in lung parenchyma, to develop less organised lesions. Type III lesions present with lymphocytes, small amounts of neutrophils, and epithelioid and foamy macrophages. In this lesion type, the bacterial burden is better controlled and progress slower, as it has been found that the number of lymphocytes is inversely related to the bacterial load (Lenaerts *et al.*, 2015). In addition, recent studies have reported that neutrophils play a major role as regulators of the inflammatory response in TB and they have also been found to be the dominant cell type in *Mtb*-infected C3HeB/FeJ mice (especially in Type I and II lesions) (Lenaerts *et al.*, 2015; Marzo *et al.*, 2014).

This mouse model can, therefore, be described as more representative of the human pathology than other mouse strains that are commonly used in TB experiments, and is effective in investigating the interaction between TB lesion pathology and treatment (Lanoix *et al.*, 2015). By using a model that is more closely related to the human TB clinical picture and that can accurately predict treatment effects, long and costly phase 3 trials for TB drug development that provide useless results, can be avoided (Lanoix *et al.*, 2015).

2.9 Conclusion

From the information provided, it is clear that TB is still a substantial problem globally and in SA. It is further evident that the TB cure and mortality rate is still unfavourable and leaves room for supportive treatment to enhance current treatment regimes. It is vital to consider new therapeutic interventions, such as targeting the host inflammatory processes or iron metabolism. Previous studies have found anti-inflammatory treatment beneficial in TB outcomes, which merits further investigation into whether n-3 LCPUFAs elicits similar improvements.

In addition, it is clear that anaemia of infection and iron-deficiency anaemia is a major problem in TB patients. However, the treatment of anaemia of infection and iron-deficiency anaemia in TB is complicated. As there is evidence that combination treatment of iron and n-3 LCPUFA may be a better way to deliver iron more safely and that n-3 LCPUFAs may aid in inflammation resolution,

this may be worth investigating. In the current research study, the C3HeB/FeJ mouse model was chosen for its close resemblance to human TB. The following chapters will provide insight into our findings.

2.10 References

Abedin, L., Lien, E., Vingrys, A. & Sinclair, A. 1999. The effects of dietary α-linolenic acid compared with docosahexaenoic acid on brain, retina, liver, and heart in the guinea pig. *Lipids*, 34(5):475-482.

Adedapo, K., Arinola, O., Ige, O., Adedapo, A. & Salimonu, L. 2006. Combination of reduced levels of serum albumin and αlpha-2-macroglobulin differentiates newly diagnosed pulmonary tuberculosis patients from patients on chemotherapy. *African Journal of Biomedical Research*, 9(3): 23-38.

Adkins, Y., Laugero, K.D., Mackey, B. & Kelley, D.S. 2019. Accretion of dietary docosahexaenoic acid in mouse tissues did not differ between its purified phospholipid and triacylglycerol forms. *Lipids*, 54(1):25-37.

Agoro, R., Benmerzoug, S., Rose, S., Bouyer, M., Gozzelino, R., Garcia, I., Ryffel, B., Quesniaux, V.F. & Mura, C. 2017. An iron-rich diet decreases the mycobacterial burden and correlates with hepcidin upregulation, lower levels of proinflammatory mediators, and increased T-cell recruitment in a model of Mycobacterium bovis Bacille Calmette-Guerin infection. *The Journal of infectious diseases*, 216(7):907-918.

Agoro, R. & Mura, C. 2019. Iron supplementation therapy, a friend and foe of Mycobacterial infections? *Pharmaceuticals*, 12(2):75.

Aguilo, J., Alonso, H., Uranga, S., Marinova, D., Arbues, A., Martino, A., Anel, A., Monzon, M., Badiola, J. & Pardo, J. 2013. ESX-1-induced apoptosis is involved in cell-to-cell spread of Mycobacterium tuberculosis. *Cellular microbiology*, 15(12):1994-2005.

Allen, M.J., Fan, Y.Y., Monk, J.M., Hou, T.Y., Barhoumi, R., McMurray, D.N. & Chapkin, R.S. 2014. n–3 PUFAs reduce T-helper 17 cell differentiation by decreasing responsiveness to interleukin-6 in isolated mouse splenic CD4+ T cells. *The Journal of nutrition*, 144(8):1306-1313.

Almeida, P.E., Carneiro, A.B., Silva, A.R. & Bozza, P.T. 2012. PPARγ expression and function in mycobacterial infection: roles in Lipid metabolism, immunity, and bacterial killing. *PPAR Research*, 2012:383829.

Alpert, P.T. 2017. The role of vitamins and minerals on the immune system. *Home Health Care Management & Practice*:1084822317713300.

Anand, R. & Kaithwas, G. 2014. Anti-inflammatory potential of alpha-linolenic acid mediated through selective COX inhibition: computational and experimental data. *Inflammation*, 37(4):1297-1306.

Anand, S.P. & Selvaraj, P. 2009. Effect of 1, 25 dihydroxyvitamin D3 on matrix metalloproteinases MMP-7, MMP-9 and the inhibitor TIMP-1 in pulmonary tuberculosis. *Clinical immunology*, 133(1):126-131.

Andersen, L.F., Solvoll, K., Johansson, L.R., Salminen, I., Aro, A. & Drevon, C.A. 1999. Evaluation of a food frequency questionnaire with weighed records, fatty acids, and alpha-tocopherol in adipose tissue and serum. *American journal of epidemiology*, 150(1):75-87.

Anderson, G.J. & Frazer, D.M. 2017. Current understanding of iron homeostasis. *The American journal of clinical nutrition*, 106(suppl_6):1559S-1566S.

Anel, A., Naval, J., González, B., Torres, J.M., Mishal, Z., Uriel, J. & Piñeiro, A. 1990a. Fatty acid metabolism in human lymphocytes. I. Time-course changes in fatty acid composition and membrane fluidity during blastic transformation of peripheral blood lymphocytes. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 1044(3):323-331.

Anel, A., Naval, J., González, B., Uriel, J. & Piñeiro, A. 1990b. Fatty acid metabolism in human lymphocytes. II. Activation of fatty acid desaturase-elongase systems during blastic transformation. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 1044(3):332-339.

Apt, A. & Kramnik, I. 2009. Man and mouse TB: contradictions and solutions. *Tuberculosis (Edinburgh, Scotland)*, 89(3):195.

Arbues, A., Lugo-Villarino, G., Neyrolles, O., Guilhot, C. & Astarie-Dequeker, C. 2014. Playing hide-and-seek with host macrophages through the use of mycobacterial cell envelope phthiocerol dimycocerosates and phenolic glycolipids. *Frontiers in cellular and infection microbiology*, 4:173.

Arita, M., Bianchini, F., Aliberti, J., Sher, A., Chiang, N., Hong, S., Yang, R., Petasis, N.A. & Serhan, C.N. 2005. Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. *Journal of Experimental Medicine*, 201(5):713-722.

Armitage, A.E., Eddowes, L.A., Gileadi, U., Cole, S., Spottiswoode, N., Selvakumar, T.A., Ho, L.P., Townsend, A.R. & Drakesmith, H. 2011. Hepcidin regulation by innate immune and infectious stimuli. *Blood*, 118(15):4129-4139.

Armitage, A.E., Stacey, A.R., Giannoulatou, E., Marshall, E., Sturges, P., Chatha, K., Smith, N.M., Huang, X., Xu, X. & Pasricha, S.R. 2014. Distinct patterns of hepcidin and iron regulation during HIV-1, HBV, and HCV infections. *Proceedings of the National Academy of Sciences*, 111(33):12187-12192.

Arnardottir, H., Orr, S.K., Dalli, J. & Serhan, C.N. 2016. Human milk proresolving mediators stimulate resolution of acute inflammation. *Mucosal immunology*, 9(3):757-763.

Arterburn, L.M., Hall, E.B. & Oken, H. 2006. Distribution, interconversion, and dose response of n−3 fatty acids in humans. *The American journal of clinical nutrition*, 83(6):1467S-1476S.

Augenstreich, J., Arbues, A., Simeone, R., Haanappel, E., Wegener, A., Sayes, F., Le Chevalier, F., Chalut, C., Malaga, W. & Guilhot, C. 2017. ESX-1 and phthiocerol dimycocerosates of Mycobacterium tuberculosis act in concert to cause phagosomal rupture and host cell apoptosis. *Cellular microbiology*, 19(7):e12726.

Axelrod, S., Oschkinat, H., Enders, J., Schlegel, B., Brinkmann, V., Kaufmann, S.H.E., Haas, A. & Schaible, U.E. 2008. Delay of phagosome maturation by a mycobacterial lipid is reversed by nitric oxide. *Cellular Microbiology*, 10(7):1530-1545.

Bafica, A., Scanga, C.A., Serhan, C., Machado, F., White, S., Sher, A. & Aliberti, J. 2005. Host control of Mycobacterium tuberculosis is regulated by 5-lipoxygenase–dependent lipoxin production. *The Journal of Clinical Investigation*, 115(6):1601-1606.

Baindara, P. 2019. Host-directed therapies to combat tuberculosis and associated non-communicable diseases. *Microbial Pathogenesis*, 130:156-168.

Baker, E.J., Miles, E.A., Burdge, G.C., Yaqoob, P. & Calder, P.C. 2016. Metabolism and functional effects of plant-derived omega-3 fatty acids in humans. *Progress in Lipid Research*, 64:30-56.

Bannenberg, G.L., Chiang, N., Ariel, A., Arita, M., Tjonahen, E., Gotlinger, K.H., Hong, S. & Serhan, C.N. 2005. Molecular circuits of resolution: formation and actions of resolvins and protectins. *The Journal of Immunology*, 174(7):4345-4355.

Bapat, P.R., Satav, A.R., Husain, A.A., Shekhawat, S.D., Kawle, A.P., Chu, J.J., Purohit, H.J., Daginawala, H.F., Taori, G.M. & Kashyap, R.S. 2015. Differential levels of alpha-2-macroglobulin, haptoglobin and sero-transferrin as adjunct markers for TB diagnosis and disease progression in the malnourished tribal population of Melghat, India. *PloS one*, 10(8):e0133928.

Barber, D.L., Mayer-Barber, K.D., Feng, C.G., Sharpe, A.H. & Sher, A. 2011. CD4 T cells promote rather than control tuberculosis in the absence of PD-1–mediated inhibition. *The Journal of Immunology*, 186(3):1598-1607.

Barceló-Coblijn, G. & Murphy, E.J. 2009. Alpha-linolenic acid and its conversion to longer chain n−3 fatty acids: Benefits for human health and a role in maintaining tissue n−3 fatty acid levels. *Progress in lipid research*, 48(6):355-374.

Barceló-Coblijn, G., Collison, L.W., Jolly, C.A. & Murphy, E.J. 2005. Dietary α-linolenic acid increases brain but not heart and liver docosahexaenoic acid levels. *Lipids*, 40(8):787-798.

Barden, A.E., Mas, E. & Mori, T.A. 2016. n-3 Fatty acid supplementation and proresolving mediators of inflammation. *Current opinion in lipidology*, 27(1):26-32.

Barry, S., Breen, R., Lipman, M., Johnson, M. & Janossy, G. 2009. Impaired antigen-specific CD4+ T lymphocyte responses in cavitary tuberculosis. *Tuberculosis*, 89(1):48-53.

Basil, M.C. & Levy, B.D. 2016. Specialized pro-resolving mediators: endogenous regulators of infection and inflammation. *Nature reviews. Immunology*, 16(1):51-67.

Baumgartner, J., Smuts, C.M., Malan, L., Kvalsvig, J., van Stuijvenberg, M.E., Hurrell, R.F. & Zimmermann, M.B. 2012. Effects of iron and n-3 fatty acid supplementation, alone and in combination, on cognition in school children: a randomized, double-blind, placebo-controlled intervention in South Africa. *The American journal of clinical nutrition*, 96(6):1327-1338.

Bazinet, R.P., Douglas, H., McMillan, E.G., Wilkie, B.N. & Cunnane, S.C. 2004. Dietary 18: 3ω3 influences immune function and the tissue fatty acid response to antigens and adjuvant. *Immunology letters*, 95(1):85-90.

Behar, S., Martin, C., Booty, M., Nishimura, T., Zhao, X. & Gan, H. 2011. Apoptosis is an innate defense function of macrophages against Mycobacterium tuberculosis. *Mucosal Immunol*, 4.

Behar, S.M., Divangahi, M. & Remold, H.G. 2010. Evasion of innate immunity by Mycobacterium tuberculosis: is death an exit strategy? *Nature Reviews Microbiology*, 8(9):668.

Bhargava, A. 2016. Undernutrition, nutritionally acquired immunodeficiency, and tuberculosis control. *BMJ (Clinical Research Ed.)*, 355:i5407-i5407.

Bilal, S., Haworth, O., Wu, L., Weylandt, K.H., Levy, B.D. & Kang, J.X. 2011. Fat-1 transgenic mice with elevated omega-3 fatty acids are protected from allergic airway responses. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1812(9):1164-1169.

Blasbalg, T.L., Hibbeln, J.R., Ramsden, C.E., Majchrzak, S.F. & Rawlings, R.R. 2011. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. *The American journal of clinical nutrition*, 93(5):950-962.

Bloch, K. 1975. Fatty acid synthases from Mycobacterium phlei. *Methods in enzymology*, 35:84-90.

Block, R.C., Harris, W.S. & Pottala, J.V.J.T.o.b.j. 2008. Determinants of blood cell omega-3 fatty acid content. *Open biomarkers journal,* 1:1-6.

Blomgran, R. & Ernst, J.D. 2011. Lung neutrophils facilitate activation of naive antigen-specific CD4+ T cells during Mycobacterium tuberculosis infection. *The Journal of Immunology*, 186(12):7110-7119.

Boelaert, J.R., Vandecasteele, S.J., Appelberg, R. & Gordeuk, V.R. 2007. The effect of the host's iron status on tuberculosis. *The Journal of infectious diseases*, 195(12):1745-1753.

Boilard, E. 2018. Extracellular vesicles and their content in bioactive lipid mediators: more than a sack of microRNA. *Journal of lipid research*, 59(11):2037-2046.

Bolton-Smith, C., Woodward, M. & Tavendale, R. 1997. Evidence for age-related differences in the fatty acid composition of human adipose tissue, independent of diet. *European journal of clinical nutrition*, 51(9):619.

Bonilla, D.L., Fan, Y.-Y., Chapkin, R.S. & McMurray, D.N. 2010b. Transgenic mice enriched in omega-3 fatty acids are more susceptible to pulmonary tuberculosis: impaired resistance to tuberculosis in fat-1 mice. *The Journal of infectious diseases*, 201(3):399-408.

Bonilla, D.L., Ly, L.H., Fan, Y.-Y., Chapkin, R.S. & McMurray, D.N. 2010a. Incorporation of a Dietary Omega 3 Fatty Acid Impairs Murine Macrophage Responses to Mycobacterium. *PloS one,* 5(5):e10878.

Bork, C.S., Baker, E.J., Lundbye-Christensen, S., Miles, E.A. & Calder, P.C. 2019. Lowering the linoleic acid to alpha-linoleic acid ratio decreases the production of inflammatory mediators by cultured human endothelial cells. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 141:1-8.

Borthwick, L. 2016. The IL-1 cytokine family and its role in inflammation and fibrosis in the lung. *Seminars in immunopathaolgy,* 38(4):517-534.

Bourre, J.-M., Dumont, O., Pascal, G. & Durand, G. 1993. Dietary α-linolenic acid at 1.3 g/kg maintains maximal docosahexaenoic acid concentration in brain, heart and liver of adult rats. *The Journal of nutrition*, 123(7):1313-1319.

Bowen, R.A. & Clandinin, M.T. 2000. High dietary 18: 3n− 3 increases the 18: 3n− 3 but not the 22: 6n− 3 content in the whole body, brain, skin, epididymal fat pads, and muscles of suckling rat pups. *Lipids*, 35(4):389-394.

Brand, A., Schonfeld, E., Isharel, I. & Yavin, E. 2008. Docosahexaenoic acid-dependent iron accumulation in oligodendroglia cells protects from hydrogen peroxide-induced damage. *Journal of neurochemistry*, 105(4):1325-1335.

Brash, A.R. 1999. Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. *Journal of Biological Chemistry*, 274(34):23679-23682.

Brenna, J.T. 2002. Efficiency of conversion of α-linolenic acid to long chain n-3 fatty acids in man. *Current Opinion in Clinical Nutrition & Metabolic Care*, 5(2):127-132.

Brenna, J.T., Plourde, M., Stark, K.D., Jones, P.J. & Lin, Y.-H. 2018. Best practices for the design, laboratory analysis, and reporting of trials involving fatty acids. *The American journal of clinical nutrition*.

Brenna, J.T., Salem Jr, N., Sinclair, A.J. & Cunnane, S.C. 2009a. α-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. *Prostaglandins, leukotrienes and essential fatty acids*, 80(2-3):85-91.

Brenna, J.T., Salem, N., Sinclair, A.J. & Cunnane, S.C. 2009b. α-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 80(2):85-91.

Bresnahan, K.A., Chileshe, J., Arscott, S., Nuss, E., Surles, R., Masi, C., Kafwembe, E. & Tanumihardjo, S.A. 2014. The acute phase response affected traditional measures of micronutrient status in rural Zambian children during a randomized, controlled feeding trial. *The Journal of nutrition*, 144(6):972-978.

Brock, J.H. 2018. Iron and the immune system. Iron and human disease. CRC Press. p. 161-178).

Browning, L.M., Walker, C.G., Mander, A.P., West, A.L., Madden, J., Gambell, J.M., Young, S., Wang, L., Jebb, S.A. & Calder, P.C. 2012. Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish–. *The American journal of clinical nutrition*, 96(4):748-758.

Bru, A. & Cardona, P.-J. 2010. Mathematical modeling of tuberculosis bacillary counts and cellular populations in the organs of infected mice. *PLoS One*, 5(9):e12985.

Brzóska, K., Meczynska, S. & Kruszewski, M. 2006. Iron-sulfur cluster proteins: electron transfer and beyond. *Acta biochimica polonica-english edition,* 53(4):685.

Buckley, C.D., Gilroy, D.W. & Serhan, C.N. 2014. Proresolving lipid mediators and mechanisms in the resolution of acute inflammation. *Immunity*, 40(3):315-327.

Burdge, G. 2004. α-Linolenic acid metabolism in men and women: nutritional and biological implications. *Current Opinion in Clinical Nutrition & Metabolic Care*, 7(2):137-144.

Burdge, G.C. 2019. Is essential fatty acid interconversion an important source of PUFA in humans? *British Journal of Nutrition*, 121(6):615-624.

Burdge, G.C. & Calder, P.C. 2005a. Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reproduction Nutrition Development*, 45(5):581-597.

Burdge, G.C. & Calder, P.C. 2005b. α-Linolenic acid metabolism in adult humans: the effects of gender and age on conversion to longer-chain polyunsaturated fatty acids. *European Journal of Lipid Science and Technology*, 107(6):426-439.

Burdge, G.C. & Calder, P.C. 2006. Dietary α-linolenic acid and health-related outcomes: a metabolic perspective. *Nutrition research reviews*, 19(1):26-52.

Burdge, G.C. & Calder, P.C. 2015. Introduction to fatty acids and lipids. Intravenous Lipid Emulsions. *Karger Publishers*, 1-16.

Burdge, G.C., Finnegan, Y.E., Minihane, A.M., Williams, C.M. & Wootton, S.A. 2003. Effect of altered dietary n-3 fatty acid intake upon plasma lipid fatty acid composition, conversion of [13 C] α-linolenic acid to longer-chain fatty acids and partitioning towards β-oxidation in older men. *British Journal of Nutrition*, 90(2):311-321.

Burdge, G.C., Jones, A.E. & Wootton, S.A. 2002. Eicosapentaenoic and docosapentaenoic acids are the principal products of α-linolenic acid metabolism in young men. *British Journal of Nutrition*, 88(4):355-363.

Burdge, G.C. & Wootton, S.A. 2002. Conversion of α-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. *British Journal of Nutrition*, 88(4):411-420.

Byrd, T.F. 1997. Tumor necrosis factor alpha (TNFalpha) promotes growth of virulent Mycobacterium tuberculosis in human monocytes iron-mediated growth suppression is correlated with decreased release of TNFalpha from iron-treated infected monocytes. *The Journal of clinical investigation*, 99(10):2518-2529.

Byrne, S.T., Denkin, S.M. & Zhang, Y. 2006. Aspirin and ibuprofen enhance pyrazinamide treatment of murine tuberculosis. *Journal of antimicrobial chemotherapy*, 59(2):313-316.

Byrne, S.T., Denkin, S.M. & Zhang, Y. 2007. Aspirin Antagonism in Isoniazid Treatment. *Antimicrobial agents in chemotherapy*, 51(2):794.

Calder, P., Bevan, S. & Newsholme, E. 1992. The inhibition of T-lymphocyte proliferation by fatty acids is via an eicosanoid-independent mechanism. *Immunology*, 75(1):108.

Calder, P.C. 2008. The relationship between the fatty acid composition of immune cells and their function. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 79(3-5):101-108.

Calder, P.C. 2009a. Fatty Acids and Immune Function: Relevance to Inflammatory Bowel Diseases. *International Reviews of Immunology*, 28(6):506-534.

Calder, P.C. 2010. Rationale and use of n-3 fatty acids in artificial nutrition. *Proceedings of the Nutrition society*, 69(4):565-573.

Calder, P.C. 2012. Mechanisms of action of (n-3) fatty acids. *The Journal of nutrition*, 142(3):592S-599S.

Calder, P.C. 2013a. Long chain fatty acids and gene expression in inflammation and immunity. *Current Opinion in Clinical Nutrition & Metabolic Care*, 16(4):425-433.

Calder, P.C. 2013b. Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology? *British journal of clinical pharmacology*, 75(3):645-662.

Calder, P.C. 2014. Very long chain omega-3 (n-3) fatty acids and human health. *European journal of lipid science and technology*, 116(10):1280-1300.

Calder, P.C. 2015a. Functional roles of fatty acids and their effects on human health. *Journal of Parenteral and Enteral Nutrition*, 39:18S-32S.

Calder, P.C. 2015b. Marine omega-3 fatty acids and inflammatory processes: effects, mechanisms and clinical relevance. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1851(4):469-484.

Calder, P.C. 2017. Omega-3 fatty acids and inflammatory processes: from molecules to man. *Biochemical Society Transactions*, 45(5):1105-1115.

Calder, P.C. 2018. Metabolism of polyunsaturated fatty acids by cells of the immune system. *(In* Burdge, G.C., *ed.* Fatty acid metabolism. London, UK: Elsevier Inc. p. 136-151).

Calder, P.C., Ahluwalia, N., Albers, R., Bosco, N., Bourdet-Sicard, R., Haller, D., Holgate, S.T., Jönsson, L.S., Latulippe, M.E. & Marcos, A. 2013a. A consideration of biomarkers to be used for evaluation of inflammation in human nutritional studies. *British Journal of Nutrition*, 109(S1):S1-S34.

Calder, P.C., Bond, J.A., Bevan, S.J., Hunt, S.V. & Newsholme, E.A. 1991. Effect of fatty acids on the proliferation of concanavalin A-stimulated rat lymph node lymphocytes. *International Journal of Biochemistry*, 23(5-6):579-588.

Calder, P.C., Bond, J.A., Harvey, D.J., Gordon, S. & Newsholme, E.A. 1990. Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochemical Journal*, 269(3):807-814.

Calder, P.C. & Newsholme, E.A. 1992. Polyunsaturated fatty acids suppress human peripheral blood lymphocyte proliferation and interleukin-2 production. *Clinical science (London, England: 1979)*, 82(6):695-700.

Calder, P.C. & Yaqoob, P. 2007. Lipid rafts—composition, characterization, and controversies. *The Journal of nutrition*, 137(3):545-547.

Calder, P.C., Yaqoob, P., Harvey, D.J., Watts, A. & Newsholme, E.A. 1994. Incorporation of fatty acids by concanavalin A-stimulated lymphocytes and the effect on fatty acid composition and membrane fluidity. *Biochemical Journal*, 300(2):509-518.

Cambier, C., Takaki, K.K., Larson, R.P., Hernandez, R.E., Tobin, D.M., Urdahl, K.B., Cosma, C.L. & Ramakrishnan, L. 2014. Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. *Nature*, 505(7482):218.

Campbell, G.R. & Spector, S.A. 2012. Vitamin D inhibits human immunodeficiency virus type 1 and Mycobacterium tuberculosis infection in macrophages through the induction of autophagy. *PLoS pathogens*, 8(5):e1002689.

Cannetti, G. 1955. The tubercle bacillus: Springer Publishing Company, Inc., New York (NY).

Carpenter, C.E. & Mahoney, A.W. 1992. Contributions of heme and nonheme iron to human nutrition. *Critical Reviews in Food Science & Nutrition*, 31(4):333-367.

Carrillo-Alvarado, M.A., Pardo, A., Alsaihati, L., Noveron, J.C. & Ouellet, H. 2019. Deciphering the mechanism of action of cholesterol analogs as inhibitors of Mycobacterium tuberculosis. *The FASEB Journal*, 33(1_supplement):639.632-639.632.

Casanova, J.-L. & Abel, L. 2002. Genetic dissection of immunity to mycobacteria: the human model. *Annu Rev Immunol*, 20.

Caughey, G.E., Mantzioris, E., Gibson, R.A., Cleland, L.G. & James, M.J. 1996. The effect on human tumor necrosis factor alpha and interleukin 1 beta production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. *The American journal of clinical nutrition*, 63(1):116-122.

Chan, J.K., McDonald, B.E., Gerrard, J.M., Bruce, V.M., Weaver, B.J. & Holub, B.J. 1993. Effect of dietary α-linolenic acid and its ratio to linoleic acid on platelet and plasma fatty acids and thrombogenesis. *Lipids*, 28(9):811-817.

Chao, A., Sieminski, P.J., Owens, C.P. & Goulding, C.W. 2019. Iron Acquisition in Mycobacterium tuberculosis. *Chem Rev*, 119(2):1193-1220.

Chapkin, R., Somers, S. & Erickson, K.L. 1988. Inability of murine peritoneal macrophages to convert linoleic acid into arachidonic acid. Evidence of chain elongation. *The Journal of Immunology*, 140(7):2350-2355.

Chapkin, R.S., Akoh, C.C. & Lewis, R.E. 1992. Dietary fish oil modulation of in vivo peritoneal macrophage leukotriene production and phagocytosis. *The Journal of Nutritional Biochemistry*, 3(11):599-604.

Chen, M., Divangahi, M., Gan, H., Shin, D.S., Hong, S., Lee, D.M., Serhan, C.N., Behar, S.M. & Remold, H.G. 2008. Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death. *Journal of Experimental Medicine*, 205(12):2791-2801.

Chen, M., Gan, H. & Remold, H.G. 2006. A mechanism of virulence: virulent Mycobacterium tuberculosis strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis. *The Journal of Immunology*, 176(6):3707-3716.

Cherayil, B.J. 2010. Iron and immunity: immunological consequences of iron deficiency and overload. *Archivum immunologiae et therapiae experimentalis*, 58(6):407-415.

Chiang, N., Fredman, G., Bäckhed, F., Oh, S.F., Vickery, T., Schmidt, B.A. & Serhan, C.N. 2012. Infection regulates pro-resolving mediators that lower antibiotic requirements. *Nature*, 484(7395):524.

Chiaranunt, P., Ferrara, J.L. & Byersdorfer, C.A. 2015. Rethinking the paradigm: How comparative studies on fatty acid oxidation inform our understanding of T cell metabolism. *Molecular immunology*, 68(2):564-574.

Childs, C.E., Romeu-Nadal, M., Burdge, G.C. & Calder, P.C. 2008. Gender differences in the n-3 fatty acid content of tissues. *Proceedings of the Nutrition Society*, 67(1):19-27.

Cho, H.P., Nakamura, M. & Clarke, S.D. 1999a. Cloning, expression, and fatty acid regulation of the human Δ-5 desaturase. *Journal of Biological Chemistry*, 274(52):37335-37339.

Cho, H.P., Nakamura, M.T. & Clarke, S.D. 1999b. Cloning, expression, and nutritional regulation of the mammalian Δ-6 desaturase. *Journal of Biological Chemistry*, 274(1):471-477.

Chowdhury, I.H., Ahmed, A.M., Choudhuri, S., Sen, A., Hazra, A., Pal, N.K., Bhattacharya, B. & Bahar, B. 2014. Alteration of serum inflammatory cytokines in active pulmonary tuberculosis following anti-tuberculosis drug therapy. *Molecular immunology*, 62(1):159-168.

Clark, R.J., Tan, C.C., Preza, G.C., Nemeth, E., Ganz, T. & Craik, D.J. 2011. Understanding the structure/activity relationships of the iron regulatory peptide hepcidin. *Chemistry & biology*, 18(3):336-343.

Clauss, M., Dierenfeld, E.S., Bigley, K., Wang, Y., Ghebremeskel, K., Hatt, J.M., Flach, E., Behlert, O., Castell, J. & Streich, W. 2008. Fatty acid status in captive and free-ranging black rhinoceroses (Diceros bicornis). *Journal of animal physiology and animal nutrition*, 92(3):231-241.

Clay, H., Volkman, H.E. & Ramakrishnan, L. 2008. Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. *Immunity*, 29(2):283-294.

Clayton, K., Polak, M.E., Woelk, C.H. & Elkington, P. 2017. Gene expression signatures in tuberculosis have greater overlap with autoimmune diseases than with infectious diseases. *American journal of respiratory and critical care medicine*, 196(5):655-656.

Codagnone, M., Cianci, E., Lamolinara, A., Mari, V., Nespoli, A., Isopi, E., Mattoscio, D., Arita, M., Bragonzi, A. & Iezzi, M. 2018. Resolvin D1 enhances the resolution of lung inflammation caused by long-term Pseudomonas aeruginosa infection. *Mucosal immunology*, 11(1):35-45.

Colas, R.A., Nhat, L.T.H., Thuong, N.T.T., Gómez, E.A., Ly, L., Thanh, H.H., Mai, N.T.H., Phu, N.H., Thwaites, G.E. & Dalli, J. 2019. Proresolving mediator profiles in cerebrospinal fluid are linked with disease severity and outcome in adults with tuberculous meningitis. *The FASEB Journal*, 33(11):13028-13039.

Colas, R.A., Shinohara, M., Dalli, J., Chiang, N. & Serhan, C.N. 2014. Identification and signature profiles for pro-resolving and inflammatory lipid mediators in human tissue. *American Journal of Physiology-Cell Physiology*, 307(1):C39-C54.

Cole, S., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S., Eiglmeier, K., Gas, S. & Barry Iii, C. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature*, 393(6685):537.

Condos, R., Rom, W.N., Liu, Y.M. & Schluger, N.W. 1998. Local immune responses correlate with presentation and outcome in tuberculosis. *American journal of respiratory and critical care medicine*, 157(3):729-735.

Cooper, A.M. 2009. Cell-mediated immune responses in tuberculosis. *Annu Rev Immunol*, 27.

Cooper, A.M., Dalton, D.K., Stewart, T.A., Griffin, J.P., Russell, D.G. & Orme, I.M. 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *Journal of Experimental Medicine*, 178(6):2243-2247.

Cooper, A.M. & Khader, S.A. 2008. The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis. *Immunological reviews*, 226(1):191-204.

Cooper, A.M., Magram, J., Ferrante, J. & Orme, I.M. 1997. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with Mycobacterium tuberculosis. *Journal of Experimental Medicine*, 186(1):39-45.

Cooper, A.M., Mayer-Barber, K.D. & Sher, A. 2011. Role of innate cytokines in mycobacterial infection. *Mucosal immunology*, 4(3):252.

Cooper, A.M. & Torrado, E. 2012. Protection versus pathology in tuberculosis: recent insights. *Current Opinion in Immunology*, 24(4):431-437.

Cotogni, P., Muzio, G., Trombetta, A., Ranieri, V.M. & Canuto, R.A. 2011. Impact of the ω-3 to ω-6 polyunsaturated fatty acid ratio on cytokine release in human alveolar cells. *Journal of parenteral and enteral nutrition*, 35(1):114-121.

Coussens, A.K., Wilkinson, R.J., Hanifa, Y., Nikolayevskyy, V., Elkington, P.T., Islam, K., Timms, P.M., Venton, T.R., Bothamley, G.H. & Packe, G.E. 2012. Vitamin D accelerates resolution of inflammatory responses during tuberculosis treatment. *Proceedings of the National Academy of Sciences*, 109(38):15449-15454.

Covar, R., Gleason, M., Macomber, B., Stewart, L., Szefler, P., Engelhardt, K., Murphy, J., Liu, A., Wood, S. & DeMichele, S. 2010. Impact of a novel nutritional formula on asthma control and biomarkers of allergic airway inflammation in children. *Clinical & Experimental Allergy*, 40(8):1163-1174.

Critchley, J.A., Young, F., Orton, L. & Garner, P. 2013. Corticosteroids for prevention of mortality in people with tuberculosis: a systematic review and meta-analysis. *The Lancet infectious diseases*, 13(3):223-237.

Cronjé, L., Edmondson, N., Eisenach, K.D. & Bornman, L. 2005. Iron and iron chelating agents modulate Mycobacterium tuberculosis growth and monocyte-macrophage viability and effector functions. *FEMS Immunology & Medical Microbiology*, 45(2):103-112.

Cunnane, S.C. & Anderson, M.J. 1997. The majority of dietary linoleate in growing rats is β-oxidized or stored in visceral fat. *The Journal of nutrition*, 127(1):146-152.

Cunnane, S.C. & McAdoo, K.R. 1987. Iron intake influences essential fatty acid and lipid composition of rat plasma and erythrocytes. *The Journal of nutrition*, 117(9):1514-1519.

Cunnane, S.C., Ryan, M.A., Nadeau, C.R., Bazinet, R.P., Musa-Veloso, K. & McCloy, U. 2003. Why is carbon from some polyunsaturates extensively recycled into lipid synthesis? *Lipids*, 38(4):477-484.

Cunningham-Rundles, S., McNeeley, D.F. & Moon, A. 2005. Mechanisms of nutrient modulation of the immune response. *Journal of Allergy and Clinical Immunology*, 115(6):1119-1128.

Dallenga, T., Repnik, U., Corleis, B., Eich, J., Reimer, R., Griffiths, G.W. & Schaible, U.E. 2017. M. tuberculosis-induced necrosis of infected neutrophils promotes bacterial growth following phagocytosis by macrophages. *Cell host & microbe*, 22(4):519-530. e513.

Dalli, J., Chiang, N. & Serhan, C.N. 2015. Elucidation of novel 13-series resolvins that increase with atorvastatin and clear infections. *Nature medicine*, 21(9):1071.

Dalli, J. & Serhan, C. 2012. Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators. *Blood,* 120(15):e60-e72.

Damsgaard, C.T., Frøkiær, H. & Lauritzen, L. 2008. The effects of fish oil and high or low linoleic acid intake on fatty acid composition of human peripheral blood mononuclear cells. *British Journal of Nutrition*, 99(1):147-154.

Dartois, V. 2014. The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. *Nature Reviews Microbiology*, 12(3):159-167.

Dasa, F. & Abera, T. 2018. Factors affecting iron absorption and mitigation mechanisms: a review. *Int J Agric Sc Food Technol*, 4(1):024-030.

Davis, J.M. & Ramakrishnan, L. 2009. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell*, 136(1):37-49.

De La Mora, I.L., Martinez-Oceguera, D. & Laniado-Laborin, R. 2015. Chronic airway obstruction after successful treatment of tuberculosis and its impact on quality of life. *The International Journal of Tuberculosis and Lung Disease*, 19(7):808-810.

Del Bò, C., Contino, D., Riso, P., Simonetti, P. & Ciappellano, S. 2009. Effect of horse meat consumption on iron status, lipid profile and fatty acid composition of red blood cell membrane: preliminary study. Societa Italiana di Radiologia Medica, Milan, Italy.

Dennis, E.A., Cao, J., Hsu, Y.-H., Magrioti, V. & Kokotos, G.J.C.r. 2011. Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chemicl reviews,* 111(10):6130-6185.

Denys, A., Hichami, A. & Khan, N.A. 2005. n-3 PUFAs modulate T-cell activation via protein kinase C-α and-ε and the NF-κB signaling pathway. *Journal of lipid research*, 46(4):752-758.

Devi, U., Rao, C.M., Srivastava, V.K., Rath, P.K. & Das, B.S. 2003. Effect of iron supplementation on mild to moderate anaemia in pulmonary tuberculosis. *British Journal of Nutrition*, 90(3):541-550.

Dheda, K., Gumbo, T., Maartens, G., Dooley, K.E., McNerney, R., Murray, M., Furin, J., Nardell, E.A., London, L. & Lessem, E. 2017. The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. *The lancet Respiratory medicine*, 5(4):291-360.

Dietzold, J., Gopalakrishnan, A. & Salgame, P. 2015. Duality of lipid mediators in host response against Mycobacterium tuberculosis: good cop, bad cop. *F1000Prime Reports*, 7:29.

Dinarello, C.A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annual review of immunology*, 27:519-550.

Dittrich, M., Jahreis, G., Bothor, K., Drechsel, C., Kiehntopf, M., Blüher, M. & Dawczynski, C. 2015. Benefits of foods supplemented with vegetable oils rich in α-linolenic, stearidonic or docosahexaenoic acid in hypertriglyceridemic subjects: a double-blind, randomized, controlled trail. *European journal of nutrition*, 54(6):881-893.

Divangahi, M., Behar, S.M. & Remold, H. 2013. Dying to live: how the death modality of the infected macrophage affects immunity to tuberculosis. The New Paradigm of Immunity to Tuberculosis. Springer. p. 103-120.

Divangahi, M., Chen, M., Gan, H., Desjardins, D., Hickman, T.T., Lee, D.M., Fortune, S., Behar, S.M. & Remold, H.G. 2009. Mycobacterium tuberculosis evades macrophage defenses by inhibiting plasma membrane repair. *Nature immunology*, 10(8):899.

Divangahi, M., Desjardins, D., Nunes-Alves, C., Remold, H.G. & Behar, S.M. 2010. Eicosanoid pathways regulate adaptive immunity to Mycobacterium tuberculosis. *Nature immunology*, 11(8):751.

Domenichiello, A.F., Chen, C.T., Trepanier, M.-O., Stavro, P.M. & Bazinet, R.P. 2014. Whole body synthesis rates of DHA from α-linolenic acid are greater than brain DHA accretion and uptake rates in adult rats. *Journal of lipid research*, 55(1):62-74.

Domingo-Gonzalez, R., Prince, O., Cooper, A. & Khader, S.A. 2016. Cytokines and Chemokines in Mycobacterium tuberculosis Infection. *Microbiology spectrum*, 4(5):10.1128/microbiolspec. TBTB1122-0018-2016.

Donovan, A., Lima, C.A., Pinkus, J.L., Pinkus, G.S., Zon, L.I., Robine, S. & Andrews, N.C. 2005. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell metabolism*, 1(3):191-200.

Dorhoi, A. & Kaufmann, S. 2016. Pathology and immune reactivity: understanding multidimensionality in pulmonary tuberculosis. *Seminars in Immunopathology*, 38(2):153-166.

Driver, E.R., Ryan, G.J., Hoff, D.R., Irwin, S.M., Basaraba, R.J., Kramnik, I. & Lenaerts, A.J. 2012. Evaluation of a mouse model of necrotic granuloma formation using C3HeB/FeJ mice for testing of drugs against Mycobacterium tuberculosis. *Antimicrobial agents and chemotherapy*, 56(6):3181-3195.

Dubos, R.J. & Dubos, J. 1952. The white plague: tuberculosis, man, and society. *Bulletin of medical library association,* 42(1):142-143.

Duda, M.K., O'Shea, K.M., Tintinu, A., Xu, W., Khairallah, R.J., Barrows, B.R., Chess, D.J., Azimzadeh, A.M., Harris, W.S. & Sharov, V.G. 2008. Fish oil, but not flaxseed oil, decreases inflammation and prevents pressure overload-induced cardiac dysfunction. *Cardiovascular research*, 81(2):319-327.

Dülger, H., Arik, M., Şekeroğlu, M.R., Tarakçioğlu, M., Noyan, T., Cesur, Y. & Balahoroğlu, R. 2002. Pro-inflammatory cytokines in Turkish children with protein-energy malnutrition. *Mediators of inflammation*, 11(6):363-365.

Durry, F.D., Wirjatmadi, B. & Adriani, M. 2018. The role of zinc sulphate and omega 3 on the improvement of weight and speed conversion conversion on tuberculosis parent patients at Surabaya Park Hospital, 2015. *Jurnal Ilmiah Kedokteran Wijaya Kusuma*, 7(1):62-74.

Dutta, N., Kumar, K.A., Mazumdar, K., Dastidar, S.G., Ray, R. & Chakrabarty, A. 2004. In vitro and in vivo antimycobacterial activity of antiinflammatory drug, diclofenac sodium. *Indian journal of experimental biology,* 42:922-927.

Dutta, N.K., Mazumdar, K., Dastidar, S.G. & Park, J.-H. 2007. Activity of diclofenac used alone and in combination with streptomycin against Mycobacterium tuberculosis in mice. *International journal of antimicrobial agents*, 30(4):336-340.

Edwards, I.J. & O'Flaherty, J.T. 2008. Omega-3 fatty acids and PPAR in cancer. *PPAR research*, 2008.

Egen, J.G., Rothfuchs, A.G., Feng, C.G., Winter, N., Sher, A. & Germain, R.N. 2008. Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. *Immunity*, 28(2):271-284.

Ekiz, C., Agaoglu, L., Karakas, Z., Gurel, N. & Yalcin, I. 2005. The effect of iron deficiency anemia on the function of the immune system. *The Hematology Journal*, 5(7):579-583.

El-Ahmady, O., Mansour, M., Zoeir, H. & Mansour, O. 1997. Elevated concentrations of interleukins and leukotriene in response to Mycobacterium tuberculosis infection. *Annals of clinical biochemistry*, 34(2):160-164.

Elagizi, A., Lavie, C.J., Marshall, K., DiNicolantonio, J.J., O'Keefe, J.H. & Milani, R.V. 2018. Omega-3 Polyunsaturated Fatty Acids and Cardiovascular Health: A Comprehensive Review. *Progress in Cardiovascular Diseases*, 61(1):76-85.

Erdinest, N., Shmueli, O., Grossman, Y., Ovadia, H. & Solomon, A. 2012. Anti-inflammatory effects of alpha linolenic acid on human corneal epithelial cells. *Investigative ophthalmology & visual science*, 53(8):4396-4406.

Ernst, J.D. 2012. The immunological life cycle of tuberculosis. *Nature Reviews Immunology*, 12(8):581-591.

Eum, S.-Y., Kong, J.-H., Hong, M.-S., Lee, Y.-J., Kim, J.-H., Hwang, S.-H., Cho, S.-N., Via, L.E. & Barry III, C.E. 2010. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. *Chest*, 137(1):122-128.

Faber, J., Berkhout, M., Vos, A.P., Sijben, J.W., Calder, P.C., Garssen, J. & van Helvoort, A. 2011. Supplementation with a Fish Oil-Enriched, High-Protein Medical Food Leads to Rapid Incorporation of EPA into White Blood Cells and Modulates Immune Responses within One Week in Healthy Men and Women, 2. *The Journal of nutrition*, 141(5):964-970.

FAO. 2010. Fats and fatty acids in human nutrition. Report of an expert consultation, 10-14 November 2008, Geneva.

Fekete, K., Marosvölgyi, T., Jakobik, V. & Decsi, T. 2009. Methods of assessment of n–3 long-chain polyunsaturated fatty acid status in humans: a systematic review. *The American journal of clinical nutrition*, 89(6):2070S-2084S.

Fielding, B.A., Calder, P.C., Irvine, N.A., Miles, E.A., Lillycrop, K.A., von Gerichten, J. & Burdge, G.C. 2019. How does polyunsaturated fatty acid biosynthesis regulate T-lymphocyte function? *Nutrition Bulletin*, DOI: 10.1111/nbu.12404.

Flock, M.R., Harris, W.S. & Kris-Etherton, P.M. 2013a. Long-chain omega-3 fatty acids: time to establish a dietary reference intake. *Nutrition Reviews*, 71(10):692-707.

Flock, M.R., Skulas-Ray, A.C., Harris, W.S., Etherton, T.D., Fleming, J.A. & Kris-Etherton, P.M.J.J.o.t.A.H.A. 2013b. Determinants of erythrocyte omega-3 fatty acid content in response to fish oil supplementation: a dose–response randomized controlled trial. *Journal of the American Heart association,* 2(6):e000513.

Flynn, J., Chan, J. & Lin, P. 2011. Macrophages and control of granulomatous inflammation in tuberculosis. *Mucosal immunology*, 4(3):271.

Ford, R., Faber, M., Kunneke, E. & Smuts, C.M. 2016. Dietary fat intake and red blood cell fatty acid composition of children and women from three different geographical areas in South Africa. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 109:13-21.

Fratazzi, C., Arbeit, R.D., Carini, C. & Remold, H.G. 1997. Programmed cell death of Mycobacterium avium serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. *The Journal of Immunology*, 158(9):4320-4327.

Frediani, J.K., Jones, D.P., Tukvadze, N., Uppal, K., Sanikidze, E., Kipiani, M., Tran, V.T., Hebbar, G., Walker, D.I. & Kempker, R.R. 2014. Plasma metabolomics in human pulmonary tuberculosis disease: a pilot study. *PloS one*, 9(10):e108854.

Frediani, J.K., Sanikidze, E., Kipiani, M., Tukvadze, N., Hebbar, G., Ramakrishnan, U., Jones, D.P., Easley, K.A., Shenvi, N., Kempker, R.R., Tangpricha, V., Blumberg, H.M. & Ziegler, T.R. 2016. Macronutrient intake and body composition changes during anti-tuberculosis therapy in adults. *Clinical Nutrition*, 35(1):205-212.

Friis, H., Range, N., Kristensen, C.B., Kæstel, P., Changalucha, J., Malenganisho, W., Krarup, H., Magnussen, P. & Andersen, Å.B. 2009. Acute-phase response and iron status markers among pulmonary tuberculosis patients: a cross-sectional study in Mwanza, Tanzania. *British journal of nutrition*, 102(2):310-317.

Fritsche, K. 2007. Important differences exist in the dose–response relationship between diet and immune cell fatty acids in humans and rodents. *Lipids*, 42(11):961-979.

Fullerton, J.N., O'Brien, A.J. & Gilroy, D.W. 2014. Lipid mediators in immune dysfunction after severe inflammation. *Trends in immunology*, 35(1):12-21.

Fulton, A.S., Hill, A.M., Williams, M.T., Howe, P.R. & Coates, A.M. 2015. Paucity of evidence for a relationship between long-chain omega-3 fatty acid intake and chronic obstructive pulmonary disease: a systematic review. *Nutrition reviews*, 73(9):612-623.

Fuqua, B.K., Vulpe, C.D. & Anderson, G.J. 2012. Intestinal iron absorption. *Journal of Trace Elements in Medicine and Biology*, 26(2-3):115-119.

Gago, G., Diacovich, L. & Gramajo, H. 2018. Lipid metabolism and its implication in mycobacteria–host interaction. *Current Opinion in Microbiology*, 41:36-42.

Gangaidzo, I.T., Moyo, V.M., Mvundura, E., Aggrey, G., Murphree, N.L., Khumalo, H., Saungweme, T., Kasvosve, I., Gomo, Z.A. & Rouault, T. 2001. Association of pulmonary tuberculosis with increased dietary iron. *The Journal of infectious diseases*, 184(7):936-939.

Ganz, T. 2018. Iron and infection. *International journal of hematology*, 107(1):7-15.

Ganz, T. 2019. Anemia of inflammation. *New England Journal of Medicine*, 381(12):1148-1157.

Ganz, T. & Nemeth, E. 2015. Iron homeostasis in host defence and inflammation. *Nature Reviews Immunology*, 15(8):500.

Geerling, B., Houwelingen, A.v., Stockbrügger, R. & Brummer, R.-J. 1999. Fat intake and fatty acid profile in plasma phospholipids and adipose tissue in patients with Crohn's disease, compared with controls. *The American journal of gastroenterology*, 94(2):410-417.

Geldmacher, C., Ngwenyama, N., Schuetz, A., Petrovas, C., Reither, K., Heeregrave, E.J., Casazza, J.P., Ambrozak, D.R., Louder, M. & Ampofo, W. 2010. Preferential infection and depletion of Mycobacterium tuberculosis–specific CD4 T cells after HIV-1 infection. *Journal of Experimental Medicine*:jem. 20100090.

Gelvan, D., Fibach, E., Meyron-Holtz, E.G. & Konijn, A. 1996. Ferritin uptake by human erythroid precursors is a regulated iron uptake pathway. *Blood*, 88(8):3200-3207.

Geng, E., Kreiswirth, B., Burzynski, J. & Schluger, N.W. 2005. Clinical and radiographic correlates of primary and reactivation tuberculosis: a molecular epidemiology study. *Jama*, 293(22):2740-2745.

Georgiadi, A., Boekschoten, M.V., Müller, M. & Kersten, S. 2012. Detailed transcriptomics analysis of the effect of dietary fatty acids on gene expression in the heart. *Physiological genomics*, 44(6):352-361.

Gibson, R.A., Muhlhausler, B. & Makrides, M. 2011. Conversion of linoleic acid and alpha-linolenic acid to long-chain polyunsaturated fatty acids (LCPUFAs), with a focus on pregnancy, lactation and the first 2 years of life. *Maternal & child nutrition*, 7:17-26.

Gibson, R.A., Neumann, M.A., Lien, E.L., Boyd, K.A. & Tu, W.C. 2013. Docosahexaenoic acid synthesis from alpha-linolenic acid is inhibited by diets high in polyunsaturated fatty acids. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 88(1):139-146.

Gilbert, N.C., Bartlett, S.G., Waight, M.T., Neau, D.B., Boeglin, W.E., Brash, A.R. & Newcomer, M.E. 2011. The structure of human 5-lipoxygenase. *Science*, 331(6014):217-219.

Gill, W.P., Harik, N.S., Whiddon, M.R., Liao, R.P., Mittler, J.E. & Sherman, D.R. 2009. A replication clock for Mycobacterium tuberculosis. *Nature medicine*, 15(2):211-214.

Gioxari, A., Kaliora, A.C., Marantidou, F. & Panagiotakos, D.P. 2018. Intake of ω-3 polyunsaturated fatty acids in patients with rheumatoid arthritis: A systematic review and meta-analysis. *Nutrition*, 45:114-124.

Girelli, D., Nemeth, E. & Swinkels, D.W. 2016. Hepcidin in the diagnosis of iron disorders. *Blood*, 127(23):2809-2813.

Gkouvatsos, K., Papanikolaou, G. & Pantopoulos, K. 2012. Regulation of iron transport and the role of transferrin. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1820(3):188-202.

Gomez, G., Dowdy, D.W., Bastos, M., Zwerling, A., Sweeney, S., Foster, N., Trajman, A., Islam, M., Kapiga, S. & Sinanovic, E. 2016. Cost and cost-effectiveness of tuberculosis treatment shortening: a model-based analysis. *BMC infectious diseases*, 16(1):726-731.

Goyens, P.L., Spilker, M.E., Zock, P.L., Katan, M.B. & Mensink, R.P. 2006. Conversion of α-linolenic acid in humans is influenced by the absolute amounts of α-linolenic acid and linoleic acid in the diet and not by their ratio. *The American journal of clinical nutrition*, 84(1):44-53.

Grobler, L., Nagpal, S., Sudarsanam, T.D. & Sinclair, D. 2016. Nutritional supplements for people being treated for active tuberculosis. *The Cochrane Library,* 6: DOI:10.1002/14651858.CD006086.pub4.

Guida, C., Altamura, S., Klein, F.A., Galy, B., Boutros, M., Ulmer, A.J., Hentze, M.W. & Muckenthaler, M.U. 2015. A novel inflammatory pathway mediating rapid hepcidin-independent hypoferremia. *Blood*, 125(14):2265-2275.

Guler, R., Parihar, S.P., Spohn, G., Johansen, P., Brombacher, F. & Bachmann, M.F. 2011. Blocking IL-1α but not IL-1β increases susceptibility to chronic Mycobacterium tuberculosis infection in mice. *Vaccine*, 29(6):1339-1346.

Guo, W., Bachman, E., Li, M., Roy, C.N., Blusztajn, J., Wong, S., Chan, S.Y., Serra, C., Jasuja, R. & Travison, T.G. 2013. Testosterone administration inhibits hepcidin transcription and is associated with increased iron incorporation into red blood cells. *Aging cell*, 12(2):280-291.

Gupta, A., Misra, A. & Deretic, V. 2016. Targeted pulmonary delivery of inducers of host macrophage autophagy as a potential host-directed chemotherapy of tuberculosis. *Advanced drug delivery reviews*, 102:10-20.

Gupta, S., Tyagi, S., Almeida, D.V., Maiga, M.C., Ammerman, N.C. & Bishai, W.R. 2013. Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. *American journal of respiratory and critical care medicine*, 188(5):600-607.

Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I. & Deretic, V. 2004. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell*, 119(6):753-766.

Hallberg, L., Brune, M., Erlandsson, M., Sandberg, A.-S. & Rossander-Hulten, L. 1991. Calcium: effect of different amounts on nonheme-and heme-iron absorption in humans. *The American journal of clinical nutrition*, 53(1):112-119.

Han, A.P., Yu, C., Lu, L., Fujiwara, Y., Browne, C., Chin, G., Fleming, M., Leboulch, P., Orkin, S.H. & Chen, J.J. 2001. Heme-regulated eIF2α kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. *The EMBO journal*, 20(23):6909-6918.

Harishankar, M., Afsal, K., Banurekha, V., Meenakshi, N. & Selvaraj, P. 2014. 1, 25-Dihydroxy vitamin D3 downregulates pro-inflammatory cytokine response in pulmonary tuberculosis. *International immunopharmacology*, 23(1):148-152.

Harms, K. & Kaiser, T. 2015. Beyond soluble transferrin receptor: old challenges and new horizons. *Best Practice & Research Clinical Endocrinology & Metabolism*, 29(5):799-810.

Harries, A., Lin, Y., Satyanarayana, S., Lönnroth, K., Li, L., Wilson, N., Chauhan, L., Zachariah, R., Baker, M. & Jeon, C. 2011. The looming epidemic of diabetes-associated tuberculosis: learning lessons from HIV-associated tuberculosis. *The international journal of tuberculosis and lung disease*, 15(11):1436-1445.

Harrington-Kandt, R., Stylianou, E., Eddowes, L.A., Lim, P.J., Stockdale, L., Pinpathomrat, N., Bull, N., Pasricha, J., Ulaszewska, M. & Beglov, Y. 2018. Hepcidin deficiency and iron deficiency do not alter tuberculosis susceptibility in a murine M. tb infection model. *PloS one*, 13(1):e0191038.

Harris, J. & Keane, J. 2010. How tumour necrosis factor blockers interfere with tuberculosis immunity. *Clin Exp Immunol*, 161.

Harris, W.S., Sands, S.A., Windsor, S.L., Ali, H.A., Stevens, T.L., Magalski, A., Porter, C.B. & Borkon, A.M. 2004. Omega-3 fatty acids in cardiac biopsies from heart transplantation patients: correlation with erythrocytes and response to supplementation. *Circulation*, 110(12):1645-1649.

Harris, W.S. & Von Schacky, C. 2004. The Omega-3 Index: a new risk factor for death from coronary heart disease? *Preventive medicine*, 39(1):212-220.

Hawn, T.R., Matheson, A.I., Maley, S.N. & Vandal, O. 2013. Host-directed therapeutics for tuberculosis: can we harness the host? *Microbiol. Mol. Biol. Rev.*, 77(4):608-627.

Hayashi, S. & Chandramohan, D. 2018. Risk of active tuberculosis among people with diabetes mellitus: systematic review and meta-analysis. *Tropical medicine & international health*, 23(10):1058-1070.

Health, D.o. 2014. National Tuberculosis Management Guidelines 2014.

Healy, D., Wallace, F., Miles, E., Calder, P. & Newsholme, P. 2000. Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. *Lipids*, 35(7):763-768.

Hella, J., Cercamondi, C.I., Mhimbira, F., Sasamalo, M., Stoffel, N., Zwahlen, M., Bodmer, T., Gagneux, S., Reither, K. & Zimmermann, M.B. 2018. Anemia in tuberculosis cases and household controls from Tanzania: Contribution of disease, coinfections, and the role of hepcidin. *PloS one*, 13(4):e0195985.

Hernandez-Pando, R., Orozco, H. & Mancilla, R. 1995. T-cell lung granulomas induced by sepharose-coupled Mycobacterium tuberculosis protein antigens: immunosuppressive phenomena reversed with cyclophosphamide and indomethacin. *Immunology*, 86(4):506.

Herzmann, C., Ernst, M., Ehlers, S., Stenger, S., Maertzdorf, J. & Sotgiu, G. 2012. Increased frequencies of pulmonary regulatory T-cells in latent Mycobacterium tuberculosis infection. *European respiratory journal*, 40:1450-1457.

Hillier, K., Jewell, R., Dorrell, L. & Smith, C. 1991. Incorporation of fatty acids from fish oil and olive oil into colonic mucosal lipids and effects upon eicosanoid synthesis in inflammatory bowel disease. *Gut*, 32(10):1151-1155.

Hodson, L., Skeaff, C.M. & Fielding, B.A.J.P.i.l.r. 2008. Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. 47(5):348-380.

Hoffmann, C., Leis, A., Niederweis, M., Plitzko, J.M. & Engelhardt, H. 2008. Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. *Proceedings of the National Academy of Sciences*, 105(10):3963-3967.

Hong, S., Gronert, K., Devchand, P.R., Moussignac, R.-L. & Serhan, C.N. 2003. Novel Docosatrienes and 17S-Resolvins Generated from Docosahexaenoic Acid in Murine Brain, Human Blood, and Glial Cells: AUTACOIDS IN ANTI-INFLAMMATION. *Journal of Biological Chemistry*, 278(17):14677-14687.

Hoppe, L.E., Kettle, R., Eisenhut, M., Abubakar, I. & Group, G.D. 2016. Tuberculosis—diagnosis, management, prevention, and control: summary of updated NICE guidance. *bmj*, 352:h6747.

Hou, Y., Zhang, S., Wang, L., Li, J., Qu, G., He, J., Rong, H., Ji, H. & Liu, S. 2012. Estrogen regulates iron homeostasis through governing hepatic hepcidin expression via an estrogen response element. *Gene*, 511(2):398-403.

Hrelia, S., Celadon, M., Rossi, C., Biagi, P. & Bordoni, A. 1990. Delta-6-desaturation of linoleic and alpha-linolenic acids in aged rats: a kinetic analysis. *Biochemistry international*, 22(4):659-667.

Hsu, D.C., Faldetta, K.F., Pei, L., Sheikh, V., Utay, N.S., Roby, G., Rupert, A., Fauci, A.S. & Sereti, I. 2015. A paradoxical treatment for a paradoxical condition: infliximab use in three cases of mycobacterial IRIS. *Clinical Infectious Diseases*, 62(2):258-261.

Hunter, R.L. 2016. Tuberculosis as a three-act play: A new paradigm for the pathogenesis of pulmonary tuberculosis. *Tuberculosis*, 97:8-17.

Ifeanyi, O.E. 2018. A Review on Iron Homeostasis and Anaemia in Pulmonary Tuberculosis. *International Journal of Healthcare and Medical Sciences*, 4(5):84-89.

Igarashi, M., DeMar, J.C., Ma, K., Chang, L., Bell, J.M. & Rapoport, S.I. 2007. Upregulated liver conversion of α-linolenic acid to docosahexaenoic acid in rats on a 15 week n-3 PUFA-deficient diet. *Journal of lipid research*, 48(1):152-164.

Innes, J.K. & Calder, P.C. 2018. Omega-6 fatty acids and inflammation. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 132:41-48.

Irwin, S.M., Driver, E., Lyon, E., Schrupp, C., Ryan, G., Gonzalez-Juarrero, M., Basaraba, R.J., Nuermberger, E.L. & Lenaerts, A.J. 2015. Presence of multiple lesion types with vastly different microenvironments in C3HeB/FeJ mice following aerosol infection with Mycobacterium tuberculosis. *Disease models & mechanisms*, 8(6):591-602.

Isanaka, S., Aboud, S., Mugusi, F., Bosch, R.J., Willett, W.C., Spiegelman, D., Duggan, C. & Fawzi, W.W. 2012. Iron status predicts treatment failure and mortality in tuberculosis patients: a prospective cohort study from Dar es Salaam, Tanzania. *PloS one*, 7(5):e37350.

Isanaka, S., Mugusi, F., Urassa, W., Willett, W.C., Bosch, R.J., Villamor, E., Spiegelman, D., Duggan, C. & Fawzi, W.W. 2011. Iron deficiency and anemia predict mortality in patients with tuberculosis. *The Journal of nutrition*, 142(2):350-357.

Isobe, Y., Arita, M., Matsueda, S., Iwamoto, R., Fujihara, T., Nakanishi, H., Taguchi, R., Masuda, K., Sasaki, K. & Urabe, D. 2012. Identification and structure determination of novel anti-inflammatory mediator resolvin E3, 17, 18-dihydroxyeicosapentaenoic acid. *Journal of Biological Chemistry*, 287(13):10525-10534.

ISSFAL. 2004. Recommendations for intake of polyunsaturated fatty acids in healthy adults. Brighton, UK.

Ivanyi, J. & Zumla, A. 2013. Nonsteroidal antiinflammatory drugs for adjunctive tuberculosis treatment. *Journal infectious diseases*, 208(2):185-188.

Jabara, H.H., Boyden, S.E., Chou, J., Ramesh, N., Massaad, M.J., Benson, H., Bainter, W., Fraulino, D., Rahimov, F. & Sieff, C. 2016. A missense mutation in TFRC, encoding transferrin receptor 1, causes combined immunodeficiency. *Nature genetics*, 48(1):74-82.

Jakiela, B., Gielicz, A., Plutecka, H., Hubalewska, M., Mastalerz, L., Bochenek, G., Soja, J., Januszek, R., Musial, J. & Sanak, M. 2013. Eicosanoid biosynthesis during mucociliary and mucous metaplastic differentiation of bronchial epithelial cells. *Prostaglandins & other lipid mediators*, 106:116-123.

Jamieson, J.A., Kuhnlein, H.V., Weiler, H.A. & Egeland, G.M. 2013. Higher n3-fatty acid status is associated with lower risk of iron depletion among food insecure Canadian Inuit women. *BMC Public Health*, 13(1):289.

Jangale, N.M., Devarshi, P.P., Bansode, S.B., Kulkarni, M.J. & Harsulkar, A.M. 2016. Dietary flaxseed oil and fish oil ameliorates renal oxidative stress, protein glycation, and inflammation in streptozotocin–nicotinamide-induced diabetic rats. *Journal of physiology and biochemistry*, 72(2):327-336.

Jaudszus, A., Gruen, M., Watzl, B., Ness, C., Roth, A., Lochner, A., Barz, D., Gabriel, H., Rothe, M. & Jahreis, G. 2013. Evaluation of suppressive and pro-resolving effects of EPA and DHA in human primary monocytes and T-helper cells. *Journal of lipid research*, 54(4):923-935.

Jenkitkasemwong, S., Wang, C.-Y., Coffey, R., Zhang, W., Chan, A., Biel, T., Kim, J.-S., Hojyo, S., Fukada, T. & Knutson, M.D. 2015. SLC39A14 is required for the development of hepatocellular iron overload in murine models of hereditary hemochromatosis. *Cell metabolism*, 22(1):138-150.

Jeong, Y.J. & Lee, K.S. 2008. Pulmonary tuberculosis: up-to-date imaging and management. *American Journal of Roentgenology*, 191(3):834-844.

Jick, S.S., Lieberman, E.S., Rahman, M.U. & Choi, H.K. 2006. Glucocorticoid use, other associated factors, and the risk of tuberculosis. *Arthritis Care & Research*, 55(1):19-26.

Johnson, E.E. & Wessling-Resnick, M. 2012. Iron metabolism and the innate immune response to infection. *Microbes and infection*, 14(3):207-216.

Jolly, C.A., Jiang, Y.-H., Chapkin, R.S. & McMurray, D.N. 1997. Dietary (n-3) polyunsaturated fatty acids suppress murine lymphoproliferation, interleukin-2 secretion, and the formation of diacylglycerol and ceramide. *The Journal of nutrition*, 127(1):37-43.

Jones, M.L., Mark, P.J., Keelan, J.A., Barden, A., Mas, E., Mori, T.A. & Waddell, B.J. 2013. Maternal dietary omega-3 fatty acid intake increases resolvin and protectin levels in the rat placenta. *Journal of lipid research*:jlr. M039842.

Jonker, F.A. & van Hensbroek, M.B. 2014. Anaemia, iron deficiency and susceptibility to infections. *Journal of Infection*, 69:S23-S27.

Jordao, L., Lengeling, A., Bordat, Y., Boudou, F., Gicquel, B., Neyrolles, O., Becker, P.D., Guzman, C.A., Griffiths, G. & Anes, E. 2008. Effects of omega-3 and-6 fatty acids on Mycobacterium tuberculosis in macrophages and in mice. *Microbes and Infection*, 10(12):1379-1386.

Jorge, J.-H., Graciela, C., Pablo, A.-P. & Luis, S.-H.J. 2012. A Life-Threatening Central Nervous System–Tuberculosis Inflammatory Reaction Nonresponsive to Corticosteroids and Successfully Controlled by Infliximab in a Young Patient With a Variant of Juvenile Idiopathic Arthritis. *JCR: Journal of Clinical Rheumatology*, 18(4):189-191.

Juffermans, N.P., Camoglio, L., van Deventer, S.J.H., Verbon, A., van der Poll, T., Speelman, P., Florquin, S. & Kolk, A.H. 2000. Interleukin-1 Signaling Is Essential for Host Defense during Murine Pulmonary Tuberculosis. *The Journal of Infectious Diseases*, 182(3):902-908.

Jump, D.B., Tripathy, S. & Depner, C.M.J.A.r.o.n. 2013. Fatty acid–regulated transcription factors in the liver. 33:249-269.

Kalinski, P. 2012. Regulation of immune responses by prostaglandin E2. *The Journal of Immunology*, 188(1):21-28.

Kant, S., Gupta, H. & Ahluwalia, S. 2015. Significance of Nutrition in Pulmonary Tuberculosis. *Critical Reviews in Food Science & Nutrition*, 55(7):955-963.

Kaplan, G., Post, F.A., Moreira, A.L., Wainwright, H., Kreiswirth, B.N., Tanverdi, M., Mathema, B., Ramaswamy, S.V., Walther, G. & Steyn, L.M. 2003. Mycobacterium tuberculosis growth at the cavity surface: a microenvironment with failed immunity. *Infection and immunity*, 71(12):7099-7108.

Karakonstantis, S., Emmanouilidou, E., Petraki, K. & Lydakis, C. 2019. Central nervous system tuberculosis reactivation following intravenous iron supplementation. *International journal of mycobacteriology*, 8(1):104.

Karyadi, E., Schultink, W., Nelwan, R.H., Gross, R., Amin, Z., Dolmans, W.M., van der Meer, J.W., Hautvast, J.G.J. & West, C.E. 2000. Poor micronutrient status of active pulmonary tuberculosis patients in Indonesia. *The Journal of nutrition*, 130(12):2953-2958.

Karyadi, E., West, C.E., Schultink, W., Nelwan, R.H., Gross, R., Amin, Z., Dolmans, W.M., Schlebusch, H. & van der Meer, J.W. 2002. A double-blind, placebo-controlled study of vitamin A and zinc supplementation in persons with tuberculosis in Indonesia: effects on clinical response and nutritional status. *The American journal of clinical nutrition*, 75(4):720-727.

Kasuga, K., Yang, R., Porter, T.F., Agrawal, N., Petasis, N.A., Irimia, D., Toner, M. & Serhan, C.N. 2008. Rapid appearance of resolvin precursors in inflammatory exudates: novel mechanisms in resolution. *The Journal of Immunology*, 181(12):8677-8687.

Katan, M.B., Deslypere, J., Van Birgelen, A., Penders, M. & Zegwaard, M. 1997. Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes, and adipose tissue: an 18-month controlled study. *Journal of lipid research*, 38(10):2012-2022.

Kaufmann, S.H. 2003. Immune response to tuberculosis: experimental animal models. *Tuberculosis*, 83(1):107-111.

Kaufmann, S.H. 2005. Robert Koch, the Nobel Prize, and the ongoing threat of tuberculosis. *New England Journal of Medicine*, 353(23):2423-2426.

Kaufmann, S.H., Dorhoi, A., Hotchkiss, R.S. & Bartenschlager, R. 2018. Host-directed therapies for bacterial and viral infections. *Nature Reviews Drug Discovery*, 17(1):35-40.

Kaufmann, S.H.E. & Dorhoi, A. 2013. Inflammation in tuberculosis: interactions, imbalances and interventions. *Current Opinion in Immunology*, 25(4):441-449.

Kaul, V., Bhattacharya, D., Singh, Y., Van Kaer, L., Peters-Golden, M., Bishai, W.R. & Das, G. 2012. An important role of prostanoid receptor EP2 in host resistance to Mycobacterium tuberculosis infection in mice. *The Journal of infectious diseases*, 206(12):1816-1825.

Kaveh, M., Eftekhar, N. & Boskabady, M.H. 2019. The effect of alpha linolenic acid on tracheal responsiveness, lung inflammation, and immune markers in sensitized rats. *Iranian journal of basic medical sciences*, 22(3):255-261.

Keane, J., Gershon, S., Wise, R.P., Mirabile-Levens, E., Kasznica, J., Schwieterman, W.D., Siegel, J.N. & Braun, M.M. 2001. Tuberculosis associated with infliximab, a tumor necrosis factor α–neutralizing agent. *New England Journal of Medicine*, 345(15):1098-1104.

Keelan, J.A., Mas, E., D'Vaz, N., Dunstan, J.A., Li, S., Barden, A., Mark, P.J., Waddell, B.J., Prescott, S.L. & Mori, T.A. 2014. Effects of maternal n-3 fatty acid supplementation on placental pro-resolving lipid mediators. *Reproduction*, 149:171-178.

Keeton, R., Allie, N., Dambuza, I., Abel, B., Hsu, N.-J., Sebesho, B., Randall, P., Burger, P., Fick, E. & Quesniaux, V.F. 2014. Soluble TNFRp75 regulates host protective immunity against Mycobacterium tuberculosis. *The Journal of clinical investigation*, 124(4):1537-1542.

Kerkhoff, A., Meintjes, G., Opie, J., Vogt, M., Jhilmeet, N., Wood, R. & Lawn, S. 2016. Anaemia in patients with HIV-associated TB: relative contributions of anaemia of chronic disease and iron deficiency. *The international journal of tuberculosis and lung disease*, 20(2):193-201.

Kerkhoff, A.D., Meintjes, G., Burton, R., Vogt, M., Wood, R. & Lawn, S.D. 2015. Relationship between blood concentrations of hepcidin and anemia severity, mycobacterial burden, and mortality among patients with HIV-associated tuberculosis. *The Journal of infectious diseases*, 213(1):61-70.

Kew, S., Banerjee, T., Minihane, A.M., Finnegan, Y.E., Muggli, R., Albers, R., Williams, C.M. & Calder, P.C. 2003. Lack of effect of foods enriched with plant-or marine-derived n− 3 fatty acids on human immune function. *The American journal of clinical nutrition*, 77(5):1287-1295.

Kew, S., Mesa, M.D., Tricon, S., Buckley, R., Minihane, A.M. & Yaqoob, P. 2004. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. *The American journal of clinical nutrition*, 79(4):674-681.

Khader, S.A., Partida-Sanchez, S., Bell, G., Jelley-Gibbs, D.M., Swain, S., Pearl, J.E., Ghilardi, N., deSauvage, F.J., Lund, F.E. & Cooper, A.M. 2006. Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection. *The Journal of Experimental Medicine*, 203(7):1805.

Khadge, S., Thiele, G.M., Sharp, J.G., McGuire, T.R., Klassen, L.W., Black, P.N., DiRusso, C.C. & Talmadge, J.E. 2018. Long-Chain Omega-3 Polyunsaturated Fatty Acids Modulate Mammary Gland Composition and Inflammation. *Journal of Mammary Gland Biology and Neoplasia*, 23(1):43-58.

Kim, J.-J., Lee, H.-M., Shin, D.-M., Kim, W., Yuk, J.-M., Jin, H.S., Lee, S.-H., Cha, G.-H., Kim, J.-M. & Lee, Z.-W. 2012. Host cell autophagy activated by antibiotics is required for their effective antimycobacterial drug action. *Cell host & microbe*, 11(5):457-468.

Kim, W., Khan, N.A., McMurray, D.N., Prior, I.A., Wang, N. & Chapkin, R.S. 2010. Regulatory activity of polyunsaturated fatty acids in T-cell signaling. *Progress in lipid research*, 49(3):250-261.

Kim, Y.-R. & Yang, C.-S. 2017. Host-directed therapeutics as a novel approach for tuberculosis treatment. *Journal of Microbiology Biotechnology*, 27(9):1549-1558.

Kiss, L., Schütte, H., Padberg, W., Weissmann, N., Mayer, K., Gessler, T., Voswinckel, R., Seeger, W. & Grimminger, F. 2010. Epoxyeicosatrienoates are the dominant eicosanoids in human lungs upon microbial challenge. *European Respiratory Journal*, 36(5):1088-1098.

Kochan, I. 1973. The role of iron in bacterial infections, with special consideration of host-tubercle bacillus interaction. *Current topics in microbiology and immunology*, 60:1-30.

Koekkoek, W.A.C., Panteleon, V. & van Zanten, A.R.H. 2019. Current evidence on ω-3 fatty acids in enteral nutrition in the critically ill: A systematic review and meta-analysis. *Nutrition*, 59:56-68.

Kolloli, A., Singh, P., Rodriguez, G.M. & Subbian, S. 2019. Effect of Iron Supplementation on the Outcome of Non-Progressive Pulmonary Mycobacterium tuberculosis Infection. *Journal of clinical medicine*, 8(8):1155.

Kota, S.K., Jammula, S., Kota, S.K., Tripathy, P.R., Panda, S. & Modi, K.D. 2011. Effect of vitamin D supplementation in type 2 diabetes patients with pulmonary tuberculosis. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, 5(2):85-89.

Koulaouzidis, A., Said, E., Cottier, R. & Saeed, A.A. 2009. Soluble transferrin receptors and iron deficiency, a step beyond ferritin. A systematic review. *Journal of gastrointestinal and liver disease*, 18(3):345-352.

Koutroubakis, I.E., Ramos-Rivers, C., Regueiro, M., Koutroumpakis, E., Click, B., Schwartz, M., Swoger, J., Baidoo, L., Hashash, J.G. & Barrie, A. 2015. The Influence of Anti–tumor Necrosis Factor Agents on Hemoglobin Levels of Patients with Inflammatory Bowel Disease. *Inflammatory bowel diseases*, 21(7):1587-1593.

Krajčovičová-Kudláčková, M., Klvanova, J. & Dušinská, M. 2004. Polyunsaturated Fatty Acid Plasma Content in Groups of General Population with lowvitamin B6 or low iron serum levels. *Annals of nutrition and metabolism*, 48(2):118-121.

Kramnik, I. & Beamer, G. 2016. Mouse models of human TB pathology: roles in the analysis of necrosis and the development of host-directed therapies. *Seminars in Immunopathology*, 38(2):221-237.

Kroesen, V.M., Gröschel, M.I., Martinson, N., Zumla, A., Maeurer, M., van der Werf, T.S. & Vilaplana, C. 2017. Non-Steroidal Anti-inflammatory Drugs As Host-Directed Therapy for Tuberculosis: A Systematic Review. *Frontiers in immunology*, 8:772.

Kroesen, V.M., Rodríguez-Martínez, P., García, E., Rosales, Y., Díaz, J., Martín-Céspedes, M., Tapia, G., Sarrias, M.-R., Cardona, P.-J. & Vilaplana, C. 2018. A Beneficial Effect of Low-Dose Aspirin in a Murine Model of Active Tuberculosis. *Frontiers in immunology*, 9:798.

Krutzik, S.R., Tan, B., Li, H., Ochoa, M.T., Liu, P.T. & Sharfstein, S.E. 2005. TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells. *Nat Med*, 11.

Kuhn, H., Saam, J., Eibach, S., Holzhütter, H.-G., Ivanov, I. & Walther, M. 2005. Structural biology of mammalian lipoxygenases: enzymatic consequences of targeted alterations of the protein structure. *Biochemical and biophysical research communications*, 338(1):93-101.

Kumar, A., Farhana, A., Guidry, L., Saini, V., Hondalus, M. & Steyn, A.J. 2011. Redox homeostasis in mycobacteria: the key to tuberculosis control? *Expert reviews in molecular medicine*, 13.

Kumar, N.P., Kadar Moideen, A.N., Viswanathan, V., Shruthi, B.S., Shanmugam, S., Hissar, S., Kornfeld, H. & Babu, S. 2019a. Plasma Eicosanoid Levels in Tuberculosis and Tuberculosis-Diabetes Co-morbidity Are Associated With Lung Pathology and Bacterial Burden. *Frontiers in Cellular and Infection Microbiology*, 9.

Kumar, N.P., Moideen, K., Banurekha, V.V., Nair, D. & Babu, S. 2019b. Plasma pro-inflammatory cytokines are markers of disease severity and bacterial burden in pulmonary tuberculosis. *Open forum infectious diseases*, DOI:10.1093/ofid/ofz257.

Kumar, P. 2016. Adult pulmonary tuberculosis as a pathological manifestation of hyperactive antimycobacterial immune response. *Clinical and Translational Medicine*, 5(1):38.

Kurthkoti, K., Amin, H., Marakalala, M.J., Ghanny, S., Subbian, S., Sakatos, A., Livny, J., Fortune, S.M., Berney, M. & Rodriguez, G.M. 2017. The capacity of Mycobacterium tuberculosis to survive iron starvation might enable it to persist in iron-deprived microenvironments of human granulomas. *MBio*, 8(4):e01092-01017.

Kuvibidila, S.R., Baliga, B.S., Warrier, R.P. & Suskind, R.M. 1998. Iron deficiency reduces the hydrolysis of cell membrane phosphatidyl inositol-4, 5-bisphosphate during splenic lymphocyte activation in C57BL/6 mice. *The Journal of nutrition*, 128(7):1077-1083.

Lands, W.E. 2005. Fish, omega-3 and human health: AOCS Publishing.

Lands, W.E., Morris, A. & Libelt, B. 1990. Quantitative effects of dietary polyunsaturated fats on the composition of fatty acids in rat tissues. *Lipids*, 25(9):505-516.

Lane, D., Bae, D.-H., Merlot, A., Sahni, S. & Richardson, D. 2015a. Duodenal cytochrome b (DCYTB) in iron metabolism: an update on function and regulation. *Nutrients*, 7(4):2274-2296.

Lane, D., Merlot, A., Huang, M.-H., Bae, D.-H., Jansson, P., Sahni, S., Kalinowski, D. & Richardson, D. 2015b. Cellular iron uptake, trafficking and metabolism: key molecules and mechanisms and their roles in disease. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1853(5):1130-1144.

Lanoix, J.-P., Lenaerts, A.J. & Nuermberger, E.L. 2015. Heterogeneous disease progression and treatment response in a C3HeB/FeJ mouse model of tuberculosis. *Disease models & mechanisms*, 8(6):603-610.

Lawn, S.D., Wood, R. & Wilkinson, R.J. 2010. Changing concepts of “latent tuberculosis infection” in patients living with HIV infection. *Clinical and developmental immunology*, 2011: doi:10.1155/2011/980594.

Le, H.D., Meisel, J.A., de Meijer, V.E., Gura, K.M. & Puder, M. 2009. The essentiality of arachidonic acid and docosahexaenoic acid. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 81(3):165-170.

LeBlanc, C.P., Fiset, S., Surette, M.E., Turgeon O'Brien, H. & Rioux, F.M. 2009. Maternal iron deficiency alters essential fatty acid and eicosanoid metabolism and increases locomotion in adult guinea pig offspring. *The Journal of nutrition*, 139(9):1653-1659.

Lee, C.R. & Zeldin, D.C. 2015. Resolvin infectious inflammation by targeting the host response. *New England Journal of Medicine*, 373(22):2183-2185.

Lee, H.-S., Lee, Y., Lee, S.-O., Choi, S.-H., Kim, Y.S., Woo, J.H. & Kim, S.-H. 2012. Adalimumab treatment may replace or enhance the activity of steroids in steroid-refractory tuberculous meningitis. *Journal of Infection and Chemotherapy*, 18(4):555-557.

Lee, P., Peng, H., Gelbart, T., Wang, L. & Beutler, E. 2005. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. *Proceedings of the National Academy of Sciences*, 102(6):1906-1910.

Lee, S.W., Kang, Y., Yoon, Y.S., Um, S.-W., Lee, S.M., Yoo, C.-G., Kim, Y.W., Han, S.K., Shim, Y.-S. & Yim, J.-J. 2006. The prevalence and evolution of anemia associated with tuberculosis. *Journal of Korean medical science*, 21(6):1028-1032.

Lee, W., VanderVen, B.C., Fahey, R.J. & Russell, D.G. 2013. Intracellular Mycobacterium tuberculosis exploits host-derived fatty acids to limit metabolic stress. *Journal of Biological Chemistry*, 288(10):6788-6800.

Lenaerts, A., Barry III, C.E. & Dartois, V. 2015. Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunological reviews*, 264(1):288-307.

Lerna, M., Kerr, A., Scales, H., Berge, K. & Griinari, M. 2010. Supplementation of diet with krill oil protects against experimental rheumatoid arthritis. *BMC musculoskeletal disorders*, 11(1):136.

Leslie, C.A., Gonnerman, W.A., Ullman, M.D., Hayes, K., Franzblau, C. & Cathcart, E.S. 1985. Dietary fish oil modulates macrophage fatty acids and decreases arthritis susceptibility in mice. *Journal of Experimental Medicine*, 162(4):1336-1349.

Levy, B.D. & Serhan, C.N. 2014. Resolution of acute inflammation in the lung. *Annual review of physiology*, 76:467-492.

Libregts, S.F., Gutiérrez, L., de Bruin, A.M., Wensveen, F.M., Papadopoulos, P., van Ijcken, W., Özgür, Z., Philipsen, S. & Nolte, M.A. 2011. Chronic IFN-γ production in mice induces anemia by reducing erythrocyte life span and inhibiting erythropoiesis through an IRF-1/PU. 1 axis. *Blood*, 118(9):2578-2588.

Lin, P.L., Ford, C.B., Coleman, M.T., Myers, A.J., Gawande, R., Ioerger, T., Sacchettini, J., Fortune, S.M. & Flynn, J.L. 2014. Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing. *Nature medicine*, 20(1):75.

Lin, P.L., Myers, A., Smith, L.K., Bigbee, C., Bigbee, M., Fuhrman, C., Grieser, H., Chiosea, I., Voitenek, N.N. & Capuano, S.V. 2010. Tumor necrosis factor neutralization results in disseminated disease in acute and latent Mycobacterium tuberculosis infection with normal granuloma structure in a cynomolgus macaque model. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, 62(2):340-350.

Lin, P.L., Plessner, H.L., Voitenok, N.N. & Flynn, J.L. 2007. Tumor necrosis factor and tuberculosis. (*In*. Journal of Investigative Dermatology Symposium Proceedings organised by: Elsevier. p. 22-25).

Lin, Y.H. & Salem, N. 2007. Whole body distribution of deuterated linoleic and α-linolenic acids and their metabolites in the rat. *Journal of Lipid Research*, 48(12):2709-2724.

Liston, A. & Gray, D.H. 2014. Homeostatic control of regulatory T cell diversity. *Nature Reviews Immunology*, 14(3):154-165.

Lorent, J.H., Diaz-Rohrer, B., Lin, X., Spring, K., Gorfe, A.A., Levental, K.R. & Levental, I. 2018. Author Correction: Structural determinants and functional consequences of protein affinity for membrane rafts. *Nature communications*, 9(1):1805.

Lounis, N., Truffot-Pernot, C., Grosset, J., Gordeuk, V.R. & Boelaert, J.R. 2001. Iron and Mycobacterium tuberculosis infection. *Journal of Clinical Virology*, 20(3):123-126.

Lovewell, R.R., Sassetti, C.M. & VanderVen, B.C. 2016. Chewing the fat: lipid metabolism and homeostasis during M. tuberculosis infection. *Current Opinion in Microbiology*, 29(Supplement C):30-36.

Lowe, D.M., Bandara, A.K., Packe, G.E., Barker, R.D., Wilkinson, R.J., Griffiths, C.J. & Martineau, A.R. 2013. Neutrophilia independently predicts death in tuberculosis. *European Respiratory Journal*, 42(6):1752-1757.

Lowe, D.M., Redford, P.S., Wilkinson, R.J., O'Garra, A. & Martineau, A.R. 2012. Neutrophils in tuberculosis: friend or foe? *Trends in immunology*, 33(1):14-25.

Lukaski, H.C., Bolonchuk, W.W., Klevay, L.M., Milne, D.B. & Sandstead, H.H. 2001. Interactions among dietary fat, mineral status, and performance of endurance athletes: a case study. *International journal of sport nutrition and exercise metabolism*, 11(2):186-198.

Lundström, S.L., Yang, J., Brannan, J.D., Haeggström, J.Z., Hammock, B.D., Nair, P., O'byrne, P., Dahlén, S.E. & Wheelock, C.E. 2013. Lipid mediator serum profiles in asthmatics significantly shift following dietary supplementation with omega-3 fatty acids. *Molecular nutrition & food research*, 57(8):1378-1389.

Ma, D.W., Seo, J., Switzer, K.C., Fan, Y.-Y., McMurray, D.N., Lupton, J.R. & Chapkin, R.S. 2004. n− 3 PUFA and membrane microdomains: a new frontier in bioactive lipid research. *The Journal of nutritional biochemistry*, 15(11):700-706.

MacAllan, D.C., McNurlan, M.A., Kurpad, A.V., De Souza, G., Shetty, P.S., Calder, A.G. & Griffin, G.E. 1998. Whole body protein metabolism in human pulmonary tuberculosis and undernutrition: evidence for anabolic block in tuberculosis. *Clinical science*, 94(3):321-331.

Macdougall, I.C. & Cooper, A.C. 2002. Erythropoietin resistance: the role of inflammation and pro-inflammatory cytokines. *Nephrology Dialysis Transplantation*, 17(S11):39-43.

Madigan, C.A., Martinot, A.J., Wei, J.-R., Madduri, A., Cheng, T.-Y., Young, D.C., Layre, E., Murry, J.P., Rubin, E.J. & Moody, D.B. 2015. Lipidomic analysis links mycobactin synthase K to iron uptake and virulence in M. tuberculosis. *PLoS pathogens*, 11(3):e1004792.

Mahan, L.K. & Raymond, J.L. 2017. Krause's Food and Nutrition therapy. 14. Missouri: Elsevier.

Malan, L., Baumgartner, J., Calder, P.C., Zimmermann, M.B. & Smuts, C.M. 2014. n–3 Long-chain PUFAs reduce respiratory morbidity caused by iron supplementation in iron-deficient South African schoolchildren: a randomized, double-blind, placebo-controlled intervention. *The American journal of clinical nutrition*, 101(3):668-679.

Malan, L., Baumgartner, J., Zandberg, L., Calder, P. & Smuts, C. 2016. Iron and a mixture of dha and epa 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. *Prostaglandins, Leukotrienes and essential fatty acids*, 105:15-25.

Manary, M.J., Yarasheski, K.E., Berger, R., Abrams, E.T., Hart, C.A. & Broadhead, R.L. 2004. Whole-body leucine kinetics and the acute phase response during acute infection in marasmic Malawian children. *Pediatric research*, 55(6):940-946.

Manca, C., Koo, M.-S., Peixoto, B., Fallows, D., Kaplan, G. & Subbian, S. 2013. Host targeted activity of pyrazinamide in Mycobacterium tuberculosis infection. *PloS one*, 8(8):e74082.

Manca, C., Tsenova, L., Freeman, S., Barczak, A.K., Tovey, M., Murray, P.J., Barry III, C. & Kaplan, G. 2005. Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. *Journal of Interferon & Cytokine Research*, 25(11):694-701.

Manji, M., Shayo, G., Mamuya, S., Mpembeni, R., Jusabani, A. & Mugusi, F. 2016. Lung functions among patients with pulmonary tuberculosis in Dar es Salaam–a cross-sectional study. *BMC pulmonary medicine*, 16(1):58.

Marakalala, M.J., Raju, R.M., Sharma, K., Zhang, Y.J., Eugenin, E.A., Prideaux, B., Daudelin, I.B., Chen, P.-Y., Booty, M.G., Kim, J.H., Eum, S.Y., Via, L.E., Behar, S.M., Barry, C.E., 3rd, Mann, M., Dartois, V. & Rubin, E.J. 2016. Inflammatory signaling in human tuberculosis granulomas is spatially organized. *Nature medicine*, 22(5):531-538.

Martens, G.W., Arikan, M.C., Lee, J., Ren, F., Vallerskog, T. & Kornfeld, H. 2008. Hypercholesterolemia Impairs Immunity to Tuberculosis. *Infection and Immunity*, 76(8):3464-3472.

Martineau, A.R., Newton, S.M., Wilkinson, K.A., Kampmann, B., Hall, B.M., Nawroly, N., Packe, G.E., Davidson, R.N., Griffiths, C.J. & Wilkinson, R.J. 2007. Neutrophil-mediated innate immune resistance to mycobacteria. *The Journal of clinical investigation*, 117(7):1988-1994.

Martineau, A.R., Timms, P.M., Bothamley, G.H., Hanifa, Y., Islam, K., Claxton, A.P., Packe, G.E., Moore-Gillon, J.C., Darmalingam, M. & Davidson, R.N. 2011. High-dose vitamin D3 during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial. *The Lancet*, 377(9761):242-250.

Marzo, E., Vilaplana, C., Tapia, G., Diaz, J., Garcia, V. & Cardona, P.-J. 2014. Damaging role of neutrophilic infiltration in a mouse model of progressive tuberculosis. *Tuberculosis*, 94(1):55-64.

Mas, E., Barden, A., Burke, V., Beilin, L.J., Watts, G.F., Huang, R.-C., Puddey, I.B., Irish, A.B. & Mori, T.A. 2016. A randomized controlled trial of the effects of n-3 fatty acids on resolvins in chronic kidney disease. *Clinical Nutrition*, 35(2):331-336.

Masi, L.N., Rodrigues, A.C. & Curi, R. 2013. Fatty acids regulation of inflammatory and metabolic genes. *Current Opinion in Clinical Nutrition & Metabolic Care*, 16(4):418-424.

Masoodi, M., Pearl, D.S., Eiden, M., Shute, J.K., Brown, J.F., Calder, P.C. & Trebble, T.M. 2013. Altered colonic mucosal polyunsaturated fatty acid (PUFA) derived lipid mediators in ulcerative colitis: new insight into relationship with disease activity and pathophysiology. *PLoS One*, 8(10):e76532.

Mayer-Barber, K.D., Andrade, B.B., Barber, D.L., Hieny, S., Feng, C.G., Caspar, P., Oland, S., Gordon, S. & Sher, A. 2011. Innate and adaptive interferons suppress IL-1α and IL-1β production by distinct pulmonary myeloid subsets during Mycobacterium tuberculosis infection. *Immunity*, 35(6):1023-1034.

Mayer-Barber, K.D., Andrade, B.B., Oland, S.D., Amaral, E.P., Barber, D.L., Gonzales, J., Derrick, S.C., Shi, R., Kumar, N.P. & Wei, W. 2014. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature*, 511(7507):99.

Mayer-Barber, K.D. & Sher, A. 2015. Cytokine and lipid mediator networks in tuberculosis. *Immunological Reviews*, 264(1):264-275.

Mayeur, S., Spahis, S., Pouliot, Y. & Levy, E. 2016. Lactoferrin, a pleiotropic protein in health and disease. *Antioxidants & redox signaling*, 24(14):813-836.

McFarland, C.T., Fan, Y.-Y., Chapkin, R.S., Weeks, B.R. & McMurray, D.N. 2008. Dietary polyunsaturated fatty acids modulate resistance to Mycobacterium tuberculosis in guinea pigs. *The Journal of nutrition*, 138(11):2123-2128.

McGlory, C., Galloway, S.D., Hamilton, D.L., McClintock, C., Breen, L., Dick, J.R., Bell, J.G. & Tipton, K.D. 2014. Temporal changes in human skeletal muscle and blood lipid composition with fish oil supplementation. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)*, 90(6):199-206.

Medlar, E. 1955. The behavior of pulmonary tuberculosis lesions: a pathological study. *Am Rev Tuberc.*, 72(1):1-244.

Meena, L. & Sharma, S. 2016. Foamy Macrophages in Mycobacterium tuberculosis Pathogenesis. *Journal of Molecular Biology and Biotechnology*, 2(3):1.

Meghji, J., Simpson, H., Squire, S.B. & Mortimer, K. 2016. A systematic review of the prevalence and pattern of imaging defined post-TB lung disease. *PloS one*, 11(8):e0161176.

Meraviglia, S., El Daker, S., Dieli, F., Martini, F. & Martino, A. 2011. γδ T cells cross-link innate and adaptive immunity in Mycobacterium tuberculosis infection. *Clinical and Developmental Immunology*, 2011:doi:10.1155/2011/587315.

Metherel, A., Armstrong, J., Patterson, A. & Stark, K. 2009. Assessment of blood measures of n-3 polyunsaturated fatty acids with acute fish oil supplementation and washout in men and women. *Prostaglandins, leukotrienes and essential fatty acids*, 81(1):23-29.

Metherel, A.H. & Stark, K.D.J.T.J.o.n. 2015. Cryopreservation prevents iron-initiated highly unsaturated fatty acid loss during storage of human blood on chromatography paper at− 20 C. 145(3):654-660.

Meydani, S.N., Endres, S., Woods, M.M., Goldin, B.R., Soo, C., Morrill-Labrode, A., Dinarello, C.A. & Gorbach, S.L. 1991. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. *The Journal of nutrition*, 121(4):547-555.

Meyer, B.J., Mann, N.J., Lewis, J.L., Milligan, G.C., Sinclair, A.J. & Howe, P.R. 2003. Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. *Lipids*, 38(4):391-398.

Micha, R., Khatibzadeh, S., Shi, P., Fahimi, S., Lim, S., Andrews, K.G., Engell, R.E., Powles, J., Ezzati, M. & Mozaffarian, D. 2014. Global, regional, and national consumption levels of dietary fats and oils in 1990 and 2010: a systematic analysis including 266 country-specific nutrition surveys. *BMJ : British Medical Journal*, 348:g2272.

Miles, E.A., Banerjee, T., Dooper, M.M., M'Rabet, L., Graus, Y.M. & Calder, P.C. 2004. The influence of different combinations of γ-linolenic acid, stearidonic acid and EPA on immune function in healthy young male subjects. *British Journal of Nutrition*, 91(6):893-903.

Miles, E.A. & Calder, P.C. 2012. Influence of marine n-3 polyunsaturated fatty acids on immune function and a systematic review of their effects on clinical outcomes in rheumatoid arthritis. *British Journal of Nutrition*, 107(S2):S171-S184.

Millonig, G., Ganzleben, I., Peccerella, T., Casanovas, G., Brodziak-Jarosz, L., Breitkopf-Heinlein, K., Dick, T.P., Seitz, H.-K., Muckenthaler, M.U. & Mueller, S. 2012. Sustained submicromolar H2O2 levels induce hepcidin via signal transducer and activator of transcription 3 (STAT3). *Journal of Biological Chemistry*, 287(44):37472-37482.

Mills, E.L. & O'Neill, L.A. 2016. Reprogramming mitochondrial metabolism in macrophages as an anti-inflammatory signal. *European journal of immunology*, 46(1):13-21.

Mily, A., Rekha, R.S., Kamal, S.M., Arifuzzaman, A.S.M., Rahim, Z., Khan, L., Haq, M.A., Zaman, K., Bergman, P. & Brighenti, S. 2015. Significant effects of oral phenylbutyrate and vitamin D3 adjunctive therapy in pulmonary tuberculosis: a randomized controlled trial. *PLoS One*, 10(9):e0138340.

Minchella, P.A., Donkor, S., Owolabi, O., Sutherland, J.S. & McDermid, J.M. 2014. Complex anemia in tuberculosis: the need to consider causes and timing when designing interventions. *Clinical Infectious Diseases*, 60(5):764-772.

Miossec, P. 2018. Reactivation of tuberculosis during treatment with inhibitors of TNF. *La Revue du praticien*, 68(5):537-540.

Miret, S., Saiz, M. & Mitjavila, M. 2003. Effects of fish oil-and olive oil-rich diets on iron metabolism and oxidative stress in the rat. *British journal of nutrition*, 89(1):11-18.

Mishra, S., Taparia, M.P., Yadav, D. & Koolwal, S. 2018. Study of Iron Metabolism in Pulmonary Tuberculosis Patients. *International journal of health sciences and research,* 8(3):70-77.

Misra, U., Kalita, J. & Nair, P. 2010. Role of aspirin in tuberculous meningitis: a randomized open label placebo controlled trial. *Journal of the neurological sciences*, 293(1-2):12-17.

Miyata, J. & Arita, M. 2015. Role of omega-3 fatty acids and their metabolites in asthma and allergic diseases. *Allergology International*, 64(1):27-34.

Mogues, T., Goodrich, M.E., Ryan, L., LaCourse, R. & North, R.J. 2001. The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice. *Journal of Experimental Medicine*, 193(3):271-280.

Mohrhauer, H. & Holman, R.T. 1963. Effect of linolenic acid upon the metabolism of linoleic acid. *The Journal of nutrition*, 81(1):67-74.

Morris, C.D., Bird, A.R. & Nell, H. 1989. The haematological and biochemical changes in severe pulmonary tuberculosis. *QJM: An International Journal of Medicine*, 73(3):1151-1159.

Morris, T., Stables, M., Hobbs, A., de Souza, P., Colville-Nash, P., Warner, T., Newson, J., Bellingan, G. & Gilroy, D.W. 2009. Effects of low-dose aspirin on acute inflammatory responses in humans. *The Journal of Immunology*, 183(3):2089-2096.

Moura-Assis, A., Afonso, M.S., de Oliveira, V., Morari, J., dos Santos, G.A., Koike, M., Lottenberg, A.M., Catharino, R.R., Velloso, L.A. & da Silva, A.S.R. 2018. Flaxseed oil rich in omega-3 protects aorta against inflammation and endoplasmic reticulum stress partially mediated by GPR120 receptor in obese, diabetic and dyslipidemic mice models. *The Journal of nutritional biochemistry*, 53:9-19.

Murphy, M.G. 1990. Dietary fatty acids and membrane protein function. *The Journal of nutritional biochemistry*, 1(2):68-79.

Murray, M.J., Murray, A.B., Murray, M.B. & Murray, C. 1978. The adverse effect of iron repletion on the course of certain infections. *Br Med J*, 2(6145):1113-1115.

Mutanen, M., Freese, R., Vessby, B., Korkalo, L., Selvester, K. & Kulathinal, S. 2016. Determinants of plasma phospholipid arachidonic and docosahexaenoic acids among adolescent girls in central Mozambique–possible roles of iron and zinc. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 115:1-7.

Nagu, T.J., Spiegelman, D., Hertzmark, E., Aboud, S., Makani, J., Matee, M.I., Fawzi, W. & Mugusi, F. 2014. Anemia at the initiation of tuberculosis therapy is associated with delayed sputum conversion among pulmonary tuberculosis patients in Dar-es-Salaam, Tanzania. *PloS one*, 9(3):e91229.

Nakamura, M.T. & Nara, T.Y. 2004. Structure, function, and dietary regulation of Δ6, Δ5, and Δ9 desaturases. *Annu. Rev. Nutr.*, 24:345-376.

Namgaladze, D. & Bruene, B. 2016. Macrophage fatty acid oxidation and its roles in macrophage polarization and fatty acid-induced inflammation. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1861(11):1796-1807.

Ndlovu, H. & Marakalala, M.J. 2016. Granulomas and Inflammation: Host-Directed Therapies for Tuberculosis. *Frontiers in Immunology*, 7:434.

Nemeth, E., Rivera, S., Gabayan, V., Keller, C., Taudorf, S., Pedersen, B.K. & Ganz, T. 2004a. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *The Journal of clinical investigation*, 113(9):1271-1276.

Nemeth, E., Tuttle, M.S., Powelson, J., Vaughn, M.B., Donovan, A., Ward, D.M., Ganz, T. & Kaplan, J. 2004b. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science*, 306(5704):2090-2093.

Nenni, V., Nataprawira, H.M. & Yuniati, T. 2013. Role of combined zinc, vitamin A, and fish oil supplementation in childhood tuberculosis. *Southeast Asian J. Trop. Med. Public Health*, 44(5):854-861.

Nestel, P., Clifton, P., Colquhoun, D., Noakes, M., Mori, T.A., Sullivan, D. & Thomas, B. 2015. Indications for omega-3 long chain polyunsaturated fatty acid in the prevention and treatment of cardiovascular disease. *Heart, Lung and Circulation*, 24(8):769-779.

Nettleton, J. 1991. Omega-3 fatty acids: comparison of plant and seafood sources in human nutrition. *Journal of the American Dietetic Association*, 91(3):331-337.

Nicolas, G., Chauvet, C., Viatte, L., Danan, J.L., Bigard, X., Devaux, I., Beaumont, C., Kahn, A. & Vaulont, S. 2002. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *The Journal of clinical investigation*, 110(7):1037-1044.

Nihues, S.d.S.E., Mancuzo, E.V., Sulmonetti, N., Sacchi, F.P.C., Viana, V.d.S., Martins Netto, E., Miranda, S.S. & Croda, J. 2015. Chronic symptoms and pulmonary dysfunction in post-tuberculosis Brazilian patients. *Brazilian Journal of Infectious Diseases*, 19(5):492-497.

Nissenson, A.R., Goodnough, L.T. & Dubois, R.W. 2003. Anemia: not just an innocent bystander? *Archives of internal medicine*, 163(12):1400-1404.

Noack, M. & Miossec, P. 2014. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmunity reviews*, 13(6):668-677.

Nolan, A., Condos, R., Huie, M.L., Dawson, R., Dheda, K., Bateman, E., Rom, W.N. & Weiden, M.D. 2013. Elevated IP-10 and IL-6 from bronchoalveolar lavage cells are biomarkers of non-cavitary tuberculosis. *The International Journal of Tuberculosis and Lung Disease*, 17(7):922-927.

Norling, L.V., Headland, S.E., Dalli, J., Arnardottir, H.H., Haworth, O., Jones, H.R., Irimia, D., Serhan, C.N. & Perretti, M. 2016. Proresolving and cartilage-protective actions of resolvin D1 in inflammatory arthritis. *JCI insight*, 1(5).

Novak, T.E., Babcock, T.A., Jho, D.H., Helton, W.S. & Espat, N.J. 2003. NF-κB inhibition by ω-3 fatty acids modulates LPS-stimulated macrophage TNF-α transcription. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 284(1):L84-L89.

Novikov, A., Cardone, M., Thompson, R., Shenderov, K., Kirschman, K.D., Mayer-Barber, K.D., Myers, T.G., Rabin, R.L., Trinchieri, G. & Sher, A. 2011. Mycobacterium tuberculosis triggers host type I IFN signaling to regulate IL-1β production in human macrophages. *The journal of immunology*, 187(5):2540-2547.

Nunes-Alves, C., Booty, M.G., Carpenter, S.M., Jayaraman, P., Rothchild, A.C. & Behar, S.M. 2014. In search of a new paradigm for protective immunity to TB. *Nature Reviews Microbiology*, 12(4):289-299.

Nursyam, E.W., Amin, Z. & Rumende, C.M. 2006. The effect of vitamin D as supplementary treatment in patients with moderately advanced pulmonary tuberculous lesion. *Acta Medicia Indonesia*, 38.

O'Garra, A., Redford, P.S., McNab, F.W., Bloom, C.I., Wilkinson, R.J. & Berry, M.P. 2013. The immune response in tuberculosis. *Annual review of immunology*, 31:475-527.

Ober, M. & Hart, C. 1998. Attenuation of oxidant-mediated endothelial cell injury with docosahexaenoic acid: the role of intracellular iron. *Prostaglandins, leukotrienes and essential fatty acids*, 59(2):127-135.

Oh, S.F., Pillai, P.S., Recchiuti, A., Yang, R. & Serhan, C.N. 2011. Pro-resolving actions and stereoselective biosynthesis of 18S E-series resolvins in human leukocytes and murine inflammation. *The Journal of clinical investigation*, 121(2):569-581.

Oliver, C. & Watson, H. 2016. Omega-3 fatty acids for cystic fibrosis. *Cochrane Database of Systematic Reviews*, doi.org/10.1002/14651858.CD002201.pub4.

Oloyede, O., Folayan, A. & Odutuga, A. 1992. Effects of low-iron status and deficiency of essential fatty acids on some biochemical constituents of rat brain. *Biochemistry international*, 27(5):913-922.

Ong, C.W., Elkington, P.T., Brilha, S., Ugarte-Gil, C., Tome-Esteban, M.T., Tezera, L.B., Pabisiak, P.J., Moores, R.C., Sathyamoorthy, T. & Patel, V. 2015. Neutrophil-derived MMP-8 drives AMPK-dependent matrix destruction in human pulmonary tuberculosis. *PLoS pathogens*, 11(5):e1004917.

Ong, C.W., Elkington, P.T. & Friedland, J.S. 2014. Tuberculosis, pulmonary cavitation, and matrix metalloproteinases. *American journal of respiratory and critical care medicine*, 190(1):9-18.

Oppenheimer, S.J. 2001. Iron and its relation to immunity and infectious disease. *The Journal of nutrition*, 131(2):616S-635S.

Palanisamy, G.S., DuTeau, N., Eisenach, K.D., Cave, D.M., Theus, S.A., Kreiswirth, B.N., Basaraba, R.J. & Orme, I.M. 2009. Clinical strains of Mycobacterium tuberculosis display a wide range of virulence in guinea pigs. *Tuberculosis*, 89(3):203-209.

Palmee, C., Jablon, S. & Edwards, P.Q. 1957. Tuberculosis morbidity of young men in relation to tuberculin sensitivity and body build. *American Review of Tuberculosis and Pulmonary Diseases*, 76(4):517-539.

Palucci, I. & Delogu, G. 2018. Host Directed Therapies for Tuberculosis: Futures Strategies for an Ancient Disease. *Chemotherapy*, 63(3):172-180.

Pancholi, P., Mirza, A., Bhardwaj, N. & Steinman, R.M. 1993. Sequestration from Immune CD47r T Cells of. *Science*, 260:14.

Pantopoulos, K., Porwal, S.K., Tartakoff, A. & Devireddy, L. 2012. Mechanisms of mammalian iron homeostasis. *Biochemistry*, 51(29):5705-5724.

Parihar, S.P., Guler, R., Khutlang, R., Lang, D.M., Hurdayal, R., Mhlanga, M.M., Suzuki, H., Marais, A.D. & Brombacher, F. 2014. Statin Therapy Reduces the Mycobacterium tuberculosis Burden in Human Macrophages and in Mice by Enhancing Autophagy and Phagosome Maturation. *The Journal of Infectious Diseases*, 209(5):754-763.

Parish, M., Valiyi, F., Hamishehkar, H., Sanaie, S., Jafarabadi, M.A., Golzari, S.E. & Mahmoodpoor, A. 2014. The effect of omega-3 fatty acids on ARDS: a randomized double-blind study. *Advanced pharmaceutical bulletin*, 4(S2):555-262.

Park, J.S., Tamayo, M.H., Gonzalez-Juarrero, M., Orme, I.M. & Ordway, D.J. 2006. Virulent clinical isolates of Mycobacterium tuberculosis grow rapidly and induce cellular necrosis but minimal apoptosis in murine macrophages. *Journal of leukocyte biology*, 79(1):80-86.

Passemar, C., Arbués, A., Malaga, W., Mercier, I., Moreau, F., Lepourry, L., Neyrolles, O., Guilhot, C. & Astarie-Dequeker, C. 2014. Multiple deletions in the polyketide synthase gene repertoire of M ycobacterium tuberculosis reveal functional overlap of cell envelope lipids in host–pathogen interactions. *Cellular microbiology*, 16(2):195-213.

Paul, K.P., Leichsenring, M., Pfisterer, M., Mayatepek, E., Wagner, D., Domann, M., Sonntag, H.G. & Bremer, H.J. 1997. Influence of n-6 and n-3 polyunsaturated fatty acids on the resistance to experimental tuberculosis. *Metabolism*, 46(6):619-624.

Pearl, J.E., Torrado, E., Tighe, M., Fountain, J.J., Solache, A., Strutt, T., Swain, S., Appelberg, R. & Cooper, A.M. 2012. Nitric oxide inhibits the accumulation of CD4+ CD44hiTbet+ CD69lo T cells in mycobacterial infection. *European journal of immunology*, 42(12):3267-3279.

Peters, W. & Ernst, J.D. 2003. Mechanisms of cell recruitment in the immune response to Mycobacterium tuberculosis. *Microbial infections*, 5(2):151-158.

Petty, T.L. & Dalrymple, G.V. 1964. Inhibition of pyrazinamide hyperuricemia by small doses of acetylsalicylic acid. *Annals of internal medicine*, 60(5):898-900.

Petursdottir, D.H. & Hardardottir, I. 2008. Dietary fish oil decreases secretion of T helper (Th) 1-type cytokines by a direct effect on murine splenic T cells but enhances secretion of a Th2-type cytokine by an effect on accessory cells. *British journal of nutrition*, 101(7):1040-1046.

Peyssonnaux, C., Nizet, V. & Johnson, R.S. 2008. Role of the hypoxia inducible factors HIF in iron metabolism. *Cell Cycle*, 7(1):28-32.

Porro, B., Songia, P., Squellerio, I., Tremoli, E. & Cavalca, V. 2014. Analysis, physiological and clinical significance of 12-HETE: A neglected platelet-derived 12-lipoxygenase product. *Journal of Chromatography B*, 964:26-40.

Preiser, J.-C., van Zanten, A.R., Berger, M.M., Biolo, G., Casaer, M.P., Doig, G.S., Griffiths, R.D., Heyland, D.K., Hiesmayr, M. & Iapichino, G. 2015. Metabolic and nutritional support of critically ill patients: consensus and controversies. *Critical care*, 19(1):35.

Py, B. & Barras, F. 2010. Building Fe–S proteins: bacterial strategies. *Nature Reviews Microbiology*, 8(6):436.

Quach, N.D., Arnold, R.D. & Cummings, B.S. 2014. Secretory phospholipase A2 enzymes as pharmacological targets for treatment of disease. *Biochemical pharmacology*, 90(4):338-348.

Quigley, J., Hughitt, V.K., Velikovsky, C.A., Mariuzza, R.A., El-Sayed, N.M. & Briken, V. 2017. The cell wall lipid PDIM contributes to phagosomal escape and host cell exit of Mycobacterium tuberculosis. *MBio*, 8(2):e00148-00117.

Rahmawaty, S., Charlton, K., Lyons-Wall, P. & Meyer, B.J. 2013. Dietary intake and food sources of EPA, DPA and DHA in Australian children. *Lipids*, 48(9):869-877.

Ralph, A.P., Waramori, G., Pontororing, G.J., Kenangalem, E., Wiguna, A., Tjitra, E., Lolong, D.B., Yeo, T.W., Chatfield, M.D. & Soemanto, R.K. 2013. L-arginine and vitamin D adjunctive therapies in pulmonary tuberculosis: a randomised, double-blind, placebo-controlled trial. *PloS one*, 8(8):e70032.

Ramakrishnan, L. 2012. Revisiting the role of the granuloma in tuberculosis. *Nature Reviews Immunology*, 12(5):352.

Rangel-Huerta, O.D., Aguilera, C.M., Mesa, M.D. & Gil, A. 2012. Omega-3 long-chain polyunsaturated fatty acids supplementation on inflammatory biomakers: a systematic review of randomised clinical trials. *British Journal of Nutrition*, 107(S2):S159-S170.

Rangel Moreno, J., Estrada Garcia, I., De La Luz García Hernández, M., Aguilar Leon, D., Marquez, R. & Hernández Pando, R. 2002. The role of prostaglandin E2 in the immunopathogenesis of experimental pulmonary tuberculosis. *Immunology*, 106(2):257-266.

Rao, G.A. & Larkin, E.C. 1984. Role of dietary iron in lipid metabolism. *Nutrition Research*, 4(1):145-151.

Rao, S. 2009. Serum cholesterol, HDL, LDL levels in pulmonary tuberculosis: A clinico-radiological correlation and implications. *Infectious diseases in clinical practice*, 17(2):99-101.

Rathinam, V.A., Vanaja, S.K. & Fitzgerald, K.A. 2012. Regulation of inflammasome signaling. *Nature immunology*, 13(4):333.

Raygan, F., Taghizadeh, M., Mirhosseini, N., Akbari, E., Bahmani, F., Memarzadeh, M.R., Sharifi, N., Jafarnejad, S., Banikazemi, Z. & Asemi, Z. 2019. A comparison between the effects of flaxseed oil and fish oil supplementation on cardiovascular health in type 2 diabetic patients with coronary heart disease: A randomized, double-blinded, placebo-controlled trial. *Phytotherapy Research*, 33:1943-1951

Rees, D., Miles, E.A., Banerjee, T., Wells, S.J., Roynette, C.E., Wahle, K.W. & Calder, P.C. 2006a. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. *The American journal of clinical nutrition*, 83(2):331-342.

Rees, D., Miles, E.A., Banerjee, T., Wells, S.J., Roynette, C.E., Wahle, K.W. & Calder, P.C. 2006b. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men–. *The American journal of clinical nutrition*, 83(2):331-342.

Reese, I. & Werfel, T. 2015. Do long-chain omega-3 fatty acids protect from atopic dermatitis? *JDDG: Journal der Deutschen Dermatologischen Gesellschaft*, 13(9):879-885.

Ren, J. & Chung, S.H. 2007. Anti-inflammatory effect of α-linolenic acid and its mode of action through the inhibition of nitric oxide production and inducible nitric oxide synthase gene expression via NF-κB and mitogen-activated protein kinase pathways. *Journal of agricultural and food chemistry*, 55(13):5073-5080.

Richter, M., Baumgartner, J., Wentzel-Viljoen, E. & Smuts, C.M. 2014. Different dietary fatty acids are associated with blood lipids in healthy South African men and women: The PURE study. *International journal of cardiology*, 172(2):368-374.

Riediger, N.D., Othman, R., Fitz, E., Pierce, G.N., Suh, M. & Moghadasian, M.H. 2008. Low n-6: n-3 fatty acid ratio, with fish-or flaxseed oil, in a high fat diet improves plasma lipids and beneficially alters tissue fatty acid composition in mice. *European journal of nutrition*, 47(3):153-160.

Robinson, R.T., Orme, I.M. & Cooper, A.M. 2015. The onset of adaptive immunity in the mouse model of tuberculosis and the factors that compromise its expression. *Immunological reviews*, 264(1):46-59.

Roca, F.J. & Ramakrishnan, L. 2013. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. *Cell*, 153(3):521-534.

Rodríguez, M.C., Sáiz, M.P., Muntané, J. & Mitjavila, M.T. 1996. Fatty acid composition of erythrocyte membranes affects iron absorption in rats. *The Journal of nutrition*, 126(12):3109-3117.

Rohner, F., Namaste, S.M., Larson, L.M., Addo, O.Y., Mei, Z., Suchdev, P.S., Williams, A.M., Sakr Ashour, F.A., Rawat, R. & Raiten, D.J. 2017. Adjusting soluble transferrin receptor concentrations for inflammation: Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project. *The American journal of clinical nutrition*, 106(s1):372S-382S.

Romagnani, S. 1997. The th1/th2 paradigm. *Immunology today*, 18(6):263-266.

Romero-Adrian, T.B., Leal-Montiel, J., Fernández, G. & Valecillo, A. 2015. Role of cytokines and other factors involved in the Mycobacterium tuberculosis infection. *World Journal of Immunology*, 5(1):16-50.

Rook, G., Steele, J., Ainsworth, M. & Champion, B. 1986. Activation of macrophages to inhibit proliferation of Mycobacterium tuberculosis: comparison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages. *Immunology*, 59(3):333.

Rosenthal, I.M., Tasneen, R., Peloquin, C.A., Zhang, M., Almeida, D., Mdluli, K.E., Karakousis, P.C., Grosset, J.H. & Nuermberger, E.L. 2012. Dose-ranging comparison of rifampin and rifapentine in two pathologically distinct murine models of tuberculosis. *Antimicrobial agents and chemotherapy*, 56(8):4331-4340.

Russell, C.D. & Schwarze, J. 2014. The role of pro-resolution lipid mediators in infectious disease. *Immunology*, 141(2):166-173.

Ryan, J.D., Altamura, S., Devitt, E., Mullins, S., Lawless, M.W., Muckenthaler, M.U. & Crowe, J. 2012. Pegylated interferon-α induced hypoferremia is associated with the immediate response to treatment in hepatitis C. *Hepatology*, 56(2):492-500.

Ryan, V.H., Primiani, C.T., Rao, J.S., Ahn, K., Rapoport, S.I. & Blanchard, H. 2014. Coordination of gene expression of arachidonic and docosahexaenoic acid cascade enzymes during human brain development and aging. *PloS one*, 9(6):e100858.

Sahiratmadja, E., Wieringa, F.T., van Crevel, R., de Visser, A.W., Adnan, I., Alisjahbana, B., Slagboom, E., Marzuki, S., Ottenhoff, T.H. & van de Vosse, E. 2007. Iron deficiency and NRAMP1 polymorphisms (INT4, D543N and 3′ UTR) do not contribute to severity of anaemia in tuberculosis in the Indonesian population. *British Journal of Nutrition*, 98(4):684-690.

Sala-Vila, A., Miles, E. & Calder, P. 2008. Fatty acid composition abnormalities in atopic disease: evidence explored and role in the disease process examined. *Clinical & Experimental Allergy*, 38(9):1432-1450.

Salahuddin, N., Ali, F., Hasan, Z., Rao, N., Aqeel, M. & Mahmood, F. 2013. Vitamin D accelerates clinical recovery from tuberculosis: results of the SUCCINCT Study [Supplementary Cholecalciferol in recovery from tuberculosis]. A randomized, placebo-controlled, clinical trial of vitamin D supplementation in patients with pulmonary tuberculosis'. *BMC infectious diseases*, 13(1):22.

Sanak, M. 2016. Eicosanoid mediators in the airway inflammation of asthmatic patients: what is new? *Allergy, asthma & immunology research*, 8(6):481-490.

Sani, M., Houben, E.N., Geurtsen, J., Pierson, J., De Punder, K., van Zon, M., Wever, B., Piersma, S.R., Jiménez, C.R. & Daffé, M. 2010. Direct visualization by cryo-EM of the mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. *PLoS pathogens*, 6(3):e1000794.

Sazawal, S., Black, R.E., Ramsan, M., Chwaya, H.M., Stoltzfus, R.J., Dutta, A., Dhingra, U., Kabole, I., Deb, S. & Othman, M.K. 2006. Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. *The Lancet*, 367(9505):133-143.

Schaible, U.E., Collins, H.L., Priem, F. & Kaufmann, S.H. 2002. Correction of the iron overload defect in β-2-microglobulin knockout mice by lactoferrin abolishes their increased susceptibility to tuberculosis. *Journal of Experimental Medicine*, 196(11):1507-1513.

Schiessel, D.L., Yamazaki, R.K., Kryczyk, M., Coelho de Castro, I., Yamaguchi, A.A., Pequito, D.C., Brito, G.A., Borghetti, G., Aikawa, J. & Nunes, E.A. 2016. Does oil rich in alpha-linolenic fatty acid cause the same immune modulation as fish oil in walker 256 tumor-bearing rats? *Nutrition and cancer*, 68(8):1369-1380.

Schloss, I., Kidd, M., Tichelaar, H.Y., Young, G.O. & O'Keefe, S.J.S.A.m.j.S.-A.t.v.g. 1997. Dietary factors associated with a low risk of colon cancer in coloured west coast fishermen. 87(2):152-158.

Schmidt, P.J. 2015. Regulation of iron metabolism by hepcidin under conditions of inflammation. *Journal of Biological Chemistry*, 290(31):18975-18983.

Schonfeld, E., Yasharel, I., Yavin, E. & Brand, A. 2007. Docosahexaenoic acid enhances iron uptake by modulating iron transporters and accelerates apoptotic death in PC12 cells. *Neurochemical research*, 32(10):1673-1684.

Schulze-Tanzil, G. 2002. de SP, Behnke B, Klingelhoefer S, Scheid A, Shakibaei M. Effects of the antirheumatic remedy hox alpha–a new stinging nettle leaf extract–on matrix metalloproteinases in human chondrocytes in vitro. *Histol Histopathol*, 17(2):477-485.

Schutz, C., Davis, A.G., Sossen, B., Lai, R.P., Ntsekhe, M., Harley, Y.X. & Wilkinson, R.J. 2018. Corticosteroids as an adjunct to tuberculosis therapy. *Expert review of respiratory medicine*, 12(10):881-891.

Scrimshaw, N.S., Taylor, C.E., Gordon, J.E. & Organization, W.H. 1968. Interactions of nutrition and infection.

Serafín-López, J., Chacón-Salinas, R., Muñoz-Cruz, S., Enciso-Moreno, J., Estrada-Parra, S.A. & Estrada-Garcia, I. 2004. The effect of iron on the expression of cytokines in macrophages infected with Mycobacterium tuberculosis. *Scandinavian journal of immunology*, 60(4):329-337.

Serhan, C.N. 2014. Pro-resolving lipid mediators are leads for resolution physiology. *Nature*, 510(7503):92.

Serhan, C.N. 2017a. Treating inflammation and infection in the 21st century: new hints from decoding resolution mediators and mechanisms. *The FASEB Journal*, 31(4):1273-1288.

Serhan, C.N., Brain, S.D., Buckley, C.D., Gilroy, D.W., Haslett, C., O'Neill, L.A., Perretti, M., Rossi, A.G. & Wallace, J.L. 2007. Resolution of inflammation: state of the art, definitions and terms. *The FASEB journal*, 21(2):325-332.

Serhan, C.N., Chiang, N. & Dalli, J. 2017b. New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration. *Molecular aspects of medicine*, http://dx.doi.org/10.1016/j.mam.2017.08.002.

Serhan, C.N., Chiang, N., Dalli, J. & Levy, B.D. 2015. Lipid mediators in the resolution of inflammation. *Cold Spring Harbor perspectives in biology*, 7(2):a016311.

Serhan, C.N., Hong, S., Gronert, K., Colgan, S.P., Devchand, P.R., Mirick, G. & Moussignac, R.-L. 2002. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *Journal of Experimental Medicine*, 196(8):1025-1037.

Serhan, C.N. & Petasis, N.A. 2011. Resolvins and protectins in inflammation resolution. *Chemical reviews*, 111(10):5922-5943.

Shanklin, J., Whittle, E. & Fox, B.G. 1994. Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. *Biochemistry*, 33(43):12787-12794.

Shaw, J.G. & Friedman, J.F. 2011. Iron deficiency anemia: focus on infectious diseases in lesser developed countries. *Anemia*, doi:10.1155/2011/260380

Shields-Cutler, R.R., Crowley, J.R., Miller, C.D., Stapleton, A.E., Cui, W. & Henderson, J.P. 2016. Human metabolome-derived cofactors are required for the antibacterial activity of siderocalin in urine. *Journal of Biological Chemistry*, 291(50):25901-25910.

Shimazaki, T., Marte, S., Saludar, N., Dimaano, E., Salva, E., Ariyoshi, K., Villarama, J. & Suzuki, M. 2013. Risk factors for death among hospitalised tuberculosis patients in poor urban areas in Manila, The Philippines. *The International Journal of Tuberculosis and Lung Disease*, 17(11):1420-1426.

Shotton, A.D. & Droke, E.A. 2004. Iron utilization and liver mineral concentrations in rats fed safflower oil, flaxseed oil, olive oil, or beef tallow in combination with different concentrations of dietary iron. *Biological trace element research*, 97(3):265-277.

Shroff, K., Sainis, K., Sengupta, S. & Kamat, R. 1990. Role of antigen-presenting cells in variation in immunogenicity of mycobacteria. *Clinical & Experimental Immunology*, 79(2):285-290.

Sia, A.K., Allred, B.E. & Raymond, K.N. 2013. Siderocalins: Siderophore binding proteins evolved for primary pathogen host defense. *Current opinion in chemical biology*, 17(2):150-157.

Sibbons, C.M., Irvine, N.A., Pérez-Mojica, J.E., Calder, P.C., Lillycrop, K.A., Fielding, B.A. & Burdge, G.C. 2018. Polyunsaturated fatty acid biosynthesis involving Δ8 desaturation and differential DNA methylation of FADS2 regulates proliferation of human peripheral blood mononuclear cells. *Frontiers in immunology*, 9:432.

Sigal, G., Segal, M., Mathew, A., Jarlsberg, L., Wang, M., Barbero, S., Small, N., Haynesworth, K., Davis, J. & Weiner, M. 2017. Biomarkers of tuberculosis severity and treatment effect: a directed screen of 70 host markers in a randomized clinical trial. *EBioMedicine*, 25:112-121.

Simeone, R., Bobard, A., Lippmann, J., Bitter, W., Majlessi, L., Brosch, R. & Enninga, J. 2012. Phagosomal rupture by Mycobacterium tuberculosis results in toxicity and host cell death. *PLoS pathogens*, 8(2):e1002507.

Simeone, R., Sayes, F., Song, O., Gröschel, M.I., Brodin, P., Brosch, R. & Majlessi, L. 2015. Cytosolic access of Mycobacterium tuberculosis: critical impact of phagosomal acidification control and demonstration of occurrence in vivo. *PLoS pathogens*, 11(2):e1004650.

Simons, K. 2018. Lipid Rafts: A Personal Account. Physics of Biological Membranes. Springer. p. 109-123).

Simons, K. & Gerl, M.J. 2010. Revitalizing membrane rafts: new tools and insights. *Nature reviews Molecular cell biology*, 11(10):688.

Simopoulos, A.P. 2008. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Experimental biology and medicine*, 233(6):674-688.

Sinclair, D., Abba, K., Grobler, L. & Sudarsanam, T.D. 2011. Nutritional supplements for people being treated for active tuberculosis. *Cochrane database for systematic reviews*, https://doi.org/10.1002/14651858.CD006086.pub4.

Singh, S., Kubler, A., Singh, U.K., Singh, A., Gardiner, H., Prasad, R., Elkington, P.T. & Friedland, J.S. 2014. Antimycobacterial drugs modulate immunopathogenic matrix metalloproteinases in a cellular model of pulmonary tuberculosis. *Antimicrobial agents and chemotherapy*, 58(8):4657-4665.

Smuts, C., Tichelaar, H., Van Jaarsveld, P., Badenhorst, C., Kruger, M., Laubscher, R., Mansvelt, E. & Benade, A. 1995. The effect of iron fortification on the fatty acid composition of plasma and erythrocyte membranes in primary school children with and without iron deficiency. *Prostaglandins, leukotrienes and essential fatty acids*, 52(1):59-67.

Soberman, R.J. & Christmas, P.J.T.J.o.c.i. 2003. The organization and consequences of eicosanoid signaling. 111(8):1107-1113.

Songane, M., Kleinnijenhuis, J., Netea, M.G. & van Crevel, R. 2012. The role of autophagy in host defence against Mycobacterium tuberculosis infection. *Tuberculosis*, 92(5):388-396.

Sorensen, L.S., Rasmussen, H.H., Aardestrup, I.V., Thorlacius-Ussing, O., Lindorff-Larsen, K., Schmidt, E.B. & Calder, P.C. 2014. Rapid Incorporation of ω-3 Fatty Acids Into Colonic Tissue After Oral Supplementation in Patients With Colorectal Cancer: A Randomized, Placebo-Controlled Intervention Trial. *Journal of Parenteral and Enteral Nutrition*, 38(5):617-624.

Spite, M., Norling, L.V., Summers, L., Yang, R., Cooper, D., Petasis, N.A., Flower, R.J., Perretti, M. & Serhan, C.N. 2009. Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature*, 461:1287-1289.

Sprecher, H., Luthria, D.L., Mohammed, B. & Baykousheva, S.P. 1995. Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *Journal of lipid research*, 36(12):2471-2477.

Sritharan, M. 2016. Iron homeostasis in Mycobacterium tuberculosis: mechanistic insights into siderophore-mediated iron uptake. *Journal of bacteriology*, 198(18):2399-2409.

Stangl, G.I. & Kirchgessner, M. 1998. Different degrees of moderate iron deficiency modulate lipid metabolism of rats. *Lipids*, 33(9):889-895.

Stark, K.D., Van Elswyk, M.E., Higgins, M.R., Weatherford, C.A. & Salem, N. 2016. Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults. *Progress in Lipid Research*, 63:132-152.

Stefanova, D., Raychev, A., Arezes, J., Ruchala, P., Gabayan, V., Skurnik, M., Dillon, B.J., Horwitz, M.A., Ganz, T. & Bulut, Y. 2017. Endogenous hepcidin and its agonist mediate resistance to selected infections by clearing non–transferrin-bound iron. *Blood*, 130(3):245-257.

Stek, C., Allwood, B., Walker, N.F., Wilkinson, R.J., Lynen, L. & Meintjes, G. 2018. The immune mechanisms of lung parenchymal damage in tuberculosis and the role of host-directed therapy. *Frontiers in microbiology*, 9:2603.

Storcksdieck, S. 2007. Dietary factors influencing non-heme iron absorption. ETH Zurich.

Stubbs, C.D. & Smith, A.D. 1984. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes*, 779(1):89-137.

Stulnig, T.M. & Zeyda, M. 2004. Immunomodulation by polyunsaturated fatty acids: impact on T-cell signaling. *Lipids*, 39(12):1171-1175.

Su, H., Liu, R., Chang, M., Huang, J., Jin, Q. & Wang, X. 2018. Effect of dietary alpha-linolenic acid on blood inflammatory markers: a systematic review and meta-analysis of randomized controlled trials. *European journal of nutrition*, 57(3):877-891.

Tam, V.C. 2013. Lipidomic profiling of bioactive lipids by mass spectrometry during microbial infections. *Seminars in immunology,* 25(3):240-248.

Theegarten, D., Kahl, B. & Ebsen, M. 2006. Frequency and morphology of tuberculosis in autopsies: increase of active forms. *Deutsche medizinische Wochenschrift (1946)*, 131(24):1371-1376.

Theil, E.C. 2013. Ferritin: the protein nanocage and iron biomineral in health and in disease. *Inorganic chemistry*, 52(21):12223-12233.

Tichelaar, H., Smuts, C., Gross, R., Jooste, P., Faber, M. & Benadé, A. 1997. The effect of dietary iron deficiency on the fatty acid composition of plasma and erythrocyte membrane phospholipids in the rat. *Prostaglandins, leukotrienes and essential fatty acids*, 56(3):229-233.

Tilley, S.L., Coffman, T.M. & Koller, B.H. 2001. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *The Journal of clinical investigation*, 108(1):15-23.

Tobin, D.M. & Ramakrishnan, L. 2013. TB: the Yin and Yang of lipid mediators. *Current opinion in pharmacology*, 13(4):641-645.

Tobin, D.M., Roca, F.J., Oh, S.F., McFarland, R., Vickery, T.W., Ray, J.P., Ko, D.C., Zou, Y., Bang, N.D. & Chau, T.T. 2012. Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. *Cell*, 148(3):434-446.

Torrado, E., Robinson, R.T. & Cooper, A.M. 2011. Cellular response to mycobacteria: balancing protection and pathology. *Trends in immunology*, 32(2):66-72.

Tsuda, Y., Takahashi, H., Kobayashi, M., Hanafusa, T., Herndon, D.N. & Suzuki, F. 2004. Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant Staphylococcus aureus. *Immunity*, 21(2):215-226.

Tu, W., Cook-Johnson, R., James, M., Mühlhäusler, B. & Gibson, R. 2010. Omega-3 long chain fatty acid synthesis is regulated more by substrate levels than gene expression. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 83(2):61-68.

Turner, D., Zlotkin, S.H., Shah, P.S. & Griffiths, A.M. 2009. Omega 3 fatty acids (fish oil) for maintenance of remission in Crohn's disease. *Cochrane database of systematic reviews,* https://doi.org/10.1002/14651858.CD006320.pub3.

Tutino, V., Caruso, M.G., De Leonardis, G., De Nunzio, V. & Notarnicola, M. 2017. Tissue fatty acid profile is differently modulated from olive oil an omega-3 polyunsaturated fatty acids in apcmin/+

mice. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders)*, 17(4):303-308.

Tyagi, A., Kumar, U., Reddy, S., Santosh, V.S., Mohammed, S.B., Ehtesham, N.Z. & Ibrahim, A. 2012. Attenuation of colonic inflammation by partial replacement of dietary linoleic acid with α-linolenic acid in a rat model of inflammatory bowel disease. *British Journal of Nutrition*, 108(9):1612-1622.

Unoda, K., Doi, Y., Nakajima, H., Yamane, K., Hosokawa, T., Ishida, S., Kimura, F. & Hanafusa, T. 2013. Eicosapentaenoic acid (EPA) induces peroxisome proliferator-activated receptors and ameliorates experimental autoimmune encephalomyelitis. *Journal of neuroimmunology*, 256(2):7-12.

van Dokkum, W., Cloughley, F.A., Hulshof, K.F. & Oosterveen, L.A. 1983. Effect of variations in fat and linoleic acid intake on the calcium, magnesium and iron balance of young men. *Annals of nutrition and metabolism*, 27(5):361-369.

Van Lettow, M., West, C., Van der Meer, J., Wieringa, F. & Semba, R. 2005. Low plasma selenium concentrations, high plasma human immunodeficiency virus load and high interleukine-6 concentrations are risk factors associated with anemia in adults presenting with pulmonary tuberculosis in Zomba district, Malawi. *Eur J Clin Nutr*, 59:526-532.

VanderVen, B.C., Fahey, R.J., Lee, W., Liu, Y., Abramovitch, R.B., Memmott, C., Crowe, A.M., Eltis, L.D., Perola, E., Deininger, D.D., Wang, T., Locher, C.P. & Russell, D.G. 2015. Novel inhibitors of cholesterol degradation in Mycobacterium tuberculosis: reveal how the bacterium's metabolism is constrained by the intracellular environment. *PLOS Pathogens*, 11(2):e1004679.

Vanek, V.W., Seidner, D.L., Allen, P., Bistrian, B., Collier, S., Gura, K., Miles, J.M., Valentine, C.J. & Kochevar, M. 2012. ASPEN position paper: clinical role for alternative intravenous fat emulsions. *Nutrition in Clinical Practice*, 27(2):150-192.

Vangaveti, V.N., Jansen, H., Kennedy, R.L. & Malabu, U.H. 2016. Hydroxyoctadecadienoic acids: oxidised derivatives of linoleic acid and their role in inflammation associated with metabolic syndrome and cancer. *European journal of pharmacology*, 785:70-76.

Verbon, A., Juffermans, N., Van Deventer, D., Speelman, P., Van Deutekom, D. & Van der Poll, P. 1999a. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. *Clinical & Experimental Immunology*, 115(1):110-113.

Verbon, A., Juffermans, N., Deventer, S., Speelman, P., Deutekom, H. & Poll, T. 1999b. Serum concentrations of cytokines in patients with active tuberculosis (TB) and after treatment. *Clin Exp Immunol*, 115.

Vergne, I., Chua, J., Lee, H.H., Lucas, M., Belisle, J. & Deretic, V. 2005. Mechanism of phagolysosome biogenesis block by viable Mycobacterium tuberculosis. *Proceedings of the National Academy of Sciences*, 102(11):4033-4038.

Vidyarani, M., Selvaraj, P., Jawahar, M. & Narayanan, P. 2007. 1, 25 Dihydroxyvitamin D3 modulated cytokine response in pulmonary tuberculosis. *Cytokine*, 40(2):128-134.

Vijayamalini, M. & Manoharan, S. 2004. Lipid peroxidation, vitamins C, E and reduced glutathione levels in patients with pulmonary tuberculosis. *Cell biochemistry and function*, 22(1):19-22.

Vilaplana, C., Marzo, E., Tapia, G., Diaz, J., Garcia, V. & Cardona, P.J. 2013. Ibuprofen therapy resulted in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. *J Infect Dis*, 208.

Villamor, E., Mugusi, F., Urassa, W., Bosch, R.J., Saathoff, E., Matsumoto, K., Meydani, S.N. & Fawzi, W.W. 2008. A trial of the effect of micronutrient supplementation on treatment outcome, T cell counts, morbidity, and mortality in adults with pulmonary tuberculosis. *The Journal of infectious diseases*, 197(11):1499-1505.

Volker, D.H., FitzGerald, P.E. & Garg, M.L. 2000. The eicosapentaenoic to docosahexaenoic acid ratio of diets affects the pathogenesis of arthritis in Lew/SSN rats. *The Journal of nutrition*, 130(3):559-565.

Von Schacky, C., Fischer, S. & Weber, P.C. 1985. Long-term effects of dietary marine omega-3 fatty acids upon plasma and cellular lipids, platelet function, and eicosanoid formation in humans. *The Journal of clinical investigation*, 76(4):1626-1631.

Vulpe, C.D., Kuo, Y.M., Murphy, T.L., Cowley, L., Askwith, C., Libina, N., Gitschier, J. & Anderson, G.J. 1999. Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nature genetics*, 21(2):195-201.

Waldvogel-Abramowski, S., Waeber, G., Gassner, C., Buser, A., Frey, B.M., Favrat, B. & Tissot, J.D. 2014. Physiology of iron metabolism. *Transfusion Medicine and Hemotherapy*, 41(3):213-221.

Walker, N.F., Clark, S.O., Oni, T., Andreu, N., Tezera, L., Singh, S., Saraiva, L., Pedersen, B., Kelly, D.L. & Tree, J.A. 2012. Doxycycline and HIV infection suppress tuberculosis-induced matrix metalloproteinases. *American journal of respiratory and critical care medicine*, 185(9):989-997.

Wallace, F.A., Miles, E.A., Evans, C., Stock, T.E., Yaqoob, P. & Calder, P.C. 2001. Dietary fatty acids influence the production of Th1-but not Th2-type cytokines. *Journal of leukocyte biology*, 69(3):449-457.

Wallgren, A. 1948. The time-table of tuberculosis. *Tubercle*, 29(11):245-251.

Wallis, R.S., Kyambadde, P., Johnson, J.L., Horter, L., Kittle, R., Pohle, M., Ducar, C., Millard, M., Mayanja-Kizza, H. & Whalen, C. 2004. A study of the safety, immunology, virology, and microbiology of adjunctive etanercept in HIV-1-associated tuberculosis. *Aids*, 18(2):257-264.

Wang, C.Y. & Babitt, J.L. 2019. Liver iron sensing and body iron homeostasis. *Blood, The Journal of the American Society of Hematology*, 133(1):18-29.

Weiss, G., Ganz, T. & Goodnough, L.T. 2019. Anemia of inflammation. *Blood, The Journal of the American Society of Hematology*, 133(1):40-50.

Weiss, G. & Goodnough, L.T. 2005. Anemia of chronic disease. *New England Journal of Medicine*, 352(10):1011-1023.

Weiss, G., Werner-Felmayer, G., Werner, E.R., Grünewald, K., Wachter, H. & Hentze, M.W. 1994. Iron regulates nitric oxide synthase activity by controlling nuclear transcription. *Journal of Experimental Medicine*, 180(3):969-976.

West, A.R. & Oates, P.S. 2008. Mechanisms of heme iron absorption: current questions and controversies. *World journal of gastroenterology: WJG*, 14(26):4101.

WHO. 2013. Guideline: nutritional care and support for patients with tuberculosis: World Health Organization.

WHO. 2019. Global Tuberculosis Report. Gineva.

Wilkinson, K.A., Seldon, R., Meintjes, G., Rangaka, M.X., Hanekom, W.A. & Maartens, G. 2009. Dissection of regenerating T-Cell responses against tuberculosis in HIV-infected adults sensitized by Mycobacterium tuberculosis. *Am J Respir Crit Care Med*, 180.

Wilkinson, N. & Pantopoulos, K. 2014. The IRP/IRE system in vivo: insights from mouse models. *Frontiers in pharmacology*, 5:176.

Winau, F., Weber, S., Sad, S., De Diego, J., Hoops, S.L., Breiden, B., Sandhoff, K., Brinkmann, V., Kaufmann, S.H. & Schaible, U.E. 2006. Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity*, 24(1):105-117.

Wood, K., Lau, A., Mantzioris, E., Gibson, R., Ramsden, C. & Muhlhausler, B. 2014. A low omega-6 polyunsaturated fatty acid (n-6 PUFA) diet increases omega-3 (n-3) long chain PUFA status in

plasma phospholipids in humans. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)*, 90(4):133-138.

Wood, K., Mantzioris, E., Gibson, R., Ramsden, C. & Muhlhausler, B. 2015. The effect of modifying dietary LA and ALA intakes on omega-3 long chain polyunsaturated fatty acid (n-3 LCPUFA) status in human adults: a systematic review and commentary. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 95:47-55.

Wrighting, D.M. & Andrews, N.C. 2006. Interleukin-6 induces hepcidin expression through STAT3. *Blood*, 108(9):3204-3209.

Wu, C.W., Wu, J.Y., Lee, M.T.G., Lai, C.C., Wu, I.L., Tsai, Y.W., Chang, S.S. & Lee, C.C. 2017a. Risk of incident active tuberculosis disease in patients treated with non-steroidal anti-inflammatory drugs: a population-based study. *BMC pulmonary medicine*, 17(1):82.

Wu, J., Wang, S., Lu, C., Shao, L., Gao, Y., Zhou, Z., Huang, H., Zhang, Y. & Zhang, W. 2017b. Multiple cytokine responses in discriminating between active tuberculosis and latent tuberculosis infection. *Tuberculosis*, 102:68-75.

Yamada, H., Mizumo, S., Horai, R., Iwakura, Y. & Sugawara, I. 2000. Protective role of interleukin-1 in mycobacterial infection in IL-1 α/β double-knockout mice. *Laboratory investigation*, 80(5):759.

Yaqoob, P. 2009. The nutritional significance of lipid rafts. *Annual review of nutrition*, 29:257-282.

Yaqoob, P., Newsholme, E. & Calder, P. 1994a. The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation. *Immunology*, 82(4):603.

Yaqoob, P., Newsholme, E. & Calder, P. 1994b. Fatty acid oxidation by lymphocytes: Portland Press Limited.

Yaqoob, P., Pala, H., Cortina-Borja, M., Newsholme, E. & Calder, P. 2000. Encapsulated fish oil enriched in alpha-tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. *European journal of clinical investigation*, 30(3):260-274.

Yen, Y.F., Chuang, P.H., Yen, M.Y., Lin, S.Y., Chuang, P., Yuan, M.J., Ho, B.L., Chou, P. & Deng, C.Y. 2016. Association of body mass index with tuberculosis mortality: a population-based follow-up study. *Medicine*, 95(1).

Young, D.B., Gideon, H.P. & Wilkinson, R.J. 2009. Eliminating latent tuberculosis. *Trends in microbiology*, 17(5):183-188.

Yuhas, Y., Azoulay-Alfaguter, I., Berent, E. & Ashkenazi, S. 2007. Rifampin inhibits prostaglandin E2 production and arachidonic acid release in human alveolar epithelial cells. *Antimicrobial agents and chemotherapy*, 51(12):4225-4230.

Yuk, J.M., Shin, D.M., Lee, H.M., Yang, C.S., Jin, H.S., Kim, K.K., Lee, Z.W., Lee, S.H., Kim, J.M. & Jo, E.K. 2009. Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell host & microbe*, 6(3):231-243.

Zachariah, R., Spielmann, M., Harries, A. & Salaniponi, F. 2002. Moderate to severe malnutrition in patients with tuberculosis is a risk factor associated with early death. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 96(3):291-294.

Zhang, G., Zhou, B., Li, S., Yue, J., Yang, H., Wen, Y., Zhan, S., Wang, W., Liao, M. & Zhang, M. 2014. Allele-specific induction of IL-1β expression by C/EBPβ and PU. 1 contributes to increased tuberculosis susceptibility. *PLoS pathogens*, 10(10):e1004426.

Zhang, P., Smith, R., Chapkin, R.S. & McMurray, D.N. 2005. Dietary (n-3) polyunsaturated fatty acids modulate murine Th1/Th2 balance toward the Th2 pole by suppression of Th1 development. *The Journal of nutrition*, 135(7):1745-1751.

Zhao, G., Etherton, T.D., Martin, K.R., Gillies, P.J., West, S.G. & Kris-Etherton, P.M. 2007. Dietary α-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects. *The American journal of clinical nutrition*, 85(2):385-391.

Zhao, G., Etherton, T.D., Martin, K.R., West, S.G., Gillies, P.J. & Kris-Etherton, P.M. 2004. Dietary α-Linolenic acid reduces inflammatory and Lipid cardiovascular risk factors in hypercholesterolemic men and women. *The Journal of Nutrition*, 134(11):2991-2997.

Zhou, Y.E., Kubow, S. & Egeland, G.M. 2011. Is iron status associated with highly unsaturated fatty acid status among Canadian Arctic Inuit? *Food & function*, 2(7):381-385.

Zhu, D., Zhang, Y., Li, S., Gan, L., Feng, H. & Nie, W. 2014. Enteral omega-3 fatty acid supplementation in adult patients with acute respiratory distress syndrome: a systematic review of randomized controlled trials with meta-analysis and trial sequential analysis. *Intensive care medicine*, 40(4):504-512.

Zumla, A., Nahid, P. & Cole, S.T. 2013. Advances in the development of new tuberculosis drugs and treatment regimens. *Nature reviews Drug discovery*, 12(5):388-298.

CHAPTER 3 MANUSCRIPT 1

Omega-3 long-chain fatty acids promote antibacterial and inflammation-resolving effects in *Mycobacterium tuberculosis*-infected C3HeB/FeJ mice, dependent on fatty acid status

A Nienaber[1], M Ozturk[2,3], RC Dolman[1], R Blaauw[4], L Zandberg[1], S King[1], M Britz[1], FEA Hayford[1,5], F Brombacher[2,3], D Loots[6], CM Smuts[1], SP Parihar[2,7], L Malan[1]

[1] Centre of Excellence for Nutrition, North-West University, Potchefstroom, South Africa
[2] Institute of Infectious Diseases and Molecular Medicine (IDM), Division of Immunology and South African Medical Research Council (SAMRC) Immunology of Infectious Diseases, University of Cape Town, Cape Town, South Africa
[3] International Centre for Genetic Engineering and Biotechnology (ICGEB), Cape Town-Component, Cape Town, South Africa
[4] Division of Human Nutrition, Stellenbosch University, Stellenbosch, South Africa
[5] Department of Nutrition and Dietetics, University of Ghana, Accra, Ghana
[6] Laboratory of Infectious Disease Metabolomics, Centre for Human Metabolomics, North-West University, Potchefstroom, South Africa
[7] Centre of Infectious Diseases Research in Africa, University of Cape Town, Cape Town, South Africa

Running head: Omega-3 improves TB outcomes

Submitted for publication in *Mucosal Immunology*

Abstract

Excessive non-resolving inflammation is characteristic of tuberculosis (TB), leading to host lung tissue destruction. Given their inflammation-resolving properties, omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs) may provide a nutritional approach to support current TB treatment. The aim of this research was to investigate the effects of n-3 LCPUFA supplementation on clinical and inflammatory outcomes of *Mycobacterium tuberculosis* (*Mtb*)-infected C3HeB/FeJ mice with normal and low n-3 polyunsaturated fatty acid (PUFA) status prior to infection. Uninfected mice were conditioned on either n-3 PUFA-sufficient (n-3FAS) or -deficient (n-3FAD) diets for six weeks. One week post-infection, mice were randomized to either n-3 LCPUFA supplemented diets (n-3FAS/n-3+ and n-3FAD/n-3+) or continued on n-3FAS or n-3FAD diets for three weeks. The n-3FAS/n-3+ group had lower bacterial loads, interferon-gamma (IFN-γ) concentrations, T cell percentage of total cells and lung pathology and a more pro-resolving lipid mediator profile compared with the n-3FAS group. In contrast, no beneficial effect on bacterial load or lung cytokines was evident in the n-3FAD/n-3+ group, which, instead, had higher interleukin (IL)-6 concentrations. Our study provides the first evidence that n-3 LCPUFA supplementation has antibacterial and inflammation-resolving benefits in TB when provided after the initial response, depending on n-3 PUFA status.

Keywords: host-directed therapy, omega-3 long-chain polyunsaturated fatty acids, tuberculosis, inflammation

Introduction

The bacterial manipulation of host responses in tuberculosis (TB) favors bacterial growth and excessive inflammation, with the resultant lung tissue damage that, unfortunately, persists in some TB patients.[1, 2] In addition, TB patients are burdened by drug side effects and toxicity, long treatment periods and poor cure rates.[3] Host-directed therapy (HDT), aimed at enhancing the host's response to infection, rather than treatment strategies directed at bacterial killing, has lately been suggested for improving current TB treatment regimens.[3] Since TB is characterized by excessive, non-resolving inflammation, various anti-inflammatory drugs have been investigated for use as possible HDT options.[4,5] These medications have been shown to reduce lung lesions and bacillary load, favoring host survival.[4,6,7] However, they are not without side effects and, therefore, a nutritional approach may be considered a safer alternative.[8]

Dietary n-3 LCPUFA consumption alters membrane phospholipid fatty acid (FA) composition of blood and tissue cells that play a role in immune and inflammatory responses.[9-11] It is well known that various lipid mediators, synthesized from omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs), contribute to inflammation resolution. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) serve as precursors for specialized pro-resolving mediators (SPMs), including resolvins, protectins, and maresins. These SPMs play a role in significantly reducing pro-inflammatory lipid mediator, chemokine and cytokine production and altering immune cell recruitment, whilst promoting anti-inflammatory cytokine release.[12] The incorporation of dietary EPA and DHA into cell membranes has also been found to enhance the phagocytosis of apoptotic cells and bacteria, whilst SPMs promote bacterial killing.[12,13] Although these functions have not been proven in TB specifically, n-3 LCPUFAs have been successfully used as anti-inflammatory and pro-resolving agents in other conditions driven by inflammation.[9]

Considering this, it is reasonable to hypothesize that n-3 LCPUFA supplementation would benefit TB patients, but research on the application of n-3 LCPUFAs as HDT in TB is rather limited at present. Moreover, the effects of n-3 LCPUFA supplementation after the acute inflammatory response in *Mycobacterium tuberculosis* (*Mtb*) infection have not yet been investigated. The aim of this study is, therefore, to determine the effects of EPA and DHA supplementation, administered one week after *Mtb* infection for a period of 28 days, on inflammatory, immune and clinical outcomes in C3HeB/FeJ mice. The well-established C3HeB/FeJ mouse model has been reported to be the closest representative murine model of human pulmonary TB lung histopathology.[14] Furthermore, the n-3 LCPUFA status of the general human adult population is not considered optimal, owing to insufficient dietary n-3 polyunsaturated fatty acid (PUFA) consumption and high dietary omega-6 (n-6)/n-3 PUFA

ratios, resulting in lower n-3 PUFA status.[15, 16] We will further mimic this scenario of possible suboptimal n-3 PUFA intakes among TB patients in order to determine whether supplementation outcomes depend on n-3 PUFA status prior to *Mtb* infection.

Results

The intervention diets altered red blood cell (RBC), peripheral blood mononuclear cell (PBMC), and crude lung homogenate fatty acid composition consistent with dietary fatty acid intake

Prior to infection, mice were conditioned for six weeks on either n-3 PUFA-sufficient (n-3FAS) or -deficient (n-3FAD) diets, whereafter the RBC phospholipid FA composition was analyzed. This was done in order to determine whether the dietary fat modifications reflected in the cell membrane compositions and mimicked the scenario of lower and more ideal n-3 PUFA status (Table 1). RBC FA composition has been reported to be representative of the FA content of other tissues.[17] Following the conditioning period, the n-3FAD group had lower EPA, DHA and total n-3 LCPUFA, and higher arachidonic acid (AA), osbond acid, total n-6 LCPUFA composition, as well as a higher n-6/n-3 LCPUFA ratio, in comparison with the n-3FAS group. These results indicate that the pre-infection dietary conditioning was effective in altering FA status accordingly.

Table 1 Phospholipid fatty acid composition of RBCs in mice receiving n-3FAS or n-3FAD diets for six weeks[1]

Fatty Acids	**n-3FAS**	**n-3FAD**	***P*-value**
20:5n-3 (EPA)	0.20 ± 0.01	0.04 ± 0.01	< 0.001
22:6n-3 (DHA)	7.84 ± 0.26	3.92 ± 0.22	< 0.001
Total n-3 LCPUFA	8.70 ± 0.20	4.12 ± 0.22	< 0.001
20:4n-6 (AA)	17.95 ± 0.38	19.80 ± 0.40	< 0.001
22:5n-6 (Osbond)	1.11 ± 0.05	4.06 ± 0.33	< 0.001
Total n-6 LCPUFA	22.86 ± 0.28	28.60 ± 0.48	< 0.001
Total n-6/n-3 LCPUFA ratio	2.63 ± 0.04	7.04 ± 0.38	< 0.001

[1] Values are reported as means ± SEM percentage of total fatty acids. Intervention effects were estimated by using the Independent Student Fischer T-test (n=6 per group).

AA, Arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LCPUFA, long-chain polyunsaturated fatty acids; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet.

One week post-infection, the group that was conditioned with the n-3FAS diet either continued on the n-3FAS diet or was switched to an n-3 LCPUFA-supplemented diet (n-3FAS/n-3+). Similarly, in the n-3FAD group, the mice were also either continued on the n-3FAD diet or switched to the n-3 LCPUFA-supplemented diet (n-3FAD/n-3+ group), as shown in Table 2. The mice in the n-3 LCPUFA-supplemented groups (n-3FAS/n-3+ and n-3FAD/n-3+) consumed approximately 1.98 mg DHA and 2.94 mg EPA daily or 1% of total energy intake when calculated on average daily food consumption. We found that there were no significant differences in the pre-infection weight (33 ± 0.47 g), percentage weight gain (n-3FAS, 6.65 ± 0.57%; n-3FAS/n-3+, 8.11 ± 0.89%; n-3FAD, 3.23 ± 1.67%; n-3FAD/n-3+, 6.98 ± 0.60%), and daily food intake per mouse (3.30 ± 0.25 g).

Table 2 Experimental study design

			Diet received				
Group	**6weeks**		**Week 1**	**Week 2**	**Week 3**	**Week 4**	
n-3FAS	n-3FAS	**Infection**	n-3FAS	n-3FAS	n-3FAS	n-3FAS	**Euthanasia**
n-3FAS/n-3+	n-3FAS		n-3FAS	n-3+	n-3+	n-3+	
n-3FAD	n-3FAD		n-3FAD	n-3FAD	n-3FAD	n-3FAD	
n-3FAD/n-3+	n-3FAD		n-3FAD	n-3+	n-3+	n-3+	

n-3FAD, omega-3 fatty acid deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet.

The FA composition of phospholipids in RBCs, PBMCs and crude lung homogenates after three weeks of dietary intervention is presented in Table 3. In addition to recruited immune cells, lung epithelium also synthesizes lipid mediators, and therefore, the modification of the FA composition of lung tissue and immune cells may exert local immune- and inflammation-modulatory effects.[11,18] A higher EPA, DHA, and total n-3 LCPUFA composition was found in the cell membranes of the n-3 LCPUFA-supplemented groups (n-3FAS/n-3+ and n-3FAD/n-3+) compared with other groups (n-3FAS and n-3FAD). EPA was higher in all RBCs and PBMCs of n-3 LCPUFA-supplemented groups, whilst DHA composition was higher in RBCs and lung homogenates. On the other hand, the n-3FAD group had the highest AA, osbond acid, and total n-6 LCPUFA phospholipid FA composition, and the highest n-6/n-3 LCPUFA ratios in RBCs, PBMCs, and lung homogenates. Our results show that three weeks of dietary intervention successfully altered the FA composition of RBCs, PBMCs, and lung tissue in accordance with dietary FA intake.

Table 3 Phospholipid fatty acid composition of RBCs, PBMCs, and crude lung homogenates in *Mtb*-infected mice receiving n-3FAS, n-3FAS/n-3+, n-3FAD or n-3FAD/n-3+ for three weeks[1]

Fatty Acids		n-3FAS	n-3FAS/n-3+	n-3FAD	n-3FAD/n-3+	*P*– value
20:5n-3 (EPA)	RBC	0.13 ± 0.00^{c}	0.51 ± 0.04^{a}	0.02 ± 0.00^{d}	0.39 ± 0.02^{b}	<0.001
	PBMC	0.21 ± 0.01^{c}	0.89 ± 0.04^{a}	0.05 ± 0.11^{d}	0.70 ±0.04^{b}	< 0.001
	Lung	0.17 ± 0.01^{a}	0.40 ± 0.01^{a}	0.23 ± 0.15^{a}	0.38 ± 0.01^{a}	0.103
22:6n-3 (DHA)	RBC	6.09 ± 0.21^{b}	7.48 ± 0.41^{a}	1.72 ± 0.15^{c}	5.41 ± 0.06^{b}	<0.001
	PBMC	9.04 ± 0.20^{a}	9.82 ± 0.22^{a}	3.21 ± 0.19^{b}	9.49 ± 0.24^{a}	< 0.001
	Lung	8.08 ± 0.15^{b}	9.68 ± 0.28^{a}	2.39 ± 0.26^{c}	9.56 ± 0.11^{a}	< 0.001
Tot n-3 LCPUFA	RBC	6.63 ±0.20^{b}	8.59±0.48^{a}	1.81 ± 0.15^{c}	6.19 ± 0.05^{b}	<0.001
	PBMC	10.60 ± 0.21^{b}	12.48 ± 0.33^{a}	3.48 ± 0.21^{c}	11.59 ± 0.31^{ab}	< 0.001
	Lung	10.03 ± 0.14^{b}	12.62 ±0.26^{a}	2.91 ± 0.24^{c}	12.33 ± 0.10^{a}	< 0.001
20:4n-6 (AA)	RBC	17.71 ± 0.27^{b}	16.42 ± 0.30^{c}	20.45 ± 0.25^{a}	16.35 ± 0.30^{c}	< 0.001
	PBMC	16.36 ± 0.39^{b}	14.76 ± 0.31^{c}	21.48 ± 0.47^{a}	16.85 ± 0.27^{b}	< 0.001
	Lung	14.40 ± 0.15^{ab}	13.32 ± 0.42^{b}	15.19 ± 0.58^{a}	13.34 ± 0.19^{b}	< 0.001
22:5n-6 (Osbond)	RBC	0.87 ± 0.10^{b}	0.37 ± 0.01^{b}	3.37 ± 0.32^{a}	0.84 ± 0.07^{b}	<0.001
	PBMC	1.42 ± 0.04^{b}	0.74 ± 0.05^{c}	5.68 ± 0.30^{a}	0.98 ± 0.05^{bc}	< 0.001
	Lung	1.13 ± 0.05^{b}	0.54 ± 0.01^{c}	5.16 ± 0.27^{a}	0.85 ± 0.03^{bc}	< 0.001
Tot n-6 LCPUFA	RBC	20.17 ± 0.32^{b}	18.66 ± 0.44^{b}	25.47 ± 0.39^{a}	18.68 ± 0.32^{b}	<0.001
	PBMC	21.83 ± 0.41^{b}	19.36 ± 0.54^{c}	32.75 ± 0.69^{a}	21.48 ± 0.27^{b}	< 0.001
	Lung	21.24 ± 0.19^{b}	18.27 ± 0.50^{c}	26.58 ± 0.65^{a}	18.81 ± 0.26^{c}	< 0.001
Tot n-6/n-3	RBC	3.05 ± 0.05^{b}	2.19 ± 0.07^{b}	14.38 ± 1.19^{a}	3.02 ± 0.05^{b}	< 0.001
LCPUFA ratio	PBMC	2.06 ± 0.02^{b}	1.55 ± 0.01^{b}	9.60 ± 0.82^{a}	1.86 ± 0.05^{b}	< 0.001
	Lung	2.11 ± 0.03^{b}	1.45 ± 0.06^{b}	9.29 ± 0.65^{a}	1.52 ± 0.02^{b}	< 0.001

[1]Values are reported as means ± SEM percentage of total fatty acids. Results repeated in two experiments, data shown for one experiment (n=5 per group). Means in a row with superscripts without a common letter differ, $P < 0.05$. One-way ANOVA, with Tukey post hoc test was used to test effects between groups.

AA, Arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LCPUFA, long-chain polyunsaturated fatty acids; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid sufficient diet; n-3+, omega-3 long-chain polyunsaturated fatty acid supplemented diet; PBMC, peripheral blood mononuclear cell; RBC, red blood cell; /, switched to.

Dietary n-3 LCPUFA supplementation reduced the bacterial load and lung pathology of n-3 PUFA-sufficient mice, but not in mice with a low n-3 PUFA status

Following three weeks of dietary intervention, the mice were euthanized to determine the effects of the experimental diets on bacterial burden. Within the n-3 PUFA-sufficient arm, the n-3FAS/n-3+ group had a lower lung bacterial load when compared with the n-3FAS group (Figure 1a). On the other hand, no significant difference in lung bacterial load was found in the n-3FAD group switched to the n-3 LCPUFA-supplemented diet (n-3FAD/n-3+) (Figure 1a). Moreover, the quantification of the percentage of free alveolar space revealed that the n-3FAS/n-3+ group had a higher percentage of free alveolar space compared with the n-3FAS group, whilst no significant differences could be detected in the n-3FAD groups (Figure 1b and 1c). This indicated a possible benefit of n-3 LCPUFAs on bacterial loads and lung pathology in mice with a prior sufficient n-3 PUFA status.

Dietary n-3 LCPUFA supplementation affected immune cell recruitment in the lungs

We further investigated the immune cell phenotyping from a single-cell suspension of the lungs determined by flow cytometry (Figure 2). The percentage T cells (Figure 2a) and $CD4^+$ T cells (Figure 2b) were lower in the n-3FAS/n-3+ group when compared with the n-3FAS group. However, the n-3FAD group presented with a higher percentage of natural killer (NK) cells (Figure 2c) compared with other groups, whilst interstitial macrophages and CD11b dendritic cell (DC) percentages were higher in the n-3FAD than in n-3FAS and n-3FAS/n-3+ groups (Figure 2d and 2e). In addition, neutrophils appeared to remain unaffected by n-3 LCPUFA supplementation in n-3FAS and n-3FAD groups (Figure 2f). Consistent with cell percentages, similar patterns were evident in the total cell counts between groups (Supplemental Figure 1). These results suggest that n-3 LCPUFA supplementation specifically lowered T cell recruitment during *Mtb* infection, whilst a low n-3 PUFA status increased cellular recruitment in the lungs.

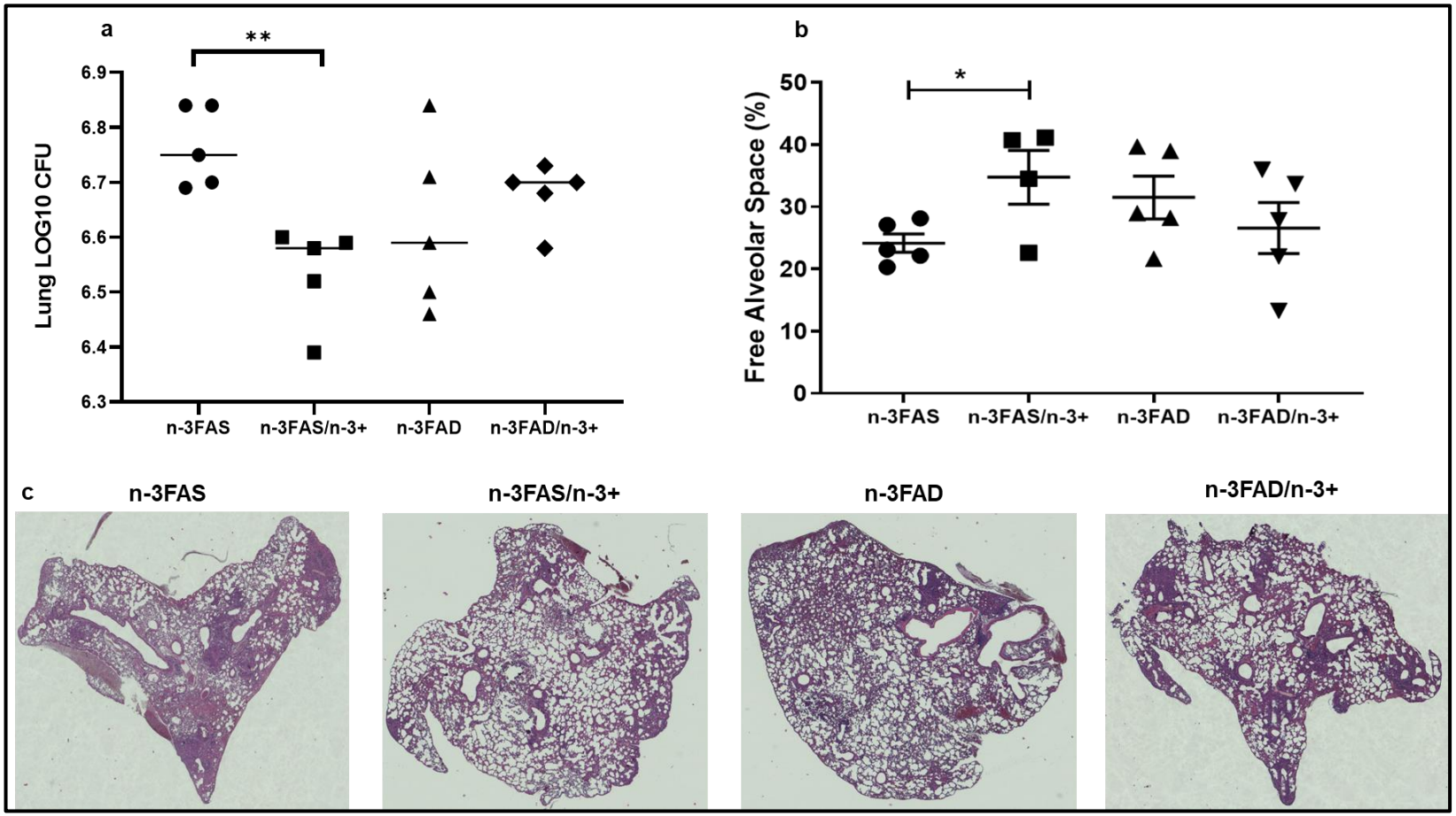


Figure 1 a) Lung bacterial loads, b) percentage free alveolar air space, and c) representative hematoxylin-eosin stained sections of the lungs after providing *Mtb*-infected mice with n-3FAS, n-3FAS/n-3+, n-3FAD, and n-3FAD/n-3+ diets for three weeks. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). One-way ANOVA followed by the Tukey post-hoc test was used to compare means, **P* < 0.05 ***P* < 0.01. CFU, colony-forming units; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet; /, switched to.

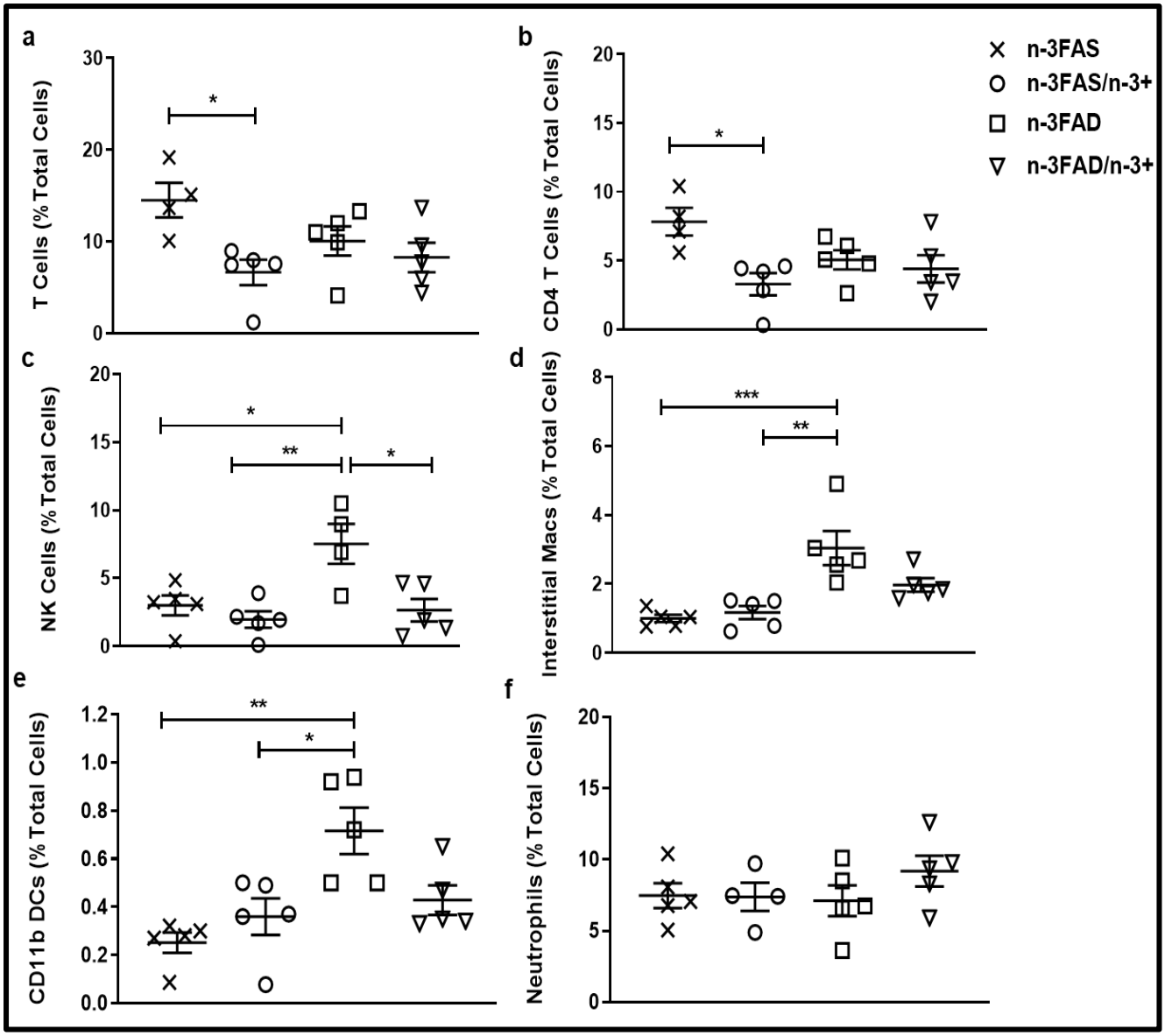


Figure 2 Immune cell phenotyping of a) T cells ($CD3^+$ CD19), b) $CD4^+$ T cells ($CD3^+$ $CD4^+$), c) natural killer cells (CD3- NK1.1+), d) interstitial macrophages (Cd64+ CDCd11b+ CD11c+ SiglecF-), e) CD11b dendritic cells (CD11b+ CD11c+ MHCII+ CD64-) and, f) neutrophils (CD11b+ Ly6G+), in crude lung homogenates after providing *Mtb*-infected mice with n-3FAS, n-3FAS/n-3+, n-3FAD, or n-3FAD/n-3+ diets for three weeks. The values represent mean ± SEM % of total cells. Results repeated in two experiments, data shown for one experiment (n=5 per group). One-way ANOVA followed by Tukey's post-hoc test was used to compare means, *P < 0.05, **P < 0.01, ***P < 0.001. DCs, dendritic cells; Macs, macrophages; NK, natural killer; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; /, switch.

Dietary n-3 LCPUFA supplementation lowered lung inflammation in n-3 PUFA-sufficient mice, but not in mice with a low n-3 PUFA status

We next assessed the effect of the dietary intervention on lung cytokine responses, measured in cell-free lung homogenates. There were lower interferon-gamma (IFN-γ) concentrations in the n-3FAS/n-3+ compared with the n-3FAS group (Figure 3a), whilst there was a trend towards (*P* = 0.072) higher chemokine ligand 3 (CCL3) (Figure 3b). In contrast with the n-3FAS/n-3+ group, mice in the n-3FAD/n-3+ group showed a higher production of interleukin-6 (IL-6) when compared with the n-3FAD group (Figure 3c). We further compared n-3FAS and n-3FAD groups and found that the n-3FAD group had lower lung IFN-γ, IL-1α, IL-1β, and IL-17 (Figure 3a, d, e, and f). These results indicate that n-3 LCPUFA supplementation potentially lowers the lung inflammatory cytokine response in mice conditioned to a sufficient n-3 PUFA status prior to supplementation. However, the opposite was true for mice in the n-3FAD groups. These results further suggest that a low n-3 PUFA status lowered inflammatory cytokines and IL-17 production during *Mtb*-infection.

Dietary n-3 LCPUFA supplementation favored the production of pro-resolving lung lipid mediators

Crude lung homogenate lipid mediators were also measured to determine the effects of the intervention diets on the local lipid mediator profile, which is influenced by n-3 LCPUFA intake. The n-3FAS/n-3+ group presented with higher lung concentrations of the less inflammatory EPA-derived prostaglandin E_3 (PGE_3), (Figure 4a), as well as the pro-resolving EPA-derived intermediates 5-, 11-, 12-, 15-, and 18-hydroxyeicosapentaenoic acid (HEPE), in comparison with the n-3FAS group (Figure 4b, 4d, and 4e; results not shown for 12- and 15-HEPE).

Similarly, when compared with the n-3FAD group, the n-3FAD/n-3+ group also showed higher lung concentrations of PGE_3, 5-, 9-, 11-, 12-, 15-, and 18-HEPE (Figure 4a, 4b, 4c, 4d, and 4e). Additionally, the n-3FAD/n-3+ group also had higher concentrations of the DHA-derived 17-hydroxydocosahexaenoic acid (17-HDHA) (Figure 4f). Nine-HEPE was higher in the n-3FAS than in the n-3 FAD group (Figure 4c), with trends for higher 12- and 18-HEPE (*P* = 0.099 for both, Figure 4e) and 17-HDHA (*P* = 0.082, Figure 4f).

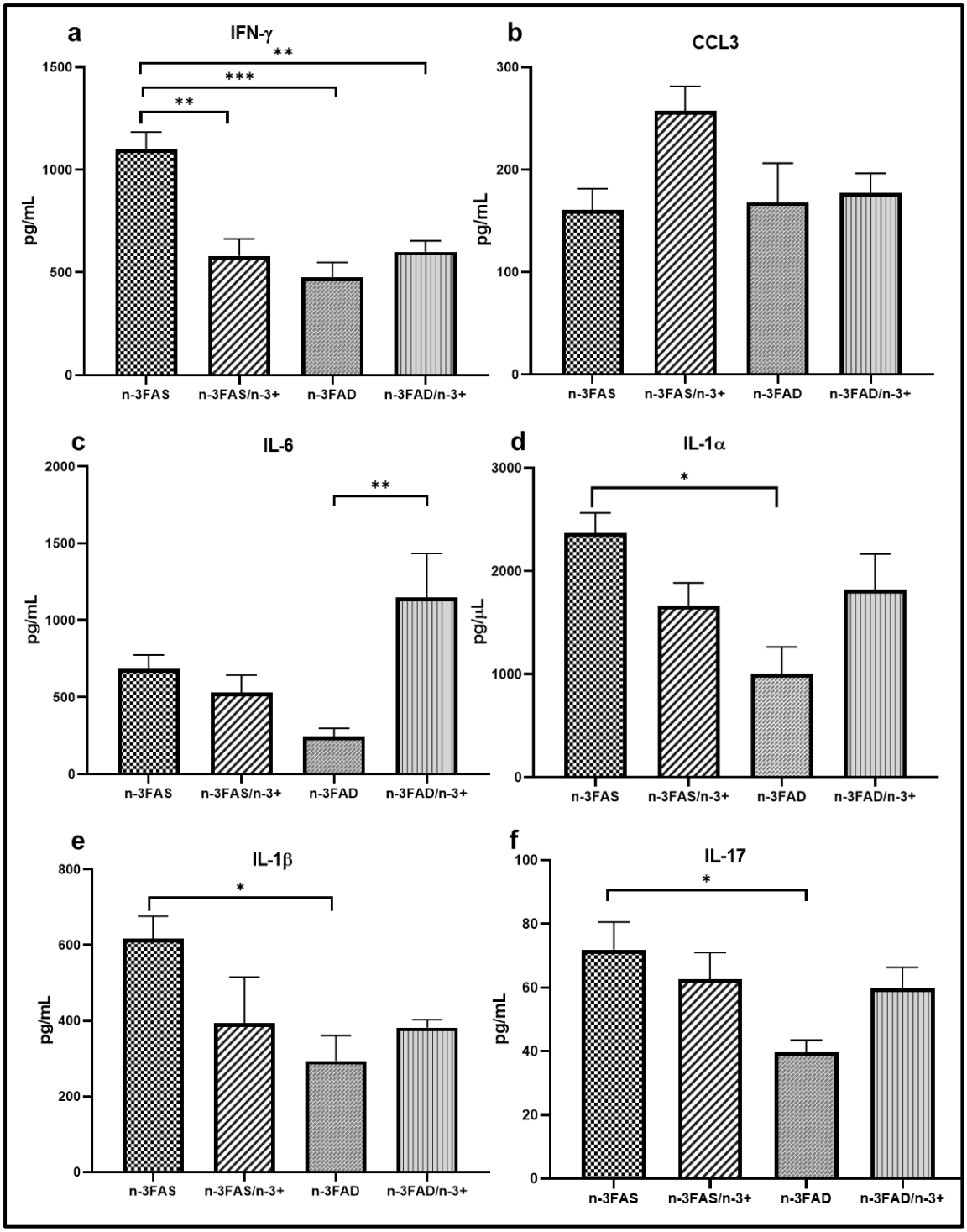


Figure 3 Cytokine concentrations, including a) IFN-γ, b) CCL3, c) IL-6, d) IL-1α, e) IL-1β, and f) IL-17 in crude lung homogenates after providing *Mtb*-infected mice with n-3FAS, n-3FAS/n-3+, n-3FAD, or n--3FAD/n-3+ diets for three weeks. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). One-way ANOVA followed by Tukey's post-hoc test was used to compare means, **P* < 0.05, ***P* < 0.01, ****P* < 0.001. CCL3, chemokine ligand 3; IFN-γ, interferon-gamma; IL, interleukin; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; /, switched to.

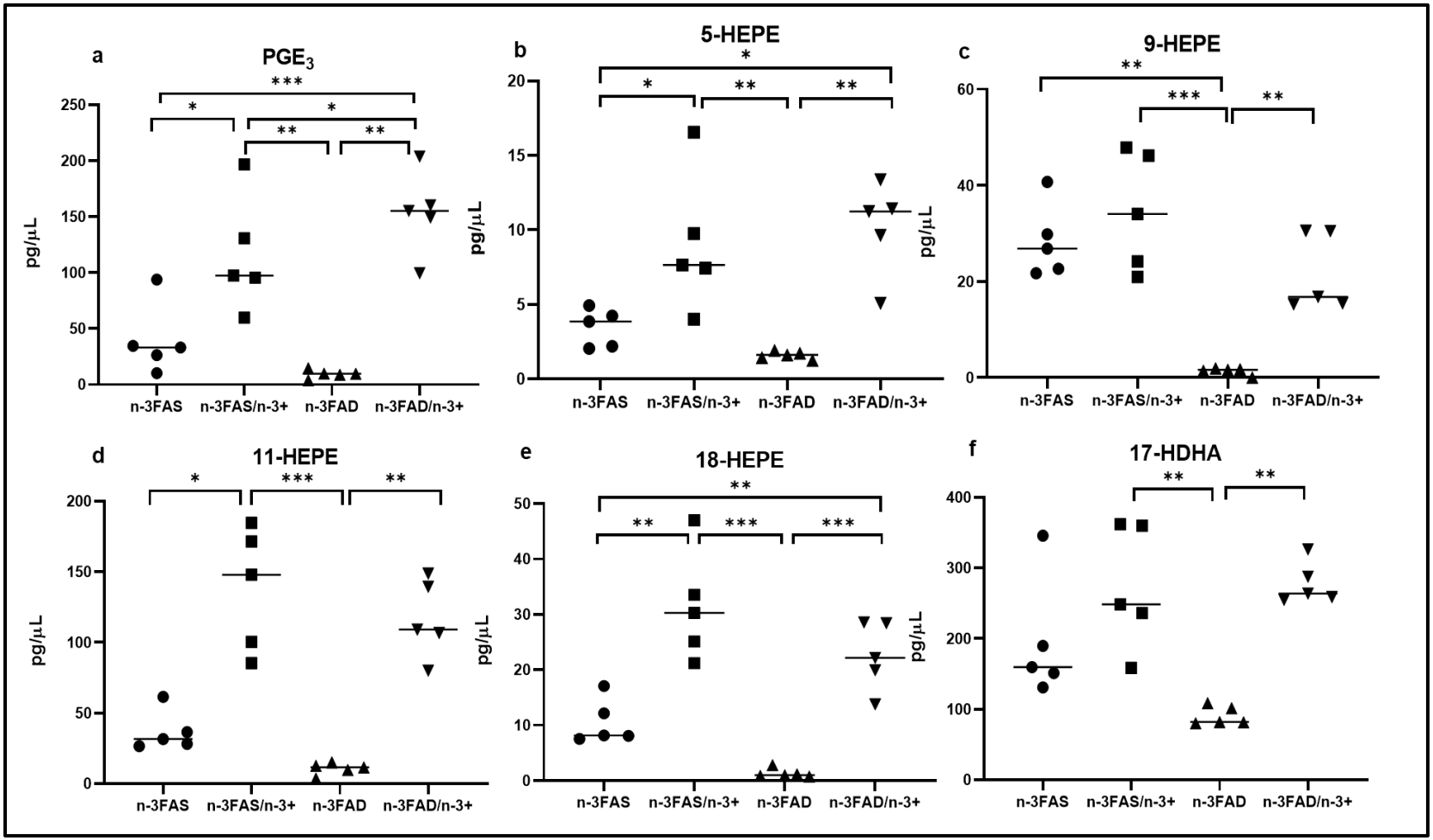


Figure 4 Pro-resolving lipid mediator concentrations of a) PGE_3, b) 5-HEPE, c) 9-HEPE, d) 11-HEPE, e) 18-HEPE, and f) 17-HDHA in crude lung homogenates after providing *Mtb*-infected mice with n-3FAS, n-3FAS/n-3+, n-3FAD, or n-3FAD/n-3+ diets for three weeks. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). One-way ANOVA followed by Tukey's post-hoc test was used to compare means, *P < 0.05, **P < 0.01, ***P < 0.001. HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; PG, prostaglandin; /, switched to.

With regard to the more pro-inflammatory AA-derived lipid mediators, n-3 LCPUFA supplementation did not significantly lower pro-inflammatory lipid mediators in the n-3FAS/n-3+ group. On the other hand, n-3 LCPUFA supplementation in the n-3FAD group resulted in lower lung concentrations of 8-hydroxyeicosatetraenoic acid (HETE) (Figure 5a) and PGD_2 (Figure 5b), and a trend towards lower PGE_2 concentrations ($P = 0.093$) (Figure 5c). Additionally, the n-3FAD group had higher concentrations of 11-HETE compared with the n-3FAS group (Figure 5d). These results suggest that dietary n-3 LCPUFA supplementation altered the lung lipid mediator production in both n-3 PUFA-sufficient and n-3 PUFA low-status groups to result in more pro-resolving lipid mediator profiles in *Mtb*-infected mice.

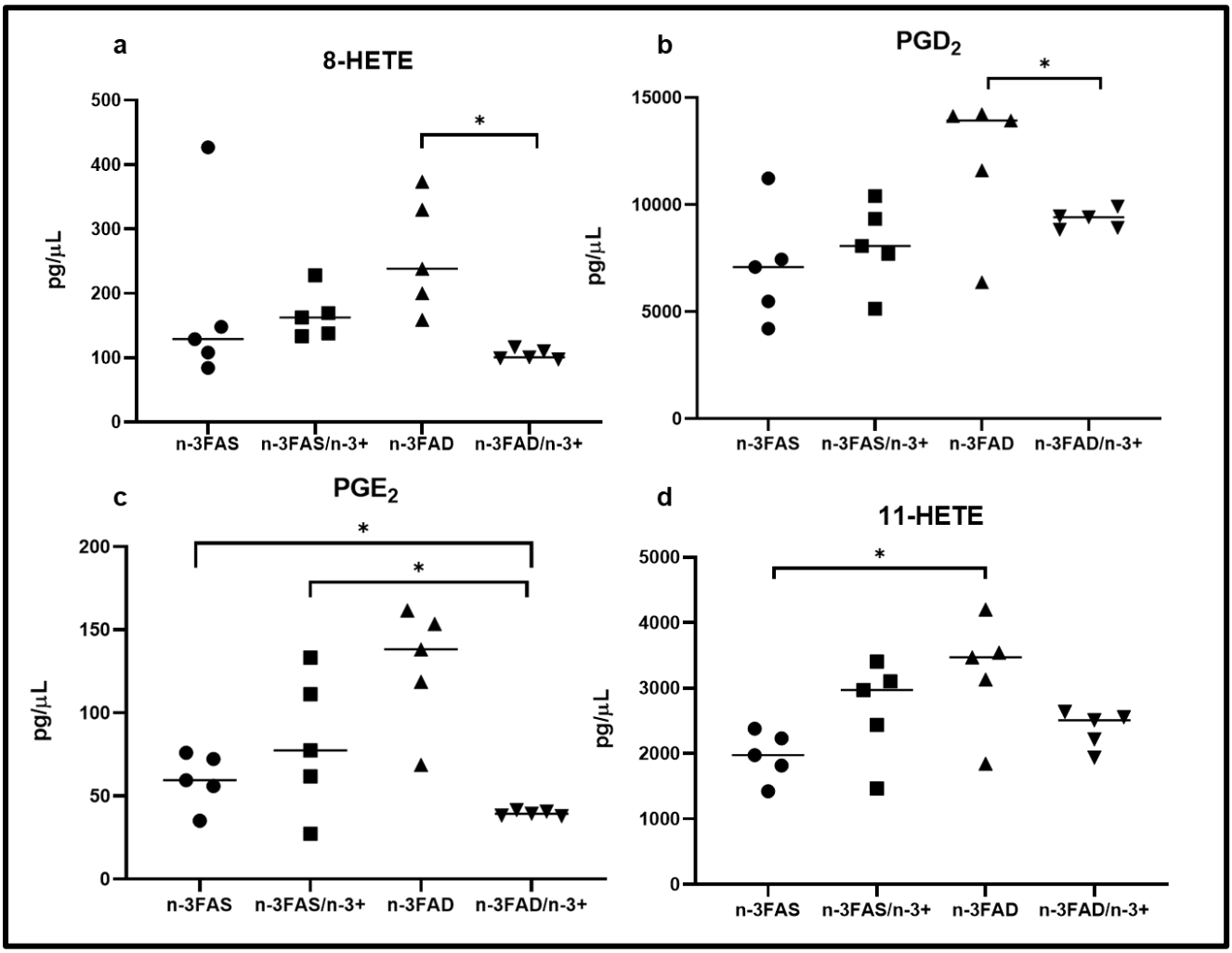


Figure 5 Pro-inflammatory lipid mediator concentrations of a) 8-HETE, b) PGD_2, c) PGE_2, and d) 11-HETE in crude lung homogenates after providing *Mtb*-infected mice with n-3FAS, n-3FAS/n-3+, n-3FAD, or n-3FAD/n-3+ diets for three weeks. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). One-way ANOVA followed by Tukey's post-hoc test was used to compare means, *P < 0.05. HETE, hydroxyeicosatetraenoic acids; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; PG, prostaglandin; /, switched to.

Discussion

This study provides evidence that n-3 LCPUFA supplementation, commenced one week post-infection, reduced bacterial burden and lung pathology and altered the local lung immune response in a C3HeB/FeJ mouse model of TB. Importantly, these findings were applicable only to mice conditioned to have an n-3 PUFA-sufficient status prior to infection. This is similar to the findings of Jordao et al., who found lower bacterial loads in the lungs and spleen of BALB/c *Mtb*-infected mice fed n-3 PUFA-rich diets than in mice that were provided with a fat-free diet.[19]

Our findings are different from those previously published, which showed that n-3 LCPUFAs inhibit immune response and worsen TB outcomes.[20-24] We hypothesize that the main reason for these discrepancies may be the timing of supplementation. Previous experiments were focused on the conditioning of the animals with n-3 LCPUFAs prior to infection or upon infection.[20-24] However, the timing of immunonutrition in any HDT approach for TB is critical and an early strong inflammatory response is essential.[4] In this study, we aimed to provide n-3 LCPUFA supplementation as therapy after the initial acute inflammatory response, by initiating the dietary intervention one week post-infection. Early ingestion of n-3 LCPUFAs, or upon infection initiation, has been shown to inhibit phagosome and phagolysosome maturation, which causes higher initial bacterial loads.[24, 25] Therefore, the timely initiation of n-3 LCPUFA supplementation was an important contributor to positive outcomes.

Furthermore, the dietary composition provided in previous studies differed from that which we used. Whilst the EPA/DHA ratio in the n-3+ diet groups was comparable to that of Jordao et al., who also found antibacterial effects of n-3 LCPUFA supplementation in TB, other studies that found negative effects provided either higher DHA concentrations or DHA only.[19,21,22,24,26] Furthermore, previous studies used *in vitro* cell culture models[24] or endogenously enriched mice (*fat-1* mice)[20], and differences in the genetic backgrounds of the mice may also have contributed.

The incorporation of n-3 LCPUFAs into phagocytic cell membranes changes membrane fluidity in addition to receptor expression, thereby enhancing bacterial phagocytosis, which has also been proven in TB.[24, 27] This is confirmed by the higher n-3 LCPUFA composition found in crude lung homogenates and PBMCs in our study, and subsequently, higher EPA incorporation would be expected in the macrophage and neutrophil phospholipid bilayers as well. Additionally, the changes in FA compositions resulted in a more pro-resolving lipid mediator profile. The n-3FAS/n-3+ group presented with higher lung concentrations of the pro-resolving 18-HEPE, which is an intermediate of the E-series resolvins (SPMs) synthesized from EPA.[28,29] Since SPMs aid in the differentiation and activation of macrophages and

neutrophils for phagocytosis and bacterial killing,[12,13,30] this further explains the bactericidal effects of n-3 LCPUFA supplementation observed in this study.

As lung inflammation is central in lesion formation, granuloma liquefication, cavity formation, and clinical outcomes, we hypothesized that the resolution of inflammation would also improve lung pathology.[2,31] Contrasting with previous research on n-3 LCPUFA supplementation in TB, we found a higher percentage of free alveolar space in the n-3FAS/n-3+ group than in the n-3FAS group.[21] This is most likely underpinned by the lower bacterial burden and lung inflammation and a more pro-resolving lipid mediator profile that was evident in the n-3FAS/n-3+ group. Furthermore, n-3 LCPUFA supplementation has previously been found to inhibit T cell proliferation, elsewhere and in TB, specifically.[21,32] Consistent with this, we also found a lower percentage of lung T cells in the n-3FAS/n-3+ group. This is driven by the effects of n-3 LCPUFAs, to cause structural changes in cell membranes, alterations in cell signaling and lipid mediator synthesis.[29] This, together with the lower bacterial burden in this group, may explain the lower T cell percentages in the n-3FAS/n-3+ mice.

Concerning lung cytokines, IFN-γ is important in the protection against TB; however, higher concentrations have been correlated with cavitary TB, higher bacterial loads and delayed culture conversion.[2,33] We found that IFN-γ concentrations were reduced in the n-3FAS/n-3+ group, which is consistent with the findings of others in TB.[21] This complements our findings on T cell numbers mentioned above.[34] In the n-3FAS/n-3+ group, CCL3 tended to be higher; however, CCL3 is not influenced by PG synthesis and cyclooxygenase (COX) activity and, therefore, n-3 LCPUFA supplementation is not expected to affect CCL3 concentrations.[35]

Supplementation of n-3 LCPUFA was successfully reflected in elevated cell membrane compositions and a pro-resolving lung lipid mediator profile. However, it was not fully translated into lower lung inflammatory lipid mediator and cytokine concentrations. A similar result to ours was found in a rat model injected with *Salmonella enteritidis* endotoxin.[36] The administration of fish oil altered pro-resolving lipid mediators without significantly changing the cytokine concentrations in the bronchoalveolar lavage fluid.[36] The fact that n-3 LCPUFAs have been reported to affect the Th1/Th2 balance mainly by inhibiting the production of Th1 type cytokines (including IFN-γ) may serve as an explanation for the current findings.[37] Furthermore, Kroesen and colleagues found a more pronounced effect on systemic (serum) cytokine production as compared with lung cytokines when administering aspirin in the same animal TB model as in our study.[4] In contrast, previous studies on n-3 LCPUFA treatment in *Mtb*-infected animals, macrophages, and peritoneal cells showed reduced PGE_2, leukotriene B_4 (LTB_4), TNF-α, IL-6, IL-1β, and monocyte chemotactic protein-1 synthesis.[19,22,24,38] Nevertheless, irrespective of the fact that immune cell recruitment and most

of the pro-inflammatory lipid mediators and cytokines were not significantly altered in the n-3FAS/n-3+ group, the increased pro-resolving lipid mediators were a positive finding, demonstrating the pro-resolving properties of n-3 LCPUFAs. Our results suggest that n-3 LCPUFA supplementation does not inhibit the host's natural immune and inflammatory responses necessary to protect against bacteria, supporting the notion that SPMs are not immunosuppressive and do not block inflammation but instead elicit pro-resolving effects.[12]

The n-3 LCPUFA supplementation in the n-3 PUFA-deficient group (n-3FAD/n-3+) did not have the same beneficial effects as in the n-3FAS/n-3+ group. This was despite the successful alteration of the n-3 LCPUFA cell membrane composition and lipid mediators towards a more pro-resolving lung profile in the n-3FAD/n-3+ group, and instead, showed increased IL-6 lung concentrations. Possible reasons why n-3 LCPUFA supplementation did not exert the same beneficial effects in the n-3FAD/n-3+ group may be related, firstly, to the dosage and duration of supplementation, which may have been too low in this deficient group, and secondly, to possible epigenetic adaptation to deficiency. Furthermore, although this was not part of the aim of this research, we found that mice that were continued on an n-3 PUFA-deficient diet (n-3FAD group) throughout the infection period presented with lower lung inflammation than the n-3 PUFA-sufficient group, which is not explained by their FA or lipid mediator profiles. Further investigation into these findings is warranted.

One of the strengths of this study was that we used a murine model that is well-established and reflective of human pulmonary TB. Furthermore, our experimental design, including the timing of supplementation, comparison of n-3 PUFA sufficiency and low status and the EPA/DHA ratio of our supplement, also strengthens our findings. However, in the n-3FAD group, specifically, the dose of n-3 LCPUFA supplementation may have been too low and/or the duration too short. Future prospects would be to perform this study with euthanasia time points at the different phases of the inflammatory and immune response, also including systemic markers of inflammation. Additionally, the possible beneficial effects of n-3 LCPUFAs, when administered in combination with standard TB treatment, are yet to be determined.

In conclusion, this study shows that n-3 LCPUFA supplementation, administered after the initial inflammatory response in *Mtb*-infected mice, lowered the bacterial burden in n-3 PUFA-sufficient mice, but not in mice with a low n-3 PUFA status. It further promoted a more pro-resolving lipid mediator profile together with reduced lung pathology and lower production of inflammatory cytokines. Considering this, n-3 LCPUFA supplementation may be a promising approach as an HDT in TB. This study emphasizes, however, that the timing, the EPA/DHA ratio administered and n-3 PUFA status prior to supplementation, are critical considerations.

Methods

Animals and ethics statement

Male C3HeB/FeJ mice (Jackson Laboratory, Bar Harbor, ME), aged 10 to 12 weeks, were housed under pathogen-free conditions in a biosafety level 3 containment facility. The experiments were performed in accordance with the South African National Guidelines and University of Cape Town practice guidelines for laboratory animal procedures. The protocol was approved by the Animal Ethics Committee, Faculty of Health Sciences, University of Cape Town (AEC 015/040) and the AnimCare Animal Research Ethics Committee of the North-West University (NWU-00260-16-A5).

Experimental design and animal diets

Mice had *ad libitum* access to food and water. The experimental design of this study is illustrated in Table 2. Mice were randomly allocated to an n-3 PUFA-deficient (n-3FAD) (n=20) or -sufficient diet (n-3FAS) (n=20) and kept on these diets for six weeks prior to infection in order to establish a sufficient or a low n-3 PUFA status. Mice were then infected *via* the aerosol route (described below) and their respective diets maintained for an additional week. One week post-infection (week 7), mice that were conditioned on the n-3 PUFA-sufficient diet (n-3FAS) were randomized to continue on this diet (n-3FAS) (n=10) or were switched the same diet supplemented with n-3 LCPUFAs (EPA plus DHA) (n-3FAS/n-3+ group, n=10) (Table 2). Similarly, the mice in the n-3FAD group either continued on the n-3FAD diet (n=10) or were switched to the n-3 LCPUFA-supplemented diet (n-3FAD/n-3+ group, n=10). The mice received these diets for an additional three weeks until euthanasia (at 28 days after infection).

All the purified experimental diets were obtained commercially (Dyets, Bethlehem, PA), and were based on the AIN-93G[39] formulation, all containing 10% fat, but with modifications in the fat source (Table 4). All the diets were isocaloric with identical macronutrient contents. The EPA- and DHA-supplemented diets (n-3+) contained commercially obtained Incromega TG4030 oil (Croda Chemicals, Snaith, Europe). Gas chromatography-mass spectrometry (GCMS) analysis was performed by the manufacturer to confirm the FA composition of the diets (Table 4). From this composition, the actual EPA and DHA intake could be calculated and was expressed as % of total energy intake. The body weight and food intake of mice were measured weekly. The daily food intake per mouse was calculated by dividing the food intake per cage by five and by seven. The results of this experiment were reproduced in a second experiment (resulting in 10 mice per treatment group). The data of one experiment (5 mice per group) are presented in this article.

Table 4 Fat source and fatty acid content of experimental diets[1]

Diet	Fat source	LA	ALA	AA	DHA	EPA
n-3FAS	70 g/kg Soybean oil	3.54	0.44	< 0.01	< 0.01	< 0.01
	30 g/kg Coconut oil					
n-3FAD	81 g/kg Coconut oil	1.30	0.01	< 0.01	< 0.01	< 0.01
	19 g/kg Safflower oil					
n-3+	70 g/kg Soybean oil	3.44	0.43	< 0.01	0.06	0.09
	27 g/kg Coconut oil				28 % FA	44 % FA
	3 g/kg Incromega TG4030					

[1]Based on gas chromatography-mass spectrometry (GCMS) analysis of diets. Values expressed as grams per 100 gram of diet.

ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LA: linoleic acid; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet.

Aerosol infection

A virulent *Mtb* H37Rv strain was cultured and stocks were prepared and stored at -80°C, as described elsewhere.[40] Mice were exposed to aerosol infection for 40 minutes by nebulizing 6 ml of a suspension that contained 2.4 x 10^7 live bacteria in an inhalation exposure system (model A4224, Glas-Col). One day following infection, four mice were euthanized to confirm the infection dose, which was 500 colony-forming units (CFU)/mouse.

Endpoint blood and tissue collection

At the end of the three weeks of receiving intervention diets, mice were euthanized by halothane exposure, followed by trunk blood collection by heart puncture. The blood was collected into EDTA coated Minivette® tubes (Minivette® POCT, 1000 µl, Sarsted), and then centrifuged. The plasma and buffy coat were removed for fatty acid analysis. The RBCs were washed twice with saline before storage at -80°C and subsequent fatty acid analysis. The lung lobes were removed aseptically and weighed prior to preparation. The left lung lobe and was homogenized in PBS for the analysis of the bacillary load and lung cytokines. The right superior and post-caval lung lobes were snap-frozen in liquid nitrogen and stored at -80°C for lung fatty acid and lipid mediator analysis. The right middle lobe was submerged in 10% formalin for histology analysis and the right inferior lobe prepared for flow cytometry.

Total phospholipid fatty acid composition analysis

FAs were extracted from ~20 mg lung tissue, homogenized in 10 µl phosphate-buffered saline with protease inhibitor (homogenization buffer) per 1 mg tissue, or from ~200 µL RBCs or PBMCs collected as buffy coat. Lipids were extracted from each lipid pool with chloroform:methanol (2:1, v:v; containing 0.01% BHT) by a modification of the method of Folch et al.[43] The lipid extracts were concentrated and the neutral lipids separated from the phospholipids by thin-layer chromatography (TLC) (silica gel 60 plates, Merck) and eluted with diethyl ether: petroleum ether: acetic acid (30:90:1, v:v:v). The lipid band containing phospholipids was removed from the TLC plate and transmethylated with methanol: sulphuric acid (95:5, v:v) at 70°C for 2 hours to form FA methyl esters (FAME). FAMEs were analyzed with an Agilent Technologies 7890A gas chromatography system equipped with an Agilent Technologies 7000B triple quad mass selective detector (Agilent Technologies, Santa Clara, CA) and quantification performed with Masshunter (B.06.00). Relative percentages of FAs (% w/w) were calculated by taking the concentration of a given FA as a percentage of the total concentration of all FAs identified in the sample.

Bacterial load determination

The bacterial loads of lungs were determined at euthanasia (28 days after infection). The left lung of each mouse was removed, weighed, homogenized and plated onto Difco™ Middlebrook 7H10 Agar (BD Biosciences, Johannesburg, South Africa) medium with OADC supplementation. The colony-forming unit counts were determined 21 days following incubation at 37°C. Data are expressed as $\log_{10}$ CFU.

Histopathology analysis

Right middle lobes of the lungs were dissected out and fixed in 4% neutral buffered formalin. The tissue was processed using the Leica TP 1020 Processor for 24 hours and subsequently embedded in paraffin wax. The Leica Sliding Microtome 2000R was used to cut 2 µm-thick sections of the embedded tissues. Three sections with 30 µm distance per section were cut, deparaffinized, and subsequently stained with the hematoxylin/eosin stain. The images were acquired in Nikon Eclipse 90i microscopes and analyzed with NIS-Elements AR software (Nikon Corporation, Tokyo, Japan) to determine the granulomatous area and alveolar space as a percentage of the total lung tissue.[41]

Flow Cytometry

Briefly, single-cell suspensions from the lung tissues were prepared by chopping them into small pieces and incubating them in Dulbecco's Modified Eagle Media (DMEM) containing 0.18 mg/ml Collagenase I (Sigma, St. Louis, MO), 0.02 mg/ml DNase I (Sigma, St. Louis, MO) for 1 hour at 37°C under constant rotation, followed by mechanically squeezing them through a 100 µm and 70 µm cell strainer sequentially. Erythrocytes were lysed using RBC lysis buffer (155 mM NH4Cl, 12 mM NaHCO3, 0.1 mM EDTA). Cells were then counted and subjected to flow cytometry. Lymphoid and myeloid compartments were investigated in the lung samples of mice on various intervention diets. Antibodies used for flow cytometry analysis were as follows: CD64-PeCy7, Ly6C-PerCPCy5.5, CD11b-V450, MHCII-APC, CD103-PE, CD11c-A700, SiglecF- APCCy7, Ly6G-FITC, PD-1-FITC, CD4-BV510, CD44-PE, NK1.1-APCCy7, CD3-A700, CD62L-V450, CD19-PerCPCy5.5, CD8-APC, KLRG1-BV786 purchased from BD Biosciences and eBioscience (BD Biosciences, Johannesburg, South Africa).[41,42]

Lipid mediator analyses

Lipid mediators in crude lung homogenates were analyzed with liquid chromatography-tandem mass spectrometry. Seventeen-HDHA; 5-, 11-, 12-, 15- and 18-HEPE; 5-, 8-, 9-, 11-, 12-, and 15-HETE; PGD_1; PGE_2; PGE_3 and PGD_2 were measured. Lipid mediators were extracted from ~50 mg lung tissue, in 10 µl/mg homogenization buffer, with solid-phase extraction using Strata-X (Phenomenex, Torrance, CA). The method was modified for Strata-XSPE columns from a previously described method [44]. Data were quantified with Masshunter B0502, using external calibration for each compound and internal standards (PGD_2-d4, PGE_2-d4, PGF_2-d4 and 5-and 12-HETE-d8; 1000 pg of each (Cayman Chemicals, Ann Arbor, MI)) to correct for losses and matrix effects.

Cytokine analyses

The left lung lobe homogenates leftover from determining bacterial load were centrifuged and the supernatant was frozen at -80ºC until analysis. The cytokines were measured in cell-free lung homogenates, using the Quansys Biosciences Q-Plex™ Mouse Cytokine Screen (West Logan, WV) Q-Plex Array 16 plex (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, monocyte chemoattractant protein-1, IFN-γ, TNF-α, chemokine CCL3, granulocyte-macrophage colony-stimulating factor (GMCSF), RANTES) according to manufacturer instructions, using the Q-View Imager Pro, Q-View Software.

Statistical analysis

Data are presented as means ± standard error of the mean. Statistical analysis was performed using IBM SPSS statistics software (version 25; IBM Corporation). To determine the differences between FA composition at baseline in the n-3FAD and n-3FAS group, the Student Fischer T-test for independent variables was used. Between-intervention group differences were examined using one-way analysis of variance (ANOVA) and the Tukey post-hoc test. A *P*-value of less than 0.05 was considered significant.

Acknowledgments

The authors thank Rodney Lucas (University of Cape Town, Cape Town, South Africa) and Kobus Venter (North-West University, South Africa) for their technical assistance with animals and Adriaan Jacobs and Cecile Cooke (North-West University, South Africa) for their assistance with laboratory analyses. Research was supported by the South African Medical Research Council under a Self-Initiated Research Grant (MRC-SIR), but the views and opinions expressed are those of the authors and not of the SAMRC. This study was also funded by the Nutricia Research Foundation.

Author contributions

A.N., L.M., R.D., C.M.S., and R.B. conceptualized study; A.N., L.M., D.L., and S.P. designed study; A.N., L.M., M.O., S.P., M.B., F.H., and S.K. conducted research; A.N., L.M., M.B., M.O., and S.K. analyzed data; A.N., L.M., L.Z., C.M.S., M.O., and S.P. assisted with data interpretation; A.N. wrote the manuscript. All authors reviewed and approved the final manuscript.

Disclosure

The authors have no conflict of interest to declare.

References

1. Meghji, J., Simpson, H., Squire, S.B. & Mortimer, K. A systematic review of the prevalence and pattern of imaging defined post-TB lung disease. *PloS one.* **11**, e0161176 (2016).

2. Kumar, N.P., Moideen, K., Banurekha, V.V., Nair, D. & Babu, S. Plasma pro-inflammatory cytokines are markers of disease severity and bacterial burden in pulmonary tuberculosis. *Open Forum Infect. Dis.* **6** (2019).

3. Stek, C., Allwood, B., Walker, N.F., Wilkinson, R.J., Lynen, L. & Meintjes, G. The immune mechanisms of lung parenchymal damage in tuberculosis and the role of host-directed therapy. *Front. Microbiol.* **9** (2018).

4. Kroesen, V.M. *et al.* A Beneficial Effect of Low-Dose Aspirin in a Murine Model of Active Tuberculosis. *Front. Immunol.* **9,** 798 (2018).

5. Critchley, J.A., Young, F., Orton, L. & Garner, P. Corticosteroids for prevention of mortality in people with tuberculosis: a systematic review and meta-analysis. *Lancet Infect. Dis.* **13**, 223-237 (2013).

6. Marzo, E. *et al*. Damaging role of neutrophilic infiltration in a mouse model of progressive tuberculosis. *Tuberculosis.* **94**, 55-64 (2014).

7. Kroesen, V.M. *et al.* Non-Steroidal Anti-inflammatory Drugs As Host-Directed Therapy for Tuberculosis: A Systematic Review. *Front. Immunol.* **8,** 772 (2017).

8. Ivanyi, J. & Zumla, A. Nonsteroidal antiinflammatory drugs for adjunctive tuberculosis treatment. *J. Infect. Dis.* **208,** 185-188 (2013).

9. Calder, P.C. Omega-3 fatty acids and inflammatory processes: from molecules to man. *Biochem. Soc. Trans.* **45**, 1105-1115 (2017).

10. Browning, L.M. *et al.* Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish–. *Am. J. Clin. Nutr.* **96**, 748-758 (2012).

11. Jakiela, B. *et al.* Eicosanoid biosynthesis during mucociliary and mucous metaplastic differentiation of bronchial epithelial cells. *Prostaglandins Other Lipid Mediat.* **106,** 116-123 (2013).

12. Serhan, C.N., Chiang, N. & Dalli, J. New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration. *Mol. Aspects Med.* **64**, 1 -17 (2017).

13. Chiang, N. *et al.* Infection regulates pro-resolving mediators that lower antibiotic requirements. *Nature.* **484**, 524 (2012).

14. Lenaerts, A., Barry, C.E. & Dartois, V. Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunol. Rev.* **264**, 288-307 (2015).

15. Stark, K.D., Van Elswyk, M.E., Higgins, M.R., Weatherford, C.A. & Salem, N. Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults. *Prog. Lipid Res.* **63,** 132-152 (2016).

16. Baker, E.J., Miles, E.A., Burdge, G.C., Yaqoob, P. & Calder, P.C. Metabolism and functional effects of plant-derived omega-3 fatty acids in humans. *Prog. Lipid Res.* **64,** 30-56 (2016).

17. Brenna, J.T., Plourde, M., Stark, K.D., Jones, P.J. & Lin, Y.H. Best practices for the design, laboratory analysis, and reporting of trials involving fatty acids. *Am. J. Clin. Nutr.* **108**, 211-227 (2018).

18. Sanak, M. Eicosanoid mediators in the airway inflammation of asthmatic patients: what is new? *Ann. Allergy Asthma Immunol.* **8**, 481-490 (2016).

19. Jordao, L. *et al.* Effects of omega-3 and-6 fatty acids on Mycobacterium tuberculosis in macrophages and in mice. *Microbes Infect.* **10**, 1379-1386 (2008).

20. Bonilla, D.L., Fan, Y.Y., Chapkin, R.S. & McMurray, D.N. Transgenic mice enriched in omega-3 fatty acids are more susceptible to pulmonary tuberculosis: impaired resistance to tuberculosis in fat-1 mice. *J. Infect. Dis.* **201**, 399-408 (2010).

21. McFarland, C.T., Fan, Y.Y., Chapkin, R.S., Weeks, B.R. & McMurray, D.N. Dietary polyunsaturated fatty acids modulate resistance to Mycobacterium tuberculosis in guinea pigs. *J. Nutr.* **138**, 2123-2128 (2008).

22. Paul, K.P. *et al.* Influence of n-6 and n-3 polyunsaturated fatty acids on the resistance to experimental tuberculosis. *Metb.* **46**, 619-624 (1997).

23. Mayatepek, E.*et al.* Influence of dietary (n-3)-polyunsaturated fatty acids on leukotriene B 4 and prostaglandin E 2 synthesis and course of experimental tuberculosis in guinea pigs. *Infection.* **22**, 106-112 (1994).

24. Bonilla, D.L., Ly, L.H., Fan, Y.Y., Chapkin, R.S. & McMurray, D.N. Incorporation of a Dietary Omega 3 Fatty Acid Impairs Murine Macrophage Responses to Mycobacterium. *PloS one.* **5**, e10878 (2010).

25. Anes, E. *et al*. Selected lipids activate phagosome actin assembly and maturation resulting in killing of pathogenic mycobacteria. *Nat. cell biol.* **5**, 793 (2003).

26. Bhattacharya, A., Sun, D., Rahman, M. & Fernandes, G. Different ratios of eicosapentaenoic and docosahexaenoic omega-3 fatty acids in commercial fish oils differentially alter pro-inflammatory cytokines in peritoneal macrophages from C57BL/6 female mice. *J. Nutr. Biochem.* **18**, 23-30 (2007).

27. Calder, P.C., Bond, J.A., Harvey, D.J., Gordon, S. & Newsholme, E.A. Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochem. J.* **269**, 807-814 (1990).

28. Oh, S.F., Pillai, P.S., Recchiuti, A., Yang, R. & Serhan, C.N. Pro-resolving actions and stereoselective biosynthesis of 18S E-series resolvins in human leukocytes and murine inflammation. *J. Clin. Invest.* **121**, 569-581 (2011).

29. Calder, P.C. Marine omega-3 fatty acids and inflammatory processes: effects, mechanisms and clinical relevance. *BBA-Mol Cell Biol.* **1851**, 469-484 (2015).

30. Codagnone, M. *et al.* Resolvin D1 enhances the resolution of lung inflammation caused by long-term Pseudomonas aeruginosa infection. *Mucosal Immunol.* **11**, 35-49 (2018).

31. Vilaplana, C. *et al*. Ibuprofen therapy resulted in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. *J. Infect. Dis.* **208**, 199-202 (2013).

32. Yaqoob, P., Newsholme, E. & Calder, P. The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation. *Immunol.* **82**, 603 (1994).

33. Mayer-Barber, K.D. *et al.* Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature.* **511**, 99 (2014).

34. Desvignes, L., Wolf, A.J. & Ernst, J.D. Dynamic roles of type I and type II IFNs in early infection with Mycobacterium tuberculosis. *J. Immun.* **188**, 6205-6215 (2012).

35. Davatelis, G. *et al*. Macrophage inflammatory protein-1: a prostaglandin-independent endogenous pyrogen. *Science.* **243**, 1066-1068 (1989).

36. Mancuso, P. *et al*. Dietary fish oil and fish and borage oil suppress intrapulmonary proinflammatory eicosanoid biosynthesis and attenuate pulmonary neutrophil accumulation in endotoxic rats. *Crit. Care Med.* **25**, 1198-1206 (1997).

37. Wallace, FA. *et al*. Dietary fatty acids influence the production of Th1-but not Th2-type cytokines. *J. Leukoc. Biol.* **69**, 449-457 (2001).

38. Bonilla, D.L., Fan, Y.Y., Chapkin, R.S. & McMurray, D.N. Transgenic mice enriched in omega-3 fatty acids are more susceptible to pulmonary tuberculosis: impaired resistance to tuberculosis in fat-1 mice. *J. Infect. Dis.* **201**, 399-408 (2010).

39. Reeves, P.G., Nielsen, F.H. & Fahey, .GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr*. **123**, 1939–1951 (1993).

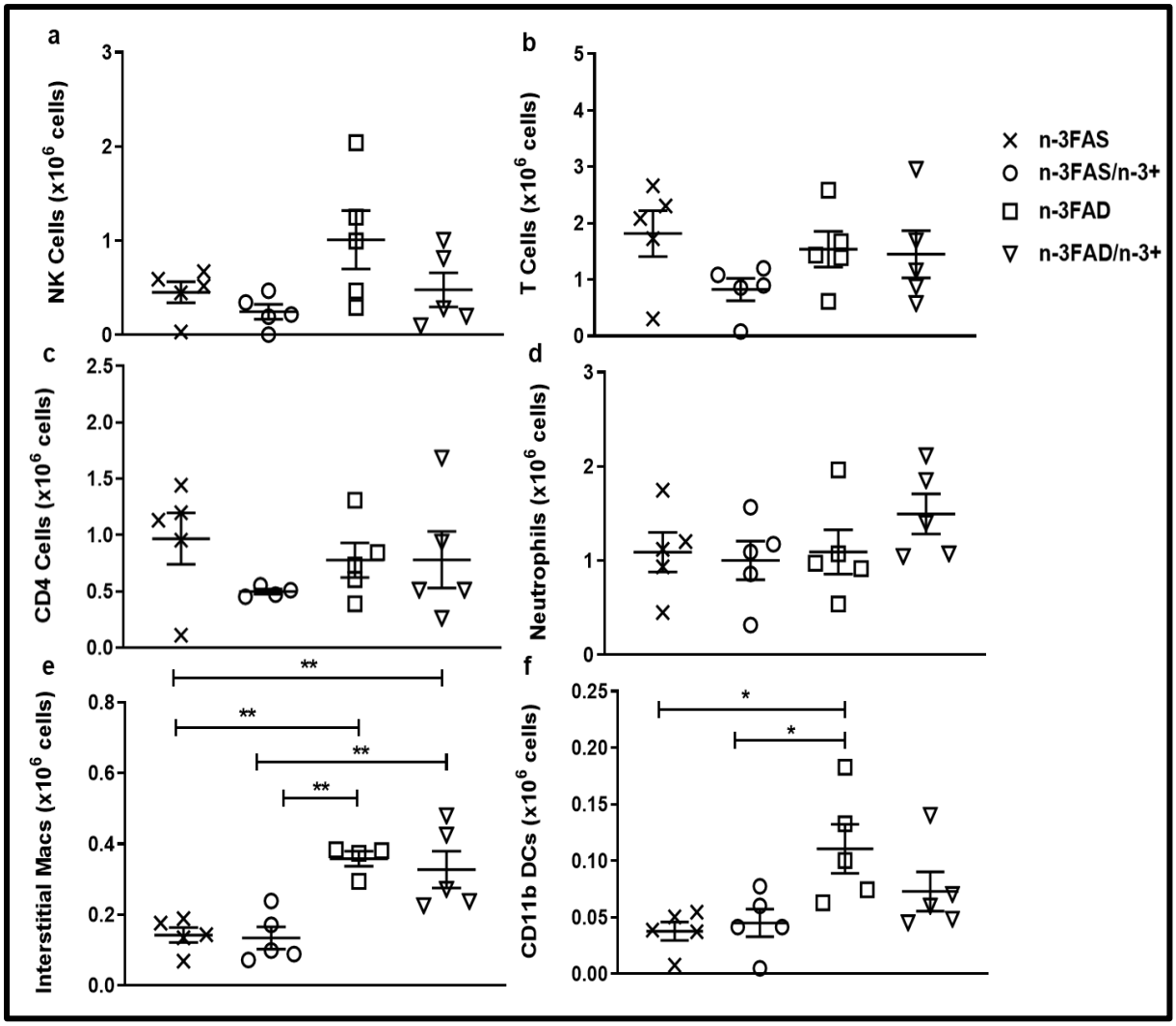
40. Guler, R. *et al*. Blocking IL-1α but not IL-1β increases susceptibility to chronic Mycobacterium tuberculosis infection in mice. *Vaccine.* **29**, 1339-1346 (2011).

41. Parihar, S. *et al.* Protein kinase C-delta (PKC delta), a marker of inflammation and tuberculosis disease progression in humans, is important for optimal macrophage killing effector functions and survival in mice. *Mucosal Immunol.* **11**, 578-579 (2018).

42. Parihar, S.P. *et al.* Statin Therapy Reduces the Mycobacterium tuberculosis Burden in Human Macrophages and in Mice by Enhancing Autophagy and Phagosome Maturation. *J. Infect. Dis.* **209**, 754-763 (2014).

43. Folch, J. Lees, M. & Stanley, G.S. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**, 497-509 (1957).

44. Malan, L., Baumgartner, J., Zandberg, L., Calder, P. & Smuts, C.M. Iron and a mixture of dha and EPA 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. *Prostaglandins Leukot. Essent. Fatty Acids.* **105**, 15-25 (2016).



Supplemental Figure 1 Immune cell phenotyping of a) natural killer cells ($CD3^-$ $NK1.1^+$), b) T cells ($CD3^+$ CD19), c) $CD4^+$ T cells ($CD3^+$ $CD4^+$), d) neutrophils ($CD11b^+$ $Ly6G^+$), e) interstitial macrophages ($Cd64^+$ $CDCd11b^+$ $CD11c^+$ $SiglecF^-$), and CD11b dendritic cells ($CD11b^+$ $CD11c^+$ $MHCII^+$ $CD64^-$) in crude lung homogenates after providing *Mtb*-infected mice with n-3FAS, n-3FAS/n-3+, n-3FAD, or n-3FAD/n-3+ diets for three weeks. The values represent mean ± SEM x 10^6. Results repeated in two experiments, data shown for one experiment (n=5 per group). One-way ANOVA followed by Tuckey post hoc test was used to compare means. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. DCs, dendritic cells; Macs, macrophages; NK, natural killer; n-3+, omega-3 long-chain polyunsaturated fatty acid supplemented diet; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid sufficient diet; /, switch.

CHAPTER 4: MANUSCRIPT 2

Both post-infection n-3 fatty acid and iron supplementation alone, but not in combination, lower inflammation and anemia of infection in *Mycobacterium tuberculosis*-infected mice

Arista Nienaber[1], Jeannine Baumgartner[1,2], Robin C Dolman[1], Mumin Ozturk[3,4], Lizelle Zandberg[1], Frank EA Hayford[1,5], Renee Blaauw[6], Frank Brombacher[3,4], Suraj Parihar[3,7], Cornelius M Smuts[1], Linda Malan[1]

[1] Centre of Excellence for Nutrition, North-West University, Potchefstroom, South Africa

[2] Laboratory of Human Nutrition, ETH, Zurich, Switzerland

[3] Institute of Infectious Diseases and Molecular Medicine (IDM), Division of Immunology and South African Medical Research Council (SAMRC) Immunology of Infectious Diseases, University of Cape Town, Cape Town, South Africa

[4] International Centre for Genetic Engineering and Biotechnology (ICGEB), Cape Town-Component, Cape Town, South Africa

[5] Department of Nutrition and Dietetics, University of Ghana, Accra, Ghana

[6] Division of Human Nutrition, Stellenbosch University, Stellenbosch, South Africa

[7] Centre of Infectious Diseases Research in Africa, University of Cape Town, Cape Town, South Africa

Running title: Omega-3 lowers anemia of infection in TB

Prepared for publication in *The Journal of Nutrition*

Abstract

Background: Anemia is a common complication of tuberculosis (TB), caused mainly by progressive inflammation that impairs iron absorption and utilization, and is linked to poor clinical outcomes. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) provide inflammation-resolving properties, whereas iron supplementation in TB may have limited efficacy and enhance bacterial growth.

Objective: We aimed to investigate the effects of iron and EPA/DHA supplementation, alone and in combination, on inflammation, iron status markers and clinical outcomes in *Mycobacterium tuberculosis* (*Mtb)*-infected C3HeB/FeJ mice.

Methods: Using a 2x2 design, mice were randomly allocated to the AIN-93 purified diet without (control) or with supplemental iron (Fe) or EPA/DHA, or Fe+EPA/DHA, 1 wk post-infection for 3 wk.

Results: We found antagonistic FexEPA/DHA interactions on soluble transferrin receptor (sTfR) (*P*=0.030), ferritin (*P*=0.045) and hepcidin (*P*=0.044) concentrations. Mice supplemented with Fe or EPA/DHA alone had lower sTfR (both *P*=0.045), ferritin (*P*=0.040 and *P*=0.06, respectively) and hepcidin (*P*=0.07 and *P*=0.047, respectively) than controls, but these lowering effects were attenuated in Fe+EPA/DHA mice. EPA/DHA supplementation promoted a more inflammation-resolving lipid mediator profile and significantly lowered plasma IL-1β and TNF-α, lung IL-1α and IFN-γ. Iron supplementation significantly lowered lung IL-1α and IL-1β, plasma IL-1β, TNF-α, and IL-6. We observed antagonistic FexEPA/DHA interactions for lung IL-1α (*P*=0.001), lL-1β (*P*=0.003), and IFN-γ (*P*=0.016); the lowering effects of Fe and EPA/DHA alone were attenuated in the Fe+EPA/DHA group. EPA/DHA significantly lowered $CD8^+$ T cell counts, whilst iron resulted in significantly higher T cell, $CD4^+$ and $CD8^+$ T cell, interstitial macrophage, CD11b dendritic cell (DC), and CD103 DC counts. We found FexEPA/DHA interactions to attenuate the effect of Fe for higher CD11b DC (*P*=0.018) and $CD8^+$ T cell (*P*=0.05) counts in the Fe+EPA/DHA group. We also found an antagonistic FexEPA/DHA interaction (*P*=0.009) on lung bacterial load. Mice supplemented with EPA/DHA alone had a significantly lower lung bacterial load than controls, but this effect was attenuated in Fe+EPA/DHA mice.

Conclusion: These results indicate that both post-infection EPA/DHA and iron supplementation individually, but not in combination, lower systemic and lung inflammation and mitigate anemia of infection in TB-infected mice. EPA/DHA also enhanced bactericidal effects, and may be an effective strategy to support inflammation resolution and treat anemia of infection.

Keywords: Anemia of infection, docosahexaenoic acid, eicosapentaenoic acid, inflammation, iron, tuberculosis

Introduction

Anemia is a common complication in tuberculosis (TB), affecting 30 to 94% of diagnosed TB patients (1-6). In addition, anemia has been linked to poor TB outcomes, such as delayed sputum conversion, higher mortality rates and TB reoccurrence (3, 7-9). Exaggerated progressive inflammation is characteristic of TB, and induces hepatic hepcidin synthesis (2, 10). Hepcidin is the main regulator of iron homeostasis and mediates the degradation and internalization of the iron exporter ferroportin in macrophages, hepatocytes, and enterocytes (11). This leads to intracellular sequestration of iron and the inhibition of iron absorption (12, 13). Consequently, hepcidin restricts iron availability for pathogens that rely on host iron stores (14, 15). The persistent inflammation in TB patients also restricts iron availability for erythropoiesis and results in anemia of infection (6, 16). Anemia of inflammation (which includes anemia of infection), and iron-deficiency anemia are the two most common anemias worldwide and often co-exist (17, 18). Although less common in TB, iron-deficiency anemia resulting from low dietary iron intake, amongst other causes, also affects TB patients and may contribute to disease susceptibility and progression (1-3, 19). Iron supplementation may be essential for those patients with iron-deficiency anemia (1-3, 19, 20). However, the treatment of anemia with iron is complicated by impaired absorption and the risk of promoting bacterial growth and inflammation, resulting in poor clinical outcomes (6, 14).

Omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs) have anti-inflammatory and pro-resolving properties, and our group and others have demonstrated that these fatty acids lower the excessive inflammation in TB (Nienaber et al., submitted manuscript) (21). Omega-3 LCPUFAs serve as precursors for lipid mediator (LM) synthesis including the inflammation-resolving protectins, resolvins, and maresins that are metabolized from docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (22). Therefore, we hypothesized that n-3 LCPUFA administration would result in a shift towards a more pro-resolving LM profile and a reduction in inflammation, which might lower the burden of anemia of infection in TB patients.

In addition, our group has previously shown that iron supplementation resulted in a pro-inflammatory LM profile and increased respiratory morbidity in iron-deficient South African (SA) school-children (23, 24). However, in children supplemented with a combination of iron and EPA plus DHA a pro-resolving LM profile was maintained and the iron-induced increase in respiratory morbidity attenuated (23, 24). We, therefore, hypothesized that providing a combination treatment of iron and n-3 LCPUFAs might potentially be an approach to deliver iron more safely in TB. Thus, the aim of this study was to investigate the effect of EPA plus DHA and iron supplementation, alone and in combination, on inflammation, anemia and iron

status markers, as well as bacterial burden, in *Mycobacterium* tuberculosis (*Mtb*)-infected C3HeB/FeJ mice.

Methods

Animals and ethical statement

Male C3HeB/FeJ mice (Jackson Laboratory, Bar Harbor, ME), aged 10 to 12 weeks, were bred and housed at the Institute of Infectious Diseases and Molecular Medicine (IDM), University of Cape Town (UCT), Cape Town, SA. Following infection, mice were housed in a biosafety level 3 containment facility, five per individually ventilated cage with filter tops (type 2 long), as well as dried wood shavings and shredded filter paper as floor coverings. The temperature range was set at 22 to 24 °C and 12-to-12 hour light cycles. The experiments were performed in accordance with the SA National Guidelines and the UCT practice guidelines for laboratory animal procedures. The protocol was approved by the Animal Ethics Committee, Faculty of Health Sciences, UCT (AEC 015/040) and the AnimCare Animal Research Ethics Committee of the North-West University (NWU) (NWU-00260-16-A5).

Experimental design and diets

Mice had *ad libitum* access to food and water and were all conditioned on the same standardized AIN-93G purified rodent diet for six weeks prior to infection. Baseline hemoglobin (Hb) concentrations in all mice, and the fatty acid (FA) composition of red blood cells (RBCs) in a sub-sample of mice (n=6), were measured prior to infection from tail vein blood. Mice were then infected with *Mtb via* the aerosol route (described below). One week post-infection, the mice were randomly allocated to continue on the AIN-93G control diet, (control, n=10); or to receive the AIN-93G control diet supplemented with EPA and DHA (EPA/DHA, n=10); or the AIN-93G control diet supplemented with iron (Fe, n=10); or the AIN-93G control diet supplemented with iron and EPA plus DHA (Fe+EPA/DHA) (n=10). The mice were fed these diets for three weeks, whereafter they were euthanized at 28 days post-infection. An uninfected reference group was kept on the same AIN-93G control diet as the control group throughout the study period (n=3). All the purified experimental diets were obtained commercially (Dyets, Bethlehem, USA), and were based on the AIN-93G formulation (25), all containing 10% fat, but with modifications in fat source and/or iron content.

The dietary FA and iron composition of the experimental diets is presented in **(Table 1).** All the diets were isocaloric with the same macronutrient content. The EPA/DHA supplemented diets (EPA/DHA and Fe+EPA/DHA) contained commercially obtained Incromega TG4030 oil

(Croda Chemicals, Europe) with a minimum EPA at 44% of total FA and DHA at 28% of total FA. Gas chromatography-mass spectrometry (GCMS) analysis was performed by the manufacturer to confirm the FA and iron composition of the diets (Table 1). The body weight and food intake of mice were measured weekly. Food intake per mouse was calculated by dividing the food intake per cage by five and seven. The results of this experiment were reproduced in a second experiment (resulting in 10 mice per treatment group). The data of one experiment (5 mice per group) are presented in this article.

Table 1 Iron content, fat source, and fatty acid composition of the experimental diets[1]

Group	Iron[2]	Fat source[3]	ALA[3]	AA[3]	DHA[3]	EPA[3]
Control and uninfected	40	7 g soybean oil 3 g Coconut oil	0.44	< 0.01	< 0.007	< 0.01
Fe	130	7 g Soybean oil 3 g Coconut oil	0.44	< 0.01	< 0.007	< 0.01
EPA/DHA	40	7 g Soybean oil 2.7 g Coconut oil 3 g Incromega TG4030	0.43	< 0.01	0.06	0.09
Fe+EPA/DHA	123	7 g Soybean oil 2.7 g Coconut oil 3 g Incromega TG4030	0.43	< 0.01	0.06	0.09

[1]Based on gas chromatography-mass spectrometry (GCMS) analysis of diets. [2]Values expressed as ppm iron per 100 g diet. [3] Values expressed as g per 100 g diet.

AA, arachidonic acid; ALA, alpha-linolenic acid; EPA/DHA, eicosapentaenoic and docosahexaenoic acid supplemented group; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Fe, iron supplemented group; Fe+EPA/DHA, iron and eicosapentaenoic and docosahexaenoic acid supplemented group.

Aerosol infection

A virulent *Mtb* H37Rv strain was cultured and stocks were prepared and stored at -80°C, as described elsewhere (26). Mice were exposed to aerosol infection for 40 minutes by nebulizing 6 ml of a suspension that contained 2.4 x 10^7 live bacteria in an inhalation exposure system (model A4224, Glas-Col). One day following infection, four mice were euthanized to confirm the infection dose, which was 500 colony-forming units (CFU)/mouse.

Endpoint blood and tissue collection

At the end of the three weeks of receiving intervention diets, mice were euthanized by halothane exposure, followed by trunk blood collection by heart puncture. The blood was collected into EDTA-coated Minivette® tubes (Minivette® POCT, 1000 µl, Sarsted), the Hb measured in whole blood and then centrifuged. The plasma and buffy coat were removed for fatty acid (buffy coat), ferritin, soluble transferrin receptor (sTfR), hepcidin and cytokine (plasma) analyses. The RBCs were washed twice with saline before storage at -80°C and subsequent fatty acid analysis. The lung lobes and spleen were removed aseptically and weighed prior to preparation. The left lung lobe was homogenized in PBS for the analysis of the bacillary load and lung cytokines. The right superior and post-caval lung lobes were snap-frozen in liquid nitrogen and stored at -80°C for lung fatty acid and lipid mediator analysis. The right middle lobe was submerged in 10% formalin for histology analysis and the right inferior lobe prepared for flow cytometry.

Total phospholipid fatty acid composition analysis

Lipids were extracted from ~20 mg lung tissue, homogenized in 10 µl phosphate-buffered saline with protease inhibitor (homogenization buffer) per 1 mg tissue; and from ~200 µL RBCs and peripheral blood mononuclear cells (PBMCs) collected as buffy coat, with chloroform: methanol (2:1, v:v; containing 0.01% BHT) by a modification of the method of Folch et al. (27). The lipid extracts were concentrated and the neutral lipids separated from the phospholipids by thin-layer chromatography (TLC) (silica gel 60 plates, Merck) and eluted with diethyl ether: petroleum ether: acetic acid (30:90:1, v:v:v). The lipid band containing phospholipids was removed from the TLC plate and transmethylated with methanol: sulphuric acid (95:5, v:v) at 70°C for 2 h to form FA methyl esters (FAME). FAMEs were analyzed with an Agilent Technologies 7890A gas chromatography system equipped with an Agilent Technologies 7000B triple quad mass selective detector (Agilent Technologies, Santa Clara, USA) and quantification performed with Masshunter (B.06.00). Relative percentages of FAs (% w/w) were calculated by taking the concentration of a given FA as a percentage of the total concentration of all FAs identified in the sample.

Lipid mediator analyses

LMs in crude lung homogenates were analyzed with liquid chromatography-tandem mass spectrometry. Seventeen-hydroxydocosahexaenoic acid (17-HDHA); 5-, 11-, 12-, 15- and 18-hydroxyeicosapentaenoic acid (HEPE); 5-, 8-, and 9-hydroxyeicosatetraenoic acids (HETE); prostaglandin D_1 (PD_1); PGE_2; PGE_3 and PGD_2 were measured. LMs were extracted

from ~50 mg lung tissue, in 10 µl/mg homogenization buffer, with solid-phase extraction (SPE) using Strata-X (Phenomenex, Torrance, USA). The method was modified for Strata-XSPE columns from a previously described method (24). Data were quantified with Masshunter B0502, using external calibration for each compound and internal standards (PGD_2-d4, PGE_2-d4, PGF_2-d4 and 5- and 12-HETE-d8; 1000 pg of each, Cayman Chemicals, Ann Arbor, USA) to correct for losses and matrix effects.

Cytokine analyses

The cytokines interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, monocyte chemoattractant protein, interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), chemokine ligand 3 (CCL3), granulocyte-macrophage colony-stimulating factor (GMCSF), chemokine ligand 5 (CCL5) were measured in cell-free lung homogenates and plasma. Cytokines were analyzed using the Quansys Biosciences Q-Plex™ Mouse Cytokine Screen (West Logan, USA) 16-plex Array for mouse cytokines according to manufacturer instructions. Arrays were analyzed using the Q-View Imager Pro and the Q-View Software.

Markers of iron status and anemia of infection

Hb concentrations were measured prior to infection in a tail vein whole blood and directly after blood collection in whole blood using a portable HemoCue® Hb 201+ photometer (HemoCue AB, Angelholm, Sweden). Mice-specific enzyme-linked immunosorbent assays (ELISA) kits from ELAB Science (Houston, USA) were used for the analysis of ferritin, soluble transferrin receptor (sTfR) and hepcidin concentrations in plasma.

Lung histopathology

Right middle lobes of the lungs were dissected out and fixed in 4% neutral buffered formalin. The tissue was processed using the Leica TP 1020 Processor for 24 hours and subsequently embedded in paraffin wax. The Leica Sliding Microtome 2000R was used to cut 2 µm-thick sections of the embedded tissues. Three sections with 30 µm distance per section were cut, deparaffinized, and subsequently stained with the hematoxylin/eosin stain. The images were acquired in Nikon Eclipse 90i microscopes and analyzed with NIS-Elements AR software (Nikon Corporation, Tokyo, Japan) to determine the granulomatous area and alveolar space as a percentage of the total lung tissue.

Flow cytometry

Briefly, single-cell suspensions from the lung tissues were prepared by chopping them into small pieces and incubating them in Dulbecco's Modified Eagle Media (DMEM) containing

0.18 mg/ml Collagenase I (Sigma, St. Louis, MO), 0.02 mg/ml DNase I (Sigma, St. Louis, MO) for 1 hour at 37°C under constant rotation, followed by mechanically squeezing them through a 100 µm and 70 µm cell strainer sequentially. Erythrocytes were lysed using RBC lysis buffer (155 mM NH4Cl, 12 mM NaHCO3, 0.1 mM EDTA). Cells were then counted and subjected to flow cytometry. Lymphoid and myeloid compartments were investigated in the lung samples from mice on various intervention diets. Antibodies used for flow cytometry analysis were as follows: CD64-PeCy7, Ly6C-PerCPCy5.5, CD11b-V450, MHCII-APC, CD103-PE, CD11c-A700, SiglecF- APCCy7, Ly6G-FITC, PD-1-FITC, CD4-BV510, CD44-PE, NK1.1-APCCy7, CD3-A700, CD62L-V450, CD19-PerCPCy5.5, CD8-APC, and KLRG1-BV786 purchased from BD Biosciences and eBioscience (BD Biosciences, Johannesburg, South Africa) (28).

Bacterial load

The bacterial loads of lungs were determined at euthanasia (28 days after infection). The left lung of each mouse was removed, weighed, homogenized and plated onto Difco™ Middlebrook 7H10 Agar (BD Biosciences) medium with OADC supplementation. The colony-forming unit counts were determined 21 days following incubation at 37°C. Data are expressed as $\log_{10}$ CFU.

Statistical analysis

Data are presented as mean ± standard error of the mean. Statistical analysis was performed using IBM SPSS statistics software (version 25; IBM Corporation). The main effects of EPA/DHA (EPA/DHA & Fe+EPA/DHA versus control & Fe) and iron (Fe & Fe+EPA/DHA versus EPA/DHA & control) supplementation, and their interaction (Fe x EPA/DHA), on all outcome variables were analyzed by using two-way factor analysis of variance (ANOVA). Significant treatment effects in the absence of a significant interaction effect indicate additive effects of the treatments, whereas a significant interaction implies synergism or antagonism. In the presence of a significant main effect or interaction, between-group differences were examined using one-way ANOVA and the Tukey post-hoc test. For iron parameters Fisher's least significant difference post-hoc test was used. Between-group analysis was only conducted when there was a significant main effect or treatment interaction for the variable. Data for ferritin and hepcidin concentrations were log-transformed prior to statistical analysis to improve normality. The differences in indices of iron status and anemia of infection between the infected intervention groups and the uninfected reference group were determined by the Dunnett two-sided t-test. A *P*-value of less than 0.05 was considered significant.

Results

Bodyweight gain and food intake

There was no difference in food intake between groups. However, iron supplementation lowered the percentage of body weight gain of the *Mtb*-infected mice ($P < 0.001$). The control (6.70 ± 0.63%) and EPA/DHA (8.12 ± 1.19%) groups had a significantly higher percentage weight gain compared with the groups supplemented with iron (Fe, 0.88 ± 1.50% and Fe+EPA/DHA, 0.16 ± 1.50%) (control vs. Fe, $P < 0.001$; control vs. Fe+EPA/DHA, $P = 0.001$; EPA/DHA vs. Fe, $P = 0.002$; and EPA/DHA vs. Fe+EPA/DHA, $P = 0.005$).

Total phospholipid fatty acid composition of RBCs, PBMCs, and crude lung homogenates

Table 2 presents the phospholipid FA composition of RBCs, PBMCs, and crude lung homogenates of all infected groups, measured at the endpoint. From Table 2 it is evident that EPA/DHA supplementation resulted in higher phospholipid EPA, DHA, and total n-3 LCPUFA (all $P < 0.001$). On the other hand, iron supplementation lowered EPA ($P = 0.041$ and $P = 0.038$) and total n-3 LCPUFAs ($P = 0.046$ and $P = 0.011$) in RBCs and PBMCs, as well as DHA in PBMCs ($P = 0.008$).

With regards to n-6 PUFAs, EPA/DHA supplementation lowered arachidonic acid (AA), osbond acid, total n-6 LCPUFAs and total n-6/n-3 LCPUFA ratios in RBCs, PBMCs and crude lung homogenates (all $P < 0.001$, except for AA in PBMCs $P = 0.001$ and AA in lungs $P = 0.023$). Iron also lowered osbond acid in RBCs and PBMCs ($P = 0.008$ and $P = 0.025$). Additionally, there were Fe x EPA/DHA interactions in RBCs and PBMCs ($P < 0.021$ and $P = 0.005$) to lower osbond acid. However, there was an effect of iron supplementation for higher AA ($P < 0.001$ and $P = 0.013$) and n-6 LCPUFAs ($P = 0.001$ and $P = 0.022$) in PBMCs and lung homogenates and n-6/n-3 LCPUFA ratios in RBCs, PBMCs, and lung homogenates ($P = 0.004$, $P < 0.001$, $P = 0.039$). Respective differences between groups are shown in Table 2.

Table 2 Phospholipid FA composition of RBCs, PBMCs, and crude lung homogenates in *Mtb* infected mice receiving control, EPA/DHA, Fe, or Fe+EPA/DHA diets for 3wk[1]

% of total FA	Control	EPA/DHA	Fe	Fe+EPA/DHA	*P* value[2]		
					EPA/DHA	Fe	Fe x EPA/DHA
20:5(n-3) (EPA)							
RBC	0.13 ± 0.00^{b}	0.51 ± 0.04^{a}	0.12 ± 0.00^{b}	0.43 ± 0.02^{a}	< 0.001	0.041	0.17
PBMC	0.21 ± 0.01^{b}	0.89 ± 0.04^{a}	0.16 ± 0.01^{b}	0.55 ± 0.11^{a}	< 0.001	0.038	0.19
Lung	0.17 ± 0.01^{b}	0.40 ± 0.01^{a}	0.17 ± 0.03^{b}	0.38 ± 0.01^{a}	< 0.001	0.74	0.63
22:6(n-3) (DHA)							
RBC	6.09 ± 0.21^{bc}	7.48 ± 0.41^{a}	5.58 ± 0.22^{c}	6.83 ± 0.25^{ab}	< 0.001	0.06	0.82
PBMC	9.04 ± 0.20^{a}	9.82 ± 0.22^{a}	7.57 ± 0.29^{b}	9.52 ± 0.39^{a}	< 0.001	0.008	0.06
Lung	8.08 ± 0.15^{b}	9.68 ± 0.28^{a}	7.99 ± 0.08^{b}	9.86 ± 0.13^{a}	< 0.001	0.75	0.38
Total n-3 LCPUFAs							
RBC	6.63 ±0.20^{bc}	8.59±0.48^{a}	6.06 ± 0.24^{c}	7.80 ± 0.27^{ab}	< 0.001	0.046	0.74
PBMC	10.6 ± 0.21^{b}	12.4 ± 0.33^{a}	8.76 ± 0.42^{c}	11.8 ± 0.64^{ab}	< 0.001	0.011	0.20
Lung	10.0 ± 0.14^{b}	12.6 ±0.26^{a}	9.73 ± 0.10^{b}	12.9 ± 0.12^{a}	< 0.001	0.96	0.07
20:4(n-6) AA							
RBC	17.71 ± 0.27^{a}	16.42 ± 0.30^{ab}	17.68 ± 0.40^{a}	15.88 ± 0.28^{b}	< 0.001	0.38	0.43
PBMC	16.36 ± 0.39^{b}	14.76 ± 0.31^{b}	20.94 ± 0.70^{a}	17.34 ± 0.94^{b}	0.001	< 0.001	0.14
Lung	14.40 ± 0.15^{ab}	13.32 ± 0.42^{b}	14.76 ± 0.27^{a}	14.31 ± 0.23^{ab}	0.023	0.013	0.25

% of total FA	Control	EPA/DHA	Fe	Fe+EPA/DHA	*P* value[2]		
					EPA/DHA	Fe	Fe x EPA/DHA
22:5(n-6) (osbond)							
RBC	0.87 ± 0.10[a]	0.37 ± 0.01[c]	0.49 ± 0.01[b]	0.35 ± 0.00[c]	< 0.001	0.008	0.021
PBMC	1.42 ± 0.04[a]	0.74 ± 0.05[b]	1.01 ± 0.08[b]	0.80 ± 0.10[b]	< 0.001	0.025	0.005
Lung	1.13 ± 0.05[a]	0.54 ± 0.01[b]	1.01 ± 0.02[a]	0.54 ± 0.01[b]	< 0.001	0.27	0.05
Total n-6 LCPUFAs							
RBC	20.2 ± 0.32[a]	18.7 ± 0.44[a]	20.1 ± 0.47[ab]	18.0 ± 0.33[b]	< 0.001	0.33	0.45
PBMC	21.8 ± 0.41[b]	19.4 ± 0.54[b]	25.7 ± 0.78[a]	22.0 ± 1.16[b]	0.001	0.00	0.45
Lung	21.2 ± 0.19[ab]	18.3 ± 0.50[c]	21.5 ± 0.37[a]	19.9 ± 0.35[b]	< 0.001	0.022	0.07
Total n-6/ n-3 LCPUFA ratio							
RBC	3.05 ± 0.05[b]	2.19 ± 0.07[c]	3.32 ± 0.07[a]	2.31 ± 0.04[c]	< 0.001	0.004	0.19
PBMC	2.06 ± 0.02[b]	1.55 ± 0.01[b]	2.94 ± 0.14[a]	1.91 ± 0.23[b]	< 0.001	< 0.001	0.07
Lung	2.12 ± 0.19[a]	1.45 ± 0.50[b]	2.21 ± 0.03[a]	1.54 ± 0.04[b]	< 0.001	0.039	0.97

[1] Values are presented as mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group, except for Fe group PBMC n=4 and Fe+EPA/DHA group lung n=4). [2] Two-way ANOVA was used to test effects of EPA/DHA (control & Fe vs. EPA/DHA & Fe+EPA/DHA), Fe (control & EPA/DHA vs. Fe & Fe+EPA/DHA), and Fe x EPA/DHA interactions. One-way ANOVA followed by Tuckey post-hoc test was used to compare groups. Means in a row without common superscript letters differ significantly, $P < 0.05$.

AA, Arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EPA/DHA, eicosapentaenoic acid and docosahexaenoic acid-supplemented group; FA, fatty acid; Fe, iron-supplemented group; Fe+EPA/DHA, iron plus eicosapentaenoic acid and docosahexaenoic acid-supplemented group; LCPUFA, long-chain polyunsaturated fatty acid; lung, crude lung homogenates; PBMC, peripheral blood mononuclear cell; RBC, red blood cell.

Biomarkers of iron status and anemia of infection

We further evaluated indices of iron status as well as hepcidin concentrations to determine the effects of intervention diets on biomarkers that have been linked to hypoferremia and anemia in TB. We observed a significant effect of EPA/DHA supplementation for lower Hb concentrations ($P = 0.031$), and a trend for an Fe x EPA/DHA interaction ($P = 0.08$) **(Table 3).** The Hb concentrations in the EPA/DHA supplemented mice did not differ from the control group but were significantly lower in the Fe+EPA/DHA ($P = 0.009$) compared with the Fe supplemented mice (Table 3). However, when compared with a non-infected reference group fed the control diet (n=3), *Mtb*-infection did not result in lower Hb concentrations in control mice nor in the mice supplemented with Fe and/or EPA/DHA.

Mtb-infection significantly increased sTfR, ferritin and hepcidin concentrations in the control group compared with a non-infected reference group ($P = 0.002$, $P = 0.001$, and $P = 0.002$) (Table 3). We found antagonistic Fe x EPA/DHA interactions on sTfR ($P = 0.030$), ferritin ($P = 0.045$) and hepcidin ($P = 0.044$) concentrations. The mice receiving the diets supplemented with EPA/DHA or Fe alone presented with significantly lower sTfR concentrations compared with the control group (both $P = 0.045$), but this sTfR lowering effect of EPA/DHA and iron alone was attenuated in mice supplemented with Fe+EPA/DHA (Table 3). Similarly, the mice supplemented with Fe alone had significantly lower ferritin concentrations ($P = 0.040$) and the mice supplemented with EPA/DHA tended ($P = 0.06$) to have lower concentrations than the control mice. The ferritin concentrations of the combined Fe+EPA/DHA group did not differ from any of the other groups. Additionally, the mice supplemented with EPA/DHA presented with significantly lower ($P = 0.047$) and the mice supplemented with Fe tended to have lower ($P = 0.07$) hepcidin concentrations compared with the control group, with the Fe+EPA/DHA group not having significantly different hepcidin concentrations from the other groups. When compared with the non-infected reference group, sTfR, ferritin and hepcidin concentrations remained significantly higher in the Fe+EPA/DHA group ($P = 0.027$, $P = 0.007$, $P = 0.029$) but did not differ from the mice supplemented with EPA/DHA or Fe alone

Table 3 Biomarkers of iron status and anaemia of infection in *Mtb*-infected mice receiving control, EPA/DHA, Fe, or Fe+EPA/DHA diets for 3wk[1]

Iron parameter	Non-infected reference (n=3)[2]	Control (n=5)	EPA/DHA (n=5)	Fe (n=5)	Fe+EPA/DHA (n=5)	*P* value[3]		
						EPA/DHA	Fe	Fe x EPA/DHA
Hb (g/dL)	14.40 ± 0.82	13.58 ± 0.27^{ab}	13.40 ± 0.32^{ab}	14.3 ± 0.37^{a}	12.96 ± 0.38^{b}	0.031	0.59	0.08
Ferritin (ng/mL)	26.1 ± 2.52	222 ± 43.91^{a**}	136 ± 32.52^{ab}	125 ± 23.41^{b}	164 ± 13.80^{ab*}	0.51	0.63	0.045
sTfR (ng/mL)	2.34 ± 0.15	17.5 ± 2.77^{a**}	10.8 ± 2.43^{b}	10.8 ± 2.17^{b}	14.5 ± 0.97^{ab**}	0.49	0.50	0.030
Hepcidin (µg/mL)	0.92 ± 1.12	14.3 ± 2.68^{a**}	8.05 ± 2.23^{b}	8.67 ± 2.06^{ab}	10.1 ± 4.65^{ab**}	0.71	0.36	0.044

[1] Values are means ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group).

[2] Different from the uninfected reference group determined by Dunnett's two-sided t-test. $^{*}P$ <0.05, $^{**}P$ < 0.01, $^{***}P$ < 0.001.

[3] Two-way ANOVA was used to test the effects of EPA/DHA (control & Fe vs. EPA/DHA & Fe+EPA/DHA), Fe (control & EPA/DHA vs. Fe & Fe+EPA/DHA), and Fe x EPA/DHA interactions. One-way ANOVA followed by Tuckey post-hoc test was used to compare groups. Means in a row without common superscript letters differ significantly, P < 0.05. EPA/DHA, eicosapentaenoic acid and docosahexaenoic acid supplemented-group; Fe, iron supplemented-group; Fe+EPA/DHA, iron plus eicosapentaenoic acid and docosahexaenoic acid supplemented-group; Hb, hemoglobin; sTfR, soluble transferrin receptor.

Tuberculosis related clinical outcomes

Figure 1 shows the lung- and spleen-weight-indexes amongst the different groups. This represents the weight of either the lungs or spleen in relation to the bodyweight of the mice (spleen or lung weight divided by endpoint bodyweight). A higher index represents worse disease severity. There were antagonistic Fe x EPA/DHA interactions on lung- and spleen-weight indexes (P = 0.009 and P = 0.002) in *Mtb*-infected mice **(Figure 1).** The mice supplemented with Fe alone tended to have a lower lung-weight-index when compared with the control mice (P = 0.08), but this lung-weight-index lowering trend of iron supplementation was attenuated in the mice supplemented with Fe+EPA/DHA (Fe+EPA/DHA vs. control, P = 0.99) (Figure 1a). In contrast, only the mice supplemented with EPA/DHA presented with a lower spleen-weight-index compared with the control group (P = 0.012), but this decrease was attenuated in the mice supplemented with Fe+EPA/DHA (Fe+DHA/EPA vs. control, P = 0.99) (Figure 1b).

Figure 2 shows the lung bacterial loads, percentage of free alveolar space and lung histology images, which represent the disease severity and lung pathology of the various groups. We found a significant antagonistic Fe x EPA/DHA interaction on lung bacterial load (P = 0.009) (Figure 2a) and a trend for an interaction on free alveolar space (%) (P = 0.08) (Figure 2b). Supplementation of EPA/DHA alone lowered lung bacterial load compared with the control mice (P = 0.008), but this effect was attenuated in the mice supplemented with EPA/DHA and Fe in combination (Fe+EPA/DHA vs. control, P =0.69).

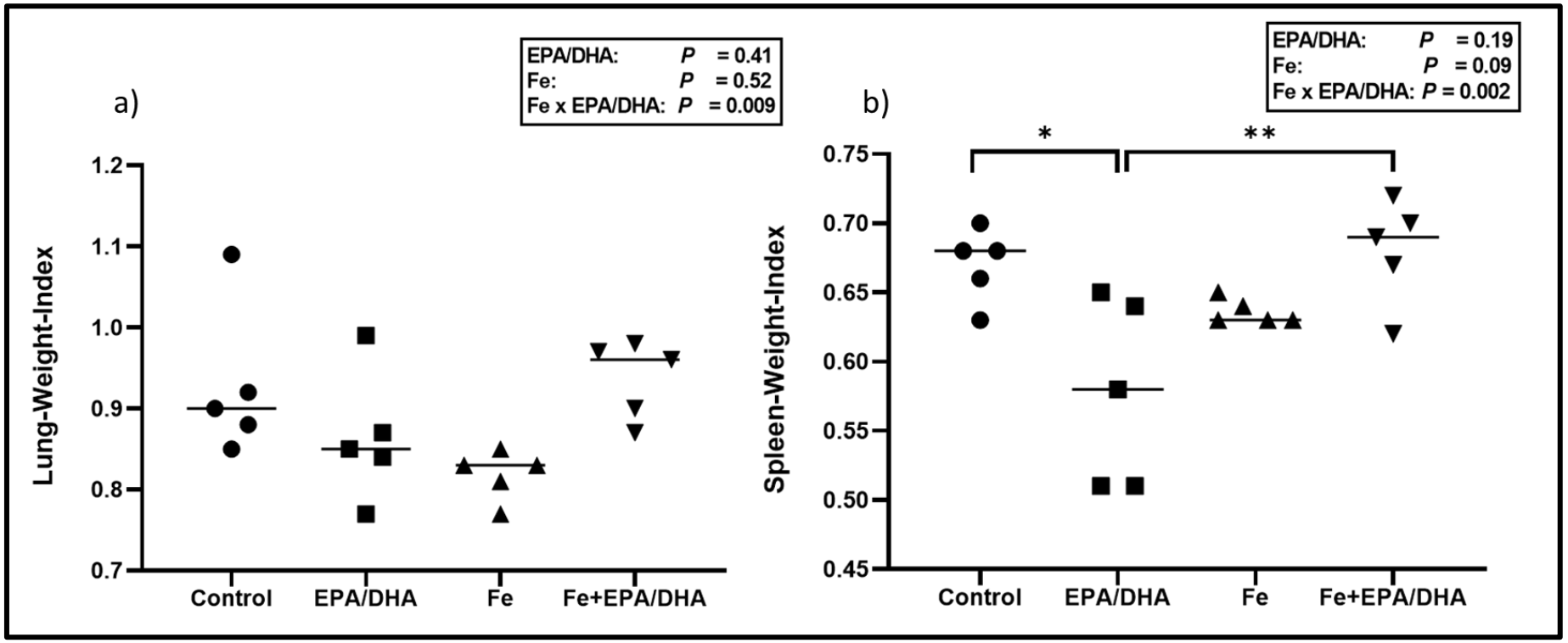


Figure 1 Mean a) lung-weight-index and b) spleen-weight-index after providing *Mtb*-infected mice with control, EPA/DHA, Fe, or Fe+EPA/DHA diets for 3wk. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). Two-way ANOVA was used to test effects of EPA/DHA (control & Fe vs. EPA/DHA & Fe+EPA/DHA), Fe (control & EPA/DHA vs. Fe & Fe+EPA/DHA), and Fe x EPA/DHA interactions. One-way ANOVA followed by Tuckey post-hoc test was used to compare groups. *P < 0.05, **P < 0.01. EPA/DHA, eicosapentaenoic and docosahexaenoic acid supplemented-group; Fe, iron-supplemented group; Fe+EPA/DHA, iron plus eicosapentaenoic and docosahexaenoic acid-supplemented group.

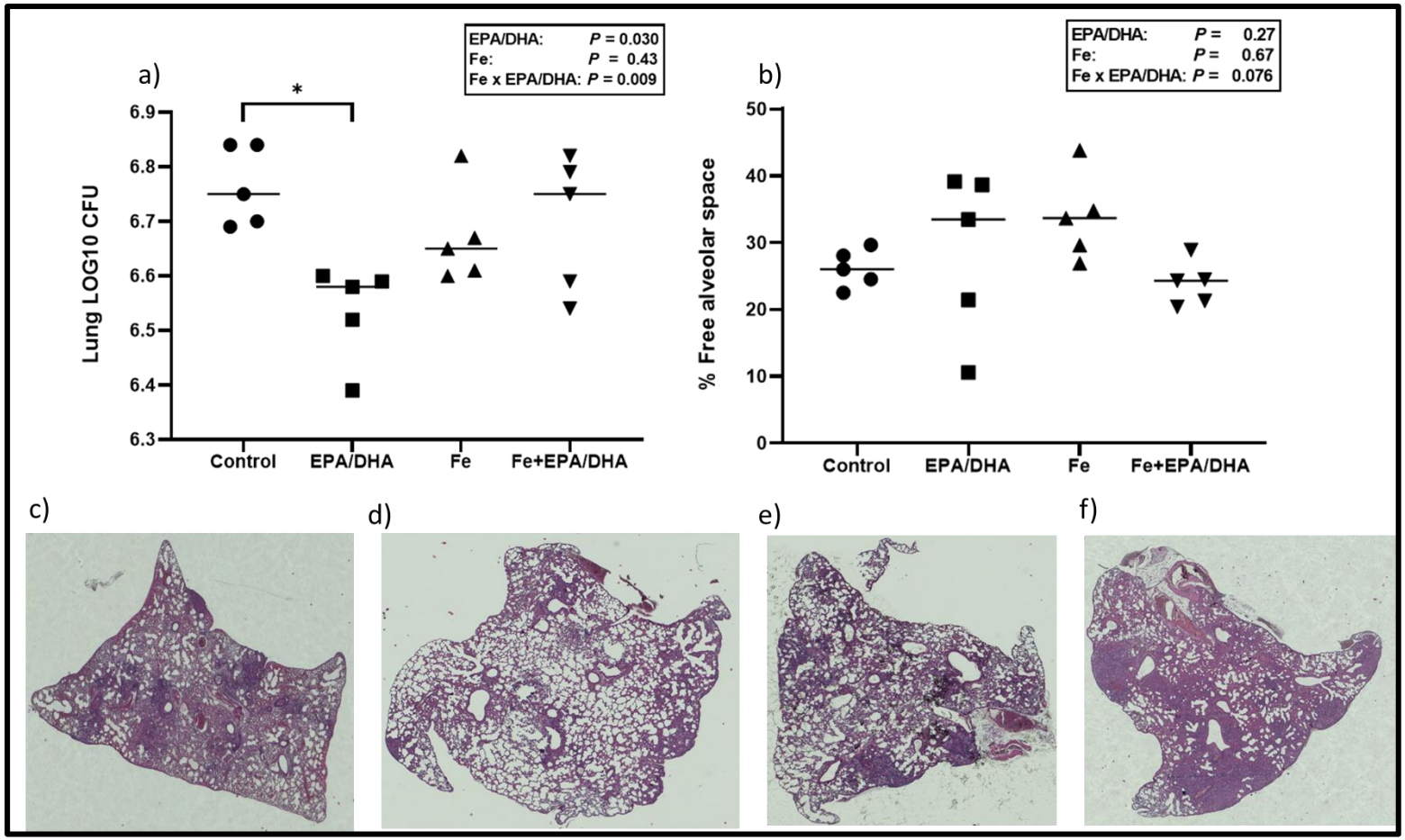


Figure 2 a) Mean lung bacterial load, b) free alveolar space (%), and representative hematoxylin-eosin staining of lungs for c) control, d) EPA/DHA, e) Fe, f) Fe+EPA/DHA groups after providing *Mtb*-infected mice with control, EPA/DHA, Fe, or Fe+EPA/DHA diets for 3 wk. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). Two-way ANOVA was used to test effects of EPA/DHA (control & Fe vs. EPA/DHA & Fe+EPA/DHA), Fe (control & EPA/DHA vs. Fe & Fe+EPA/DHA), and Fe x EPA/DHA interactions. One-way ANOVA followed by Tuckey post-hoc test was used to compare means. *P < 0.05. CFU, colony-forming units; EPA/DHA, eicosapentaenoic and docosahexaenoic acid-supplemented group; Fe, iron-supplemented group; Fe+EPA/DHA, iron plus eicosapentaenoic and docosahexaenoic acid-supplemented group.

Lipid mediators in crude lung homogenates

Next, we measured the lung LM concentrations, as this was the target site of our intervention and we hypothesized that the LM production would be altered according to the FA composition of the intervention diets. As shown in **Figure 3,** EPA/DHA supplementation of *Mtb*-infected mice resulted in higher lung concentrations of the EPA-derived PGE_3 ($P < 0.001$) and pro-resolving LM intermediates 5- ($P = 0.047$), 11- ($P < 0.001$), 12- ($P = 0.002$), 15- ($P < 0.001$), and 18-HEPE ($P < 0.001$) and the DHA-derived 17-HDHA ($P = 0.023$) (Figure 3a, 3b, 3c, 3d, 3e, 3f). There was an antagonistic Fe x EPA/DHA interaction for the AA-derived pro-inflammatory intermediate 11-HETE ($P = 0.047$) and a tendency towards an antagonistic interaction for 15-HETE ($P = 0.08$), but there were no significant differences between groups (Supplemental Figure 1a and 1b). No treatment effects could be found on the other pro-inflammatory LMs that were measured.

Other markers of the immune and inflammatory response

Plasma and lung cytokines were measured in order to determine systemic and local inflammatory effects, respectively **(Figures 4 and 5)**. We observed antagonistic Fe x EPA/DHA interactions for lung IL-1α ($P = 0.001$), lL-1β ($P = 0.003$) and IFN-γ ($P = 0.016$) (Figure 4a, 4b, and 4c). The *Mtb*-infected mice supplemented with EPA/DHA or Fe alone had significantly lower lung IL-1α concentrations than the control mice ($P = 0.039$ and $P = 0.049$), but the IL-1α-lowering effect was attenuated in mice supplemented with Fe+EPA/DHA (Figure 4a). The mice supplemented with Fe had lower lung lL-1β compared with control mice ($P = 0.022$), but not the mice supplemented with Fe+EPA/DHA (Figure 4b). Compared with the control groups, EPA/DHA supplementation significantly lowered lung IFN-γ concentrations only in the mice receiving EPA/DHA alone ($P = 0.003$), but not in the mice receiving EPA/DHA in combination with Fe (Figure 4c). Furthermore, there was a main effect of EPA/DHA supplementation for higher lung CCL3 concentrations ($P = 0.004$), but only the mice receiving EPA/DHA alone had significantly higher CCL3 compared with the control mice ($P = 0.026$) (Figure 4d).

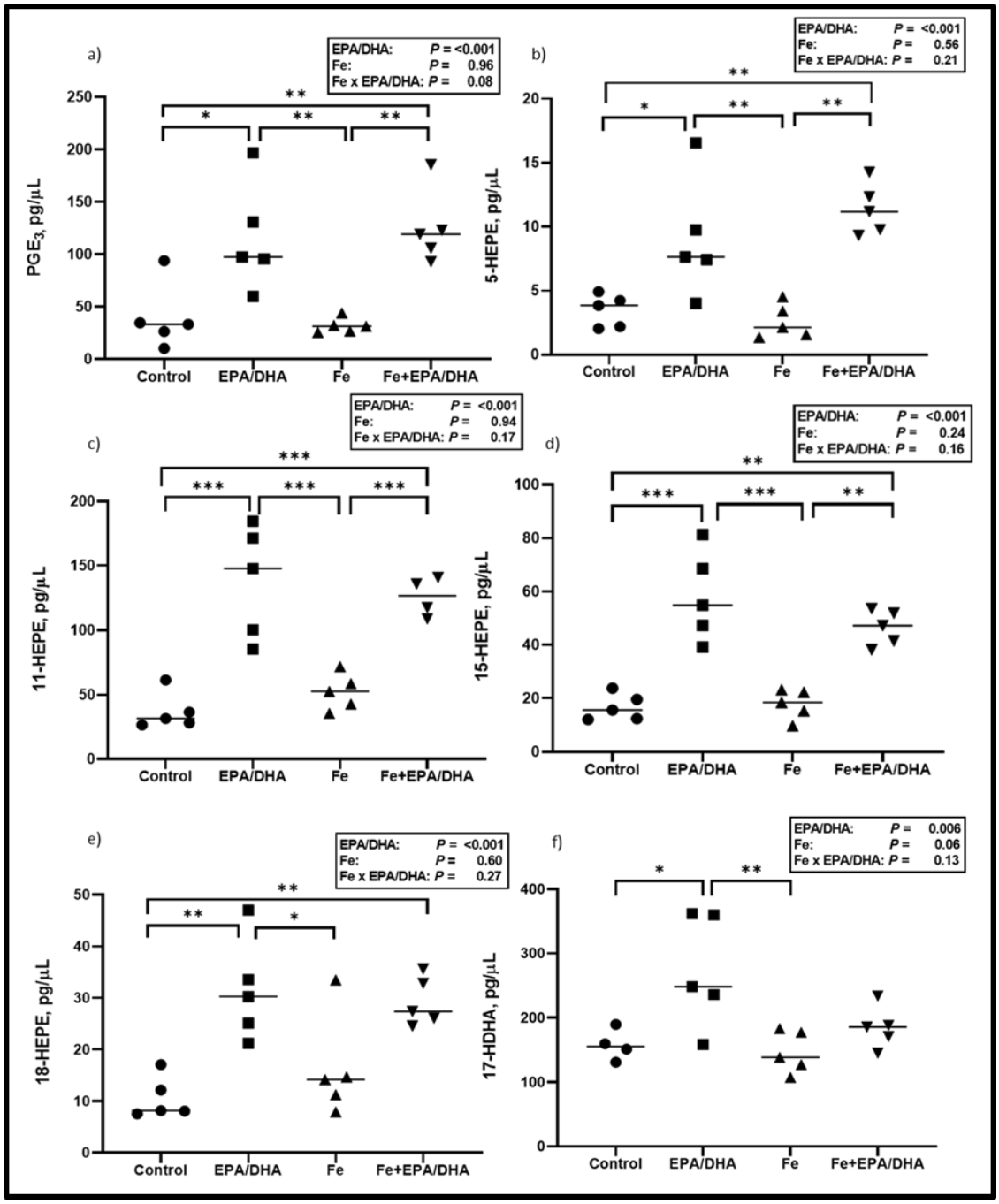


Figure 3 Lipid mediator concentrations including a) PGE3, b) 5-HEPE, c) 11-HEPE, d) 15-HEPE, e) 17-HDHA and f) 18-HEPE after providing *Mtb*-infected mice with control, EPA/DHA, Fe, or Fe+EPA/DHA diets for 3wk. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). Two-way ANOVA was used to test effects of EPA/DHA (control & Fe vs. EPA/DHA & Fe+EPA/DHA), Fe (control & EPA/DHA vs. Fe & Fe+EPA/DHA), and Fe x EPA/DHA interactions. One-way ANOVA followed by Tuckey post-hoc test was used to compare groups. $P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. EPA/DHA, eicosapentaenoic and docosahexaenoic acid-supplemented group; Fe, iron-supplemented group; Fe+EPA/DHA, iron plus eicosapentaenoic and docosahexaenoic acid-supplemented group; HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid.

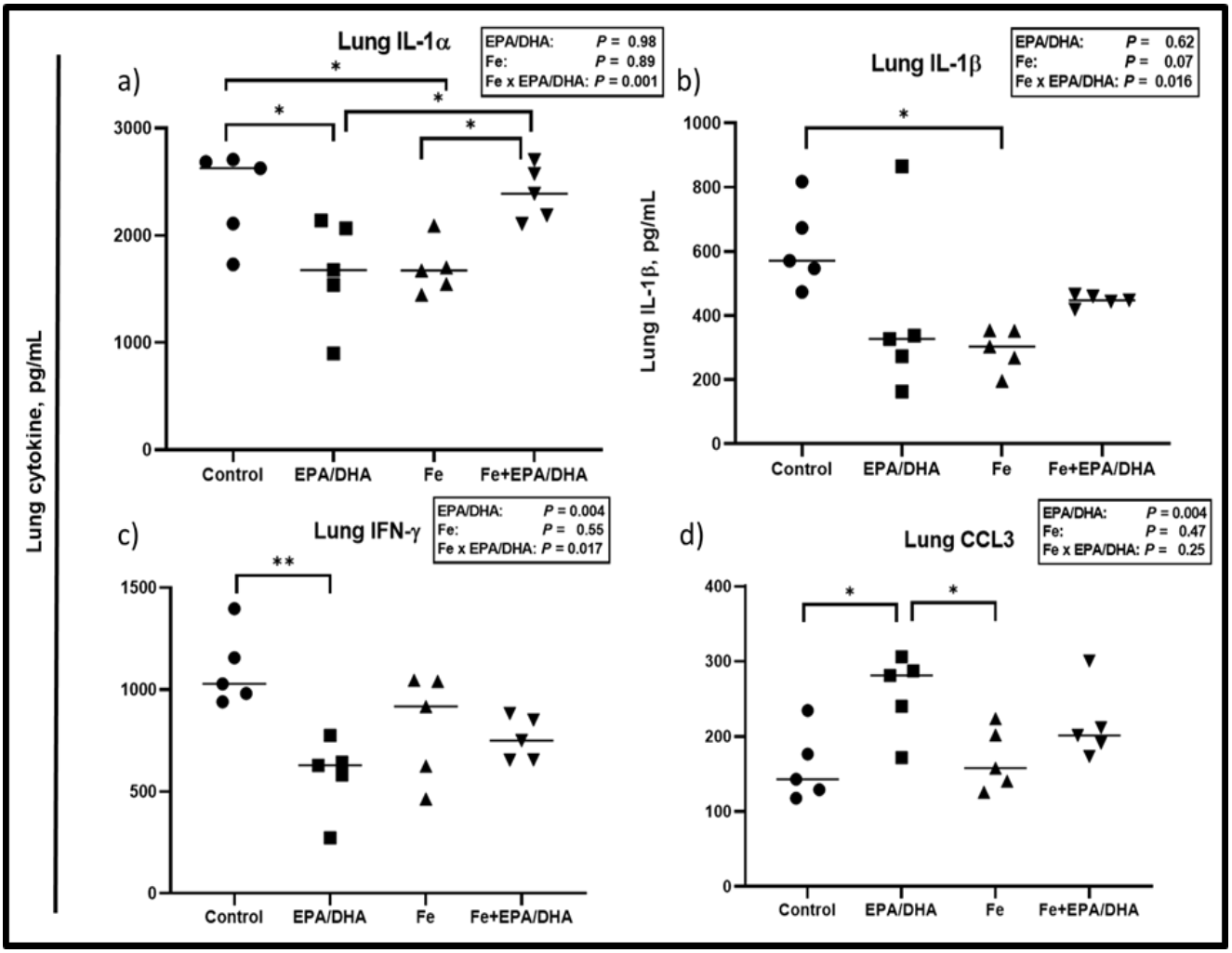


Figure 4 Lung cytokine concentrations after providing *Mtb*-infected mice with control, EPA/DHA, Fe, or Fe+EPA/DHA diets for 3wk, including a) IL-1α, b) IL-1β, c) IFN-γ, and d) CCL3. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). Two-way ANOVA was used to test the effects of EPA/DHA (control & Fe vs. EPA/DHA & Fe+EPA/DHA), Fe (control & EPA/DHA vs. Fe & Fe+EPA/DHA), and Fe x EPA/DHA interactions. One-way ANOVA followed by Tuckey post-hoc test was used to compare means. *P < 0.05, **P < 0.01. CCL3, chemokine ligand 3; EPA/DHA, eicosapentaenoic and docosahexaenoic acid-supplemented group; Fe, iron-supplemented group; Fe+EPA/DHA, iron plus eicosapentaenoic and docosahexaenoic acid-supplemented group; IFN-γ, interferon-gamma; IL, interleukin.

Concerning plasma cytokines (Figure 5), we observed Fe x EPA/DHA interactions for the inflammatory plasma cytokines IL-1α, IL-1β, and TNF-α ($P = 0.044$, $P = 0.029$, and $P = 0.014$) (Figure 5a, b, and c). EPA/DHA and Fe supplementation significantly and equally lowered plasma IL-1β when provided alone or in combination ($P = 0.030$, $P = 0.007$, and $P = 0.029$) (Figure 5b). A similar pattern was observed for IL-1α, TNF-α, and IL-6. There were no significant main effects or between-group differences for plasma IL-1α (Figure 5a). TNF-α was significantly lower in the mice supplemented with EPA/DHA ($P = 0.040$) and Fe ($P = 0.009$) when compared with the control mice, but only tended to be lower in the Fe+DHA/EPA group ($P = 0.06$) (Figure 5c). There were also main effects of EPA/DHA and Fe for lower plasma IL-6 concentrations ($P = 0.022$ and $P = 0.011$), and significantly lower plasma IL-6 in the Fe and Fe+EPA/DHA groups compared to the control mice ($P = 0.038$ and $P = 0.006$, Figure 5d).

We also compared the lung immune cell phenotypes determined by flow cytometry and showed absolute counts **(Figure 6)** and percentages of total cells **(Supplemental Figure 2)** of a single cell suspension of the lungs. We found main effects of Fe supplementation for higher counts of T cells ($P = 0.011$), $CD4^+$ T cells ($P = 0.001$), $CD8^+$ T cells ($P = 0.007$), interstitial macrophages ($P = 0.004$), alveolar macrophages ($P = 0.035$), CD103 dendritic cells (DCs) ($P = 0.035$), and CD11b DCs ($P = 0.001$) (Figure 6a, 6b, 6d, 6e, data not shown for interstitial macrophages), as well as percentage of total cells in neutrophils ($P = 0.022$), monocyte-derived DCs (mo DCs) ($P = 0.002$), natural killer (NK) cells ($P = 0.006$), T cells ($P = 0.001$), $CD4^+$ T cells ($P < 0.001$), $CD8^+$ T cells ($P = 0.003$), interstitial macrophages ($P = 0.003$), and CD11b DCs ($P < 0.001$) (Supplemental Figure 2a, 2b, 2c, 2d, 2e, 2f, 2h) in *Mtb*-infected mice. On the other hand, there were main effects or trends of EPA/DHA supplementation for lower T cell counts ($P = 0.07$), as well as T cells ($P = 0.045$), and $CD4^+$ T cells ($P = 0.07$) as percentages of total cells.

Furthermore, we found attenuating Fe x EPA/DHA interactions on $CD8^+$ T cell counts ($P = 0.05$) and CD11b DC counts ($P = 0.018$) (Figure 6f, 6c). For both cell types, supplementation of iron alone resulted in significantly higher cell counts compared to control mice ($P = 0.001$ and $P = 0.010$), but this increase was attenuated in the Fe+EPA/DHA mice (Fe+EPA/DHA vs. control, $P = 0.19$ and $P = 0.98$).

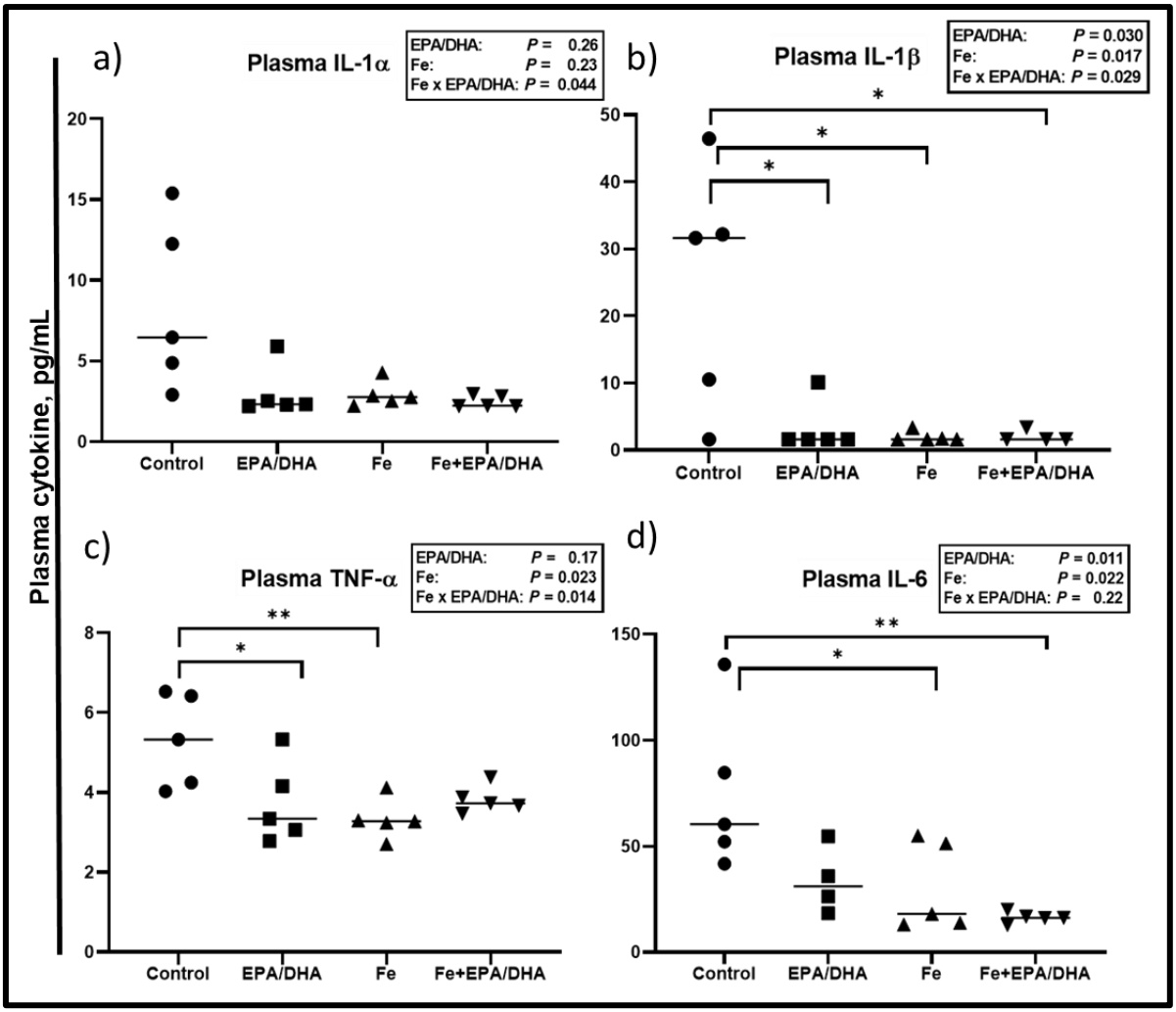


Figure 5 Plasma cytokine levels after providing *Mtb*-infected mice with control, EPA/DHA, Fe, or Fe+EPA/DHA diets for 3wk, including a) IL-1α, b) IL-1β, c) TNF-α, and d) IL-6. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). Two-way ANOVA was used to test effects of EPA/DHA (control & Fe vs. EPA/DHA & Fe+EPA/DHA), Fe (control & EPA/DHA vs. Fe & Fe+EPA/DHA), and Fe x EPA/DHA interactions. One-way ANOVA followed by Tuckey post-hoc test was used to compare means. *P < 0.05, **P < 0.01. EPA/DHA, eicosapentaenoic and docosahexaenoic acid-supplemented group; Fe, iron-supplemented group; Fe+EPA/DHA, iron plus eicosapentaenoic and docosahexaenoic acid-supplemented group; IL, interleukin; TNF-α, tumor necrosis factor-alpha.

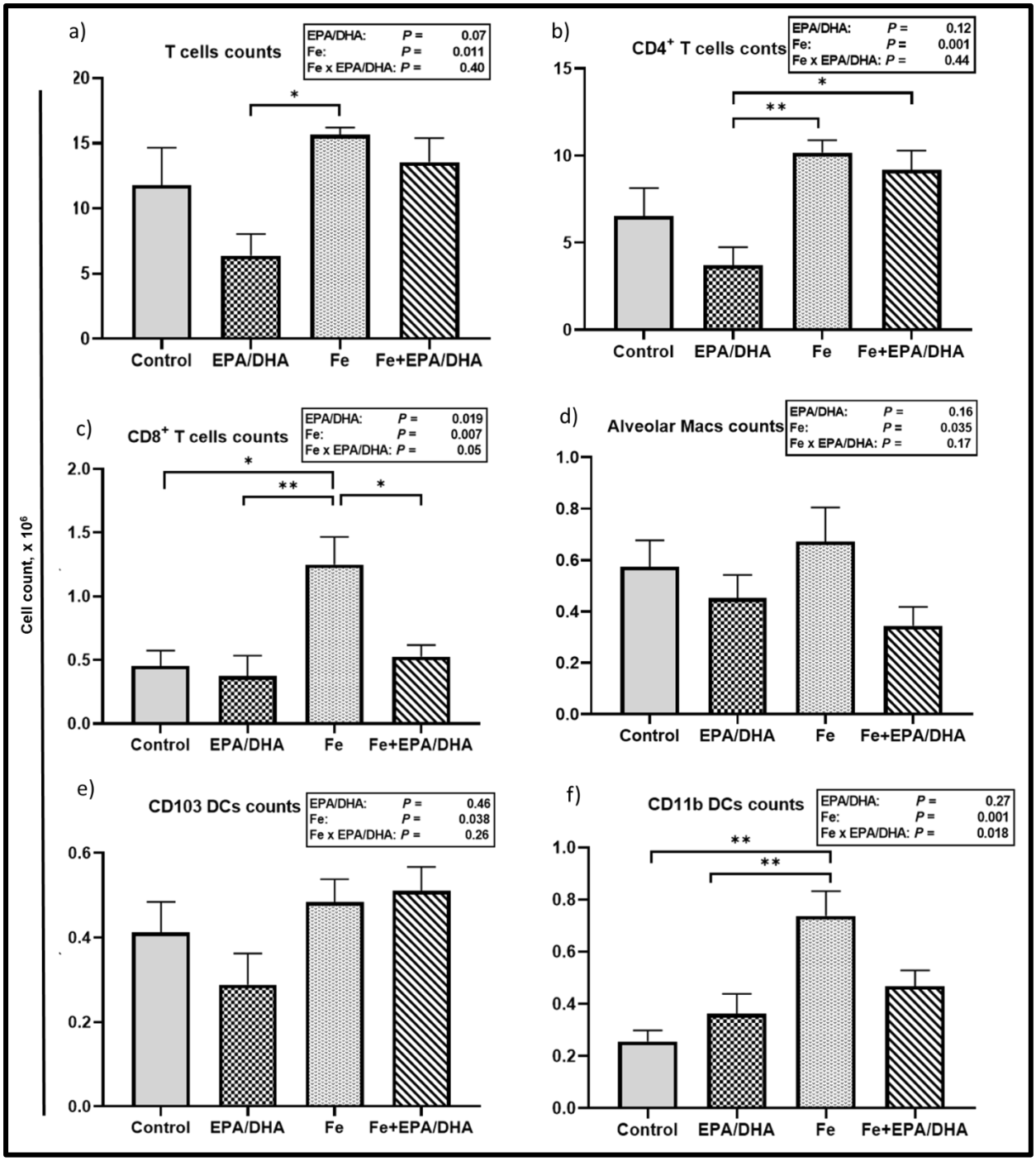


Figure 6 Lung immune cell counts after providing *Mtb*-infected mice with control, EPA/DHA, Fe, or Fe+EPA/DHA diets for 3wk, including a) T cells, b) $CD4^+$ T cells, c) $CD8^+$ T cells, d) alveolar macrophages, e) CD103 DCs, and f) CD11b DCs. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). Two-way ANOVA was used to test effects of EPA/DHA (control & Fe vs. EPA/DHA & Fe+EPA/DHA), Fe (control & EPA/DHA vs. Fe & Fe+EPA/DHA), and Fe x EPA/DHA interactions. One-way ANOVA followed by Tuckey post hoc test was used to compare means. *P < 0.05, **P < 0.01. DCs, dendritic cells; EPA/DHA, eicosapentaenoic and docosahexaenoic acid-supplemented group; Fe, iron-supplemented group; Fe+EPA/DHA, iron plus eicosapentaenoic and docosahexaenoic acid-supplemented group; macs, macrophages.

Discussion

This study provides evidence for the first time to our knowledge, that both EPA/DHA and iron, independently administered to *Mtb*-infected mice post-infection, lowered inflammation and improved indices of anemia of infection. Additionally, EPA/DHA also lowered bacterial load, which may be related to enhanced phagocytic ability of immune cells and increased synthesis of LMs (22). Interestingly, iron neither improved nor reduced bactericidal effects, but when combined with EPA/DHA supplementation, the bactericidal effect of EPA/DHA was attenuated.

When compared with the uninfected reference group, it was clear that the infected control mice presented with a typical profile of anemia of infection showing increased sTfR, ferritin, and hepcidin levels (18). Higher sTfR concentrations have been reported in inflammatory situations and in TB specifically, owing to the higher iron demand and erythropoietic stimulus resulting from inflammation-induced hypoferremia (2, 18, 29). This occurrence is underpinned by the higher levels of circulating hepcidin that we found, which functions to reduce iron absorption and iron exportation from cells into plasma (18, 30). In essence, there is an intracellular shift of iron as part of host defense to reduce iron availability for pathogens, which require iron for growth (14, 31, 32). However, both EPA/DHA and iron supplementation lowered hepcidin levels. This may be explained by the fact that high circulating hepcidin is mainly stimulated by IL-6, but also IL-1 (30, 33-35). Both EPA/DHA and iron supplementation had a lowering effect on IL-1β and IL-6. Furthermore, hepcidin is also regulated by the infection itself and has known antimicrobial properties (30, 36, 37). This further elucidates the lower hepcidin concentrations in the EPA/DHA group that also presented with a lower bacterial burden. Additionally, sTfR concentrations increase during iron deficiency but are lower where there is iron abundance, also explaining the results in the Fe group (38).

Our findings that ferritin concentrations were lower in the Fe group and tended to be lower in the EPA/DHA group compared with the control group support that ferritin rather acts as an acute-phase protein and not a marker of stored iron in TB (6, 16, 39). However, we found antagonistic interactions of iron and EPA/DHA on ferritin, TfR and hepcidin concentrations, as the lowering effect of supplementing iron or EPA/DHA individually was attenuated in mice receiving the combination treatment. Additionally, when comparing the ferritin, sTfR, and hepcidin concentrations of the uninfected reference group with the EPA/DHA and Fe groups, no significant differences could be found, whilst, in the control and Fe+EPA/DHA groups, significantly higher concentrations were evident. Our results clearly suggest that the provision of iron or EPA/DHA alone has similar effects of reducing the exacerbated effects of

inflammation on biomarkers indicative of anemia of infection but that Fe+EPA/DHA treatment did not.

However, Hb concentrations did not react similarly. The Hb concentrations of the infected groups were not found to be significantly different from the uninfected reference group, which theoretically may indicate that there was no true anemia in these mice. Nevertheless, this was likely related to the low sample size of the uninfected group and the short duration of the infection period. The erythrocyte life span in mice is ~ 40 days, which may explain why Hb concentrations remained more stable compared to other markers and may also be related to Hb's essential biological functions (40).

The improvements of the above biomarkers in the EPA/DHA and Fe groups were mediated by inflammation. Mutually, TNFα, IFN-γ, and IL-1 are important in host defense against TB but higher concentrations of these markers have also been correlated with worsened lung pathology together with disease severity (41-43). We found that EPA/DHA therapy lowered the pro-inflammatory plasma cytokines IL-1β and TNF-α as well as lung IL-1α and IFN-γ. This is in congruence with the lower bacterial load (43) and more pro-resolving lung LM profile found in the EPA/DHA group, resulting from changes in the FA composition evident in all FA pools measured.

Contrasting these findings, iron supplementation resulted in a higher n-6 LCPUFA composition of PBMCs and lung tissue membranes. This opposes previous research indicating that higher iron intakes favor higher n-3 LCPUFA membrane composition by affecting desaturase activities and/or membrane incorporation (44, 45). The reason for the discrepancies may be that these studies were conducted in healthy humans and not under infectious conditions. Nevertheless, these FA composition changes did not influence the LM profile or cytokines of the Fe group which presented with lower plasma IL-1β, IL-6 and TNF-α, as well as lung IL-1α and IL-1β concentrations compared with the control group. Our results agree with gene-expression studies which found that the expression of *IL-1α, IL-1β,* and *TNF-α* were downregulated in the macrophages, blood and livers of TB infected rabbits and mice that were supplemented with iron (46-48).

Similar to the EPA/DHA group, a pro-resolving LM profile was found in the Fe+EPA/DHA group, together with lower pro-inflammatory plasma cytokines. Our group previously also established that when providing iron with EPA/DHA in iron-deficient school children, the pro-resolving LM profile produced by EPA and DHA was maintained (24).

We further investigated the effects of our intervention diets on lung immune cell phenotyping. It was found that EPA/DHA lowered T cell recruitment, probably by decreasing T cell

proliferation that is induced by EPA and DHA, together with the lower bacterial burden that was found in the EPA/DHA group (49-51). This is supported by previous research on n-3 PUFA supplementation in TB (49). In contrast, dietary iron supplementation led to higher counts and percentages of various immune cells in the lungs. Iron has previously been shown to affect T cell numbers, where iron deficiency reduces the proliferation of T cells (52, 53). The Fe group presented with higher counts of total T cells, $CD4^+$ T cells, and $CD8^+$ T cells. This finding is consistent with previous research, where higher $CD8^+$ T cell recruitment was also reported in the granulomas of *M. Bovis* BCG-infected mice that received iron-rich diets (46). When provided in combination with EPA/DHA, these effects of iron were attenuated, which was probably related to the effects of EPA/DHA, described above. Furthermore, phagocytic cells are produced at infection sites to compete with the *Mtb* for iron acquisition (54, 55). This partly explains the higher macrophages, monocytes, and DCs that were evident in the lungs of mice in the Fe group, which is consistent with the findings of others (56). However, again when supplemented together with EPA/DHA, this effect of iron was attenuated.

Even though there were alterations in lung inflammation and immune cell phenotyping amongst the different intervention groups, there were no main treatment effects of EPA/DHA or iron on lung pathology, which is consistent with the findings of others (46, 49). However, it seemed that combination treatment again did not deliver favorable clinical outcomes, which elicited a trend to reduce free alveolar space, and therefore worsen lung pathology.

Iron supplementation did not affect bacterial load in our study. These findings, although unexpected because iron is crucial for *Mtb* growth and virulence (32), are consistent with previous research in *Mtb*-infected animals and humans (1, 46, 47, 57, 58). Our findings and those of previous research are partly explained by the fact that *Mtb* is an intracellular bacteria able to obtain iron, irrespective of the host iron regulatory mechanisms and iron status (1, 58). Furthermore, iron has important functions in the antibacterial activity of macrophages and T cells, and serves as co-factor for various enzymes (14, 59). This suggests its importance in host resistance, rather than promoting bacterial growth. Contrasting our findings, others suggested that iron abundance enhanced *Mtb* growth in mice (60-62), which may be related to the use of genetically modified mice (62) or intraperitoneally administered iron, resulting in high circulatory iron concentrations (60, 61). A recent review by Agoro and Mura (14) suggested an “iron benefit window” where moderate iron supplementation may enhance the immune response to lower inflammation and bacterial burden. However, beyond this threshold, iron instead augments inflammation and bacterial load (14). Interestingly, in our study iron lowered body weight gain in male C3HeB/FeJ mice. This is in contrast with previous studies, which showed no effect of iron on body weight changes in *Mtb*-infected BALB/C mice

and humans and is not explained by the inflammatory profile or bacterial burden in the iron-supplemented group (1, 61).

This study is strengthened by the use of a well-established TB rodent (C3HeB/FeJ) model that closely reflects human pulmonary TB pathology closely (63). This is also the first study, to our knowledge, to compare iron supplementation directly with an inflammation-resolving therapy in TB. This study is limited by the fact that we measured outcomes only at one time point in the inflammatory response. Furthermore, it could be recommended that iron concentrations should be measured in the lungs and other tissue, such as the liver, as well as to include mean corpuscular volume and mean corpuscular hemoglobin to assess effects on iron-deficiency anemia specifically.

The findings of this research indicate that EPA/DHA or iron supplementation exerted similar benefits with regard to systemic and lung inflammation and markers of anemia of infection in TB in the absence of standard TB drugs. Additionally, EPA/DHA supplementation induced a more pro-resolving lung LM profile that was maintained with combination treatment. It also enhanced bactericidal activity without influencing lung immune cell recruitment or body weight gain, as was the case with iron supplementation. However, the combination of Fe+EPA/DHA treatment did not exert the beneficial effects observed in the case of EPA/DHA or iron supplementation alone, on clinical outcomes, markers of anemia of infection, or lung inflammation. Therefore, EPA/DHA supplementation after infection may be a strategy to consider in order to support inflammation resolution and mitigate anemia of infection without compromising host immunity in TB. Additionally, contrary to current belief, iron supplementation may not worsen the bacterial burden in TB and may even contribute to lowering inflammation, which warrants further investigation.

Acknowledgments

The authors thank Rodney Lucas (UCT, Cape Town, South Africa) for his assistance with data collection and Adriaan Jacobs and Cecile Cooke (NWU, South Africa) for their assistance with laboratory analyses.

A.N., L.M., R.D., C.M.S., and R.B. conceptualized study; A.N., L.M., D.L., J.B., and S.P. designed study; A.N., L.M., M.O., S.P., and F.H., and S.K. conducted research; A.N., L.M., and M.O. analyzed data; A.N., L.M., L.Z., S.P., M.O., J.B., and C.M.S. assisted with data interpretation; A.N. wrote the manuscript. All authors reviewed and approved the final manuscript.

References

1. Devi U, Rao CM, Srivastava VK, Rath PK, Das BS. Effect of iron supplementation on mild to moderate anaemia in pulmonary tuberculosis. Br J Nutr 2003;90:541-50.

2. Hella J, Cercamondi CI, Mhimbira F, Sasamalo M, Stoffel N, Zwahlen M, Bodmer T, Gagneux S, Reither K, Zimmermann MB. Anemia in tuberculosis cases and household controls from Tanzania: Contribution of disease, coinfections, and the role of hepcidin. PloS on. 2018;13:e0195985.

3. Isanaka S, Mugusi F, Urassa W, Willett WC, Bosch RJ, Villamor E, Spiegelman D, Duggan C, Fawzi WW. Iron deficiency and anemia predict mortality in patients with tuberculosis. J Nutr 2011;142:350-7.

4. Karyadi E, Schultink W, Nelwan RH, Gross R, Amin Z, Dolmans WM, van der Meer JW, Hautvast JGJ, West CE. Poor micronutrient status of active pulmonary tuberculosis patients in Indonesia. J Nutr 2000;130:2953-8.

5. Lee SW, Kang Y, Yoon YS, Um S-W, Lee SM, Yoo C-G, Kim YW, Han SK, Shim Y-S, Yim J-J. The prevalence and evolution of anemia associated with tuberculosis. J Korean Med Sci 2006;21:1028-32.

6. Mishra S, Taparia MP, Yadav D, Koolwal S. Study of Iron Metabolism in Pulmonary Tuberculosis Patients. Int J Health Sci Res 2018:8:70-7.

7. Isanaka S, Aboud S, Mugusi F, Bosch RJ, Willett WC, Spiegelman D, Duggan C, Fawzi WW. Iron status predicts treatment failure and mortality in tuberculosis patients: a prospective cohort study from Dar es Salaam, Tanzania. PloS one 2012;7:e37350.

8. Nagu TJ, Spiegelman D, Hertzmark E, Aboud S, Makani J, Matee MI, Fawzi W, Mugusi F. Anemia at the initiation of tuberculosis therapy is associated with delayed sputum conversion among pulmonary tuberculosis patients in Dar-es-Salaam, Tanzania. PloS one 2014;9:e91229.

9. Shimazaki T, Marte S, Saludar N, Dimaano E, Salva E, Ariyoshi K, Villarama J, Suzuki M. Risk factors for death among hospitalised tuberculosis patients in poor urban areas in Manila, The Philippines. Int J Tuberc Lung Dis 2013;17:1420-6.

10. Kerkhoff AD, Meintjes G, Burton R, Vogt M, Wood R, Lawn SD. Relationship between blood concentrations of hepcidin and anemia severity, mycobacterial burden, and mortality among patients with HIV-associated tuberculosis. J Infect Dis 2015;213:61-70.

11. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004;306:2090-3.

12. Ganz T, Nemeth E. Hepcidin and iron homeostasis. Biochim Biophys Acta Mol Cell Res 2012;1823:1434-43.

13. Aschemeyer S, Qiao B, Stefanova D, Valore EV, Sek AC, Ruwe TA, Vieth KR, Jung G, Casu C, Rivella S. Structure-function analysis of ferroportin defines the binding site and an alternative mechanism of action of hepcidin. Blood. 2018;131:899-910.

14. Agoro R, Mura C. Iron Supplementation Therapy, A Friend and Foe of Mycobacterial Infections? Pharmaceuticals. 2019;12:75.

15. Kurthkoti K, Amin H, Marakalala MJ, Ghanny S, Subbian S, Sakatos A, Livny J, Fortune SM, Berney M, Rodriguez GM. The capacity of Mycobacterium tuberculosis to survive iron starvation might enable it to persist in iron-deprived microenvironments of human granulomas. MBio 2017;8:e01092-17.

16. Weiss G, Ganz T, Goodnough LT. Anemia of inflammation. Blood 2019;133:40-50.

17. Shaw JG, Friedman JF. Iron deficiency anemia: focus on infectious diseases in lesser developed countries. Anemia 2011;2011-22.

18. Ganz T. Anemia of inflammation. N Eng J Med 2019;381:1148-57.

19. Kerkhoff A, Meintjes G, Opie J, Vogt M, Jhilmeet N, Wood R, Lawn S. Anaemia in patients with HIV-associated TB: relative contributions of anaemia of chronic disease and iron deficiency. Int J Tuberc Lung Dis 2016;20:193-201.

20. Minchella PA, Donkor S, Owolabi O, Sutherland JS, McDermid JM. Complex anemia in tuberculosis: the need to consider causes and timing when designing interventions. Clin Infect Dis 2014;60:764-72.

21. Jordao L, Lengeling A, Bordat Y, Boudou F, Gicquel B, Neyrolles O, Becker PD, Guzman CA, Griffiths G, Anes E. Effects of omega-3 and-6 fatty acids on Mycobacterium tuberculosis in macrophages and in mice. Microbes Infect 2008;10:1379-86.

22. Serhan CN, Chiang N, Dalli J. New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration. Mol Aspects Med 2018:64:1-17.

23. Malan L, Baumgartner J, Calder PC, Zimmermann MB, Smuts CM. n–3 Long-chain PUFAs reduce respiratory morbidity caused by iron supplementation in iron-deficient South African schoolchildren: a randomized, double-blind, placebo-controlled intervention. Am J Clin Nutr 2014;101:668-79.

24. Malan L, Baumgartner J, Zandberg L, Calder P, Smuts C. Iron and a mixture of dha and epa 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. Prostaglandins Leukot Essent. Fatty Acids 2016;105:15-25.

25. Reeves PG, Nielsen FH, Fahey Jr GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;11:1939–51.

26. Guler R, Parihar SP, Spohn G, Johansen P, Brombacher F, Bachmann MF. Blocking IL-1α but not IL-1β increases susceptibility to chronic Mycobacterium tuberculosis infection in mice. Vaccine 2011;29:1339-46.

27. Folch J, Lees M, Stanley GS. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497-509.

28. Parihar SP, Guler R, Khutlang R, Lang DM, Hurdayal R, Mhlanga MM, Suzuki H, Marais AD, Brombacher F. Statin Therapy Reduces the Mycobacterium tuberculosis Burden in Human Macrophages and in Mice by Enhancing Autophagy and Phagosome Maturation. J Infect Dis 2014;209:754-63.

29. Rohner F, Namaste SM, Larson LM, Addo OY, Mei Z, Suchdev PS, Williams AM, Sakr Ashour FA, Rawat R, Raiten DJ. Adjusting soluble transferrin receptor concentrations

for inflammation: Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project. Am J Clin Nutr 2017;106:372S-82S.

30. Schmidt PJ. Regulation of iron metabolism by hepcidin under conditions of inflammation. J Biol Chem 2015;290:18975-83.

31. Ganz T. Iron and infection. Int J Hematol 2018;107:7-15.

32. Cole S, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon S, Eiglmeier K, Gas S, Barry C. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 1998;393:537-543.

33. Lee P, Peng H, Gelbart T, Wang L, Beutler E. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. Proc Natl Acad Sci 2005;102:1906-10.

34. Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 2004;113:1271-6.

35. Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 2002;110:1037-44.

36. Millonig G, Ganzleben I, Peccerella T, Casanovas G, Brodziak-Jarosz L, Breitkopf-Heinlein K, Dick TP, Seitz H-K, Muckenthaler MU, Mueller S. Sustained submicromolar H2O2 levels induce hepcidin via signal transducer and activator of transcription 3 (STAT3). J Biol Chem 2012;287:37472-82.

37. Armitage AE, Eddowes LA, Gileadi U, Cole S, Spottiswoode N, Selvakumar TA, Ho L-P, Townsend AR, Drakesmith H. Hepcidin regulation by innate immune and infectious stimuli. Blood 2011;118:4129-39.

38. Harms K, Kaiser T. Beyond soluble transferrin receptor: Old challenges and new horizons. Best Pract Res Clin Endocrinol 2015;29:799-810.

39. Truman-Rosentsvit M, Berenbaum D, Spektor L, Cohen LA, Belizowsky-Moshe S, Lifshitz L, Ma J, Li W, Kesselman E, Abutbul-Ionita I. Ferritin is secreted via 2 distinct nonclassical vesicular pathways. Blood 2018;131:342-52.

40. Anderson GJ, Frazer DM. Current understanding of iron homeostasis. Am J Clin Nutr 2017;106:1559S-66S.

41. Borthwick L. The IL-1 cytokine family and its role in inflammation and fibrosis in the lung. Semin Immunopatho/ 2016:38:517-34.

42. Roca FJ, Ramakrishnan L. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. Cell 2013;153:521-34.

43. Kumar NP, Moideen K, Banurekha VV, Nair D, Babu S. Plasma pro-inflammatory cytokines are markers of disease severity and bacterial burden in pulmonary tuberculosis. Open Forum Infect 2019;7.

44. Del Bò C, Contino D, Riso P, Simonetti P, Ciappellano S. Effect of horse meat consumption on iron status, lipid profile and fatty acid composition of red blood cell membrane: preliminary study. Nutrizione, la pietra d'angolo 2009:3:1-1.

45. Smuts C, Tichelaar H, Van Jaarsveld P, Badenhorst C, Kruger M, Laubscher R, Mansvelt E, Benade A. The effect of iron fortification on the fatty acid composition of plasma and erythrocyte membranes in primary school children with and without iron deficiency. Prostaglandins Leukot Essent Fatty Acids 1995;52:59-67.

46. Agoro R, Benmerzoug S, Rose S, Bouyer M, Gozzelino R, Garcia I, Ryffel B, Quesniaux VF, Mura C. An iron-rich diet decreases the mycobacterial burden and correlates with hepcidin upregulation, lower levels of proinflammatory mediators, and increased T-cell recruitment in a model of mycobacterium bovis Bacille Calmette-Guerin infection. J Infect Dis 2017;216:907-18.

47. Kolloli A, Singh P, Rodriguez GM, Subbian S. Effect of Iron Supplementation on the Outcome of Non-Progressive Pulmonary Mycobacterium tuberculosis Infection. J Clin Med 2019;8:1155.

48. Serafín-López J, Chacón-Salinas R, Muñoz-Cruz S, Enciso-Moreno J, Estrada-Parra SA, Estrada-Garcia I. The effect of iron on the expression of cytokines in macrophages infected with Mycobacterium tuberculosis. Scand J Immunol 2004;60:329-37.

49. McFarland CT, Fan Y-Y, Chapkin RS, Weeks BR, McMurray DN. Dietary polyunsaturated fatty acids modulate resistance to Mycobacterium tuberculosis in guinea pigs. J Nutr 2008;138:2123-8.

50. Calder PC, Newsholme EA. Polyunsaturated fatty acids suppress human peripheral blood lymphocyte proliferation and interleukin-2 production. Clin Science 1992;82:695-700.

51. Calder PC. The relationship between the fatty acid composition of immune cells and their function. Prostaglandins Leukot Essent Fatty Acids 2008;79:101-8.

52. Cherayil BJ. Iron and immunity: immunological consequences of iron deficiency and overload. Arch Immunol Ther Exp 2010;58:407-15.

53. Jabara HH, Boyden SE, Chou J, Ramesh N, Massaad MJ, Benson H, Bainter W, Fraulino D, Rahimov F, Sieff C. A missense mutation in TFRC, encoding transferrin receptor 1, causes combined immunodeficiency. Nature Gen 2016;48:74.

54. Bresnahan KA, Chileshe J, Arscott S, Nuss E, Surles R, Masi C, Kafwembe E, Tanumihardjo SA. The acute phase response affected traditional measures of micronutrient status in rural Zambian children during a randomized, controlled feeding trial. J Nutr 2014;144:972-8.

55. Ifeanyi OE. A Review on Iron Homeostasis and Anaemia in Pulmonary Tuberculosis. Int J Healthc Med Scien 2018;4:84-9.

56. Azcárate IG, Sánchez-Jaut S, Marín-García P, Linares M, Pérez-Benavente S, García-Sánchez M, Uceda J, Kamali AN, Morán-Jiménez M-J, Puyet A. Iron supplementation in mouse expands cellular innate defences in spleen and defers lethal malaria infection. BBA Mol Basis Dis 2017;1863:3049-59.

57. Stefanova D, Raychev A, Arezes J, Ruchala P, Gabayan V, Skurnik M, Dillon BJ, Horwitz MA, Ganz T, Bulut Y. Endogenous hepcidin and its agonist mediate resistance to selected infections by clearing non–transferrin-bound iron. Blood 2017;130:245-57.

58. Harrington-Kandt R, Stylianou E, Eddowes LA, Lim PJ, Stockdale L, Pinpathomrat N, Bull N, Pasricha J, Ulaszewska M, Beglov Y. Hepcidin deficiency and iron deficiency do not

alter tuberculosis susceptibility in a murine M. tb infection model. PloS one 2018;13:e0191038.

59. Cronjé L, Edmondson N, Eisenach KD, Bornman L. Iron and iron chelating agents modulate Mycobacterium tuberculosis growth and monocyte-macrophage viability and effector functions. FEMS Immunol Med Microbiol 2005;45:103-12.

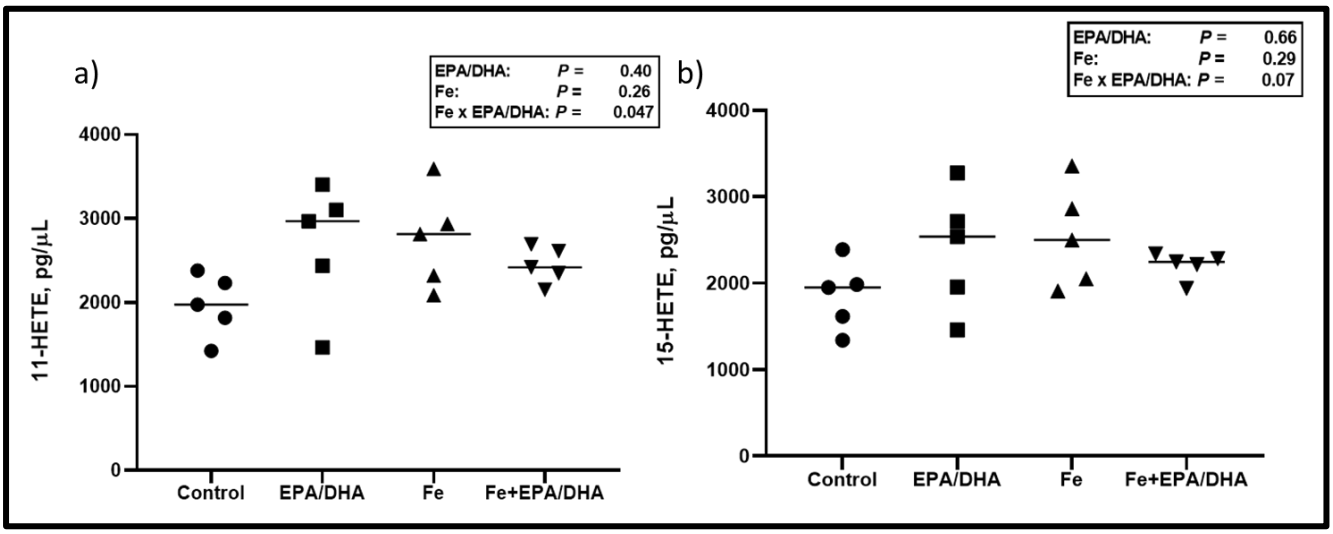
60. Kochan I. The role of iron in bacterial infections, with special consideration of host-tubercle bacillus interaction. Curr Top Microbiol Immunol 1973;60:1-30.

61. Lounis N, Truffot-Pernot C, Grosset J, Gordeuk VR, Boelaert JR. Iron and Mycobacterium tuberculosis infection. J Clin Virol 2001;20:123-6.

62. Schaible UE, Collins HL, Priem F, Kaufmann SH. Correction of the iron overload defect in β-2-microglobulin knockout mice by lactoferrin abolishes their increased susceptibility to tuberculosis. J Exp Med 2002;196:1507-13.

63. Lenaerts A, Barry III CE, Dartois V. Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. Immuno rev 2015;264:288-307.

Supplement



Supplemental Figure 1 Lipid mediator concentrations including a) 11-HETE and b) 15-HETE after providing *Mtb*-infected mice with control, EPA/DHA, Fe, or Fe+EPA/DHA diets for 3wk. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). Two-way ANOVA was used to test effects of EPA/DHA (control & Fe vs. EPA/DHA & Fe+EPA/DHA), Fe (control & EPA/DHA vs. Fe & Fe+EPA/DHA), and Fe x EPA/DHA interactions. One-way ANOVA followed by Tuckey post hoc test was used to compare groups. EPA/DHA, eicosapentaenoic and docosahexaenoic acid supplemented group; Fe, iron supplemented group; Fe+EPA/DHA, iron plus eicosapentaenoic and docosahexaenoic acid supplemented group; HETE, hydroxyeicosatetraenoic acid.

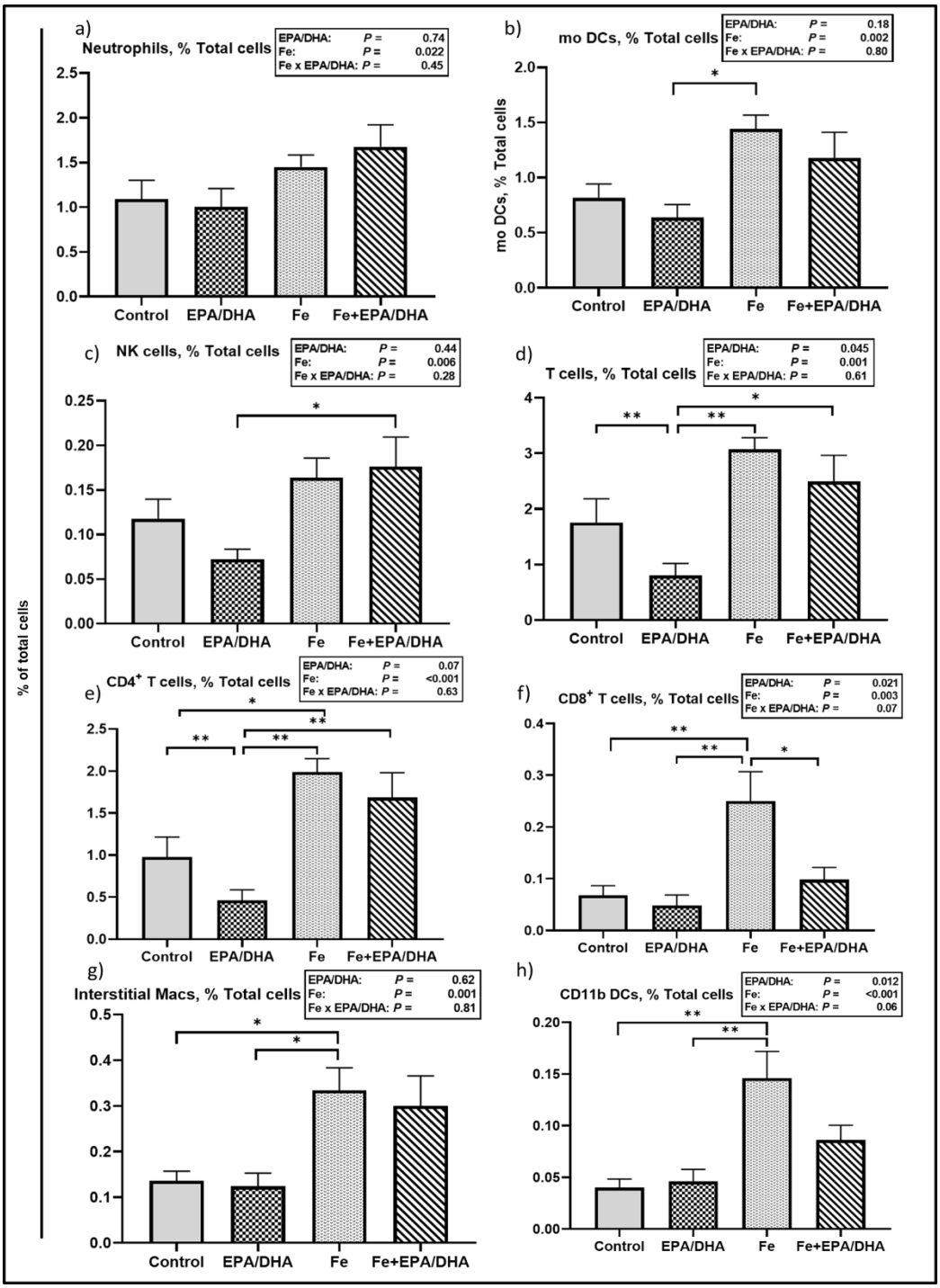


Figure 6 Lung immune cells as percentages of total cells after providing *Mtb*-infected mice with control, EPA/DHA, Fe, or Fe+EPA/DHA diets for 3wk, including a) neutrophils, b) Mo dendritic cells, c) natural killer cells, d) T cells, e)$CD4^+$ T cells, f) $CD8^+$ T cells, g) interstitial macrophages, and h).CD11b dendritic cells. The values represent mean ± SEM. Results repeated in two experiments, data shown for one experiment (n=5 per group). Two-way ANOVA was used to test effects of EPA/DHA (control & Fe vs. EPA/DHA & Fe+EPA/DHA), Fe (control & EPA/DHA vs. Fe & Fe+EPA/DHA), and Fe x EPA/DHA interactions. One-way ANOVA followed by Tuckey post hoc test was used to compare means. **P* < 0.05, ***P* < 0.01. DCs, dendritic cells; EPA/DHA, eicosapentaenoic and docosahexaenoic acid supplemented group; Fe, iron supplemented group; Fe+EPA/DHA, iron plus eicosapentaenoic and docosahexaenoic acid supplemented group; macs, macrophages; mo Dcs, monocyte-derived DCs; NK, natural killer.

CHAPTER 5 MANUSCRIPT 3

EPA and DHA provide superior clinical and inflammation-resolving benefits above sufficient essential fatty acids in omega-3 fatty acid-deficient *Mtb*-infected C3HeB/FeJ mice

Arista Nienaber[1], Robin C Dolman[1], Mumin Ozturk[2,3], Lizelle Zandberg[1], Frank EA Hayford[1,4], Renee Blaauw[5], Frank Brombacher[2,3], Cornelius M Smuts[1], Suraj P Parihar[3,6], Linda Malan[1]

[1] Centre of Excellence for Nutrition, North-West University, Potchefstroom, South Africa
[2] Institute of Infectious Diseases and Molecular Medicine (IDM), Division of Immunology and South African Medical Research Council (SAMRC) Immunology of Infectious Diseases, University of Cape Town, Cape Town, South Africa
[3] International Centre for Genetic Engineering and Biotechnology (ICGEB), Cape Town-Component, Cape Town, South Africa
[4] Department of Nutrition and Dietetics, University of Ghana, Accra, Ghana
[5] Division of Human Nutrition, Stellenbosch University, Stellenbosch, South Africa
[6] Centre of Infectious Diseases Research in Africa, University of Cape Town, Cape Town, South Africa

Running title: EPA/DHA superior to n-3 sufficient diet in TB

Prepared for submission to *The Journal of Nutrition*

Abstract

Background*:* Intakes of the omega-3 (n-3) essential fatty acid (EFA) are low in the general adult population, with high n-6/n-3 polyunsaturated fatty acid (PUFA) ratios and the accompanying suboptimal n-3 PUFA status. n-3 long-chain polyunsaturated fatty acids (LCPUFAs) have antibacterial and inflammation-resolving effects in tuberculosis (TB). However, the effect of sufficient n-3 EFA intake has not been investigated.

Objective: We aimed to compare the effects of a diet with sufficient n-3 EFA content in an acceptable n-6/n-3 PUFA ratio for rodents ((n-3)eFAS group) to those of the same diet supplemented with eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) (EPA/DHA group) in *Mycobacterium tuberculosis* (*Mtb*)-infected C3HeB/FeJ mice with a low n-3 PUFA status.

Methods*:* Mice were conditioned on an n-3 PUFA-deficient diet with a high n-6/n-3 PUFA ratio for 6 wk prior to *Mtb* infection and randomized to either the (n-3)eFAS or EPA/DHA diets 1 wk post-infection for 3 wk.

Results: At endpoint, EPA and DHA compositions were higher and arachidonic acid, osbond acid, and total n-6 LCPUFAs lower in all lipid pools measured in the EPA/DHA group (all $P < 0.001$). Percentage body weight gain was higher ($P = 0.017$) and lung bacterial load lower ($P < 0.001$) in the EPA/DHA group. Additionally, the EPA/DHA group had a more pro-resolving lung lipid mediator (LM) profile and lower lung interleukin-1 alpha (IL-1α) and -1β concentrations ($P = 0.023$ and $P = 0.049$). Strong inverse correlations were found between lung and peripheral blood mononuclear cell (PBMC) EPA and DHA and selected pro-inflammatory cytokines.

Conclusions: These findings suggest that EPA/DHA supplementation provides benefits superior to a diet with sufficient amounts of n-3 EFA with regard to bacterial killing, weight gain and lung inflammation resolution in *Mtb*-infected mice with a low n-3 PUFA status. Therefore, EPA and DHA may be worthwhile considering as adjunct TB treatment.

Keywords: eicosapentaenoic and docosahexaenoic acid supplementation, inflammation, low n-3 fatty acid status, n-3 essential fatty acid-sufficient diet, tuberculosis

Introduction

Tuberculosis (TB) is an infectious disease characterized by exaggerated and progressive inflammation, lung pathology and tissue damage (1). Controlling this excessive inflammation may lead to improved TB outcomes (2). Therefore, anti-inflammatory and pro-resolving treatments have been suggested as host-directed therapy (HDT) in TB patients (2). Dietary PUFA consumption alters membrane phospholipid fatty acid (FA) composition of cells that play a role in immune and inflammatory responses in a time- and dose-dependent manner, which affects the pro-inflammatory and inflammation-resolving capacity of these cells (3, 4). This is predominantly mediated by the conversion of long-chain PUFAs (LCPUFAs), including arachidonic acid (AA), EPA and DHA, to lipid mediators (LMs) *via* cyclooxygenase (COX) and lipoxygenase (LOX) pathways. The n-3 LCPUFAs EPA and DHA serve as substrate for the production of pro-resolving LMs, protectins, maresins and resolvins that aid in inflammation resolution, anti-inflammatory cytokine production, and alter immune cell recruitment (5). The incorporation of EPA and DHA into cell membranes also enhances the phagocytosis of apoptotic cells and bacteria and augments bacterial killing (4-6).

The precursors, linoleic acid (LA) and alpha-linolenic acid (ALA), are essential fatty acids (EFAs) and are desaturated and elongated to produce AA, as well as EPA and DHA, respectively (7). This conversion has been found to be inefficient in rodents (6% converted to DHA), and even more so in humans (8% to 12% to EPA and only 1% to DHA) (8, 9). Furthermore, the synthesis of n-3 series LCPUFAs from ALA utilizes the same desaturase enzymes for metabolism as is used for the conversion of LA to AA (10). This leads to competition between ALA and LA pathways. Despite substantial variation between countries, the general population's ratio of n-6 and n-3 PUFA intake has been shown to be skewed (7). Low n-3 PUFA intake and higher n-6 PUFA intake have been reported, consisting mostly of essential ALA and LA, and leading to intake ratios of n-6/n-3 PUFA as high as 60/1 (7, 11). This results in low n-3 PUFA status, particularly in certain settings in low-and middle-income countries (12).

Our group (Nienaber et al., unpublished data) and Jordao et al. (13) have also demonstrated the inflammation-resolving and antibacterial effects of EPA and DHA supplementation in TB if administered post-infection. However, EPA and DHA supplementation as HDT may be regarded as expensive in a public health context, particularly in lower-income countries with a high TB burden and where TB drug treatment already comprises a large portion of the national public health budget (14, 15). It may consequently be useful to determine whether dietary adjustments to enhance n-3 EFA intake and improve n-6/n-3 PUFA ratios, as a more affordable option, would be as beneficial as n-3 LCPUFA supplementation post-TB infection.

Therefore, we aimed to determine whether correcting n-3 PUFA intake after infection, with a diet containing the essential precursor ALA at sufficient amounts for optimal DHA membrane saturation in rodents, provides benefits comparable with EPA and DHA supplementation with regard to clinical and lung inflammatory outcomes. We tested this in a *Mycobacterium tuberculosis (Mtb)*-infected C3HeB/FeJ mouse model which was conditioned with an n-3 PUFA-deficient diet. It was hypothesized that, because of low n-3 PUFA status, the conversion of ALA to EPA and DHA would be upregulated in the group that did not receive EPA and DHA supplementation. Furthermore, we aimed to determine whether the FA membrane composition of peripheral mononuclear cells (PBMC) and crude lung homogenates correlated with lung cytokines in these two groups.

Methods

Animals and ethics statement

Male C3HeB/FeJ mice (Jackson Laboratory, Bar Harbor, ME), aged 10 to 12 weeks were bred and housed at the Institute of Infectious Diseases and Molecular Medicine (IDM), University of Cape Town (UCT), Cape Town, South Africa (SA). Following infection, mice were housed in a biosafety level 3 containment facility, five per individually ventilated cage with filter tops (type 2 long) and dried wood shavings and shredded filter paper as floor coverings. The temperature range was set at 22 to 24 °C and 12-to-12 hour light cycles. The experiments were performed in accordance with the SA National Guidelines and the UCT's Practice guidelines for laboratory animal procedures. The protocol was approved by the Animal Ethics Committee, Faculty of Health Sciences, University of Cape Town (AEC 015/040) and the AnimCare Animal Research Ethics Committee of the North-West University (NWU-00260-16-A5).

Animal Diets and Experimental design

Mice had *ad libitum* access to food and water. They were conditioned on an n-3 PUFA-deficient ((n-3)FAD, n-6/n-3 PUFA ratio: 130/1) diet for six weeks prior to infection in order to establish a low n-3 PUFA status (16). Thereafter, mice were infected with *Mtb via* the aerosol route (described below). One week post-infection, mice were randomized either to the AIN-93G diet containing the essential n-3 LCPUFA precursor ALA, as well as the essential n-6 LCPUFA precursor LA ((n-3)eFAS group, n=10 mice) or to a diet with the same amounts of LA and ALA, supplemented additionally with n-3 LCPUFA (EPA/DHA, n=10 mice).

All the purified experimental diets were obtained commercially (Dyets, Bethlehem, PA), and were based on the AIN-93G (17) formulation, containing 10% fat, but with modifications in the

fat source (**Table 1**). All the diets were isocaloric with the same macronutrient (including fat) content. The mice in the (n-3)eFAS group received the AIN-93G diet, which provides both the n-3 and n-6 LCPUFA precursors, ALA and LA, at amounts found to induce optimal tissue saturation of DHA and AA, respectively, in rodents (17). The (n-3)eFAS diet had an n-6/n-3 PUFA ratio of more or less 7/1 (17), which is considered normal for rodents, compared with the (n-3)FAD diet that the mice were conditioned on, which had an n-6/n-3 PUFA ratio of 130/1.

The EPA/DHA group received the AIN-93G diet containing commercially obtained Incromega TG4030 oil (Croda Chemicals, Europe). Gas chromatography-mass spectrometry (GCMS) analysis was performed by the manufacturer to confirm the FA composition of the diets (results presented in Table 1). The mice received these diets for 3 wk until euthanasia (at 28 days after infection). The body weight and food intake of mice were measured weekly. Food intake per mouse was calculated by dividing the food intake per cage by five and by seven. The results of this experiment were reproduced in a second experiment (resulting in 10 mice per treatment group). The data of one experiment (5 mice per group) are presented in this article.

Table 1 Fatty acid and oil composition of intervention diets[1]

Diet	Fat source	LA	ALA	DHA	EPA	AA
(n-3)eFAS	70 g/kg Soybean oil 30 g/kg Coconut oil	3.54	0.436	< 0.007	< 0.007	< 0.007
(n-3)FAD	81 g/kg Coconut oil 19 g/kg Safflower oil	1.3	0.01	< 0.007	< 0.007	< 0.007
EPA/DHA	70 g/kg Soybean oil 27 g/kg Coconut oil 3 g/kg Incromega TG4030	3.44	0.425	0.061 ±28% FA	0.089 ±44% FA	< 0.007

[1]Based on gas chromatography-mass spectrometry (GCMS) analysis of diets. Values expressed as grams per 100 grams of diet.

ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; (n-3)FAD, omega-3 fatty acid-deficient diet; EPA/DHA, eicosapentaenoic and docosahexaenoic acid-supplemented diet; (n-3)eFAS, omega-3 essential fatty acid-sufficient diet.

Aerosol infection

A virulent *Mtb* H37Rv strain was cultured and stocks were prepared and stored at -80°C, as described elsewhere (18). Mice were exposed to aerosol infection for 40 minutes by nebulizing

6 ml of a suspension that contained 2.4 x 10^7 live bacteria in an inhalation exposure system (model A4224, Glas-Col). One day following infection, four mice were euthanized to confirm the infection dose, which was 500 colony-forming units (CFU)/mouse

Endpoint blood and tissue collection

At the end of the three weeks of receiving intervention diets, mice were euthanized by halothane exposure, followed by trunk blood collection by heart puncture. The blood was collected into EDTA-coated Minivette® tubes (Minivette® POCT, 1000 µl, Sarsted). The plasma and buffy coat were removed for fatty acid analysis. The red blood cells (RBCs) were washed twice with saline before storage at -80°C and subsequent fatty acid analysis. The lung lobes and spleen were removed aseptically and weighed prior to preparation. The left lung lobe and spleen were homogenized in PBS for the analysis of the bacillary load and lung cytokines. The right superior and post-caval lung lobes were snap-frozen in liquid nitrogen and stored at -80 °C for lung fatty acid and lipid mediator analysis. The right middle lobe was submerged in 10% formalin for histology analysis and the right inferior lobe prepared for flow cytometry.

Total phospholipid fatty acid composition analysis

FAs were extracted from ~20 mg lung tissue, homogenized in 10 µl phosphate-buffered saline with protease inhibitor (homogenization buffer) per 1 mg tissue; or from ~200 µL RBCs or peripheral blood mononuclear cells (PBMCs) collected as buffy coat. Lipids were extracted from each lipid pool with chloroform:methanol (2:1, v:v; containing 0.01% BHT) by a modification of the method of Folch et al. (19). The lipid extracts were concentrated and the neutral lipids separated from the phospholipids by thin-layer chromatography (TLC) (silica gel 60 plates, Merck) and eluted with diethyl ether:petroleum ether: acetic acid (30:90:1, v:v:v). The lipid band containing phospholipids was removed from the TLC plate and transmethylated with methanol:sulphuric acid (95:5, v:v) at 70°C for 2 hours to form FA methyl esters (FAME). FAMEs were analyzed with an Agilent Technologies 7890A gas chromatography system equipped with an Agilent Technologies 7000B triple quad mass selective detector (Agilent Technologies, Santa Clara, USA) and quantification performed with Masshunter (B.06.00). Relative percentages of FAs (% w/w) were calculated by taking the concentration of a given FA as a percentage of the total concentration of all FAs identified in the sample.

Bacterial load determination

The bacterial loads of lungs were determined at euthanasia. The left lobes of the lung were removed, weighed, homogenized in 2 ml Saline-Tween 80 (0.04%), and 10-fold dilutions

plated onto Difco™ Middlebrook 7H10 Agar (BD Biosciences). The colony-forming unit counts were determined 21 days following incubation at 37°C. Data are expressed as $\log_{10}$ CFU.

Histopathology analysis

Right middle lobes of the lungs were dissected out and fixed in 4% neutral buffered formalin. The tissue was processed using the Leica TP 1020 Processor for 24 hours and subsequently embedded in paraffin wax. The Leica Sliding Microtome 2000R was used to cut 2 µm-thick sections of the embedded tissues. Three sections with 30 µm distance per section were cut, deparaffinized, and subsequently stained with the hematoxylin/eosin stain. The images were acquired in Nikon Eclipse 90i microscopes and analyzed with NIS-Elements AR software (Nikon Corporation, Tokyo, Japan) to determine the granulomatous area and alveolar space as a percentage of the total lung tissue.

Lipid mediator analyses

LMs in crude lung homogenates were analyzed with liquid chromatography-tandem mass spectrometry. Seventeen-hydroxy docosahexaenoic acid (17-HDHA); 5-, 11-, 12-, 15- and 18-hydroeicosapentaenoic acid (HEPE); 5-, 8-, and 9-hydroxyeicosatetraenoic acids (HETE); prostaglandin D_1 (PD_1); PGE_2; PGE_3; and PGD_2 were measured. LMs were extracted from ~50 mg lung tissue, in a 10 µl/mg homogenization buffer, with solid-phase extraction (SPE), using Strata-X (Phenomenex, Torrance, USA). The method was modified for Strata-XSPE columns from a previously described method (20). Data were quantified with Masshunter B0502, using external calibration for each compound and internal standards (PGD_2-d4, PGE_2-d4, PGF_2-d4 and 5- and 12-HETE-d8; 1000 pg of each, Cayman Chemicals, Ann Arbor, USA) to correct for losses and matrix effects.

Cytokine analyses

The left lung lobe homogenates leftover from determining the bacterial load were centrifuged and the supernatant was frozen at -80 °C until analysis. The cytokines, interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, monocyte chemoattractant protein, interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), chemokine ligand 3 (CCL3), granulocyte-macrophage colony-stimulating factor (GMCSF), and chemokine ligand 5 (CCL5) were measured in cell-free lung homogenates using the Quansys Biosciences Q-Plex™ Mouse Cytokine Screen (West Logan, USA) 16-plex Array for mouse cytokines according to manufacturer instructions. Arrays were analyzed using the Q-View Imager Pro and Q-View Software.

Statistical analysis

Data are presented as means ± standard error of the mean. Statistical analysis was performed using IBM SPSS statistics software (version 25; IBM Corporation). To determine the differences between the (n-3)eFAS and EPA/DHA groups, the Student Fischer T-test for independent variables was used. The relationships between PBMC and crude lung homogenate FA compositions and lung cytokines and LMs were determined by Pearson's correlations. A *P*-value equal to or less than 0.05 was considered significant.

Results

Bodyweight gain and food intake

There were no between-group differences in pre-infection weight (33 ± 0.47 g) and daily food intake during the experimental period (3.30 ± 0.25 g). However, the EPA/DHA group gained more weight during the 3-wk intervention period compared with the (n-3)eFAS group (6.98 ± 0.60 % and 4.18 ± 0.72 %, respectively, $P = 0.017$).

Total phospholipid fatty acid composition of RBCs, PBMCs, and crude lung homogenates

Following the 6-wk conditioning period on an n-3 PUFA-deficient diet prior to infection, the RBC phospholipid FA composition of a sub-sample (n=6) of mice was examined and is presented in **Table 2**. The phospholipid FA composition of RBCs, PBMCs and lung tissue after 3 wk of dietary intervention are presented in **Table 3**. The LA composition was higher in RBCs ($P < 0.001$) and the ALA composition in PBMCs ($P = 0.003$) of the EPA/DHA group than in the (n-3)eFAS group. There was not any significant difference in LA and ALA concentrations in crude lung homogenates (Table 3). Higher EPA, DHA and total n-3 LCPUFAs ($P < 0.001$ for all, except for DHA PBMC, $P = 0.003$) and lower AA, osbond acid and total n-6 LCPUFAs ($P < 0.001$ for all), were evident in the EPA/DHA compared with the (n-3)eFAS group.

Table 2 Red blood cell phospholipid fatty acid composition of mice prior to infection, after receiving the (n-3)FAD diet for 6 wk[1]

Fatty Acids	% of total fatty acids
18:3n-3 (ALA)	0.00 ± 0.00
20:5n3 (EPA)	0.20 ± 0.01
22:6n3 (DHA)	3.92 ± 0.22
Total n-3 LCPUFA	4.12 ± 0.22
18:2n-6 (LA)	7.25 ± 0.02
20:4n-6 (AA)	19.8 ± 0.40
22:5n-6 (Osbond)	4.06 ± 0.33
Total n-6 LCPUFA	28.6 ± 0.48
n-6/n-3 LCPUFA	7.04 ± 0.38

[1]Values are reported as means ± SEM percentage of total fatty acids (n=6).

AA, arachidonic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acid; PUFA, polyunsaturated fatty acid; n-3, omega-3; n-6, omega-6.

Table 3 Phospholipid FA composition of RBCs, PBMC, and crude lung homogenates for *Mtb*-infected mice receiving (n-3)eFAS or EPA/DHA diets for 3wk[1]

Fatty Acids	RBC			PBMC			Crude lung tissue homogenates		
	(n-3)eFAS	EPA/DHA	*P*-value	(n-3)eFAS[2]	EPA/DHA	*P*-value	(n-3)eFAS	EPA/DHA	*P*-value
18:3n-3 (ALA)	0.03 ±0.00	0.04 ±0.00	0.43	0.01 ± 0.00	0.01 ±0.00	0.003	0.06 ± 0.00[b]	0.07 ± 0.00	0.56
20:5n3 (EPA)	0.10 ±0.00	0.39 ± 0.02	<0.001	0.19 ± 0.01	0.70 ±0.04	<0.001	0.14 ± 0.01	0.38 ± 0.01	<0.001
22:6n3 (DHA)	3.99 ± 0.08	5.41 ± 0.06	<0.001	7.99 ± 0.02	9.49 ± 0.24	0.003	7.64 ± 0.15	9.56 ± 0.11	<0.001
Total n-3 PUFAs	4.39 ± 0.07	6.22 ± 0.06	<0.001	9.12 ± 0.07	11.6 ± 0.31	0.001	9.26 ± 0.18	12.4 ± 0.10	<0.001
Total n-3 LCPUFAs	4.35 ± 0.07	6.19 ± 0.05	<0.001	9.11 ± 0.07	11.6 ± 0.31	0.001	9.19 ± 0.18	12.3 ± 0.10	<0.001
18:2n-6 (LA)	7.66 ± 0.13	9.53 ± 0.27	<0.001	8.22 ± 0.30	9.24 ± 0.44	0.16	6.7 ± 0.17	7.07 ± 0.95	0.13
20:4n-6 (AA)	18.5 ± 0.15	16.4 ± 0.30	<0.001	19.9 ± 0.11	16.9 ± 0.27	<0.001	16.1 ± 0.44	13.3 ± 0.19	<0.001
22:5n-6 (Osbond)	1.00 ± 0.06	0.84 ± 0.07	<0.001	1.82 ± 0.03	0.98 ± 0.05	<0.001	1.48 ± 0.05[b]	0.85 ± 0.03	<0.001
Total n-6 PUFAs	28.6 ± 0.26	28.1 ±0.56	0.39	34.3 ± 0.41	30.8 ± 0.20	<0.001	30.1 ± 0.57	25.9 ± 0.30	<0.001
Total n-6 LCPUFAs	21.1 ± 0.18	18.7 ± 0.32	<0.001	26.0 ± 0.16	21.5 ± 0.27	<0.001	23.3 ± 0.45[b]	18.8 ± 0.26	<0.001
n-6/n-3 LCPUFA ratio	4.85 ± 0.06	3.02 ± 0.05	<0.001	2.86 ± 0.03	1.86 ± 0.05	<0.001	2.53 ± 0.03	1.52 ± 0.02	<0.001

[1]Values are reported as means ± SEM percentage of total fatty acids. Results repeated in two experiments, data shown for one experiment (n=5 per group). Intervention effects were estimated by using the Independent Student Fischer T-test. [2] n=3. AA, Arachidonic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EPA/DHA, eicosapentaenoic acid and docosahexaenoic acid-supplemented group; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acid; n-3, omega-3; n-6, omega-6; (n-3)eFAS, omega-3 essential fatty acid-sufficient group; PUFA, polyunsaturated fatty acid; PBMC, peripheral blood mononuclear cell; RBC, red blood cell.

Lung bacterial load and pathology

Figure 1 shows the bacterial loads, free alveolar space and histology images of the *Mtb*-infected mice following 3wk of dietary intervention. The EPA/DHA group had a lower bacterial load (P = 0.0001) (**Figure 1A**) and a trend towards higher free alveolar space (%) (P = 0.08) (**Figure 1B**).

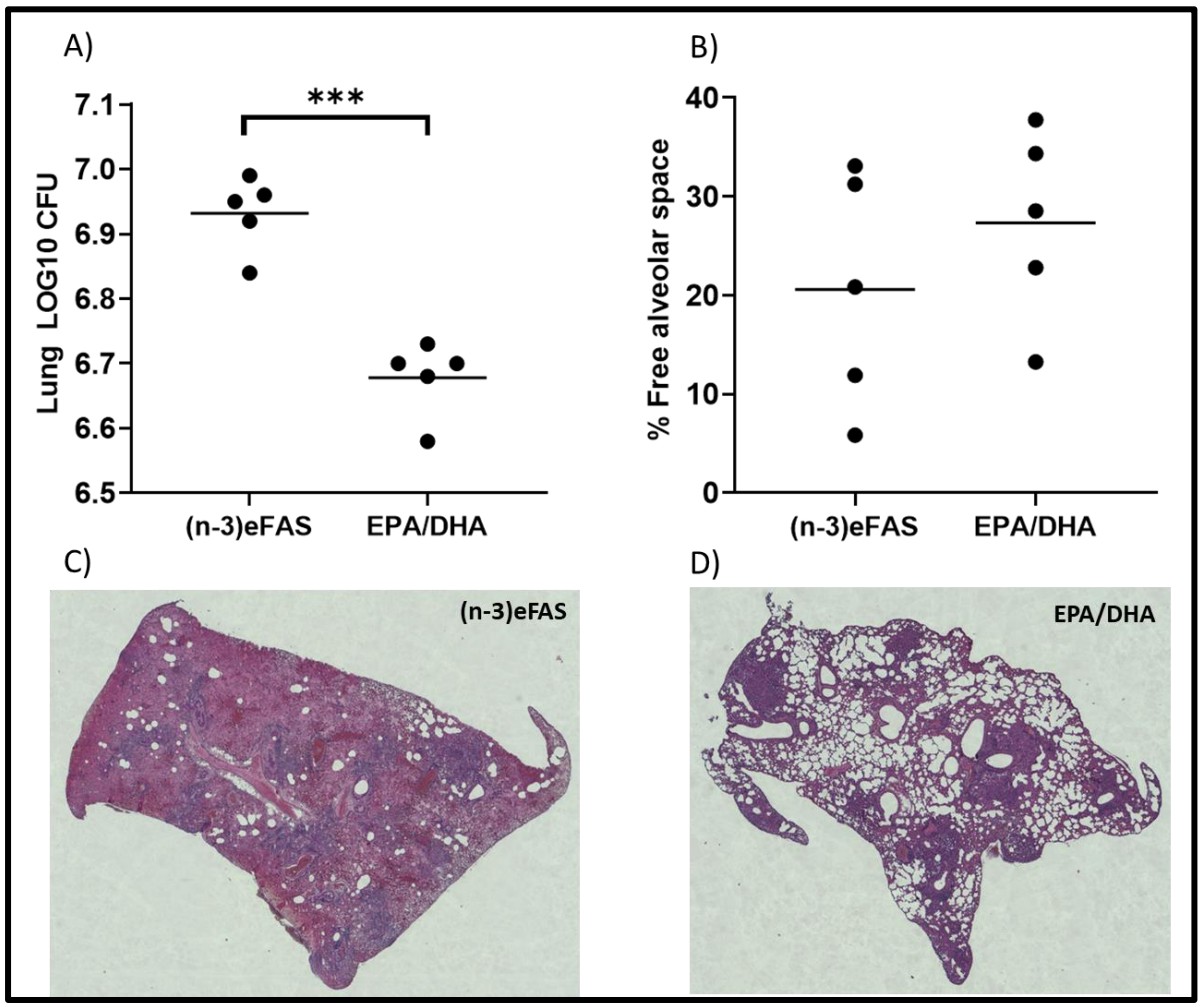


Figure 1 A) Bacterial loads in crude lung homogenates, B) percentage alveolar air space, and representative hematoxylin-eosin-stained sections of the lungs of C) (n-3)eFAS and D) EPA/DHA groups after providing *Mtb*-infected mice with (n-3)eFAS or EPA/DHA diets for 3wk. Results repeated in two experiments, data shown for one experiment (n=5 per group). The values represent mean ± SEM. Independent Student Fischer T-Test was used to compare means, ***P < 0.001. CFU, colony-forming units; EPA/DHA, eicosapentaenoic and docosahexaenoic acid-supplemented group; (n-3)eFAS, omega-3 essential fatty acid-sufficient diet group.

Lung lipid mediators and cytokines

Regarding the local lung inflammatory response, the EPA/DHA group had a more pro-resolving LM profile, with higher concentrations of the EPA-derived lung LM intermediates 5-, 11-, 12-, 15-, and 18-HEPE ($P < 0.001$, $P = < 0.001$, $P = 0.002$, $P = 0.001$, and $P = 0.003$) (**Figure 2**A, 2B, 2C, 2D, and 2E) and the DHA-derived intermediate 17-HDHA ($P = 0.023$) (Figure 2F) compared with the (n-3)eFAS group. On the other hand, the EPA/DHA group had lower concentrations of the AA-derived lung $PGF_2\alpha$ ($P = 0.002$) and intermediates 5-, and 9-HETE ($P = 0.018$ and $P = 0.012$) (**Figure 3**A, 3B and 3C), whilst PGD_2, and 8-HETE tended to be lower ($P = 0.08$ and $P = 0.08$) (Figure 3D and 3E) in comparison with the (n-3)eFAS group.

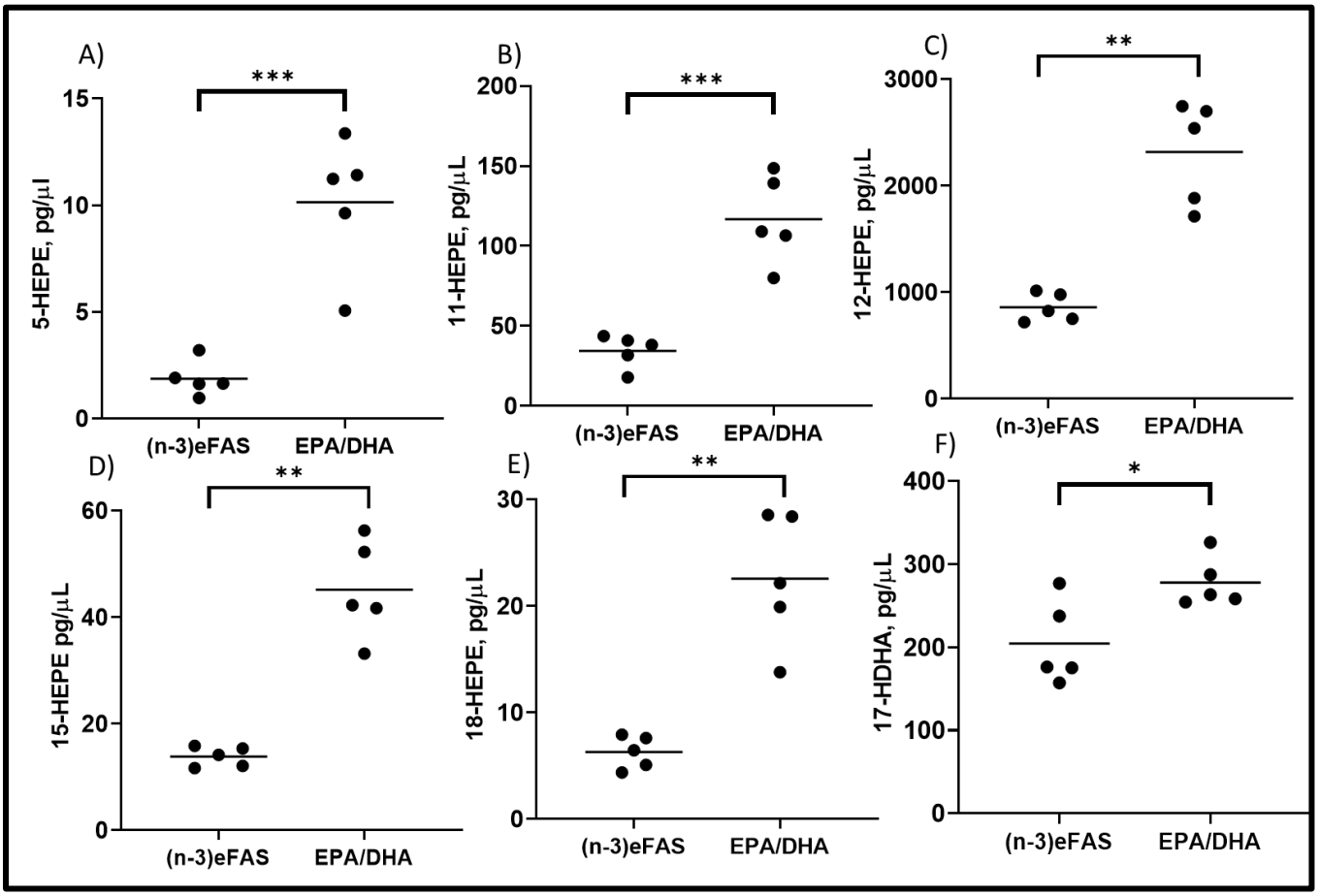


Figure 2 Pro-resolving **l**ipid mediators including A) 5-HEPE, B) 11-HEPE, C) 12-HEPE, D) 15-HEPE, E) 18-HEPE, and F) 17-HDHA in crude lung homogenates after providing *Mtb*-infected mice with (n-3)eFAS or EPA/DHA diets for 3wk. Results repeated in two experiments, data shown for one experiment (n=5 per group). The values represent mean ± SEM. Independent Student Fischer T-Test was used to compare means, *P < 0.05 **P < 0.01, ***P < 0.001. EPA/DHA, eicosapentaenoic and docosahexaenoic acid-supplemented group; HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; (n-3)eFAS, omega-3 essential fatty acid-sufficient diet group.

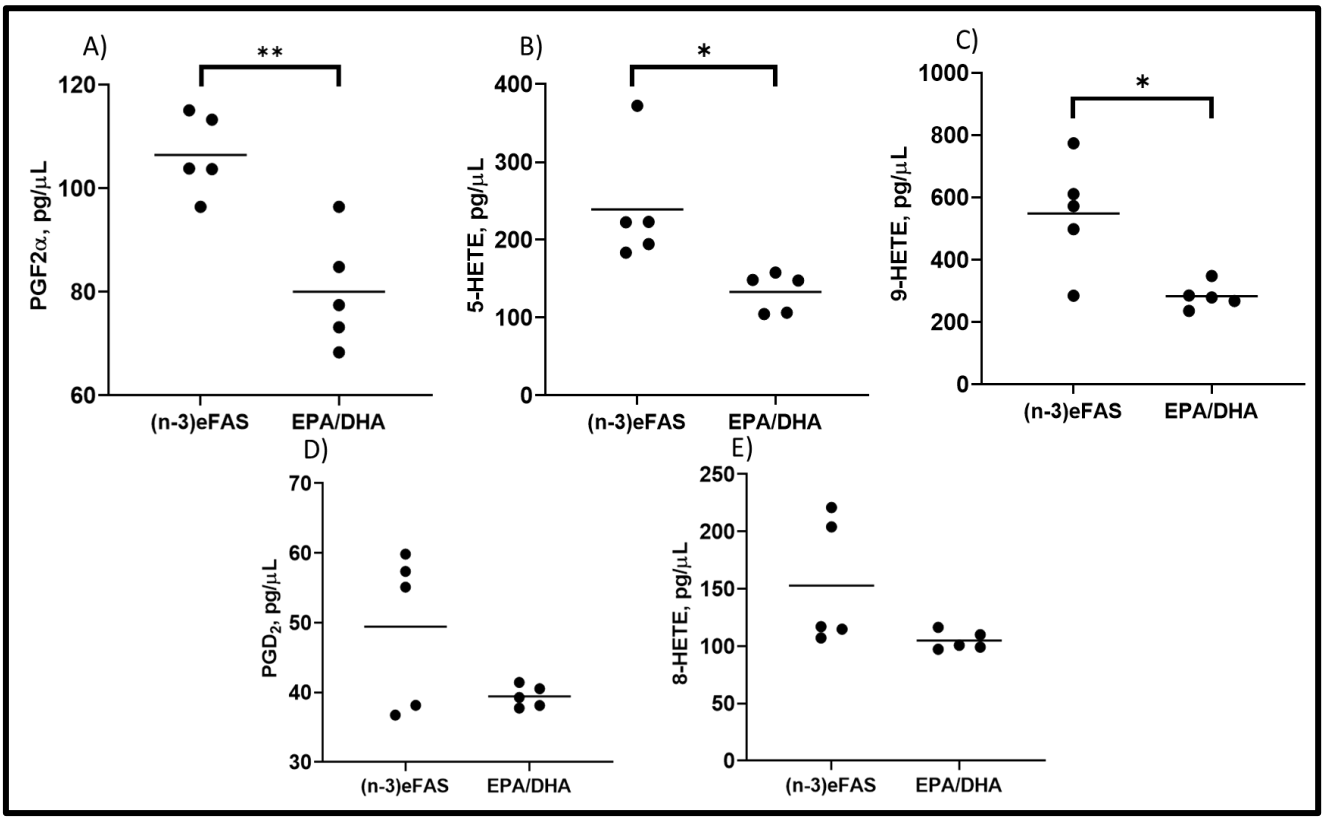


Figure 3 Pro-inflammatory lipid mediators including A) PGF2α. B) 5-HETE, C) 9-HETE, D) PGD_2, and E) 8-HETE in crude lung homogenates after providing *Mtb*-infected mice with (n-3)eFAS or EPA/DHA diets for 3wk. Results repeated in two experiments, data shown for one experiment (n=5 per group). The values represent mean ± SEM. Independent Student Fischer T-Test was used to compare means, *P < 0.05 **P < 0.01, ***P < 0.001. EPA/DHA, eicosapentaenoic and docosahexaenoic acid-supplemented group; HETE, hydroxyeicosatetraenoic acids; (n-3)eFAS, omega-3 essential fatty acid-sufficient diet group; PG, prostaglandin.

.

In addition, lung homogenate cytokine concentrations were also measured (**Figure 4**). Lung interleukin-1 alpha (IL-1α) and IL-1β (*P* = 0.023 and *P* =0.049) (Figure 4A and 4B) were lower and IL-6 tended to be lower (*P* = 0.08) (Figure 4C) in the EPA/DHA group. Furthermore, correlations between PBMC and lung FA compositions and lung homogenate cytokine concentrations were tested (**Table 4**). We found strong inverse correlations between lung EPA composition and IL-1α (r = -0.703, *P* = 0.016) and IL-1β (r = -0.684, *P* = 0.043), as well as lung DHA composition and IL-1α (r = -0.783, *P* = 0.007). In PBMC, EPA composition had a strong inverse correlation with IL-1α (r = -0.701, *P* = 0.053) and IL-6 (r = -0.700, *P* = 0.053) and DHA composition with IL-6 (r = -0.820, *P* = 0.013) (Table 4).

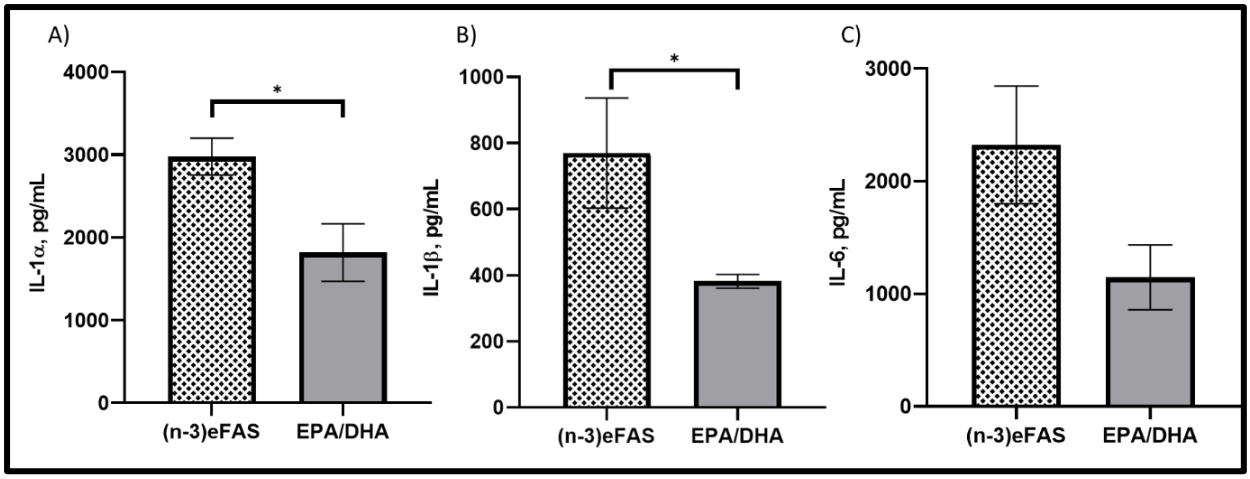


Figure 4 Cytokine concentrations of A) interleukin-1α, B) interleukin-1β, and C) interleukin-6 in crude lung homogenates after providing *Mtb*-infected mice with (n-3)eFAS or EPA/DHA diets for 3wk. Results repeated in two experiments, data shown for one experiment (n=5 per group). The values represent mean ± SEM. Independent Student Fischer T-Test was used to compare means, *P < 0.05. EPA/DHA, eicosapentaenoic and docosahexaenoic acid-supplemented group; IL; interleukin; (n-3)eFAS, omega-3 essential fatty acid-sufficient diet group.

Table 4 Correlations between the phospholipid fatty acid composition of PBMC and crude lung homogenates and lung cytokine concentrations after providing *Mtb*-infected mice with (n-3)eFAS or EPA/DHA for 3wk[1]

	PBMC			**Crude lung homogenate**		
Lung cytokine	**AA** n=8	**EPA** n=8	**DHA** n=8	**AA** n=10	**EPA** n=10	**DHA** n=10
IL-1α	0.81 (0.015)	-0.70 (0.05)	-0.61 (0.11)	0.65 (0.042)	-0.70 (0.016)	-0.78 (0.007)
IL-1β	0.57 (0.14)	-0.57 (0.14)	-0.56 (0.15)	0.77 (0.010)	-0.68 (0.043)	-0.54 (0.10)
IL-6	0.80 (0.018)	-0.70 (0.05)	-0.82 (0.013)	0.41 (0.24)	0.22 (0.53)	0.17 (0.63)

[1] Values presented as r-value (*P*-value). Results repeated in two experiments, data shown for one experiment (n=5 per group). Correlation coefficient determined by Pearson's Correlation.

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IL; interleukin; PBMC, peripheral blood mononuclear cell.

Discussion

In the current study, we demonstrated that EPA/DHA supplementation is more effective with regards to bacterial killing, weight gain, and inflammation resolution than the provision of an n-3 EFA-sufficient diet in a low n-3 PUFA status C3HeB/FeJ mouse TB model. We postulated that the conversion of ALA to its longer-chain metabolites would be increased in our experiment, owing to the low n-3 PUFA status that the mice were conditioned to. Higher liver ALA conversion rates have previously been found in n-3 PUFA-deficient rats (21). Furthermore, extrahepatic conversion is important in the immune and inflammatory response and as this was an infectious disease model, desaturase may have been upregulated in immune cells, as previously found in PBMC and lymphocytes, specifically (22-25). On the other hand, providing the preformed end products of this metabolic pathway, as in our EPA/DHA group, may reduce these conversion rates (8). Nevertheless, in our experiment, following the three-week dietary intervention period, the EPA/DHA group had higher EPA and DHA compositions in all pools measured. Although not TB-specific, this accords with previous experiments in rodents, which conveyed that providing preformed EPA and DHA amounted to higher n-3 LCPUFA cell membrane composition than did ALA provision alone (26-28).

In this study, the absolute ALA content of the (n-3)eFAS diet was calculated based on sufficient dietary ALA content to ensure optimal membrane DHA saturation in healthy rodents (17). This diet contained soybean oil, in which ALA comprises more or less 10% of FAs, compared with flaxseed oil, in which more than 50% of FAs are contributed by ALA (7). Therefore, the ALA dosage provided may not have been sufficient to increase EPA and DHA composition remarkably in this infectious disease model, where there was most likely a higher turnover of n-3 LCPUFA for pro-resolving and antibacterial functions (5). Moreover, dietary LA content also influences the desaturation and elongation of ALA and lower LA/ALA ratios will lead to better incorporation of EPA into phospholipids (10, 29). In our study, the n-6/n-3 PUFA ratio was much lower in the (n-3)eFAS diet (7/1) compared with the deficient diet ((n-3)FAD) that the mice in our experiment were conditioned on (130/1) (17). Nevertheless, the absolute ALA concentrations provided in the (n-3)eFAS diet may not have been high enough and the competition for conversion of LA still too great to have as substantial an incremental effect on n-3 LCPUFA membrane composition in the (n-3)eFAS group as in the EPA/DHA group (10, 29).

Apart from enhanced bacterial killing, the mice in the EPA/DHA group also had a higher percentage weight gain throughout the infection period, even though their food intake did not differ. It is well-known that TB is a catabolic disease, wherein a large percentage of ingested protein is oxidized for energy production, reducing its availability for lean tissue protein

synthesis (30, 31). A lower bacterial load coupled with a less inflammatory profile and its accompanying catabolism may have led to higher protein incorporation and lean tissue preservation in the mice receiving the EPA/DHA diet.

Similar to EPA and DHA, ALA has been shown to favor inflammation resolution by means of its conversion to EPA and DHA in animals. Additionally, LMs such as the anti-inflammatory 13-hydroxyoctadecatrienoic acid are also produced from ALA directly; ALA mediates COX inhibition and reduces nitric oxide production, all further contributing to inflammation resolution (32-34). Even though, to our knowledge, there is a paucity of research on the effect of ALA in TB, a dose-dependent lowering effect on several pro-inflammatory cytokines has been proven with ALA supplementation in animal models of diabetes, cancer, aortic banding and asthma-induced lung inflammation (27, 35-38). In our study, the EPA/DHA group presented with lower IL-1α and IL-1β and decreasing IL-6 concentrations. IL-1α and IL-1β are strong pro-inflammatory cytokines, important in innate immune responses and in the activation of macrophages for bacterial killing (18, 39). However, these cytokines, especially IL-1β, also exert strong inflammatory signaling that can lead to host lung tissue destruction, correlating with disease and X-ray severity, cavitary TB, and poor clinical outcomes (1, 40). IL-6 is considered an important cytokine in high bacterial loads, with high IL-6 concentrations correlating with higher bacterial burdens and chest X-ray severity in humans (41, 42). This explains our results as the (n-3)eFAS group presented with higher bacterial loads together with higher concentrations of these cytokines.

Taken together, our results showed that EPA and DHA supplementation benefited the pro-resolution of lung inflammation more than only providing a diet with sufficient amounts of ALA. Whilst clinical trials have found ALA's effects to be only modest and dependent on its conversion to EPA (43), there is some evidence in animals that the effects of ALA itself on inflammation is similar to that of preformed EPA and DHA (35, 37). However, these studies administered therapeutic ALA dosages (mostly in the form of flaxseed oil) compared with what our (n-3)eFAS diet provided, which may explain our contrasting findings (35, 37).

The importance of LMs in TB regulation, as well as the close connection between cytokine and LM networks, is well established in the literature (44-46). Whilst the PBMC FA composition represents the contribution of the immune cells' FA status, the crude lung homogenates' FA composition signifies a combination of recruited immune cells and lung tissue. As local lung epithelium also synthesizes LMs, together with recruited immune cells, lung FA composition underpins local inflammation modulatory effects (47, 48). This supports our findings that the EPA and DHA composition in cell membranes of PBMC and crude lung homogenates were strongly negatively correlated with certain pro-inflammatory lung cytokines. Additionally, the

EPA-derived HEPEs and DHA-derived 17-HDHA were higher in the EPA/DHA group. These LM intermediates are precursors of the resolvins and protectins which enhance inflammation resolution and tissue regeneration (5). In this regard, 18-HEPE is an intermediate in the LM pathway that produces the E-series resolvins, which have been shown to enhance phagocytosis and bacterial killing, supporting our findings in the EPA/DHA group (5, 6, 49, 50). The EPA/DHA group also had lower concentrations of the pro-inflammatory LMs (derived from AA) potentiating the cytokine response (51), such as 5-HETE, an intermediate for the production of leukotriene A_4 (LTA_4) which is converted to LTB_4. LTB_4 has been shown to correlate with TB severity on chest X-rays and has been implicated in granuloma cell necrosis (52, 53). This supports the tendency towards lower lung pathology that we found in the EPA/DHA group.

Our study was strengthened by the fact that *Mtb*-infected C3HeB/FeJ mice are a well-established TB model and the mouse strain that best represents human lung TB histopathology (54). We did not administer TB medication, which may have influenced our results. We also measured outcomes only at one time point, whereas in future studies, more time points should be added to determine the effects of outcomes at different stages of infection and inflammation. The provision of higher ALA dosages together with lower LA/ALA ratios (below 7/1) may also have brought about different results. Nevertheless, this experiment was designed to mimic the human situation of switching from an n-3 PUFA-deficient diet to an n-3 EFA-sufficient diet with an improved n-6/n-3 PUFA ratio, possibly brought about by dietary education.

In conclusion, we found that preformed EPA/DHA supplementation is more beneficial than only providing a diet with sufficient amounts of the n-3 EFA, ALA, with regard to reduced bacterial loads, accompanied by weight gain and lower lung inflammation in a low n-3 PUFA status TB mouse model. Therefore, switching from a deficient to a balanced diet containing sufficient essential ALA in an acceptable n-6/n-3 PUFA ratio may not be sufficient to obtain the significant effects that were found with EPA/DHA supplementation in TB. Alternatively, providing EPA/DHA supplementation as HDT in TB may be a worthwhile strategy to consider.

Acknowledgments

The authors thank Rodney Lucas (University of Cape Town, Cape Town, SA) and Kobus Venter (North-West University, SA) for their assistance with data collection, and Adriaan Jacobs and Cecile Cooke (North-West University, SA) for their assistance with laboratory analyses.

A.N., L.M., R.D., C.M.S., and R.B. conceptualized study; A.N., L.M., and S.P. designed study; A.N., L.M., M.O., S.P., and F.H., and conducted research; A.N., L.M., and M.O. analyzed data; A.N., L.M., L.Z., C.M.S., M.O., and S.P. assisted with data interpretation; A.N. wrote the manuscript. All authors reviewed and approved the final manuscript.

References

1. Kumar NP, Moideen K, Banurekha VV, Nair D, Babu S. Plasma pro-inflammatory cytokines are markers of disease severity and bacterial burden in pulmonary tuberculosis. Open Forum Infect 2019.

2. Stek C, Allwood B, Walker NF, Wilkinson RJ, Lynen L, Meintjes G. The immune mechanisms of lung parenchymal damage in tuberculosis and the role of host-directed therapy. Front micro. 2018;9.

3. Browning LM, Walker CG, Mander AP, West AL, Madden J, Gambell JM, Young S, Wang L, Jebb SA, Calder PC. Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish. Am J Clin Nutr 2012;96:748-58.

4. Rees D, Miles EA, Banerjee T, Wells SJ, Roynette CE, Wahle KW, Calder PC. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. Am J Clin Nutr 2006;83:331-42.

5. Serhan CN, Chiang N, Dalli J. New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration. Mol Aspects Med 2017;64:1-17.

6. Chiang N, Fredman G, Bäckhed F, Oh SF, Vickery T, Schmidt BA, Serhan CN. Infection regulates pro-resolving mediators that lower antibiotic requirements. Nature 2012;484:524.

7. Baker EJ, Miles EA, Burdge GC, Yaqoob P, Calder PC. Metabolism and functional effects of plant-derived omega-3 fatty acids in humans. Prog Lip Res 2016;64:30-56.

8. Brenna JT, Salem Jr N, Sinclair AJ, Cunnane SC. α-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. Prostaglandins Leukot Essent Fatty Acids 2009;80:85-91.

9. Lin YH, Salem N. Whole body distribution of deuterated linoleic and α-linolenic acids and their metabolites in the rat. J Lipid Res 2007;48:2709-24.

10. Gibson RA, Neumann MA, Lien EL, Boyd KA, Tu WC. Docosahexaenoic acid synthesis from alpha-linolenic acid is inhibited by diets high in polyunsaturated fatty acids. Prostaglandins Leukot Essent Fatty Acids 2013 2013/01/01/;88:139-46.

11. Baumgartner J, Smuts CM, Malan L, Kvalsvig J, van Stuijvenberg ME, Hurrell RF, Zimmermann MB. Effects of iron and n-3 fatty acid supplementation, alone and in combination, on cognition in school children: a randomized, double-blind, placebo-controlled intervention in South Africa. Am J Clin Nutr 2012;96:1327-38.

12. Stark KD, Van Elswyk ME, Higgins MR, Weatherford CA, Salem Jr N. Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults. Prog Lip Res 2016;63:132-52.

13. Jordao L, Lengeling A, Bordat Y, Boudou F, Gicquel B, Neyrolles O, Becker PD, Guzman CA, Griffiths G, Anes E. Effects of omega-3 and-6 fatty acids on Mycobacterium tuberculosis in macrophages and in mice. *Microb Infec* 2008;10:1379-86.

14. World Health Organization. Global tuberculosis report 2018. Geneva. Licence: CC BY-NCSA 30 IGO. 2019.

15. Massyn N, Padarath A, Peer N. District health barometer 2016/17: Health Systems Trust; 2017.

16. Baumgartner J, Smuts CM, Malan L, Arnold M, Yee BK, Bianco LE, Boekschoten MV, Müller M, Langhans W, Hurrell RF. Combined deficiency of iron and (n-3) fatty acids in male rats disrupts brain monoamine metabolism and produces greater memory deficits than iron deficiency or (n-3) fatty acid deficiency alone. J Nutr 2012;142:1463-71.

17. Reeves PG, Nielsen FH, Fahey Jr GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;11:1939–51.

18. Guler R, Parihar SP, Spohn G, Johansen P, Brombacher F, Bachmann MF. Blocking IL-1α but not IL-1β increases susceptibility to chronic Mycobacterium tuberculosis infection in mice. Vaccine 2011;29:1339-46.

19. Folch J, Lees M, Stanley GS. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497-509.

20. Malan L, Baumgartner J, Zandberg L, Calder P, Smuts C. Iron and a mixture of dha and epa 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. Prostaglandins Leukot Essent Fatty Acids 2016;105:15-25.

21. Igarashi M, DeMar JC, Ma K, Chang L, Bell JM, Rapoport SI. Upregulated liver conversion of α-linolenic acid to docosahexaenoic acid in rats on a 15 week n-3 PUFA-deficient diet. J Lip Res 2007;48:152-64.

22. Sibbons CM, Irvine NA, Pérez-Mojica JE, Calder PC, Lillycrop KA, Fielding BA, Burdge GC. Polyunsaturated fatty acid biosynthesis involving Δ8 desaturation and differential DNA methylation of FADS2 regulates proliferation of human peripheral blood mononuclear cells. Front Immuno 2018;9:432.

23. Calder PC, Yaqoob P, Harvey DJ, Watts A, Newsholme EA. Incorporation of fatty acids by concanavalin A-stimulated lymphocytes and the effect on fatty acid composition and membrane fluidity. Biochem J 1994;300:509-18.

24. Anel A, Naval J, González B, Uriel J, Piñeiro A. Fatty acid metabolism in human lymphocytes. II. Activation of fatty acid desaturase-elongase systems during blastic transformation. BBA Lip Met 1990;1044:332-9.

25. Burdge GC. Is essential fatty acid interconversion an important source of PUFA in humans? Brit J Nutr 2019;121:615-24.

26. Abedin L, Lien E, Vingrys A, Sinclair A. The effects of dietary α-linolenic acid compared with docosahexaenoic acid on brain, retina, liver, and heart in the guinea pig. Lipids 1999;34:475-82.

27. Duda MK, O'Shea KM, Tintinu A, Xu W, Khairallah RJ, Barrows BR, Chess DJ, Azimzadeh AM, Harris WS, Sharov VG. Fish oil, but not flaxseed oil, decreases inflammation and prevents pressure overload-induced cardiac dysfunction. Cardiovasc Res 2008;81:319-27.

28. Riediger ND, Othman R, Fitz E, Pierce GN, Suh M, Moghadasian MH. Low n-6: n-3 fatty acid ratio, with fish-or flaxseed oil, in a high fat diet improves plasma lipids and beneficially alters tissue fatty acid composition in mice. Eur J Clin Nutr 2008;47:153-60.

29. Goyens PL, Spilker ME, Zock PL, Katan MB, Mensink RP. Conversion of α-linolenic acid in humans is influenced by the absolute amounts of α-linolenic acid and linoleic acid in the diet and not by their ratio. Am J Clin Nutr 2006;84:44-53.

30. MacAllan DC, McNurlan MA, Kurpad AV, De Souza G, Shetty PS, Calder AG, Griffin GE. Whole body protein metabolism in human pulmonary tuberculosis and undernutrition: evidence for anabolic block in tuberculosis. Clin Sci 1998;94:321-31.

31. Kant S, Gupta H, Ahluwalia S. Significance of Nutrition in Pulmonary Tuberculosis. Crit Rev Food Sci Nutr 2015;55:955-63.

32. Anand R, Kaithwas G. Anti-inflammatory potential of alpha-linolenic acid mediated through selective COX inhibition: computational and experimental data. Inflammation 2014;37:1297-306.

33. Tam VC. Lipidomic profiling of bioactive lipids by mass spectrometry during microbial infections. Sem Immuno 2013;25:240-248.

34. Ren J, Chung SH. Anti-inflammatory effect of α-linolenic acid and its mode of action through the inhibition of nitric oxide production and inducible nitric oxide synthase gene expression via NF-κB and mitogen-activated protein kinase pathways. J Agric Food Chem 2007;55:5073-80.

35. Jangale NM, Devarshi PP, Bansode SB, Kulkarni MJ, Harsulkar AM. Dietary flaxseed oil and fish oil ameliorates renal oxidative stress, protein glycation, and inflammation in streptozotocin–nicotinamide-induced diabetic rats. J Physiol Biochem 2016;72:327-36.

36. Moura-Assis A, Afonso MS, de Oliveira V, Morari J, dos Santos GA, Koike M, Lottenberg AM, Catharino RR, Velloso LA, da Silva ASR. Flaxseed oil rich in omega-3 protects aorta against inflammation and endoplasmic reticulum stress partially mediated by GPR120 receptor in obese, diabetic and dyslipidemic mice models. J Nutr Biochem 2018;53:9-19.

37. Schiessel DL, Yamazaki RK, Kryczyk M, Coelho de Castro I, Yamaguchi AA, Pequito DC, Brito GA, Borghetti G, Aikawa J, Nunes EA. Does oil rich in alpha-linolenic fatty acid cause the same immune modulation as fish oil in walker 256 tumor-bearing rats? Nutr Can 2016;68:1369-80.

38. Kaveh M, Eftekhar N, Boskabady MH. The effect of alpha linolenic acid on tracheal responsiveness, lung inflammation, and immune markers in sensitized rats. Iran J Basic Med Sci 2019;22:255-261.

39. Romero-Adrian TB, Leal-Montiel J, Fernández G, Valecillo A. Role of cytokines and other factors involved in the Mycobacterium tuberculosis infection. World J Immunol 2015;5:16-50.

40. Borthwick L. The IL-1 cytokine family and its role in inflammation and fibrosis in the lung. Semin Immunopathol 2016;38:517-34.

41. Domingo-Gonzalez R, Prince O, Cooper A, Khader SA. Cytokines and Chemokines in Mycobacterium tuberculosis Infection. Microbiology spectrum. 2016;4:10.1128/microbiolspec.TBTB2-0018-2016.

42. Mesquita ED, Gil-Santana L, Ramalho D, Tonomura E, Silva EC, Oliveira MM, Andrade BB, Kritski A. Associations between systemic inflammation, mycobacterial loads in sputum and radiological improvement after treatment initiation in pulmonary TB patients from Brazil: a prospective cohort study. *BMC Infec Dis* 2016;16:368.

43. Zhao G, Etherton TD, Martin KR, Gillies PJ, West SG, Kris-Etherton PM. Dietary α-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects. Am J Clin Nutr 2007;85:385-91.

44. Dietzold J, Gopalakrishnan A, Salgame P. Duality of lipid mediators in host response against Mycobacterium tuberculosis: good cop, bad cop. F1000 Prime Rep 2015;7:29-37.

45. Mayer-Barber KD, Sher A. Cytokine and lipid mediator networks in tuberculosis. Immuno Rev 2015;264:264-75.

46. Colas RA, Nhat LTH, Thuong NTT, Gómez EA, Ly L, Thanh HH, Mai NTH, Phu NH, Thwaites GE, Dalli J. Proresolving mediator profiles in cerebrospinal fluid are linked with disease severity and outcome in adults with tuberculous meningitis. FASEB J 2019;33:13028-39.

47. Kiss L, Schütte H, Padberg W, Weissmann N, Mayer K, Gessler T, Voswinckel R, Seeger W, Grimminger F. Epoxyeicosatrienoates are the dominant eicosanoids in human lungs upon microbial challenge. Euro Resp J 2010;36:1088-98.

48. Sanak M. Eicosanoid mediators in the airway inflammation of asthmatic patients: what is new? Allergy Asthma Immunol Res 2016;8:481-90.

49. Codagnone M, Cianci E, Lamolinara A, Mari V, Nespoli A, Isopi E, Mattoscio D, Arita M, Bragonzi A, Iezzi M. Resolvin D1 enhances the resolution of lung inflammation caused by long-term Pseudomonas aeruginosa infection. Muc Immuno 2018;11:35.

50. Spite M, Norling LV, Summers L, Yang R, Cooper D, Petasis NA, Flower RJ, Perretti M, Serhan CN. Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. Nature 2009;461:1287.

51. Vedin I, Cederholm T, Freund-Levi Y, Basun H, Hjorth E, Irving GF, Eriksdotter-Jönhagen M, Schultzberg M, Wahlund L-O, Palmblad J. Reduced prostaglandin F2α release from blood mononuclear leukocytes after oral supplementation of ω3 fatty acids: the OmegAD study. J Lip Res 2010;51:1179-85.

52. El-Ahmady O, Mansour M, Zoeir H, Mansour O. Elevated concentrations of interleukins and leukotriene in response to Mycobacterium tuberculosis infection. Ann Clin Biochem 1997;34:160-4.

53. Kumar NP, Kadar Moideen AN, Viswanathan V, Shruthi BS, Shanmugam S, Hissar S, Kornfeld H, Babu S. Plasma Eicosanoid Levels in Tuberculosis and Tuberculosis-Diabetes Co-morbidity Are Associated With Lung Pathology and Bacterial Burden. Front Cell Infect Microbiol 2019;9 9:335.doi: 10.3389/fcimb.2019.00335.

54. Lenaerts A, Barry III CE, Dartois V. Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. Immuno Rev 2015;264:288-307.

CHAPTER 6 CONCLUSION

6.1 Introduction

This final chapter summarises the findings of this thesis and indicates how the objectives, as described in Chapter 1, have been met. Furthermore, it provides a general conclusion based on the findings that are presented in the manuscripts in Chapters 3, 4 and 5, in addition to recommendations emanating from this research. For ease of reference, the aim and objectives are repeated.

6.2 Aim of this research

The aim of this thesis was to determine the effects of omega-3 long-chain polyunsaturated fatty acid (n-3 LCPUFA) and iron supplementation, alone and in combination, on the inflammatory response and clinical outcomes in *Mycobacterium tuberculosis* (*Mtb*)-infected mice and whether these effects are dependent on n-3 polyunsaturated (PUFA) status prior to infection.

6.3 Objective 1 (Chapter 3)

To determine the effects of n-3 LCPUFA supplementation on n-3 LCPUFA status, clinical outcomes and markers of the immune and inflammatory response in *Mtb*-infected mice with a sufficient or low n-3 PUFA status prior to infection and supplementation

6.3.1 Long-chain polyunsaturated fatty acid status

The findings of this research showed that three weeks of n-3 LCPUFA supplementation was sufficient to modify membrane phospholipid n-3 LCPUFA composition of red blood cells (RBCs), peripheral blood mononuclear cells (PBMCs) and crude lung homogenates, reflecting the fatty acid (FA) composition of the respective intervention diets. This was true for both mice with a sufficient and low n-3 PUFA status. However, we found that docosahexaenoic acid (DHA) was not higher in the PBMC membranes of the group with a sufficient n-3 PUFA status that was supplemented with n-3 LCPUFAs. This may be attributed to the fact that eicosapentaenoic acid (EPA) is incorporated faster than docosahexanoic acid (DHA) into cell membranes (Yaqoob *et al.*, 2000), and that the n-3 LCPUFA-supplemented diet was dominated by EPA (44% EPA versus 28% DHA). When a higher amount of EPA is provided in combination with DHA, EPA incorporation into immune cell membranes is favoured, whilst DHA incorporation is limited (Calder, 2008). On the other hand, the crude lung homogenate EPA composition did not differ significantly between n-3 LCPUFA-supplemented and non-

supplemented groups. This may be as a result of the high turnover of EPA for the production of EPA-derived lipid mediators (LMs). In the same manner, as EPA is incorporated more rapidly into different pools, it is also mobilised faster, especially in inflammatory conditions. Conversely, DHA is structurally favoured above EPA, with mechanisms in place to preserve it in cell membranes (Calder, 2014; Calder, 2016).

6.3.2 Tuberculosis related clinical outcomes

One of the major findings of this research was that n-3 LCPUFA supplementation lowered lung bacterial burden in mice that were conditioned to an n-3 PUFA-sufficient status prior to infection and supplementation. This finding may be attributed to the known effects of n-3 LCPUFAs on the membrane FA composition of phagocytic cells. It has been shown to enhance bacterial phagocytosis by alterations in membrane fluidity and receptor expression (Bonilla *et al.*, 2010a; Calder *et al.*, 1990), together with the actions of specialised pro-resolving mediators (SPMs) to aid in the differentiation of macrophages and neutrophils for phagocytosis and to activate bacterial killing (Chiang *et al.*, 2012; Codagnone *et al.*, 2018; Serhan *et al.*, 2017b). This was supported by the higher EPA and DHA composition found in lung homogenates (consisting of both lung tissue and recruited immune cells) and PBMC membranes and also, by the higher lung concentrations of the pro-resolving 18-hydroxyeicosapentaenoic acid (HEPE), which is an intermediate of the E-series resolvins (Oh *et al.*, 2011; Serhan *et al.*, 2017b).

The aforementioned finding is supported by Jordao *et al.* (2008), similarly indicating lower bacterial loads when supplementing n-3 PUFA in tuberculosis (TB) (Jordao *et al.*, 2008). However, other research previously published on the topic disagrees, showing that n-3 LCPUFAs inhibit immune response and worsens TB outcomes (Bonilla *et al.*, 2010b; Bonilla *et al.*, 2010a; Mayatepek *et al.*, 1994; McFarland *et al.*, 2008; Paul *et al.*, 1997). The timing of immunonutrition in TB is critically important and an initial strong inflammatory response is essential for host protection (Kroesen *et al.*, 2018). Additionally, early ingestion of n-3 LCPUFAs has been shown to inhibit phagosome and phagolysosome maturation (Anes *et al.*, 2003; Bonilla *et al.*, 2010a; Kroesen *et al.*, 2018). Therefore, we postulated that the main reason for these discrepancies was the timing of supplementation. Providing n-3 LCPUFA supplementation as therapy after the initial acute inflammatory response may be crucial.

Secondly, a higher EPA:DHA ratio may also have contributed to the success of our findings and to those of Jordao *et al.* (2008) (Bhattacharya *et al.*, 2007; Jordao *et al.*, 2008). Other studies that found immunosuppressive effects with n-3 LCPUFA supplementation either provided higher DHA concentrations or DHA only (Bonilla *et al.*, 2010a; Jordao *et al.*, 2008;

McFarland *et al.*, 2008; Paul *et al.*, 1997). Moreover, the quantification of lung histology revealed that n-3 LCPUFA supplementation resulted in a higher percentage of free alveolar space in the n-3 PUFA-sufficient arm. This was probably caused by the lower bacterial burden, lung inflammation and more pro-resolving LM profile that was evident in this group. No differences could be found in lung- and spleen-weight indexes.

The current investigation was also used as an opportunity to investigate the effects of n-3 LCPUFA supplementation translatable to a TB population with a habitual sub-optimal n-3 PUFA intake. Interestingly, here the n-3 LCPUFA-supplemented group did not show the same benefits as in the sufficient group. This was irrespective of the fact that this group's membrane FA composition and LM profile were also altered in accordance with n-3 LCPUFA supplementation.

6.3.3 Lung inflammation and the immune response

We further aimed to investigate the effects of n-3 LCPUFAs on inflammation, which is central to lung pathology and clinical outcomes (Marzo *et al.*, 2014; Vilaplana *et al.*, 2013). It was found that an elevated dietary consumption of n-3 LCPUFAs was successfully reflected in a more pro-resolving lung LM profile in both n-3 PUFA-sufficient mice and those with a low status. However, this occurrence did not fully translate into lower lung pro-inflammatory LM and cytokine concentrations. One would expect that the incorporation of n-3 LCPUFAs into membranes of monocytes, lymphocytes, and macrophages may provoke a reduction of the pro-inflammatory prostaglandin (PG) (especially PGE_2) production to favour the 3-series PGs (Ivanyi & Zumla, 2013). In this study, higher PGE_3 lung concentrations were found in the n-3 LCPUFA-supplemented group; however, the differences in PGE_2 concentrations, which have been specifically implicated in lung pathology in TB, did not reach significance (Kroesen *et al.*, 2018; Kumar *et al.*, 2019a).

Furthermore, the only lung cytokine that was found to be reduced by n-3 LCPUFA supplementation was interferon-gamma (IFN-γ) in the n-3 PUFA-sufficient group. This was probably caused by the lower bacterial burden in this group, as higher IFN-γ concentrations have been correlated with higher bacterial loads (Kumar *et al.*, 2019b; Mayer-Barber *et al.*, 2014). Furthermore, this is also underpinned by the lower T cell percentages in this group, which are known to release IFN-γ (Kumar *et al.*, 2019b; Mayer-Barber *et al.*, 2014). Omega-3 LPCUFAs have been found to inhibit T cell proliferation and activity elsewhere and in TB, specifically, explaining our results (Allen *et al.*, 2014; Calder & Newsholme, 1992; Fielding *et al.*, 2019; Kim *et al.*, 2010; McFarland *et al.*, 2008; Stulnig & Zeyda, 2004). Contrary to other findings on the effect of n-3 LCPUFA supplementation on neutrophil recruitment,

which is implicated in lung damage, we found no significant differences in neutrophil counts among groups (Marzo *et al.*, 2014; Oh *et al.*, 2011).

The reason why most other pro-inflammatory cytokines were not influenced by n-3 LCPUFA supplementation may be related to the dose and duration of supplementation and the fact that we did not measure markers of systemic inflammation (Kroesen *et al.*, 2018). Taken together, these findings showed that n-3 LCPUFA supplementation resulted in a more pro-resolving LM profile, irrespective of n-3 PUFA status prior to supplementation but that lung immune cell recruitment, as well as most pro-inflammatory LMs and cytokines, were not significantly altered. This supports the notion that n-3 LCPUFAs do not inhibit the host's natural immune and inflammatory responses necessary to protect against bacteria, indicating that n-3 LCPUFAs and their LMs are not immunosuppressive and do not stop inflammation, but rather elicit pro-resolving effects (Serhan *et al.*, 2017b).

On the other hand, lung interleukin (IL)-6 concentrations were higher in the mice conditioned to a low n-3 PUFA status that was supplemented with n-3 LCPUFAs, which is not explained by their LM profile. The reason why the group with a low n-3 PUFA status responded differently to TB infection and n-3 LCPUFA treatment, may be related, firstly, to the dosage and duration of supplementation, which may have been too low for the low status of this group. Secondly, epigenetic influences may have altered this group's response to infection and n-3 LCPUFA supplementation. Concerning recommending n-3 LCPUFA supplementation in an individual with an n-3 PUFA suboptimal status, more studies are required with longer supplementation periods and higher dosages in the same TB model. In order to translate these findings to humans, the n-3 PUFA intake and status of TB patients and its relationship to clinical and inflammatory outcomes also requires further investigation.

6.3.4 Additional findings and insights

The objective of this part of the thesis was to investigate the effects of n-3 LCPUFA supplementation, specifically. However, interestingly, our results also suggested that the group that was continued on an n-3 PUFA-deficient diet presented with better clinical and inflammatory outcomes compared with the n-3 PUFA-sufficient group. Although it seemed that the low n-3 PUFA status group had a lower bacterial load and less lung pathology, these differences were not significant. Bonilla *et al*. (2010b), also reported that *fat-1 transgenic* mice (endogenous abundance of n-3 PUFAs) had a higher susceptibility to TB when compared with n-3 PUFA-deficient mice (Bonilla *et al.*, 2010b). Regarding markers of inflammation, their LM profile differences were in congruence with the FA status of the groups. In contrast, higher concentrations of IL-1α, IL-1β, and IL-17 were detected in the lungs of the n-3 PUFA-sufficient

group. All of these cytokines have been implicated in driving lung pathology at high concentrations (Guler *et al.*, 2011; Kroesen *et al.*, 2018; Kumar *et al.*, 2019b; Romero-Adrian *et al.*, 2015; Singh *et al.*, 2018). The reason for these findings may be partly explained by the essential FA (EFA) composition of the diets. The n-3 PUFA-sufficient diet contained higher amounts of the n-6 PUFA linoleic acid (LA) when compared with the deficient diet. Free FAs and LMs, such as the pro-inflammatory hydroxyoctadecadienoic acids (HODEs) derived directly from LA, in the n-3 PUFA-sufficient group may have played a role in these results (Boilard, 2018; Frediani *et al.*, 2014; Innes & Calder, 2018). Still, the n-3 PUFA-sufficient diet also contained the n-3 LCPUFA precursor, alpha-linolenic acid (ALA), and has been described as containing the optimal n-3 PUFA content for DHA membrane saturation in rodents (Reeves *et al.*, 1993). This indicates that theoretically, the diet composition should not have caused a pro-inflammatory response. Nevertheless, the clinical relevance of these findings for humans is questionable. It would unrealistic to promote low n-3 PUFA consumption in TB infection as a protective measure because of other important biological functions that n-3 PUFA elicit. With this in mind, it cannot be accepted with certainty that a low n-3 PUFA status improves TB outcomes or that n-3 LCPUFA should not be supplemented under conditions of low n-3 PUFA status.

6.4 Objective 2 (Chapter 4):

To investigate the effects of n-3 LCPUFA and iron supplementation, alone or in combination, on n-3 LCPUFA status, markers of iron status, the immune and inflammatory response, and clinical outcomes in *Mtb*-infected mice

6.4.1 Omega-3 long-chain polyunsaturated fatty acid status

Again, as mentioned above, the FA composition of membranes was altered in RBCs, PBMCs and crude lung homogenates in both groups that received n-3 LCPUFA supplementation, alone or in combination with iron. It has previously been suggested that iron consumption favours n-3 LCPUFA membrane composition by affecting desaturase activities and/or incorporation into membranes (Del Bò *et al.*, 2009; Smuts *et al.*, 1995). However, we found that iron resulted in a higher n-6 LCPUFA composition of PBMC and lung tissue membranes. The reason for our differing results may be that previous studies were conducted in healthy humans and not in an infectious disease environment where the biosynthesis of n-6 LCPUFAs and their LMs may be enhanced.

6.4.2 Markers of anaemia of infection

Our findings indicated that n-3 LCPUFA, as well as iron supplementation, individually similarly lowered the markers of anaemia of infection and inflammation in TB infection. Soluble transferrin receptor (sTfR) levels were lowered in both n-3 LCPUFA or iron-supplemented groups, which was underpinned by lower systemic inflammation in both groups, together with the higher iron availability in the iron-supplemented group (Ganz, 2019; Hella *et al.*, 2018; Rohner *et al.*, 2017). This effect of inflammation on sTfR is attributed mainly to increased hepcidin synthesis, which causes the intracellular shift of iron and reduced gastrointestinal iron absorption (Agoro & Mura, 2019; Cole *et al.*, 1998; Ganz, 2018). The fact that we found that n-3 LCPUFAs and iron both individually lowered hepcidin is probably explained by the fact that hepcidin synthesis is stimulated by IL-6 and IL-1, which was lowered in both groups (Lee *et al.*, 2005; Nemeth *et al.*, 2004; Schmidt, 2015), together with the lower bacterial load in the n-3 LCPUFA-supplemented group (Armitage *et al.*, 2011; Millonig *et al.*, 2012; Schmidt, 2015). In TB, ferritin acts rather as an acute-phase protein and not a marker of stored iron (Mishra *et al.*, 2018; Truman-Rosentsvit *et al.*, 2018; Weiss *et al.*, 2019). The fact that iron supplementation lowered, and n-3 LCPUFAs tended to lower, ferritin concentrations, is also explained by the reduced systemic and lung inflammation in these groups. However, combination treatment of n-3 LCPUFAs and iron exerted antagonistic interactions and the lowering effect of each of these nutrients, individually, on markers of anaemia of infection was attenuated. Lastly, Hb concentrations remained more stable during the infection period, possibly owing to its important functions and the duration of the infection period (Anderson & Frazer, 2017). A limitation of this study was that we did not measure actual iron content in, for example, the blood or liver, nor the mean corpuscular volume and mean corpuscular haemoglobin, which may have been clinically valuable.

6.4.3 Markers of the immune response and inflammation

As mentioned above, the effects of n-3 LCPUFAs or iron on markers of anaemia of infection were mediated by changes in inflammation. It was found that n-3 LCPUFA supplementation resulted in a more pro-resolving LM profile, whilst iron supplementation did not affect LM production. Additionally, similar to what was found in previous research in our group, the pro-resolving LM profile of n-3 LCPUFAs was maintained when combined with iron supplementation (Malan *et al.*, 2016). Furthermore, n-3 LCPUFA supplementation lowered the pro-inflammatory plasma cytokines IL-1β, IL-6, and tumour necrosis factor-alpha (TNF-α), as well as lung IL-1α and IFN-γ, which corresponds with the membrane FA composition and LM profile of this group. The iron-supplemented group also had lower plasma IL-1β, IL-6 and

TNF-α and lung IL-1α and IL-1β concentrations than the control group. This is supported by previous research in animal TB models and TB-infected macrophages, where iron supplementation was found to down-regulate the expression of *IL-1α, IL-1β* and *TNF-α* (Agoro *et al.*, 2017; Kolloli *et al.*, 2019; Serafín-López *et al.*, 2004).

The lung immune cell phenotyping investigations of this part of this research project showed that dietary iron supplementation resulted in higher counts and percentages of T cells and phagocytic cells. This was probably related to the fact that iron has been shown to induce T cell proliferation (Ekiz *et al.*, 2005; Jabara *et al.*, 2016; Kuvibidila *et al.*, 1998) and that phagocytic cells are produced at infection sites to compete with the pathogens for iron as part of the host's protective measures (Ifeanyi, 2018). These findings are also supported by previous research in TB (Agoro *et al.*, 2017). When providing n-3 LCPUFA and iron treatment in combination, this effect of iron was attenuated, indicating that n-3 LCPUFAs may provide a protective effect against the exaggerated immune cell recruitment that was caused by iron and the resultant immune cell crowding that can lead to lung tissue damage (Kroesen *et al.*, 2018).

6.4.4 Tuberculosis related clinical outcomes

As *Mtb* relies on host iron stores, the administration of iron has previously been found to enhance *Mtb* growth (Kochan, 1973; Lounis *et al.*, 2001; Schaible *et al.*, 2002). However, contrary to expectations, iron supplementation did not affect bacterial load in our study. Others support these findings, which are probably related to the fact that *Mtb* is an intracellular bacterium able to obtain iron irrespective of the host's iron regulatory mechanisms and iron status, together with the importance of iron in enhancing bactericidal activity (Agoro *et al.*, 2017; Agoro & Mura, 2019; Cronjé *et al.*, 2005; Devi *et al.*, 2003; Harrington-Kandt *et al.*, 2018; Kolloli *et al.*, 2019). An "iron benefit window" has been suggested in which moderate iron supplementation may improve TB outcomes, whereas dosages above this threshold favour inflammation and bacterial growth (Agoro & Mura, 2019). This proposes that our iron dosage may have been safe.

Again, combined n-3 LCPUFA and iron supplementation attenuated the bactericidal effects of n-3 LCPUFAs and tended to lower free alveolar space. Furthermore, although not supported by previous research, iron lowered body weight gain in this study (Devi *et al.*, 2003; Lounis *et al.*, 2001). Taken together, clinically, n-3 LCPUFAs benefited bactericidal activity, whilst iron did not affect bacterial load but lowered body weight gain, and combination treatment attenuated the beneficial effects of n-3 LCPUFAs.

6.5 Objective 3 (Chapter 5)

To compare the effects of a diet sufficient in the n-3 EFA, ALA, to an n-3 LCPUFA-supplemented diet with regard to LCPUFA status, clinical outcomes, and markers of the inflammatory response in *Mtb*-infected mice with a low n-3 PUFA-status

6.5.1 Omega-3 long-chain polyunsaturated fatty acid status

Because of the financial implications of providing EPA and DHA in the public health context, we wanted to investigate whether correcting suboptimal n-3 PUFA status by providing a diet with a more acceptable n-6:n-3 PUFA ratio would be as beneficial as supplementing n-3 LCPUFAs. We hypothesised that the fact that rodents are higher converters of ALA to EPA in comparison with humans, that the mice were conditioned to a low n-3 PUFA status, and that this was an infectious disease model, would result in higher conversion rates of ALA to n-3 LCPUFAs, especially in immune cells (Anel *et al.*, 1990; Arterburn *et al.*, 2006; Brenna *et al.*, 2009; Calder *et al.*, 1994; Igarashi *et al.*, 2007; Lin & Salem, 2007). The n-3 EFA-sufficient diet in this study contained an adequate dietary ALA content to ensure optimal membrane saturation of DHA in healthy rodents and an n-6:n-3 PUFA ratio which was acceptable for rodents and lower than the deficient diet that the mice were conditioned on (7:1 compared with 130:1) (Reeves *et al.*, 1993). Nevertheless, the findings of this study showed that, following three weeks of the provision of either an n-3 EFA-sufficient diet or an EPA/DHA-supplemented diet, the mice in the EPA/DHA-supplemented group presented with higher EPA and DHA phospholipid RBC, PBMC, and lung homogenate membrane compositions. These findings are supported by previous research that also found that providing preformed EPA and DHA increased n-3 LCPUFA cell membrane composition (especially DHA) more effectively than providing ALA alone (Abedin *et al.*, 1999; Duda *et al.*, 2008; Riediger *et al.*, 2008).

6.5.2 Tuberculosis related clinical outcomes

Our findings showed that EPA/DHA supplementation resulted in a lower bacterial load than the n-3 EFA-sufficient diet did. This was most likely due to the higher n-3 LCPUFA composition of cell membranes that mediated improved bactericidal activity, as discussed above. The EPA/DHA-supplemented group also presented with a higher percentage of body weight gain throughout the infection and dietary intervention period. This was probably related to the lower bacterial load, together with a more inflammation resolving profile and the accompanying lower catabolism anticipated in this group (MacAllan *et al.*, 1998). Completing the better clinical picture found in the EPA/DHA-supplemented group, they also had a tendency towards lower lung pathology. This is underpinned by the lower levels of lung inflammation, bacterial load, and inflammatory LMs in this group (Kumar *et al.*, 2019a; Kumar *et al.*, 2019b).

6.5.3 Markers of lung inflammation

In this study, the group supplemented with EPA/DHA had higher lung concentrations of the EPA-derived HEPEs and DHA-derived 17-hydroxydocosahexaenoic acid (17-HDHA), which was reflective of the FA composition of cell membranes. Furthermore, the EPA/DHA group also had lower concentrations of the pro-inflammatory LMs 5-hydroxyeicosatetraenoic acids (HETE), 9-HETE, and $PGF_{2\alpha}$ (derived from AA). The LM profile of the EPA/DHA group, together with their lower bacterial burden, potentiated lower lung concentrations of IL-1α, IL-1β, and IL-6, which have previously been found to correlate with $PGF_{2\alpha}$ concentrations (Vedin *et al.*, 2010). These pro-inflammatory cytokines, although important in host response, can also worsen lung pathology and clinical outcomes (Borthwick, 2016; Domingo-Gonzalez *et al.*, 2016; Kumar *et al.*, 2019b; Mesquita *et al.*, 2016; Romero-Adrian *et al.*, 2015). Although ALA has also been found to exert independent inflammation-resolving and anti-inflammatory effects in rodents and in humans, the ALA dosage that we used was not therapeutic but rather aimed at providing an acceptable intake and n-6:n-3 PUFA ratio (Anand & Kaithwas, 2014; Baker *et al.*, 2016; Duda *et al.*, 2008; Jangale *et al.*, 2016; Kaveh *et al.*, 2019; Moura-Assis *et al.*, 2018; Schiessel *et al.*, 2016; Su *et al.*, 2018). Therefore, providing preformed EPA and DHA resulted in superior effects in lowering the exacerbated lung inflammation that is characteristic of TB.

6.5.4 Additional insights and clinical implications

Gibson *et al.* (2013) reported that providing dietary LA:ALA ratios of above 7:1 in rats considerably inhibited the incorporation of ALA into phospholipids, and additionally, suppressed n-3 LCPUFA conversion (Gibson *et al.*, 2013). The conversion of ALA to EPA and DHA is also determined by the absolute amount of ALA consumed (Gibson *et al.*, 2013; Sprecher *et al.*, 1995). We wanted to mimic the human situation of providing dietary education on an acceptable n-3 PUFA intake and n-6:n-3 PUFA intake ratio. Therefore, we provided a diet with an improved n-6:n-3 PUFA ratio and what is considered sufficient ALA intake in rodents in the n-3 EFA-sufficient group. However, in the n-3 EFA-sufficient diet, the background LA content may have been still too high, and lower ratios of n-6:n-3 PUFA may be required to obtain results similar to when supplementing with EPA and DHA. This is irrespective of the fact that we used a low n-3 PUFA status rodent model in an infectious disease environment. Therefore, the diet provided may not have been optimal to answer our research question. Future studies should be aimed at providing a diet with higher absolute ALA content to further lower the n-6:n-3 PUFA ratio of 7:1. As the financial implications of providing n-3 LCPUFAs are an important public health concern, further investigations are

warranted to optimise diets in order to provide the beneficial effects of n-3 LCPUFAs in TB, but in a way that is more affordable for sustainability purposes.

6.6 Limitations and recommendations

Overall, our uninfected reference group number was too small (because of breeding difficulties) to make worthwhile comparisons with infected groups. It is recommended that a larger group, equal to that of the infected intervention groups, be included in future experiments. We repeated this study in two experiments, but we could not pool the data due to differences in infection loads, which are inevitable in studies like these. Nevertheless, we obtained significant results, even with lower numbers than planned. Lastly, the design of the last part of this study (objective 3) should be repeated, but should compare EPA and DHA supplementation with a diet with higher ALA concentrations at n-6:n-3 PUFA ratios lower than 7:1.

With regards to analyses, we did not measure plasma cytokines in all the groups, neither did we measure plasma LMs in our study, which may have provided further insights into n-3 LCPUFA's systemic effects. Furthermore, in our study, IL-10 was below the detection limit of our methodology. It may have strengthened our findings if we could have included these results in our manuscripts. It is also recommended that future investigations add tissue, particularly liver and blood iron concentrations, together with mean cell volume and mean corpuscular haemoglobin analyses, as their inclusion could have provided insights into actual iron accretion in tissues and iron status. Lastly, outcomes were measured only at the endpoint of this study, after three weeks of dietary intervention. It would be valuable to add more euthanasia time points in future studies to investigate the effects of n-3 LCPUFAs in TB at different phases of the immune and inflammatory response.

6.7 General conclusion and clinical implications

The findings of this research indicated that n-3 LCPUFA supplementation after the acute inflammatory phase is beneficial with regards to enhancing bactericidal activity, benefiting lung and systemic inflammation resolution and lowering lung pathology in *Mtb*-infected mice. The benefits of n-3 LCPUFAs seemed to be dependent on n-3 PUFA status prior to supplementation, where mice with a low n-3 PUFA status did not benefit from the same dose of n-3 LCPUFAs as the sufficient mice. Additionally, n-3 LCPUFA therapy produced similar effects as iron to lower inflammation and improve markers of anaemia of infection. On the other hand, iron enhanced immune cell recruitment and restricted body weight gain, which makes n-3 LCPUFAs a more attractive option as host-directed therapy (HDT). Nevertheless,

it remains an important finding that this dose of iron did not worsen the bacterial burden in our study. Unexpectedly, combined n-3 LCPUFA and iron treatment seemed to worsen the clinical outcomes measured in our study. Lastly, from a public health perspective, n-3 LCPUFA supplementation may be regarded as expensive, whilst dietary adjustments to improve dietary n-6:n-3 PUFA ratios may be more acceptable. However, when comparing a diet with sufficient n-3 EFA content in an acceptable n-6:n-3 PUFA ratio, n-3 LCPUFA supplementation provided superior benefits with regard to bacterial killing, lung inflammation resolution, and bodyweight changes.

In conclusion, this study provides the first evidence that n-3 LCPUFA supplementation may be a promising approach as an HDT adjunct to standard TB treatment. Additionally, it emphasises that the timing of supplementation and n-3 PUFA status are critical considerations. We further showed that iron supplementation may mitigate anaemia of infection without worsening bacterial burden in TB. However, based on the findings of this research, combined n-3 LCPUFA and iron treatment cannot be recommended in TB owing to the harmful effects that we found on clinical outcomes.

6.8 Future prospects

- The next step should be to investigate the effects of n-3 LCPUFA supplementation combined with standard TB drug treatment;
- It is recommended that the dosage and duration of n-3 LCPUFA supplementation be adjusted to further investigate the effects of n-3 LCPUFAs in a low n-3 PUFA status environment;
- Further investigation regarding the n-3 PUFA intake, status and pro-resolving LM concentrations of TB patients and how these associate with clinical outcomes is required to determine the significance of a suboptimal n-3 PUFA status in human TB. From the literature review it was evident that research in this field has been focussed mainly on pro-inflammatory LMs and their association with TB outcomes; and
- Lastly, the statistical significance that we found in our results does not ascertain the biological relevance of our findings in humans. Therefore, it is recommended that this design be repeated in TB patients, starting with a safety study.

6.9 References

Abedin, L., Lien, E., Vingrys, A. & Sinclair, A. 1999. The effects of dietary α-linolenic acid compared with docosahexaenoic acid on brain, retina, liver, and heart in the guinea pig. *Lipids*, 34(5):475-482.

Agoro, R., Benmerzoug, S., Rose, S., Bouyer, M., Gozzelino, R., Garcia, I., Ryffel, B., Quesniaux, V.F. & Mura, C. 2017. An iron-rich diet decreases the mycobacterial burden and correlates with hepcidin upregulation, lower levels of proinflammatory mediators, and increased T-cell recruitment in a model of mycobacterium bovis Bacille Calmette-Guerin infection. *The Journal of infectious diseases*, 216(7):907-918.

Agoro, R. & Mura, C. 2019. Iron supplementation therapy: a friend and foe of mycobacterial infections? *Pharmaceuticals*, 12(2):75.

Allen, M.J., Fan, Y.Y., Monk, J.M., Hou, T.Y., Barhoumi, R., McMurray, D.N. & Chapkin, R.S. 2014. n–3 PUFAs reduce T-helper 17 cell differentiation by decreasing responsiveness to interleukin-6 in isolated mouse splenic CD4+ T cells. *The Journal of nutrition*, 144(8):1306-1313.

Anand, R. & Kaithwas, G. 2014. Anti-inflammatory potential of alpha-linolenic acid mediated through selective COX inhibition: computational and experimental data. *Inflammation*, 37(4):1297-1306.

Anderson, G.J. & Frazer, D.M. 2017. Current understanding of iron homeostasis. *The American journal of clinical nutrition*, 106(S6):1559S-1566S.

Anel, A., Naval, J., González, B., Uriel, J. & Piñeiro, A. 1990. Fatty acid metabolism in human lymphocytes. II. Activation of fatty acid desaturase-elongase systems during blastic transformation. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 1044(3):332-339.

Anes, E., Kühnel, M.P., Bos, E., Moniz-Pereira, J., Habermann, A. & Griffiths, G. 2003. Selected lipids activate phagosome actin assembly and maturation resulting in killing of pathogenic mycobacteria. *Nature cell biology*, 5(9):793-800.

Armitage, A.E., Eddowes, L.A., Gileadi, U., Cole, S., Spottiswoode, N., Selvakumar, T.A., Ho, L.-P., Townsend, A.R. & Drakesmith, H. 2011. Hepcidin regulation by innate immune and infectious stimuli. *Blood*, 118(15):4129-4139.

Arterburn, L.M., Hall, E.B. & Oken, H. 2006. Distribution, interconversion, and dose response of n−3 fatty acids in humans. *The American journal of clinical nutrition*, 83(6):1467S-1476S.

Baker, E.J., Miles, E.A., Burdge, G.C., Yaqoob, P. & Calder, P.C. 2016. Metabolism and functional effects of plant-derived omega-3 fatty acids in humans. *Progress in Lipid Research*, 64:30-56.

Bhattacharya, A., Sun, D., Rahman, M. & Fernandes, G. 2007. Different ratios of eicosapentaenoic and docosahexaenoic omega-3 fatty acids in commercial fish oils differentially alter pro-inflammatory cytokines in peritoneal macrophages from C57BL/6 female mice. *The Journal of nutritional biochemistry*, 18(1):23-30.

Boilard, E. 2018. Extracellular vesicles and their content in bioactive lipid mediators: more than a sack of microRNA. *Journal of lipid research*, 59(11):2037-2046.

Bonilla, D.L., Fan, Y.-Y., Chapkin, R.S. & McMurray, D.N. 2010b. Transgenic mice enriched in omega-3 fatty acids are more susceptible to pulmonary tuberculosis: impaired resistance to tuberculosis in fat-1 mice. *The Journal of infectious diseases*, 201(3):399-408.

Bonilla, D.L., Ly, L.H., Fan, Y.-Y., Chapkin, R.S. & McMurray, D.N. 2010a. Incorporation of a Dietary Omega-3 Fatty Acid Impairs Murine Macrophage Responses to Mycobacterium. *PloS one,* 5(5):e10878.

Borthwick, L. 2016. The IL-1 cytokine family and its role in inflammation and fibrosis in the lung. *Seminars in immunopathaolgy,* 38(4):517-534.

Brenna, J.T., Salem Jr, N., Sinclair, A.J. & Cunnane, S.C. 2009. α-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. *Prostaglandins, leukotrienes and essential fatty acids*, 80(2-3):85-91.

Calder, P.C. 2008. The relationship between the fatty acid composition of immune cells and their function. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 79(3-5):101-108.

Calder, P.C. 2014. Very long chain omega-3 (n-3) fatty acids and human health. *European journal of lipid science and technology*, 116(10):1280-1300.

Calder, P.C. 2016. Docosahexaenoic acid. *Annals of Nutrition and Metabolism*, 69(S1):8-21.

Calder, P.C., Bond, J.A., Harvey, D.J., Gordon, S. & Newsholme, E.A. 1990. Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochemical Journal*, 269(3):807-814.

Calder, P.C. & Newsholme, E.A. 1992. Polyunsaturated fatty acids suppress human peripheral blood lymphocyte proliferation and interleukin-2 production. *Clinical science*, 82(6):695-700.

Calder, P.C., Yaqoob, P., Harvey, D.J., Watts, A. & Newsholme, E.A. 1994. Incorporation of fatty acids by concanavalin A-stimulated lymphocytes and the effect on fatty acid composition and membrane fluidity. *Biochemical Journal*, 300(2):509-518.

Chiang, N., Fredman, G., Bäckhed, F., Oh, S.F., Vickery, T., Schmidt, B.A. & Serhan, C.N. 2012. Infection regulates pro-resolving mediators that lower antibiotic requirements. *Nature*, 484(7395):524.

Codagnone, M., Cianci, E., Lamolinara, A., Mari, V., Nespoli, A., Isopi, E., Mattoscio, D., Arita, M., Bragonzi, A. & Iezzi, M. 2018. Resolvin D1 enhances the resolution of lung inflammation caused by long-term Pseudomonas aeruginosa infection. *Mucosal immunology*, 11(1):35-45.

Cole, S., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S., Eiglmeier, K., Gas, S. & Barry Iii, C. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature*, 393(6685):537.

Cronjé, L., Edmondson, N., Eisenach, K.D. & Bornman, L. 2005. Iron and iron chelating agents modulate mycobacterium tuberculosis growth and monocyte-macrophage viability and effector functions. *FEMS Immunology & Medical Microbiology*, 45(2):103-112.

Del Bò, C., Contino, D., Riso, P., Simonetti, P. & Ciappellano, S. 2009. Effect of horse meat consumption on iron status, lipid profile and fatty acid composition of red blood cell membrane: preliminary study. (*In* Societa Italiana di Radiologia Medica, Milan, Italy, organised by: SINU. p. 1-1).

Devi, U., Rao, C.M., Srivastava, V.K., Rath, P.K. & Das, B.S. 2003. Effect of iron supplementation on mild to moderate anaemia in pulmonary tuberculosis. *British Journal of Nutrition*, 90(3):541-550.

Domingo-Gonzalez, R., Prince, O., Cooper, A. & Khader, S.A. 2016. Cytokines and Chemokines in mycobacterium tuberculosis infection. *Microbiology spectrum*, 4(5):10.1128/microbiolspec.TBTB1122-0018-2016.

Duda, M.K., O'Shea, K.M., Tintinu, A., Xu, W., Khairallah, R.J., Barrows, B.R., Chess, D.J., Azimzadeh, A.M., Harris, W.S. & Sharov, V.G. 2008. Fish oil, but not flaxseed oil, decreases inflammation and prevents pressure overload-induced cardiac dysfunction. *Cardiovascular research*, 81(2):319-327.

Ekiz, C., Agaoglu, L., Karakas, Z., Gurel, N. & Yalcin, I. 2005. The effect of iron deficiency anemia on the function of the immune system. *The Hematology Journal*, 5(7):579-583.

Fielding, B.A., Calder, P.C., Irvine, N.A., Miles, E.A., Lillycrop, K.A., von Gerichten, J. & Burdge, G.C. 2019. How does polyunsaturated fatty acid biosynthesis regulate T-lymphocyte function? *Nutrition Bulletin*, DOI: 10.1111/nbu.12404

Frediani, J.K., Jones, D.P., Tukvadze, N., Uppal, K., Sanikidze, E., Kipiani, M., Tran, V.T., Hebbar, G., Walker, D.I. & Kempker, R.R. 2014. Plasma metabolomics in human pulmonary tuberculosis disease: a pilot study. *PloS one*, 9(10):e108854.

Ganz, T. 2018. Iron and infection. *International journal of hematology*, 107(1):7-15.

Ganz, T. 2019. Anemia of inflammation. *New England Journal of Medicine*, 381(12):1148-1157.

Gibson, R.A., Neumann, M.A., Lien, E.L., Boyd, K.A. & Tu, W.C. 2013. Docosahexaenoic acid synthesis from alpha-linolenic acid is inhibited by diets high in polyunsaturated fatty acids. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 88(1):139-146.

Guler, R., Parihar, S.P., Spohn, G., Johansen, P., Brombacher, F. & Bachmann, M.F. 2011. Blocking IL-1α but not IL-1β increases susceptibility to chronic Mycobacterium tuberculosis infection in mice. *Vaccine*, 29(6):1339-1346.

Harrington-Kandt, R., Stylianou, E., Eddowes, L.A., Lim, P.J., Stockdale, L., Pinpathomrat, N., Bull, N., Pasricha, J., Ulaszewska, M. & Beglov, Y. 2018. Hepcidin deficiency and iron deficiency do not alter tuberculosis susceptibility in a murine M. tb infection model. *PloS one*, 13(1):e0191038.

Hella, J., Cercamondi, C.I., Mhimbira, F., Sasamalo, M., Stoffel, N., Zwahlen, M., Bodmer, T., Gagneux, S., Reither, K. & Zimmermann, M.B. 2018. Anemia in tuberculosis cases and household controls from Tanzania: contribution of disease, coinfections, and the role of hepcidin. *PloS one*, 13(4):e0195985.

Ifeanyi, O.E. 2018. A review on iron homeostasis and anaemia in pulmonary tuberculosis. *International Journal of Healthcare and Medical Sciences*, 4(5):84-89.

Igarashi, M., DeMar, J.C., Ma, K., Chang, L., Bell, J.M. & Rapoport, S.I. 2007. Upregulated liver conversion of α-linolenic acid to docosahexaenoic acid in rats on a 15 week n-3 PUFA-deficient diet. *Journal of lipid research*, 48(1):152-164.

Innes, J.K. & Calder, P.C. 2018. Omega-6 fatty acids and inflammation. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 132:41-48.

Ivanyi, J. & Zumla, A. 2013. Nonsteroidal antiinflammatory drugs for adjunctive tuberculosis treatment. *Journal infectious diseases*, 208(2):185-188.

Jabara, H.H., Boyden, S.E., Chou, J., Ramesh, N., Massaad, M.J., Benson, H., Bainter, W., Fraulino, D., Rahimov, F. & Sieff, C. 2016. A missense mutation in TFRC, encoding transferrin receptor 1, causes combined immunodeficiency. *Nature genetics*, 48(1):74.

Jangale, N.M., Devarshi, P.P., Bansode, S.B., Kulkarni, M.J. & Harsulkar, A.M. 2016. Dietary flaxseed oil and fish oil ameliorates renal oxidative stress, protein glycation, and inflammation in streptozotocin–nicotinamide-induced diabetic rats. *Journal of physiology and biochemistry*, 72(2):327-336.

Jordao, L., Lengeling, A., Bordat, Y., Boudou, F., Gicquel, B., Neyrolles, O., Becker, P.D., Guzman, C.A., Griffiths, G. & Anes, E. 2008. Effects of omega-3 and-6 fatty acids on Mycobacterium tuberculosis in macrophages and in mice. *Microbes and Infection*, 10(12):1379-1386.

Kaveh, M., Eftekhar, N. & Boskabady, M.H. 2019. The effect of alpha linolenic acid on tracheal responsiveness, lung inflammation, and immune markers in sensitized rats. *Iranian journal of basic medical sciences*, 22(3):255.

Kim, W., Khan, N.A., McMurray, D.N., Prior, I.A., Wang, N. & Chapkin, R.S. 2010. Regulatory activity of polyunsaturated fatty acids in T-cell signaling. *Progress in lipid research*, 49(3):250-261.

Kochan, I. 1973. The role of iron in bacterial infections, with special consideration of host-tubercle bacillus interaction. *Current topics in microbiology and immunology*, 60:1-30.

Kolloli, A., Singh, P., Rodriguez, G.M. & Subbian, S. 2019. Effect of iron supplementation on the outcome of non-progressive pulmonary mycobacterium tuberculosis infection. *Journal of clinical medicine*, 8(8):1155.

Kroesen, V.M., Rodríguez-Martínez, P., García, E., Rosales, Y., Díaz, J., Martín-Céspedes, M., Tapia, G., Sarrias, M.-R., Cardona, P.-J. & Vilaplana, C. 2018. A beneficial effect of low-dose aspirin in a Murine model of active tuberculosis. *Frontiers in immunology*, 9:798.

Kumar, N.P., Kadar Moideen, A.N., Viswanathan, V., Shruthi, B.S., Shanmugam, S., Hissar, S., Kornfeld, H. & Babu, S. 2019a. Plasma eicosanoid levels in tuberculosis and tuberculosis-diabetes co-morbidity are associated with lung pathology and bacterial burden. *Frontiers in Cellular and Infection Microbiology*, 9.

Kumar, N.P., Moideen, K., Banurekha, V.V., Nair, D. & Babu, S. 2019b. Plasma pro-inflammatory cytokines are markers of disease severity and bacterial burden in pulmonary tuberculosis. *Open forum infectious diseases*, DOI:10.1093/ofid/ofz257.

Kuvibidila, S.R., Baliga, B.S., Warrier, R.P. & Suskind, R.M. 1998. Iron deficiency reduces the hydrolysis of cell membrane phosphatidyl inositol-4, 5-bisphosphate during splenic lymphocyte activation in C57BL/6 mice. *The Journal of nutrition*, 128(7):1077-1083.

Lee, P., Peng, H., Gelbart, T., Wang, L. & Beutler, E. 2005. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. *Proceedings of the National Academy of Sciences*, 102(6):1906-1910.

Lin, Y.H. & Salem, N. 2007. Whole body distribution of deuterated linoleic and α-linolenic acids and their metabolites in the rat. *Journal of Lipid Research*, 48(12):2709-2724.

Lounis, N., Truffot-Pernot, C., Grosset, J., Gordeuk, V.R. & Boelaert, J.R. 2001. Iron and Mycobacterium tuberculosis infection. *Journal of Clinical Virology*, 20(3):123-126.

MacAllan, D.C., McNurlan, M.A., Kurpad, A.V., De Souza, G., Shetty, P.S., Calder, A.G. & Griffin, G.E. 1998. Whole body protein metabolism in human pulmonary tuberculosis and undernutrition: evidence for anabolic block in tuberculosis. *Clinical science*, 94(3):321-331.

Malan, L., Baumgartner, J., Zandberg, L., Calder, P. & Smuts, C. 2016. Iron and a mixture of dha and epa 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. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)*, 105:15-25.

Marzo, E., Vilaplana, C., Tapia, G., Diaz, J., Garcia, V. & Cardona, P.-J. 2014. Damaging role of neutrophilic infiltration in a mouse model of progressive tuberculosis. *Tuberculosis*, 94(1):55-64.

Mayatepek, E., Paul, K., Leichsenring, M., Pfisterer, M., Wagner, D., Domann, M., Bremer, H. & Sonntag, H. 1994. Influence of dietary (n-3)-polyunsaturated fatty acids on leukotriene B 4 and prostaglandin E 2 synthesis and course of experimental tuberculosis in guinea pigs. *Infection*, 22(2):106-112.

Mayer-Barber, K.D., Andrade, B.B., Oland, S.D., Amaral, E.P., Barber, D.L., Gonzales, J., Derrick, S.C., Shi, R., Kumar, N.P. & Wei, W. 2014. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature*, 511(7507):99.

McFarland, C.T., Fan, Y.-Y., Chapkin, R.S., Weeks, B.R. & McMurray, D.N. 2008. Dietary polyunsaturated fatty acids modulate resistance to Mycobacterium tuberculosis in guinea pigs. *The Journal of nutrition*, 138(11):2123-2128.

Mesquita, E.D., Gil-Santana, L., Ramalho, D., Tonomura, E., Silva, E.C., Oliveira, M.M., Andrade, B.B. & Kritski, A. 2016. Associations between systemic inflammation, mycobacterial loads in sputum and radiological improvement after treatment initiation in pulmonary TB patients from Brazil: a prospective cohort study. *BMC infectious diseases*, 16(1):368.

Millonig, G., Ganzleben, I., Peccerella, T., Casanovas, G., Brodziak-Jarosz, L., Breitkopf-Heinlein, K., Dick, T.P., Seitz, H.-K., Muckenthaler, M.U. & Mueller, S. 2012. Sustained submicromolar H2O2 levels induce hepcidin via signal transducer and activator of transcription 3 (STAT3). *Journal of Biological Chemistry*, 287(44):37472-37482.

Mishra, S., Taparia, M.P., Yadav, D. & Koolwal, S. 2018. Study of Iron Metabolism in Pulmonary Tuberculosis Patients. *International journal of health sciences and research,* 8(3):70-77.

Moura-Assis, A., Afonso, M.S., de Oliveira, V., Morari, J., dos Santos, G.A., Koike, M., Lottenberg, A.M., Catharino, R.R., Velloso, L.A. & da Silva, A.S.R. 2018. Flaxseed oil rich in omega-3 protects aorta against inflammation and endoplasmic reticulum stress partially mediated by GPR120 receptor in obese, diabetic and dyslipidemic mice models. *The Journal of nutritional biochemistry*, 53:9-19.

Nemeth, E., Rivera, S., Gabayan, V., Keller, C., Taudorf, S., Pedersen, B.K. & Ganz, T. 2004. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *The Journal of clinical investigation*, 113(9):1271-1276.

Oh, S.F., Pillai, P.S., Recchiuti, A., Yang, R. & Serhan, C.N. 2011. Pro-resolving actions and stereoselective biosynthesis of 18S E-series resolvins in human leukocytes and murine inflammation. *The Journal of clinical investigation*, 121(2):569-581.

Paul, K.P., Leichsenring, M., Pfisterer, M., Mayatepek, E., Wagner, D., Domann, M., Sonntag, H.G. & Bremer, H.J. 1997. Influence of n-6 and n-3 polyunsaturated fatty acids on the resistance to experimental tuberculosis. *Metabolism*, 46(6):619-624.

Reeves, P.G., Nielsen, F.H. & Fahey Jr, G.C. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet: Oxford University Press.

Riediger, N.D., Othman, R., Fitz, E., Pierce, G.N., Suh, M. & Moghadasian, M.H. 2008. Low n-6: n-3 fatty acid ratio, with fish-or flaxseed oil, in a high fat diet improves plasma lipids and beneficially alters tissue fatty acid composition in mice. *European journal of nutrition*, 47(3):153-160.

Rohner, F., Namaste, S.M., Larson, L.M., Addo, O.Y., Mei, Z., Suchdev, P.S., Williams, A.M., Sakr Ashour, F.A., Rawat, R. & Raiten, D.J. 2017. Adjusting soluble transferrin receptor concentrations for inflammation: Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project. *The American journal of clinical nutrition*, 106(suppl_1):372S-382S.

Romero-Adrian, T.B., Leal-Montiel, J., Fernández, G. & Valecillo, A. 2015. Role of cytokines and other factors involved in the Mycobacterium tuberculosis infection. *World Journal of Immunology*, 5(1):16-50.

Schaible, U.E., Collins, H.L., Priem, F. & Kaufmann, S.H. 2002. Correction of the iron overload defect in β-2-microglobulin knockout mice by lactoferrin abolishes their increased susceptibility to tuberculosis. *Journal of Experimental Medicine*, 196(11):1507-1513.

Schiessel, D.L., Yamazaki, R.K., Kryczyk, M., Coelho de Castro, I., Yamaguchi, A.A., Pequito, D.C., Brito, G.A., Borghetti, G., Aikawa, J. & Nunes, E.A. 2016. Does oil rich in alpha-linolenic fatty acid cause the same immune modulation as fish oil in walker 256 tumor-bearing rats? *Nutrition and cancer*, 68(8):1369-1380.

Schmidt, P.J. 2015. Regulation of iron metabolism by hepcidin under conditions of inflammation. *Journal of Biological Chemistry*, 290(31):18975-18983.

Serafín-López, J., Chacón-Salinas, R., Muñoz-Cruz, S., Enciso-Moreno, J., Estrada-Parra, S.A. & Estrada-Garcia, I. 2004. The effect of iron on the expression of cytokines in macrophages infected with Mycobacterium tuberculosis. *Scandinavian journal of immunology*, 60(4):329-337.

Serhan, C.N., Chiang, N. & Dalli, J. 2017b. New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration. *Molecular aspects of medicine*.

Singh, S., Maniakis-Grivas, G., Singh, U.K., Asher, R.M., Mauri, F., Elkington, P.T. & Friedland, J.S. 2018. Interleukin-17 regulates matrix metalloproteinase activity in human pulmonary tuberculosis. *The Journal of pathology*, 244(3):311-322.

Smuts, C., Tichelaar, H., Van Jaarsveld, P., Badenhorst, C., Kruger, M., Laubscher, R., Mansvelt, E. & Benade, A. 1995. The effect of iron fortification on the fatty acid composition of plasma and erythrocyte membranes in primary school children with and without iron deficiency. *Prostaglandins, leukotrienes and essential fatty acids*, 52(1):59-67.

Sprecher, H., Luthria, D.L., Mohammed, B. & Baykousheva, S.P. 1995. Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *Journal of lipid research*, 36(12):2471-2477.

Stulnig, T.M. & Zeyda, M. 2004. Immunomodulation by polyunsaturated fatty acids: impact on T-cell signaling. *Lipids*, 39(12):1171-1175.

Su, H., Liu, R., Chang, M., Huang, J., Jin, Q. & Wang, X. 2018. Effect of dietary alpha-linolenic acid on blood inflammatory markers: a systematic review and meta-analysis of randomized controlled trials. *European journal of nutrition*, 57(3):877-891.

Truman-Rosentsvit, M., Berenbaum, D., Spektor, L., Cohen, L.A., Belizowsky-Moshe, S., Lifshitz, L., Ma, J., Li, W., Kesselman, E. & Abutbul-Ionita, I. 2018. Ferritin is secreted via 2 distinct nonclassical vesicular pathways. *Blood*, 131(3):342-352.

Vedin, I., Cederholm, T., Freund-Levi, Y., Basun, H., Hjorth, E., Irving, G.F., Eriksdotter-Jönhagen, M., Schultzberg, M., Wahlund, L.-O. & Palmblad, J. 2010. Reduced prostaglandin F2α release from blood mononuclear leukocytes after oral supplementation of ω3 fatty acids: the OmegAD study. *Journal of lipid research*, 51(5):1179-1185.

Vilaplana, C., Marzo, E., Tapia, G., Diaz, J., Garcia, V. & Cardona, P.-J. 2013. Ibuprofen therapy resulted in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. *J Infect Dis*, 208.

Weiss, G., Ganz, T. & Goodnough, L.T. 2019. Anemia of inflammation. *Blood, The Journal of the American Society of Hematology*, 133(1):40-50.

Yaqoob, P., Pala, H., Cortina-Borja, M., Newsholme, E. & Calder, P. 2000. Encapsulated fish oil enriched in alpha-tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. *European journal of clinical investigation*, 30(3):260-274.

ANNEXURE A ETHICS APPROVAL CERTIFICATE ANIMCARE NORTH-WEST UNIVERSITY

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

Private Bag X6001, Potchefstroom,
South Africa, 2520

Tel: (018) 299-4900
Faks: (018) 299-4910
Web: http://www.nwu.ac.za

Institutional Research Ethics Regulatory Committee
Tel: +27 18 299 4849
Email : Ethics@nwu.ac.za

2016/09/12

ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by **AnimCare Animal Research Ethics Committee (AREC-130913-015)** after being reviewed at the meeting held on **21/06/2016**, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby **approves** your study as indicated below. This implies that the NWU-IRERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: The immune modulatory effects of omega-3 polyunsaturated fatty acids and iron as applied in an animal pulmonary tuberculosis model.

Study Leader/Supervisor: **Dr Linda Malan**
Student: **Ms Arista Nienaber**

Ethics number:

N	W	U	-	0	0	2	6	0	-	1	6	-	A	5
Institution				Study Number						Year			Status	

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

Application Type: New Application - Standard Project

Commencement date: 2016-09-12 **Category:** 4

Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years.

Special conditions of the approval (if applicable):

- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the AnimCare. Ethics approval is required BEFORE approval can be obtained from these authorities.

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 study leader (principle investigator) must report in the prescribed format to the NWU-IRERC via AnimCare:*
  - *annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study.*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *Annually a number of studies may be randomly selected for an external audit.*
- *The approval applies strictly to the proposal as stipulated in the application form. Would any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the AnimCare, prior to implementation. Would there be deviated from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility the NWU-IRERC and AnimCare retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the AnimCare or that information has been false or misrepresented,*
    - *the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *AnimCare can be contacted for further information or any report templates via Ethics-AnimCare@nwu.ac.za or 018 299 2197.*

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

Yours sincerely

Prof LA Du Plessis

Digitally signed by Prof LA Du Plessis
Date: 2016.09.13 09:12:01 +02'00'

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

ANNEXURE B ETHICS APPROVAL CERTIFICATE ANIMAL ETHICS COMMITTEE UNIVERSITY OF CAPE TOWN

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Animal Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: sidney.engelbrecht@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/animalethics/forms

20 August 2019

A/Prof R Guler
Division of Immunology
IDM and ICGEB
Faculty of Health Sciences

Dear A/Prof Guler

PROTOCOL TITLE: Identification of host and pathogen-directed drug targets against M. tuberculosis infection in mice

FHS AEC REF NO: 019_023

Thank you for submitting your protocol to the Faculty of Health Sciences (FHS) Animal Ethics Committee (AEC) for review.

I am pleased to inform you that the FHS AEC has **approved** your protocol, which will terminate on **31 August 2022.**

Number of animals & species: **2083 / Mouse**

Please quote the FHS AEC REF NO (above) in all future correspondence.

Please note that the approval of this protocol imposes the following obligations on the principal investigator (PI):

1. To submit an annual mandatory progress report. The first annual report for this protocol is due on **28 February 2020.** The forms can be accessed from http://www.health.uct.ac.za/fhs/research/animalethics/forms
2. To submit a final mandatory report on the **31 August 2022**, please access the final report form from: http://www.health.uct.ac.za/fhs/research/animalethics/forms
3. Ensuring that all study participants perform within the confines of the procedures and experimental design of the protocol as approved, or as amended.
4. Ensuring that all study participants comply with all applicable national legislation, UCT policies, FHS AEC policies and standard operating procedures (SOPs) and national standards (SANS 10386: 2008).

5. Ensuring that you as the PI immediately alert the FHS AEC to any event involving the welfare of the animals which has occurred during the course of the study, as well as the actions that were taken to respond to these events.
6. Ensuring that you as the PI alert the FHS AEC to any new or unexpected ethical issues that arose during the course of the study, and how these issues were addressed.
7. Ensuring that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals or will be performing the procedures under the direct and continuous supervision of SAVC-registered veterinary professionals or SAVC-registered para-veterinary professionals.
8. If the PI or any study participant is in any way uncertain how to respond to any of these obligations or deal with any of the issues referred to above, they must consult with FHS AEC.
9. All animals found dead must be reported to the RAF on the appropriate form: http://www.health.uct.ac.za/fhs/research/animalethics/forms
10. All animals found in distress must be reported to the RAF on the appropriate form.

My best wishes for a successful research and /or teaching endeavour.

Yours sincerely

PROF PJ COMMERFORD
CHAIR, FHS AEC

AEC REF# 019_027

ANNEXURE C MUCOSAL IMMUNOLGY AUTHORS GUIDELINES

Mucosal Immunology Guide for Authors

ABOUT THE JOURNAL

Aims and Scope

Mucosal Immunology is the official publication of the Society of Mucosal Immunology (SMI). It aims to provide a forum for both basic and clinical scientists to discuss all aspects of immunity and inflammation involving mucosal tissues. The journal reflects the interests of scientists studying gastrointestinal, pulmonary, nasopharyngeal, oral, ocular, and genitourinary immunology through the publication of original research articles, scholarly reviews, and timely commentaries, editorials and letters. Publication of basic, translational, and clinical studies will all be given equal consideration.

In addition, *Mucosal Immunology* aims to provide a primary method of communication for the SMI governing board and its members through the publication of society news, announcements of planned meetings and conferences, discussions of policy concerns, and advertisements for job and training opportunities.

Journal Details

Send an e-mail to the journal

Submission of manuscripts

Please visit the online submission site to submit a manuscript to the journal.

Images Illustrative images which are unique or highly illustrative of specific occurrences in mucosal immunology. They will be reviewed by the Editorial Board prior to acceptance.	No Abstract required	Yes	Should be accompanied by a brief one-paragraph description of max 200 words of relevant clinical or basic scientific information.
News Timely briefings and analysis on a wide range of topics important to the field of Immunology.	No Abstract required	Only by invitation of Editors.	1,000 words excluding References, Tables and Figures; References: 10 max; Figures/Tables: 1 max

KEY CONSIDERATIONS AND PREPARATION OF ARTICLES

Key factors for publication of original research

Mucosal Immunology receives many more submissions than it can publish each month. Therefore, submitted manuscripts are critically evaluated according to the following criteria:

- *Data reproducibility.* Original data must include adequate evidence of reproducibility. An accurate description of each set of data that is shown must be provided and must include the number of biological replicates, the number of experiments performed, and the description and use of appropriate statistical methods. The editors will not accept representative single experiments without the author's written agreement to make available all of the replicate data upon request. Manuscripts submitted without evidence of reproducibility will be rejected without formal review.
- *Novelty.* Original findings should represent a major advance in the respective field. Data providing only an incremental advance or extension of prior work will be given low priority.
- *Scope.* Only manuscripts that include a substantive body of work will be considered. In most cases this will include a primary observation together with data providing mechanistic insight. Purely descriptive studies will be given low priority.
- *In vivo validation.* Higher priority for publication will be given to findings that are validated in well-established animal models and/or by studies of relevant immune processes in humans or patient cohorts. Purely in vitro experiments will be given low priority and may be immediately rejected without formal review.
- *Human studies.* Manuscripts providing novel insights into human disease processes will be given high priority. These include studies of basic immunological and/or disease mechanisms, as well as functional studies of gene mutations or polymorphisms. Manuscripts providing primary results of clinical trials are currently not within the scope of the journal.
- *Vaccine studies.* Manuscripts evaluating novel vaccine candidates will be considered. However, acceptable studies will need to include evidence of in vivo protection in humans or a relevant animal model of infection, direct experimental evidence providing a valid comparison with existing vaccines, and/or insights into basic immunological mechanisms.
- *Invertebrate immunology.* Studies that provide novel insight into human or vertebrate immunology and disease will be considered.
- *Genetic studies.* Studies of gene expression patterns or identification of disease-associated polymorphisms or mutations without experiments exploring the biological relevance of the findings will be given low priority.

General manuscript preparation

Manuscripts must be typed in English and double-spaced. All manuscript pages must be numbered.

In terms of file formats, please use a common word-processing package (such as Microsoft Word*) for the text. Tables should be provided at the end of the Word document. PDFs are not acceptable formats for any manuscript files.

*Microsoft Office 2007 saves files in an XML format by default (file extensions .docx, .pptx and xlsx). Files saved in this format cannot be accepted for publication. **Save Word documents using the file extension .doc**

- Select the Office Button in the upper left corner of the Word 2007 Window and choose "Save As"
- Select "Word 97-2003 Document"
- Enter a file name and select "Save"

These instructions also apply for the new versions of Excel and PowerPoint. **Equations in Word must be created using Equation Editor 3.0.** Equations created using the new equation editor in Word 2007 and saved as a "Word 97-2003 Document" (.doc) are converted to graphics and can no longer be edited. To insert or change an equation with the previous equation editor:

- Select "Object" on the "Text" section of the "Insert" tab
- In the drop-down menu - select "Equation Editor 3.0"

Do not use the "Equation" button in the "Symbols" section of the "Insert" tab.

Title page

This should include (a) the complete manuscript title; (b) all authors' names (listed as first and middle initials followed by last name), and affiliations; (c) the name and address for correspondence, fax number, telephone number, and e-mail address; and (d) any conflict of interest that could be perceived to bias the work, making known all financial support and any other personal connections.

Text

For contributions requiring Abstracts, the lengths are defined in "Article Type Specifications." For contributions that do not require an Abstract, introductory paragraphs may contain references to cited work. Manuscripts should nominally be organized under following main headings: Introduction, Results, Discussion, Methods, Acknowledgment, Author Contributions (as of May 2016), Disclosure, and References.

Abbreviations

Abbreviations should be defined at the first mention in the text and in each table and figure and must be in accordance with accepted international guidelines for mucosal immunology terms. For required guidelines, please see this article. Common mucosal immunology abbreviations are:

BAL	bronchoalveolar lavage
BALT	bronchus-associated lymphoid tissue
CLN	cervical lymph node
FAE	follicle-associated epithelium
GALT	gut-associated lymphoid tissue
ILF	isolated lymphoid follicle
J chain	joining chain
LP	lamina propria
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MALT	mucosa-associated lymphoid tissue
MLN	mesenteric lymph node
MMP	mucous membrane pemphigoid
NALT	nose- [or nasopharynx]-associated lymphoid tissue (query author if unclear which expansion to use)
pIgA	polymeric IgA
pIgR	polymeric Ig receptor
PP	Peyer's patch
SC	secretory component
SIgA	secretory IgA
SIgM	secretory IgM

For further instructions and an extended list of standard abbreviations, please consult the Council of Science Editors Style Guide (available from the Council of Science Editors, 9650 Rockville Pike, Bethesda, MD 20814) or other standard sources. Write out the full term for each abbreviation at its first use unless it is a standard unit of measure.

In terms of file formats, please use a common word-processing package (such as Microsoft Word*) for the text. Tables should be provided at the end of the Word document. PDFs are not acceptable formats for any manuscript files.

*Microsoft Office 2007 saves files in an XML format by default (file extensions .docx, .pptx and xlsx). Files saved in this format cannot be accepted for publication. **Save Word documents using the file extension .doc**

- Select the Office Button in the upper left corner of the Word 2007 Window and choose "Save As"
- Select "Word 97-2003 Document"
- Enter a file name and select "Save"

These instructions also apply for the new versions of Excel and PowerPoint. **Equations in Word must be created using Equation Editor 3.0.** Equations created using the new equation editor in Word 2007 and saved as a "Word 97-2003 Document" (.doc) are converted to graphics and can no longer be edited. To insert or change an equation with the previous equation editor:

- Select "Object" on the "Text" section of the "Insert" tab
- In the drop-down menu - select "Equation Editor 3.0"

Do not use the "Equation" button in the "Symbols" section of the "Insert" tab.

Title page

This should include (a) the complete manuscript title; (b) all authors' names (listed as first and middle initials followed by last name), and affiliations; (c) the name and address for correspondence, fax number, telephone number, and e-mail address; and (d) any conflict of interest that could be perceived to bias the work, making known all financial support and any other personal connections.

Text

For contributions requiring Abstracts, the lengths are defined in "Article Type Specifications." For contributions that do not require an Abstract, introductory paragraphs may contain references to cited work. Manuscripts should nominally be organized under following main headings: Introduction, Results, Discussion, Methods, Acknowledgment, Author Contributions (as of May 2016), Disclosure, and References.

Abbreviations

Abbreviations should be defined at the first mention in the text and in each table and figure and must be in accordance with accepted international guidelines for mucosal immunology terms. For required guidelines, please see this article. Common mucosal immunology abbreviations are:

BAL	bronchoalveolar lavage
BALT	bronchus-associated lymphoid tissue
CLN	cervical lymph node
FAE	follicle-associated epithelium
GALT	gut-associated lymphoid tissue
ILF	isolated lymphoid follicle
J chain	joining chain
LP	lamina propria
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MALT	mucosa-associated lymphoid tissue
MLN	mesenteric lymph node
MMP	mucous membrane pemphigoid
NALT	nose- [or nasopharynx]-associated lymphoid tissue (query author if unclear which expansion to use)
pIgA	polymeric IgA
pIgR	polymeric Ig receptor
PP	Peyer's patch
SC	secretory component
SIgA	secretory IgA
SIgM	secretory IgM

For further instructions and an extended list of standard abbreviations, please consult the Council of Science Editors Style Guide (available from the Council of Science Editors, 9650 Rockville Pike, Bethesda, MD 20814) or other standard sources. Write out the full term for each abbreviation at its first use unless it is a standard unit of measure.

Style

The American Medical Association Manual of Style (9th edition), Stedman's Medical Dictionary (28th edition) and Merriam Webster's Collegiate Dictionary (11th edition) should be used as standard references. Refer to drugs and therapeutic agents by their accepted generic or chemical name, and do not abbreviate them (a proprietary name may be given only with the first use of the generic name). Code names should be used only when a generic name is not yet available (the chemical name and a figure giving the chemical structure of the drug are required). Copyright or trade names of drugs should be capitalized and placed in parentheses after the name of the drug. Names and locations (city and state in United States; city and country outside United States) of manufacturers of drugs, supplies, or equipment cited in a manuscript are required to comply with trademark law and should be provided in parentheses. Quantitative data may be reported in the units used in the original measurement, but SI units are strongly preferred, including those applicable to body weight, mass (weight) and temperature.

Commas, not spaces, should be used to separate thousands.

Acknowledgments

This should include sources of support, including federal and industry support.

Author Contributions

Mucosal Immunology authors are required to include an "Author Contributions" section which follows the Acknowledgements section of the manuscript. This section should specify the contributions of every author either explicitly or by the definition of authorship to which the journal adheres (see "Authorship" section).

Examples:

"PML conceived of the study. BT, WK, EY, CC, ZS, JT, and JK initiated the study design and CH and ES helped with implementation. JT and WK provided statistical expertise in clinical trial design and JT is conducting the primary statistical analysis. All authors contributed to refinement of the study protocol and approved the final manuscript."

"BA, JK, MS and VR provided substantial contributions to the conception of the work. All authors substantially contributed to the acquisition, analysis or interpretation of data for the manuscript and drafting, revising and critically reviewing the manuscript for important intellectual content. All authors approved the final version of this manuscript to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. *Mucosal Immunology* also allows one set of up to six coauthors to be specified as having contributed equally to the work or having jointly supervised the work. Other equal contributions are best described in author contributions statements."

Disclosure/ Conflict of Interest

All authors are responsible for recognizing and disclosing any conflict of interest that could be perceived to bias their work, making known all financial support and any other personal connections. This includes, but is not limited to: funding, such as salaries, equipment, supplies, reimbursement for attending symposia, etc, from organizations that may gain or lose financially through the publication of the paper; personal financial interests, such as stocks and shares in companies that may gain or lose financially from publication, consultation fees or forms of remuneration from organizations that may gain or lose financially, or patent and patent applications whose value may be affected; and, employment, whether recent, present or anticipated, by an organization that may gain or lose from publication of the paper. Such relationships will be reviewed, and further clarification may be requested if deemed necessary by the editors or reviewers. This information should be included in the title page and in the article under the heading "Disclosure." This section must appear after the "Methods" section (below Supplementary Material, Acknowledgments, and Author Contributions). If you have no conflict of interest to declare, please state so in the "Disclosure" section.

References

The *Mucosal Immunology* reference style can be obtained from Endnote. In the text, cite references in order of appearance (Vancouver style) using superscript numbers. The reference list should start on a separate page. Only published works and manuscripts in press should be included in the reference list; articles that are submitted or in preparation should be referred to as "unpublished data" in the text (for which all authors up to six total should be listed, then "et al."). For publications in the reference list, all authors should be included unless there are more than five, in which case only the first author should be given, followed by "et al." Authors should be listed last name first, followed by a comma and initials of given names. Titles of articles should be in Roman text and titles of books or journals in italics. Journal names are italicized and abbreviated (with periods after each abbreviated word) according to common usage; refer to *Index Medicus* (PubMed) for details. Volume numbers appear in bold. For book citations, the publisher and city of publication are required; include the country (and state for US) for lesser-known cities or where any ambiguity is possible (e.g., John Wiley & Sons, Hoboken, NJ, USA, 2003; MIT Press, Cambridge, MA, USA).

The following examples demonstrate correct reference style:

Journal articles

Mentink-Kane, M. M. & Wynn, T. A. Opposing roles for IL-13 and IL-13 receptor alpha 2 in health and disease. *Immunol. Rev.* **202**, 191–202 (2004).

Kashuba, A.D. *et al.* Effect of fluvoxamine therapy on the activities of CYP1A2, CYP2D6, and CYP3A as determined by phenotyping. *Clin. Pharmacol. Ther.* **64**, 257-268 (1998).

Books

Eisen, H.N. *Immunology: An Introduction to Molecular and Cellular Principles of the Immune Response* 5th edn. (Harper & Row, New York, 1974).

Chapters in books

Weinstein, L. & Schwartz, M.N. Pathogenic properties of invading microorganisms. In *Pathologic Physiology: Mechanisms of Disease* (Sodeman, W.A. Jr. & Sodeman, W.A., eds) 457-473 (W.B. Saunders, Philadelphia, 1974).

Figures

Figures should be labeled sequentially, numbered and cited in the text. If a table, figure or any other previously published material is included, the authors must obtain written permission to reproduce the material in both print and electronic formats from the copyright owner. The original source should be cited. Legends should be brief and specific, and should appear on a separate manuscript page after the Reference section. Figure legends must include the number of biological and technical replicates, and the number of experiments that have been performed for the studies shown. Statistical notation in the figures must be explained in the figure legends, and the statistical methods used indicated in the figure legends or the methods section.

Detailed guidelines for submitting artwork can be found by downloading the artwork guidelines PDF. **Using the guidelines, please submit production quality artwork with your initial online submission. Please also see here for our policy on image manipulation.**

Minimum Resolutions

- Halftone images 300 dpi (dots per inch)
- Color images 300 dpi saved as CMYK
- Images containing text 400 dpi
- Line art 1000 dpi

Sizes

- Figure Width - Single Image
  - 86 mm (Should be able to fit into a single column of the printed journal)
- Figure Width - Multi-part Image
  - 178 mm (Should be able to fit into a double column of the printed journal)
- Text Size
  - 8 point (Should be readable after reduction - avoid large type or thick lines)
- Line Width between 0.5 and 1 point

Color

Upon accept, authors are asked to return an "Artwork and Supplementary Information Production Form," on which authors can indicate where color is necessary in the manuscript. This refers to color in the PDF/print hard copy versions, for which there is a cost (See "Costs"). SMI members receive a discount on these charges, but if they do not return the "Artwork..." form, the non-member rates apply.

There are three published formats of articles:

- **Full-text HTML:** The online HTML version. Color figures in the full-text HTML published paper incur no charges.
- **PDF:** An online version, visually identical to the print version. The PDF is a representation of how your paper will appear in print. Any figures that appear in color in the PDF will also appear in color in print, and thus incur a charge.
- **Print:** The printed version of an article in the hardcopy issue. As above, color figures that appear in the PDF and print are at cost.

Black and white figures in all versions (full-text HTML, PDF, and print) are free of charge. Note: Figures must be able to be clearly interpreted in black and white; otherwise, authors must choose color in the PDF/print version.

Tables

Each table should be double-spaced on a separate sheet and numbered consecutively in the order of first citation in the text. Minimize empty space. Supply a brief title for each, but place explanatory matter in the footnotes (not in the heading). Do not use internal horizontal and vertical lines. Please do not upload images of tables. All tabular content must be editable. An image of a table, such as a scan, is not acceptable for publication.

Supplementary material

Supplementary material is material directly relevant to the conclusion of an article that cannot be included in the printed version owing to space or format constraints. It is posted on the journal's web site and linked to the article when the article is published and may include data files, graphics, movies or extensive tables. The printed article must be complete and self-explanatory without the supplementary information. Supplementary material must be supplied to the editorial office in its final form for peer review. On acceptance, the final version of the peer-reviewed supplementary material should be submitted with the accepted paper. To ensure that the contents of the supplementary material files can be viewed by the editor(s), referees and readers, please also submit a 'read-me' file containing brief instructions on how to use the file.

Supplementary file charges

There is a charge per supplementary information file, outlined in the "Costs" section below.

Supplementary material files

Authors should ensure that supplementary material is supplied in its FINAL format because it is not copy edited and will appear online exactly as originally submitted. It cannot be altered, nor new supplementary material added, after the paper has been accepted for publication. Please supply the supplementary material through the electronic manuscript submission and tracking system, in an acceptable file format (see below). Authors should include a text summary (no more than 50 words) to describe the contents of each file; identify the types of files (file formats) submitted and include the text "Supplementary Material is linked to the online version of the paper at http://www.nature.com/mi" at the end of the body of text (before Acknowledgments and Disclosure).

Accepted file formats

Quick Time files (.mov), graphical image files (.gif), HTML files (.html), MPEG movie files (.mpg), JPEG image files (.jpg), sound files (.wav), plain ASCII text (.txt), Acrobat files (.pdf), MS Word documents (.doc), Postscript files (.ps), MS Excel spreadsheet documents (.xls) and PowerPoint files (.ppt). We cannot accept TeX and LaTeX. File sizes must be as small as possible, so that they can be downloaded quickly. Images should not exceed 640 x 480 pixels but we would recommend 480 x 360 pixels as the maximum frame size for movies. We would also recommend a frame rate of 15 frames per second. If applicable to the presentation of the supplementary material, use a 256-color palette. Please consider the use of lower specification for all of these points if the supplementary material can still be represented clearly. Our recommended maximum data rate is 150 KB/s

The number of files should be limited to eight, and the total file size should not exceed 8 MB. Individual files should not exceed 1 MB. Please seek advice from the editorial office before sending files larger than our maximum size to avoid delays in publication. PDFs are not acceptable formats for any manuscript files. Further questions about the submission or preparation of supplementary material should be directed to the editorial office.

Please note: We do not allow the resupplying of Supplementary Information files for style reasons after a paper has been exported in production, unless there is a serious error that affects the science and, if by not replacing, it would lead to a formal correction once the paper has been published. In these cases, we would make an exception and replace the file; however, there are very few instances where a Supplementary Information file would be corrected post publication.

English Language Support

For editors and reviewers to accurately assess the work presented in your manuscript you need to ensure the English language is of sufficient quality to be understood. If you need help with writing in English, you should consider:

- Asking a colleague who is a native English speaker to review your manuscript for clarity.
- Visiting the English language tutorial which covers the common mistakes when writing in English.
- Using a professional language editing service where editors will improve the English to ensure that your meaning is clear and identify problems that require your review. Two such services are provided by our affiliates Nature Research Editing Service and American Journal Experts.

English language editing services

- Nature Research Editing Service
- American Journal Experts
- Inter-Biotech
- SPI Professional Editing Services
- Write Science Right

Please note that the use of a language editing service is not a requirement for publication in this journal and does not imply or guarantee that the article will be selected for peer review or accepted.

If your manuscript is accepted, it will be checked by our copyeditors for spelling and formal style before publication.

POST-ACCEPTANCE

License to Publish form

The corresponding author must complete and sign the License to Publish form upon submission of the manuscript. Failure to complete and sign this form will result in delays to the publication of your paper.

With regards to the form, the Society for Mucosal Immunology does not require authors of original research papers to assign copyright of their published contributions. Authors grant the Society for Mucosal Immunology an exclusive license to publish, in return for which they can re-use their papers in their future printed work.

Government employees from the United States or Canada should sign a government employee License to Publish form. For a copy of this form, should your article be accepted, please contact the journal production editor.

Standard publication

Manuscripts published under the standard method of publication will be behind a paywall, requiring readers to pay to view the article, either via their institutional or personal subscription or on a pay-per-view basis.

Open access publication

Upon acceptance, authors can indicate whether they wish to make their article open access online immediately upon publication, an option which will incur a fee or "article processing charge" (APC). Editors and reviewers will be blind to your choice of this option, ensuring an impartial decision on your paper. Open access articles are published under Creative Commons licenses, which allow authors to retain copyright to their work while making it open to readers.

To facilitate self-archiving NPG deposits open access articles in PubMed Central (PMC) and Europe PMC on publication. Authors are also permitted to post the final, published PDF of their article on a website, institutional repository or other free public server, immediately on publication.

Visit our open research site for further information about:

- Creative Commons licensing
- Creative Commons license options and APC prices for *Mucosal Immunology*
- APC payment FAQs
- Help in identifying funding for APCs
- Editorial process for OA publication in hybrid journals
- Self-archiving and deposition of papers published OA

If authors opt to publish via the open access route, then the corresponding author must complete and sign the APC payment form and an open access LTP form on behalf of all authors, and return these to the editorial office. These forms will be provided upon acceptance of the article. Failure to promptly return forms will result in delay of publication.

Government employees from the United States, UK, and Canada are required to sign and submit the relevant government open access licence to publish form. To receive a copy of this, please contact the Editorial Office.

Please note with regards to payment that usual credit terms are 30 days from receipt of invoice. Failure to pay your invoice within the stated credit term may result in the Open Access status of the paper being rescinded, with the paper being placed behind the paywall. You may also be subject to such penalties as restrictions on your ability to publish with NPG in the future, involvement of a third party debt collection agency, and legal proceedings.

Please note that editors and reviewers will be blind to your choice to ensure an impartial decision on your paper.

Self-archiving and Manuscript Deposition (Green Open Access)

Authors of original research articles are encouraged to submit the author's version of the accepted paper (the unedited manuscript) to a repository (eg, PMC or Europe PMC) for public release six months after publication. NPG also offers a free, opt-in Manuscript Deposition Service for original research articles in order to help authors fulfill funder and institutional mandates.

Learn more about self-archiving and manuscript deposition.

Proofs

The Springer Nature e-proofing system is a unique solution that will enable authors to remotely edit /correct your article proofs.

The corresponding author will receive an e-mail containing a URL linking to the e-proofing site. Proof corrections must be returned within 48 hours of receipt. Failure to do so may result in delayed publication. Extensive corrections cannot be made at this stage.

For more information and instructions on how to use the e-proofing too please see here.

Advance Online Publication

All original articles, reviews, and letters to the editor are published ahead of print on Advance Online Publication. This is the final version of the manuscript that will subsequently appear, unchanged, in print.

COSTS

Open Access Publication (Gold Open Access)

Upon acceptance, authors can indicate whether they wish to make their article open access online immediately upon publication, an option which will incur a fee or "article processing charge" (APC). For information about the Creative Commons license options and APC prices for Mucosal Immunology, visit this site.

Page charges

Manuscripts accepted for publication in *Mucosal Immunology* will incur page charges to cover, in part, the cost of publication. The charge per journal page is $46 for members of the Society for Mucosal Immunology and $77 per page for nonmembers. To take advantage of this discount, sign up to become a society member here. If an author is already a member, authors need to indicate such on the "Offprint and Page Charge Authorization Form," delivered with proofs. Failure to return this form or indicate as such in the document will entail levying of nonmember rates. *

Supplementary file charges

There is a charge of $130 per supplementary information file. Authors are strongly encouraged to consolidate their files into a single file in order to avoid multiple supplemental charges. *

Color charges

Authors will be expected to contribute towards the cost of publication of color figures Upon accept, authors are asked to return an "Artwork and Supplementary Information Production Form," on which authors can indicate where color is necessary in the manuscript. This refers to color in the PDF/print hard copy versions, for which there is a cost. Charges are $618 per page for members of the Society for Mucosal Immunology and $721 for nonmembers. If members do not return the "Artwork..." form, the non-member rates apply. *

Offprints

Offprints may be ordered on the form accompanying the proofs. The charges are necessarily higher if orders for offprints are received after the issue has gone to press.

*Effective for manuscripts with a final accept date of February 9th or later, no additional page, supplementary information, or color charges apply in addition to the APC.

EDITORIAL POLICIES

Review policies

Mucosal Immunology is committed to maintaining high standards for the integrity of the published scientific record. Authors should take note and adhere to the journal editorial policies noted below. The journal will investigate any instances of suspected scientific fraud, image manipulation, plagiarism, duplicate publication, undocumented sources of funding or conflicts of interest, and other cases that compromise research ethics or the journal's scientific integrity. Depending on the investigation, the journal may opt to publish corrections, or in serious cases of scientific misconduct, request that the authors retract their paper or impose a retraction on the paper.

Review process

Manuscripts are initially evaluated by the senior editors (Editor-in-chief, and/or Deputy Editors). Those deemed to fall within the scope of the journal, to address important scientific questions, to present reproducible experiments with statistical analysis, and to provide significant advances over the current literature are then assigned to an Associate Editor with expertise in the subject area of the study. The Associate Editor then makes an independent decision as to whether the manuscript should be sent out for peer review, based on the above criteria. This initial evaluation should take no longer than 7 business days, and authors should be aware that their manuscripts might be returned without detailed reviews. The initial decision to reject a manuscript without review is made only for papers judged most unlikely to obtain favorable outside reviews and overall is meant to save the time of both our reviewers and the authors, who can prepare for submission elsewhere.

For manuscripts deemed appropriate for further evaluation, the Associate Editor then solicits reviews from 2-3 reviewers, which are normally returned within 2 weeks. The reviews are evaluated by the Associate Editor who decides on the appropriateness of the review and whether, because of inconsistency between the reviewers whether additional reviews are required. We make all attempts to secure appropriate reviews from investigators who are both expert in their respective fields of study and have proven to be unbiased and timely in their prior reviews for the journal. The Associate Editor returns a recommended decision to one of the senior editors, who reviews the decision process and makes a final decision on the manuscript. On average, an author should expect a first decision within 7 business days of submission if it is not sent for detailed review, and within 30 business days for fully reviewed manuscripts. A decision on some manuscripts will take more time, primarily because of difficulty in securing appropriate reviewers.

Manuscripts resubmitted for major revisions will be immediately sent for re-review to the original reviewers, and a recommendation for acceptance, rejection, or further revisions will be made by the Associate Editor after obtaining the comments of the reviewers, with the senior editors making the final decision. It is rare for manuscripts to returned for a second round of major revisions. Manuscripts returned for only minor revisions may or may

not be sent back to the original reviewers for review. The time for re-review is generally much shorter than the initial review, and authors can expect a decision within 2 weeks of resubmission, and often much sooner.

Anonymity

Reviewers' identities are not released to authors, except when reviewers specifically ask to be identified. If reviewers wish to reveal their identities while the manuscript is under consideration, this should be done through the Editor. Should a reviewer contact an author directly, we ask authors to inform the Editor as soon as possible. We prohibit any attempt by authors to confront reviewers or determine their identities. Our own policy is to neither confirm nor deny any speculation about reviewers' identities, and we encourage reviewers to adopt a similar policy.

Clinical trials and informed consent

Registration in a public trials registry is required for publication in *Mucosal Immunology*. A clinical trial is defined as any research project that prospectively assigns human subjects to intervention or comparison groups to study the cause-and-effect relationship between a medical intervention and a health outcome. Studies designed for other purposes, including exploring pharmacokinetics or safety and tolerability (e.g., phase 1 trials) are exempt.

Registration must be with a registry that meets the following criteria: (1) accessible to the public at no charge; (2) searchable by electronic methods; (3) open to all prospective registrants free of charge or at minimal cost; (4) validates registered information; (5) identifies trials with a unique number; and (6) includes information on the investigator(s), research question or hypothesis, methodology, intervention and comparisons, eligibility criteria, primary and secondary outcomes measured, date of registration, anticipated or actual start date, anticipated or actual date of last follow-up, target number of subjects, status (anticipated, ongoing or closed) and funding source(s).

Examples of registries that meet these criteria include (1) The registry sponsored by the United States National Library of Medicine (www.clinicaltrials.gov); (2) The International Standard Randomized Controlled Trial Number Registry (http://www.controlled-trials.com); (3) The Cochrane Renal Group Registry (http://www.cochrane-renal.org/trialsubmissionform.php); (4) The National (United Kingdom) Research Register (http://www.update-software.com/national/); and (5) European Clinical Trials Database (http://eudract.emea.eu.int/).

When reporting experiments on human subjects, it must be indicated whether the procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional or regional) or with the Helsinki Declaration of 1975 (as revised in 1983). Include Institutional Review Board or Animal Care and Use Committee approvals.

NPG endorses the toolkits and guidelines produced by the following bodies:

- Committee on Publication Ethics: http://publicationethics.org/
- Good Publication Practice: http://www.gpp-guidelines.org/
- Medical Publishing Insights and Practices Initiative: http://www.mpip-initiative.org/

Authorship

Requirements for all categories of articles largely conform to the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals," developed by the ICMJE. A manuscript will be considered for publication with the understanding that:

1. all named authors have agreed to its submission
2. it is not currently being considered for publication by another journal
3. if the paper is accepted, it will not subsequently be published in the same or similar form in any language without the consent of publisher

Each author must have contributed sufficiently to the intellectual content of the submission. The corresponding author should list all authors and their contributions to the work. Any changes to the author list after submission, such as a change in the order of the authors, or the deletion or addition of authors, must be approved by a signed letter from every author. The corresponding author must confirm that he or she has had full access to the data in the study and final responsibility for the decision to submit for publication. To qualify as a contributing author, one must meet all of the following four criteria:

1. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; and
2. Drafting the work or revising it critically for important intellectual content; and
3. Final approval of the version to published; and
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Contributions by individuals who made direct contributions to the work but do not meet all of the above criteria should be noted in the Acknowledgments section.

Mucosal Immunology also requires the inclusion of an Author Contribution section. The details of this section can be found in Author Contributions in this guide.

Medical writers and industry employees can be authors or contributors. Concurrent with their contribution to the work submitted, their roles, affiliations, and potential conflicts of interest should be included in the author list and/or noted in the Acknowledgments section. Failure to acknowledge these contributors is considered inappropriate, which conflicts with *Mucosal Immunology*'s editorial policy.

Plagiarism and fabrication

Plagiarism is when an author attempts to pass off someone else's work as his or her own. Duplicate publication, sometimes called self-plagiarism, occurs when an author reuses substantial parts of his or her own published work without providing the appropriate references. Plagiarism without dishonest intent is relatively frequent, for example, when an author reuses parts of an introduction from an earlier paper.

If plagiarism is suspected, as a member journal of the Committee on Publication Ethics (COPE), *Mucosal Immunology* will follow best industry practice to investigate the integrity of the research in question. Based on the outcome of this investigation, the journal may opt to publish a correction, or in serious cases of scientific misconduct, impose a retraction on the paper.

Duplicate publication

Papers must be original and not previously published or submitted for publication elsewhere. This rule also applies to non-English language publications. Although the editors, editorial board, and referees make every effort to ensure the validity of published manuscripts, the final responsibility rests with the authors.

NPG allows and encourages prior publication on recognized community preprint servers for review by other scientists before formal submission to a journal. The details of the preprint server concerned and any accession numbers should be included in the cover letter accompanying manuscript submission. This policy does not extend to preprints available to the media or that are otherwise publicized outside the scientific community before or during the submission and consideration process.

Image manipulation

Images submitted with a manuscript for review should be minimally processed. Authors should retain their unprocessed data and metafiles, as Editors may require them to aid in manuscript evaluation. If unprocessed data is available, manuscript evaluation may be stalled until the issue is resolved.

A certain degree of image processing is acceptable for publication (and for some experiments, fields and techniques is unavoidable), but the final image must correctly represent the original data and conform to community standards. The guidelines below will aid in accurate data presentation at the image processing level.

- Authors should list all image acquisition tools and image processing software packages used. Authors should document key image-gathering settings and processing manipulations in the Materials and Methods section.
- Images gathered at different times or from different locations should not be combined into a single image, unless it is stated that the resultant image is a product of time-averaged data or a time-lapse sequence. If juxtaposing images is essential, the borders should be clearly demarcated in the figure and described in the legend.
- Touch-up tools, such as cloning and healing tools in Photoshop, or any feature that deliberately obscures manipulations are to be avoided.
- Processing (such as changing brightness and contrast) is appropriate only when it is applied equally across the entire image and is applied equally to controls. Contrast should not be adjusted so that data disappears. Excessive manipulations, such as processing to emphasize one region in the image at the expense of others (for example, through the use of a biased choice of threshold settings), is inappropriate, as is emphasizing experimental data relative to the control.

Positive and negative controls, as well as molecular size markers, should be included on each gel and blot - either in the main figure or an expanded data supplementary figure. The display of cropped gels and blots in the main paper is encouraged if it improves the clarity and conciseness of the presentation. In such cases, the cropping must be mentioned in the figure legend.

- Vertically sliced gels that juxtapose lanes that were not contiguous in the experiment must have a clear separation or a black line delineating the boundary between the gels.
- Cropped gels in the paper must retain important bands.
- Cropped blots in the body of the paper should retain at least six band widths above and below the band.
- High-contrast gels and blots are discouraged, as overexposure may mask additional bands. Authors should strive for exposures with gray backgrounds. Immunoblots should be surrounded by a black line to indicate the borders of the blot, if the background is faint.
- For quantitative comparisons, appropriate reagents, controls and imaging methods with linear signal ranges should be used.

Microscopy adjustments should be applied to the entire image. Threshold manipulation, expansion or contraction of signal ranges and the altering of high signals should be avoided. If pseudo-coloring and nonlinear adjustment (for example, "gamma changes") are used, this must be disclosed. Adjustments of individual color channels are

sometimes necessary on merged images, but this should be noted in the figure legend.

Corrections and retraction policy

We recognize our responsibility to correct errors. Content published online (as Advance Online Publication) or in a print issue is final and cannot be amended without formal notice. The online and print versions are both part of the published record therefore the original version must be preserved and changes to the paper should be made as a formal correction. If an error is noticed in an AOP article, a correction should accompany the article when it publishes in print. An HTML (or full-text) version of the correction will also be created and linked to the original article. If the error is found in an article after print publication, the correction will be published online and in the next available print issue.

Please note the following policy for making corrections to print and online versions of peer-reviewed content:

- **Erratum.** Notification of an important error made by the journal or publisher that affects the publication record or the scientific integrity of the paper or the reputation of the authors, or of the journal.
- **Corrigendum.** Notification of an important error made by the author that affects the publication record or the scientific integrity of the paper or the reputation of the authors or the journal.
- **Retraction.** Notification of invalid results. All coauthors must sign a retraction specifying the error and stating briefly how the conclusions are affected.

Decisions about corrections are made by the Editor (sometimes with advice of peer reviewers) and this sometimes involves author consultation. Requests to make corrections that do not affect the paper in a significant way or impair the reader's understanding of the contribution (a spelling mistake or grammatical error, for example) are not considered.

Research Data Policy

We strongly encourage that all datasets on which the conclusions of the paper rely should be available to readers. We encourage authors to ensure that their datasets are either deposited in publicly available repositories (where available and appropriate) or presented in the main manuscript or additional supporting files whenever possible. Where one does not exist, the information must be made available to referees at submission and to readers promptly upon request. Any restrictions on material availability or other relevant information must be disclosed in the manuscript's Methods section and should include details of how materials and information may be obtained. Please see the journal's guidelines on Research Data policy here.

Communication

Communication with the media
Material submitted must not be discussed with the media. We reserve the right to halt the consideration or publication of a paper if this condition is broken. If a paper is particularly newsworthy, NPG may send a press release to our list of journalists in advance of publication with an embargo that forbids any coverage of the manuscript, or the findings of the manuscript, until the time and date clearly stated, which will coincide with when the paper is publishing online. Authors whose papers are scheduled for publication may also arrange their own publicity (for instance, through their institution's press offices), but they must strictly adhere to the online publication press embargo and are advised to coordinate their own publicity with NPG's press office.

Communication between scientists
Mucosal Immunology does not wish to hinder communication between scientists. You are free to communicate with other researchers as much as you wish, whether on a recognized community preprint server by discussion at scientific meetings or by online collaborative sites such as wikis, but we do not encourage premature publication by discussion with the press (beyond a formal presentation, if at a conference).

FURTHER INFORMATION

For inquiries related to submission requirements, please contact the editorial office. For inquiries related to advertising, subscriptions, permissions, papers in production or publishing a supplement, please contact the publisher's office.

ANNEXURE D THE JOURNAL OF NUTRITION AUTHORS GUIDELINES

Instructions to Authors

General Information

The Journal of Nutrition (*JN/J Nutr*) publishes nutrition research that addresses human, animal, cellular, and molecular nutrition. In particular, *JN* draws attention to research findings that identify the nutritional problems and possible solutions for various populations. *JN* advances our understanding of nutritional mechanisms and the complex interactions of nutrition with genetics, physiology, health, and behavior.

In their commitment to furthering nutrition science, the *JN* editors, reviewers, and staff have provided a consistently fast peer review and efficient publication process. Average time to first decision is 1 month, and most manuscripts are accepted for publication after thorough and expert peer review in about 3 months. Manuscripts undergo formatting and copyediting and are posted online as articles in about 1 month after acceptance. Final versions are published in print approximately 1 month later and are optimized for search engine recognition, indexed in multiple databases, and archived in LOCKSS. The most recent Google Scholar metric placed *JN* 2nd in the field of nutrition, behind only *The American Journal of Clinical Nutrition* (*AJCN*), another American Society for Nutrition (ASN) journal. Although *JN* represents only 1 of 79 journals in the ISI's field of Nutrition and Dietetics, authors of *JN* manuscripts garnered over 9% of all citations in the field in 2013, earning *JN* an Impact Factor of over 4.2. Between 60% and 70% of online visits from the researchers, clinicians, educators, public health officials, and others who visit *JN* for current developments in experimental nutrition come from outside of the United States.

Statement of Scope

JN publishes peer-reviewed original research papers covering all aspects of experimental nutrition in humans and other animal species; special articles such as reviews and biographies of prominent nutrition scientists; and issues, opinions, and commentaries on controversial issues in nutrition. Supplements are frequently published to provide extended discussion of topics of special interest.

Teresa A. Davis, Ph.D. is Editor-in-Chief of *JN*. With the assistance and recommendation of Associate Editors (selected for their expertise in specific nutrition disciplines), the Editor-in-Chief accepts or rejects submitted manuscripts. Submitted manuscripts must comply with *JN*'s Instructions to Authors.

The premier journal in its field, *JN* has been in circulation since 1928. *JN* is an official monthly publication of the American Society for Nutrition published by Oxford University Press. Membership in ASN includes a subscription to the online version of *JN*. *JN* publishes original research reported by authors in over 50 countries and is distributed worldwide to more than 1000 institutions, in addition to personal subscribers and ASN members. To provide expert evaluation of the various segments of the broad spectrum of nutrition research, the editorial office is assisted by scientists who serve as Associate Editors, editorial board members, and ad hoc reviewers chosen for their nutritional science expertise. They provide constructive evaluation and fair and rapid editorial processing. The frequency of citations to articles published in *JN* by scientists, clinicians, and others increases each year.

JN focuses on the review of manuscripts that address human, animal, cellular, and molecular nutrition. *JN* is especially interested in publishing manuscripts that advance the understanding of nutritional mechanisms in human populations, in animals, in relevant model organisms, in appropriate primary cells or cell culture models, and/or at the molecular level. Manuscripts addressing interactions of nutrition with genetics, physiological regulation, health outcomes, and behavioral outcomes are generally well suited for review by *JN*. Because the pages that can be published annually are limited, suitability is an important consideration in the review process. To facilitate review, the Editor-in-Chief previews all submitted manuscripts and independently or in consultation with an Associate Editor, decides if a manuscript is appropriate for review by members of *JN*'s editorial board and/or ad hoc reviewers. Manuscripts outside of the scope of *JN* are returned without the delay of a full review, generally within a week of submission. Authors may contact the Editor-in-Chief in advance to inquire about the potential suitability of their research topic for review.

Manuscript submissions and inquiries are encouraged. Manuscript style and formatting are described in the "Instructions to Authors" posted online. Manuscript submissions should be made using *JN* online manuscript submission system. Direct inquiries to: jnsubmit@nutrition.org.

Types of Manuscripts

Research articles

Reports of studies of interest to the community of nutritional scientists will be published as research articles. Manuscripts should have a well-defined hypothesis and report on reproducible, quantifiable, and previously unconfirmed outcomes. Manuscripts related to nutrition in any

species may be submitted. Substances studied should have clear nutritional value at doses that are not considered pharmacological and through normal process of nutrient assimilation.

The Subject Headings are:

- Biochemical, Molecular, and Genetic Mechanisms in Nutrition
- Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions
- Nutrition and Disease
- Nutrient Requirements and Optimal Nutrition
- Genomics, Proteomics, and Metabolomics
- Nutritional Epidemiology
- Community and International Nutrition
- Nutritional Immunology
- Ingestive Behavior and Nutritional Neuroscience
- Nutritional Methodologies and Mathematical Modeling

Reviews

Most reviews published in *JN* will be part of a series entitled "Recent Advances in Nutritional Sciences (RANS).” These reviews will be published in a five-page (1) format and provide a recent rather than historical review of the subject matter. These reviews are published to provide current information relative to the wide range of research topics of interest to the readers of *JN* and to serve as a useful resource for instructors of advanced nutrition courses. Authors are encouraged to cover the general background and history of the research area in a limited amount of space and to update the reader by citing good previously published comprehensive reviews. The majority of the text should deal with recent (last few years) information.

More comprehensive reviews and commentaries are published as "Critical Reviews." Critical Reviews are meant to address and discuss published literature. They are not meant for presentation of new systematic reviews or an extensive analysis or reanalysis of data. *JN* will review suitable systematic reviews as regular research papers. Critical Reviews may be invited by the Editor-in-Chief or initiated by authors. For author-initiated reviews, it is strongly advised that an outline of the proposed review be submitted to the Editor-in-Chief prior to writing so that suitability for *JN* and the scope of the review can be determined before submission. *JN* does not publish comprehensive or categorical reviews of the literature, only the focused reviews as described above.

Issues and Opinions

Short essays presenting scientific analyses and viewpoints on issues in nutrition may be submitted to *JN*. These articles are generally limited to four printed pages (1). Additional Supplementary data may be included, if essential.

Commentaries

Commentaries relative to concurrently published papers containing noteworthy findings will be published at the invitation of the Editor-in-Chief. The Commentary section may also be used to present ASN-related articles on nutritional science and education.

Biographies and historical perspectives

Biographical manuscripts are invited by the Biographical and Historical Editor after consultation with the ASN History Committee, but suggestions of subjects and authors are welcomed. Authors of potential articles on some historical aspect of nutritional science should consult with the Biographical and Historical Editor prior to submission of a manuscript, concerning its appropriateness for *JN*. Biographical sketches, including the References section, should be 2–4 published pages (1) in length with the number of cited publications limited to no more than 10. A complete bibliography of the writings of the subject, if available, can be published as Supplementary data (see page 11 for instructions). A photograph of the subject should be included.

Letters to the Editor

Letters to the Editor may be submitted. Letters are expected to provide substantive comments on papers published in *JN* in the 6 months prior to the time a letter is submitted, or other subjects that are of broad interest to the nutrition research community. *JN* does not permit unpublished data to be presented in letters. The letter and a reply, if appropriate, are published together whenever possible. Letters, including up to 10 references, are limited to one *JN* page (1) and will be reviewed prior to acceptance.

Errata/Corrigenda

When necessary to correct an error in a published manuscript, the corresponding author may submit an erratum or corrigendum for publication in *JN*. Oxford University Press defines an erratum as publisher error and a corrigendum as an author introduced error. The correction must be submitted using the *JN* online manuscript submission system.

The erratum or corrigendum will be reviewed by the Editor-in-Chief and, if approved, published in the next available issue. The correction should include the full citation of the original article (authors, title, J Nutr year;volume:page range) and explain the change or correction needed to the original published version. Page charges will not be assessed unless the correction is lengthy and due to authors' errors. For more information, please refer to the post-production changes section. Please see previously published errata for format (go to the Journal website; select Advanced Search; enter *erratum* in "Build a query" field.

Other

JN also publishes supplements, based on extensive reports of research, monographs, compendia, proceedings of symposia, etc. Proposals for supplements must be submitted in advance and will be reviewed for appropriateness by the Editor-in-Chief. One or more Supplement Editors, approved by the Editor-in-Chief, serve as the editor(s) for each supplement issue.

Initial correspondence to submit a supplement proposal, contact the Editorial Office at jn.editorialoffice@oup.com). For more information on supplement fees, please contact Gina Farago (gina.farago@oup.com). More information on supplements in Journal of Nutrition.

Submission Procedures

Manuscript submissions to *JN* must be made using the online system. Users are required to register when accessing the system for the first time. Detailed instructions and help files are also available online from the registration and submission areas of the manuscript submission system. If you experience serious problems, you can contact the *JN* manuscript office: email: jn.editorialoffice@oup.com. Questions related to the submission of a manuscript or changes in a manuscript submission should be submitted by the manuscript's corresponding author. All correspondence from journal staff regarding a manuscript submission will be directed to the manuscript's corresponding author.

The ASN journals will allow the submission of manuscripts that have been previously posted on preprint servers. Such manuscripts will undergo the same rigorous peer review as manuscripts that were not posted on preprint servers. In addition to meeting the standard requirements for submitted manuscripts (e.g., that the manuscript not be under editorial consideration for publication elsewhere), preprint submissions must satisfy the following conditions: *1*) Upon first submission to an ASN journal, the author must inform the journal via the cover letter that the manuscript has been posted to a preprint server and provide the name of the preprint server, the copyright license under which the manuscript is posted, and a link to the preprint; *2*) No additional versions of the manuscript may be posted to preprint servers at any time after initial submission to an ASN journal; *3*) the copyright terms of the preprint must not be changed after submission of the manuscript to an ASN journal ; *4*) A preprint DOI must be assigned to the preprint; *5*) Once the article has been published in its final form on the journal website, the preprint server on which the preprint is hosted must link to the article on the journal's website (the link should appear as follows: This article has been accepted for publication in [Journal Title] Published by Oxford University Press.); *6*) If the preprint is posted to a preprint server under an open access license, publication of the article in the ASN journal must also be under the same type of open access license, and the author must agree to pay the journal's open access fee.

Before submitting your manuscript, please make sure your manuscript has been formatted according to instructions below and in the "Manuscript Preparation" on Page 5 of this document. Please do not use Internet Explorer 5 to upload your manuscript.

Having the following information ready before starting your submission will save time:

1. If your paper is a resubmission, the previous manuscript ID# and a Response to Reviewers;
2. Your manuscript's title, abstract and keywords;
3. Your cover letter (see below);
4. All author names, affiliations and email addresses;
5. If you plan to suggest reviewers, their names, affiliation and email addresses.

Cover letter

A letter of submission from the corresponding author is a required field in the submission site. The cover letter may include information about Supplementary data or auxiliary files submitted.

Manuscript file format

Word (.doc or .docx) files are the preferred format for manuscript text source files. Tables should be included after the references.

Fonts. Standard fonts, including Arial, Helvetica, Times Roman, Symbol, Mathematical PI, and European PI, are recommended in order to avoid potential problems with font substitution or embedding problems. All other fonts, if not embedded, may be replaced, resulting in data loss or realignment.

Supplemental file upload. Supplemental files for upload may include articles published/in press elsewhere, Supplementary data, cover art submissions, reports or technical briefs related to manuscript submission, questionnaires, permissions, videos, etc. Clearly label each file as "Supplemental Data for Reviewers Only" or as "Supplementary data" if it is submitted for online publication.

Cover images. Authors are invited to submit color images for use on *JN* cover. Images can be figures included in a submitted manuscript, images that are representative of research reported in a submitted manuscript, or images that illustrate an aspect of nutrition research in general. Images should be 20.0 cm wide by 14.5 cm high (47 picas x 34.5 picas high). Images can be submitted in one of the following ways: 1) online during the manuscript submission process: load as a Supplemental File and, on the file upload page, indicate the file is a cover art submission; or 2) by email to the JN Editor-in-Chief, Dr. Teresa A. Davis: jnutr@bcm.edu. Please include the manuscript number in all correspondence.

If an image is included in a submitted manuscript, copyright will transfer to the American Society for Nutrition. When an image is selected for a JN cover, authors will be asked to complete the Cover Illustration Permission and Description Form (4).

License to Publish

As publisher of *JN,* ASN holds the copyright on all *JN* articles. As recommended by the Council of Science Editors, when a coauthor dies or is incapacitated during the writing, submission, or peer review process, coauthors should obtain disclosure and copyright documentation from a familial or legal proxy (4).

Open access publication option
Authors of accepted original research manuscripts can choose to pay an Open Access fee to allow their articles to become freely accessible on the journal website immediately upon publication. The open access charges are:

Non-member charges for CC BY: £3676 / $5000 / €4449
Member charges for CC BY: £3309 / $4500 / €4004
Non-member charges for CC BY-NC: £3309 / $4500 / €4004
Member charges for CC BY-NC: £2610 / $3550 / €3158

Members who receive a discount are American Society for Nutrition (ASN) members. Members will be required to enter their member number (which is the e-mail address they used to register with the ASN) in order to receive the discount.

Open Access fees are charged in addition to other author charges (page charges, color reproduction charges, author alteration charges, and charges for reprint orders). For authors who elect not to pay the open access fee, articles will have a standard license and remain under access

control for the first 12 months after publication. More information about Open Access licenses. All Open Access fees can also be paid using OUP's Author Services site.

Permissions. Authors are responsible for obtaining permission to adapt or reproduce any copyrighted material. Authors are also responsible for obtaining written permission from any person mentioned in a personal communication or acknowledgment and for providing to the Editor a copy of the permission, if requested. Please include your manuscript number on all correspondence sent to jn.editorialoffice@oup.com.

Manuscript Preparation

JN is limited in the number of pages that can be published each year, and article length is a consideration in the editorial process. Manuscripts up to 5000 words maximum from Introduction through Discussion, will be considered. Maximum word count does not include the Title Page, Abstract, Acknowledgments, Author Contributions, References, Figure Legends, or Tables. Authors are encouraged to be clear and concise. Papers must be completely double spaced. Number the lines continuously (not per page) beginning with the abstract and ending before the references, tables, and figures. Number pages consecutively in the upper right-hand corner of each page, beginning with the title page. Manuscript submissions that are not formatted correctly may be returned to authors. For a succinct list of formatting requirements, please see the Quick List Formatting on page 14. Foreign authors are advised to have their manuscripts reviewed by a colleague who is fluent in English.

JN encourages authors to provide the names, fields of interest, addresses, telephone and fax numbers, and e-mail addresses of 4–6 unbiased and qualified potential expert reviewers who do not have a conflict of interest.

Include in your research manuscript:

1. *Title Page*
2. *Abstract page*
3. *Introduction*
4. *Methods*
5. *Results*
6. *Discussion*
7. *Acknowledgments and statement of authors' contributions to manuscript*
8. *References*

1. Title page

The title page must include:

a. A title that is composed as a single declarative statement and focused on the results presented in the manuscript. The title should include the animals, participants, or cells studied. Please do not use a colon or semicolon in the title. Keep the title as generally applicable as possible. It usually is not necessary to include the exact study location or a specific study name in the title, because this information can be included in the abstract.

b. The names of all authors (first name, middle initial, last name)

c. Author Affiliations (departmental and institutional) at the time the research was done.

d. Sources of support
   The word count for the entire manuscript (introduction through discussion).

e. Conflict of Interest and Funding Disclosure - List any existing financial arrangements between an author and a company whose product figures prominently in the submitted manuscript or between the author and any company or organization sponsoring the research reported in the submitted manuscript. If an author has no conflicts of interest, list the author's name, followed by "no conflicts of interest." For detailed guidelines on possible conflicts of interest, see the ASN Journals Conflict of Interest Guidelines (5).

f. Corresponding Author name, mailing address, telephone number, and e-mail address

g. The word count for the entire manuscript (introduction through discussion).

h. The number of figures (to print, not Supplementary data).

i. The number of tables (to print, not Supplementary data).

j. Supplementary data submitted.

k. A running title of 50 or fewer characters and spaces.

l. a list of abbreviations and their definitions for all abbreviations used in the text if there are 3 or more

2. Abstract page

A properly constructed and informative abstract is helpful for the initial editorial review of the submitted manuscript. Research articles must include a structured abstract that contains no more than 300 words, is written in complete sentences, includes information pertinent to any clinical trial registry in which a trial is registered, and uses the following headings:

Background. Provide 1 or 2 sentences that explain the context of the study.
Objective. State the precise objective, the specific hypothesis to be tested, or both.
Methods. Describe the study design, including the use of cells, animal models, or human subjects. Identify specific methods and procedures.
Results. Report the most important findings, including key data and results of statistical analyses.
Conclusions. Summarize in 1 or 2 sentences the primary outcomes of the study, including their potential importance (avoid generalizations). Include the participants, animals, or cells studied.

Review articles, special articles, and reports should include an unstructured abstract (no more than 300 words) that states the purpose of the article and emphasizes the major concepts and conclusions. Any abbreviations used in the abstract should be defined in the abstract at first mention.

Below the abstract, provide and identify 5–10 keywords or short phrases, including the subject group, that will help to increase the discoverability of your manuscript; do not use adjectives. Terms that are fundamental to your manuscript but are not included in your manuscript title or abstract are especially important to include to increase discoverability by indexing services such as PubMed.

Please note that during manuscript submission, you will be asked to supply keywords to assist the editors in locating suitable reviewers for your manuscript. Keywords for reviewer searches should include the terms most fundamental to your manuscript, and may differ from your list of keywords for publication.

3. Introduction

Describe clearly the background to the research conducted and the specific objectives. This should not be a comprehensive review of the literature, however. State the specific objective or hypothesis of the study.

4. Methods

Documentation of methods and materials used should be sufficient to permit replication of the research. Describe clearly the experimental design including the control and experimental groups. State the source of specialized materials, diets, chemicals, and instruments and other equipment, with model or catalog numbers, where appropriate. Specify kits, analyzers, and commercial laboratories used. Cite references for methods whenever possible and briefly explain any modifications made.

Human and animal research. Reports of human studies must include a statement that the protocol was approved by the appropriate institutional committee or that it complied with the Helsinki Declaration as revised in 1983. Registration is required for all clinical trials that began after July 1, 2010. When preparing reports of randomized, clinical trials, refer to the checklist published in the CONSORT Statement (6). Include a CONSORT flow diagram as a manuscript figure summarizing participant flow with the sizes (n) of initial (recruited, enrolled) and final groups. Indicate in both the abstract and the manuscript text whether the outcomes reported are primary or secondary outcomes of the study. For systematic reviews and meta-analyses, refer to the PRISMA checklist and include a PRISMA flow diagram as a figure in the manuscript (7). CONSORT and PRISMA checklists can be uploaded as supplemental material for the benefit of reviewers and editors.

Research on animals should include a statement that the protocol was approved by the appropriate committee or complied with the Guide for the Care and Use of Laboratory Animals (8). Compliance with the ARRIVE guidelines is encouraged and the checklist can be uploaded as supplemental material (9). Describe how animals were euthanized. Describe control and experimental animals or participants, giving age, weight, sex, race, and for animals, breed or strain. Include the supplier of experimental animals.

Diets. Composition of control and experimental diets must be presented. When a diet composition is published for the first time in *JN*, provide complete information on all components in a table. If previously described in *JN* or *AJCN*, a reference may be used. State specifically any modifications made to the published diet compositions. The proximate composition of closed formula diets should be given as amounts of protein, energy, fat, and fiber. Express components as g/kg diet. Vitamin and mineral mixture compositions should be included using *JN* units and nomenclature. For a discussion of the formulation of purified animal diets, refer to Baker (10) and to a series of ASN publications (11–14). The experimental diets should differ from the control diets only in the nutrient(s) being investigated. Nonpurified diets generally should not be used as control diets; animals fed these diets should be included for reference only and their data should not be included in the statistical analysis.

Statistical methods. Describe all statistical tests utilized and indicate the probability level (P) at which differences were considered significant. If data are presented in the text, state what they represent (e.g., mean ± SEM). Indicate whether data were transformed before analysis. Specify any statistical computer programs used.

Present the results of the statistical analysis of data in the body of each table and on figures per se. Use letters or symbols to indicate significant differences; define these in a table footnote or the figure legend. Provide the appropriate statistics of variability with an estimate of the error variance (SD or SEM) of group means. Standard ANOVA methodology assumes a homogeneous variance. If error variance is tested and found to be heterogeneous, transform data before ANOVA, or use nonparametric tests. For a discussion of variability calculations and curve-fitting procedures, see Baker (10).

If non-significant P values are reported, use only 2 digits past the decimal (e.g., P=0.15). Present significant P values to a maximum of 4 decimal places (e.g., P<0.0001); using fewer is acceptable. Present coefficients to a maximum of 2 decimal places (e.g., r=0.87, R^2=0.16, etc.).

5. Results

Report the results of the study without repeating the methodology, Introduction, or content in the Discussion section. Do not duplicate data from tables or figures in the text.

6. Discussion

In the Discussion, explain the importance of the findings, putting them into the context of the existing literature. Clearly state the overall conclusions.

7. Acknowledgments

Technical assistance and advice may be acknowledged in a section at the end of the text. Only named individuals should be included in this section. Authors are responsible for obtaining written permission from everyone providing a personal communication or acknowledged by name in the manuscript and for providing to the Editor a copy of the permission, if requested.

Statement of authors' contributions to manuscript. Authors must indicate their contribution(s) to the manuscript in the Acknowledgments section. Use the relevant descriptors listed below unless the author performed a function that clearly is not covered by one of these. All manuscripts, including reviews, must indicate who is responsible for design, writing, and final content and must include a statement affirming that all authors have read and approved the manuscript. The initials of all authors must be included.

1. designed research (project conception, development of overall research plan, and study oversight).
2. conducted research (hands-on conduct of the experiments and data collection).
3. provided essential reagents, or provided essential materials (applies to authors who contributed by providing animals, constructs, databases, etc., necessary for the research).
4. analyzed data or performed statistical analysis.
5. wrote paper (only authors who made a major contribution).
6. had primary responsibility for final content.
7. other (use only if categories above are not applicable; describe briefly).
8. All authors have read and approved the final manuscript. For single-authored research papers and reviews, please state: The sole author had responsibility for all parts of the manuscript.

Please do not include "obtained funding." The initials of authors who received grants may be included in the footnote on the title page regarding Support.

An example is:
A. X., R. F. G., and P. G. Y. designed research; R. F. G. and Q. C. conducted research; P. T. analyzed data; and A. X., P. G. Y. and Q. C. wrote the paper. P. G. Y. had primary responsibility for final content. All authors read and approved the final manuscript.

8. References

Consecutively number references, including web citations, in the order in which they are first mentioned in the text. Number references cited for the first time in tables or figure legends in order, based on the first citation of the table or figure in the text. Identify references in the text, tables, and legends for figures by Arabic numbers in parentheses.

Only published papers and accepted papers that are "in press" may be included in the References section. "In press" papers must be submitted as supplemental files in PDF format at the time of manuscript submission. Personal communications from others and unpublished data of the authors, including submitted manuscripts, should appear parenthetically in the text. Include the full name and affiliation of the person providing a personal communication.

JN reference format is consistent with the International Committee of Medical Journal Editors (ICMJE) recommended format for bibliographic citations (15) with the following exception: list the names of all authors, unless there are more than ten, in which case list the first ten plus "et al." The ICMJE states, "as an option, if a journal carries continuous pagination throughout a volume (as many medical journals do) the month and issue number may be omitted." *JN* follows this optional style. If you are using software such as EndNote or Reference Manager that inserts this additional material, it will be automatically deleted during production of accepted manuscripts. Abbreviate journal names according to the National Library of Medicine (NLM) journal abbreviations list (16).

Authors may add to a reference, the DOI ("digital object identifier" number unique to the publication) for articles in pressIt should be included immediately after the citation in the References. An example is:

Kimokoti RW, Judd SE, Shikany JM, Newby PK. Metabolically healthy obesity is not associated with food intake in White or Black Men. J Nutr 2015 Sep 30 (Epub ahead of print; DOI: *doi:10.3945/jn.115.221283*).

Examples of citations to sources on the internet and to books can be found in the References in JN Instructions for Authors (page 13). Monographs can be cited in the following format:

Gibson RS, Ferguson EL. An interactive 24-hour recall for assessing the adequacy of iron and zinc intakes in developing countries. HarvestPlus Technical Monograph 8: Washington, DC and Cali, Colombia: International Food Policy Research Institute and International Center for Tropical Agriculture. 2008.

There is no limit on the number of citations allowed; cite recent literature comprehensively. Begin the list of references on a new page. Note that there should be no line numbers on the row with the “References” heading or throughout the References section.

Units of Measure

Metric units are required (e.g., m, kg, and L for height, weight, and volume, respectively), as is the Celsius scale (°C). For reporting data, use of SI units (le Systeme Internationale d'Unites) (17) is preferred (e.g., mmol/L,g/L) but not mandatory. Conventional units such as mg/dL and mg/mL are acceptable, using L, not l, for liter. Use units for the same analyte/compound consistently throughout the manuscript. Placing an alternate unit parenthetically in the text or giving conversion factors in table footnotes or figure legends is acceptable. Units should not be pluralized (e.g., wk, not wks) or followed by a period.

Useful websites are:
SI conversions: Online Conversion
SI conversions: Wikipedia - Conversion of Units
Clinical SI conversions: JCS Clinical Laboratory Units Conversion

Significant Digits

The number of significant figures presented for a variable should be correct and consistent. Use no more than 3 significant figures (fewer, if appropriate) or justify the greater precision.

Base the number on the precision of the analytical method and round accordingly. In some cases, change the submultiple; e.g. change 1038 mg/d to 1.04 g/d.

Five rules govern significant figures (18):

1. Non-zero digits are always significant; 1.121 has four significant digits.
2. Any zeros between two significant digits are significant; 1.08701 has six significant digits.
3. Zeros before the digits are placeholders and not significant; in the number 0.00254 , only the 2, 5, and 4 are significant, meaning the number has 3 significant digits.
4. Zeros after the decimal point and after figures are significant; in the number 0.2540, the 2, 4, 5, and last 0 are significant digits5. Exponential digits in scientific notation are not significant; $1.12x10^{6}$ has three significant digits, 1, 1, and 2.

A tutorial on the use of significant digits is available (19).

Abbreviations

Use standard abbreviations in JN papers without definition in the text. Standard abbreviations, however, should be defined at first mention in the abstract. An abridged list is in Table 1 (20). Other common standard abbreviations are listed in Scientific Style and Format (4).

Each nonstandard (author-defined) abbreviation should be defined in the abstract and text at first mention. If three or more nonstandard abbreviations are used in the text, prepare an abbreviation footnote. The footnote should be associated with the first abbreviated term in the text and should be an alphabetized listing of all author-defined abbreviations and their definitions. Group designations should be defined parenthetically at first mention [for example, "control (CON) and high-fat (HF) groups"] and included in the abbreviation footnote. Abbreviations (other than units

such as min, h, m, kg) should be pluralized where appropriate (e.g., The n-3 PUFAs are…) but should not be followed by a period. Use the standard abbreviations for SI prefixes found in Young (19) and in Table 2 and those for units of measure in Table 3 (21).

All nonstandard abbreviations, including group or treatment designations, used in a table or table title must be defined alphabetically in a footnote to the table title. If the footnote to the table title contains multiple items, the definitions of the abbreviations should be the last item. If a table contains only one abbreviated term in the body of the table, then a separate footnote placed after that abbreviation should be used to define that term. Similarly, all nonstandard abbreviations, including group or treatment designations, used in a figure or figure legend must be defined alphabetically at the end of the figure legend.

Genes and Proteins

Full gene names are not required for tables and figures in which a database identifier number is given. A full citation to the database used should be in the References and the sequential reference number to the citation provided in the text, figure legend, or table footnote. If the genes are listed in online Supplementary data (e.g., supplemental tables and figures), the citation can be given as a table footnote or in the figure legend [e.g., National Center for Biotechnology Information (NCBI) Entrez Gene (22) or Unigene (23)].

All gene symbols should be italicized throughout the text, tables, and figures. The use of prefixes to designate species is not allowed. For rodent genes, the first letter should be uppercase with the rest in lowercase letters (e.g., for PPARγ, Pparg). For human genes, all letters should be uppercase (e.g., PPARG). Messenger RNA (mRNA) and complementary DNA (cDNA) use the same gene symbol and formatting conventions. Protein designations are the same as the gene symbols, are in all uppercase letters (even rodents), and are not italicized (e.g., PPARG). For the genes of other species, follow the convention for abbreviating human gene and protein names. Further information on gene and protein nomenclature rules can be found as indicated in the list below.

- Human: HUGO Gene Nomenclature Committee (HGNC) (24)
- Mouse: Mouse Genome Nomenclature Committee (MGNC) (25)
- Rat: Rat Genome and Nomenclature Committee (RGNC) (26)
- Bovine: Bovine Genome Database (BGD) (27) Chicken and other avians: Chicken Gene
- Nomenclature Committee (CGNC) (28)
- *Xenopus* and other amphibian (29)
- Zebrafish and other piscine: Zebrafish Model Organism Database (ZFIN) (30)
- *Drosophila*: (31)

Porcine: No official genome nomenclature committee statements or annotation resources are available online to date. The International Society for Animal Genetics (ISAG) publishing guidelines defer to the HUGO Name (24) when applicable. Use NIH Gene (22) or HUGO (24) to confirm names. Where one-to-one human orthology cannot be established, an unofficial gene symbol can be used if it is supported by a previous literature assignment. Clade- specific or species-specific genes will be designated with an official gene symbol upon completion of the genome.

Equine, ovine, canine: No official genome nomenclature committee statements or annotation resources are available online to date; see porcine guidelines.

With respect to defining gene and protein symbols, please follow the instructions in the list below.

1. Text: Define all gene and protein symbols (abbreviations) at first use in the abstract and text.
2. Tables and figures: Define protein symbols or abbreviations. Full gene names are not required for tables and figures in which a database identifier number is given. Therefore, either use NM_ or other database identification numbers or define gene symbols.
3. Abbreviation footnote: Define protein symbols (abbreviations). Do not define genes for which a database identifier number is given in the text, tables, or figures. Define gene names for which a database identifier number is not given.

Nomenclature

Chemical and biochemical terms and abbreviations and identification of enzymes generally should conform to the recommended usage of the International Union of Biochemistry and Molecular Biology (32). Names for vitamins, related compounds, and abbreviations for amino acids should follow the ASN nomenclature policy (33, 34).

For fatty acids, use the ω or n system consistently with a colon to separate the chain length and number of bonds (e.g.,18:2n–6, 20:5n–3, 18:2ω-6, 20:5ω-3). In the text, refer to n–3 fatty acids, ω3 PUFA, the ratio of n–3/n–6 fatty acids, etc. Regardless of which system is used in the text, include the alternative form in parentheses in the abstract. Use common names and systematic names together at first mention, and then use the common name throughout. In general, there is no need to use the abbreviations “c” and “t” to denote cis and trans after first usage [e.g., cis-9, trans-11 CLA (18:2c9,t11)]. If, however, the article includes many references to systematic names including cis and trans designators, it may be more economical to use the “c” and “t” designators.

Ethical Considerations

Individuals who are asked to review a manuscript should decline the solicitation if they have a conflict of interest. Detailed guidelines on conflicts of interest for reviewers can be found at ASN Journals Conflict of Interest Guidelines (5).

JN strongly encourages registration in an appropriate public trials registry of all clinical trials and observational studies. Beginning in 2015, this will be required.

Before acceptance, all papers will be screened for similarity to previously published papers using iThenticate. Selected papers will be screened at earlier stages of the review process. Those with disproportionate similarity to published papers will, at the Editor's discretion, be rejected outright or returned to authors for rewriting followed by re-review before a final decision is made.

The following are considered inappropriate re-use of material (plagiarism):

1. Copying the published words of other authors or modifying only slightly, with or without citation of the original work.
2. Reusing the author's own previously published words, with or without citation (self-plagiarism).
3. Failure to quote and/or acknowledge by citation substantially similar ideas, content, tables, or illustrations that have been published or copyrighted by others.

Most published work is copyrighted. Thus, all text in the submitted paper must be original, including the Methods section. Frequently, the previous publication can be cited and thereby the length of the Methods section can be reduced. Review articles also must be original; they cannot repeat verbatim or include only minimally changed words from previous reviews or original research papers by the author or others. The NIH's Office of Scientific Integrity's piece, "Avoiding Plagiarism, Self-Plagiarism, and Other Questionable Writing Practices: A Guide to Ethical Writing" (35) is highly recommended.

As recommended in the Committee on Publication Ethics Code of Conduct for Journal Publishers (36), and supported by the International Committee of Medical Journal Editors (37), when ASN is made aware of cases of suspected research and publication misconduct, ASN holds the right to publish an Expression of Concern during an investigation, and, depending on the outcome of the investigation, to retract articles.

Tables and Figures

See current print or electronic papers in *JN* for examples of table and figure styles. Cite tables and figures sequentially in the text with the first citation of each table and figure in bold font. Tables or figures adapted or reproduced from another source must acknowledge that source in a table footnote or the figure legend and be accompanied by written proof that the copyright bearer has granted permission to reproduce or adapt the table or figure. To obtain permission, authors may need to reference the information found at the page Permission to Re-Publish Copyrighted Content in *JN* (38).

Authors of supplement and symposium manuscripts

Include 1 of 3 statements in all figure and table captions:

1. Reproduced with permission from (reference X),
2. Adapted with permission from (reference X), or
3. Original to this manuscript.

Tables

Tables must be included in the text file, and each table should begin on a new page. Please do not upload tables as separate files. Each table should have a title that clearly but concisely describes the treatments and experimental animals or participants. Information concerning methods or explanatory material can be included in footnotes to the table, but repetition of methodology should be minimized. Clearly indicate units of measure after the variable in rows, above the first value in each column, or centered over all columns to which the unit applies. Show statistics of variability (e.g., SD, pooled SEM) and the significance of differences among the data. Omit internal horizontal and vertical rules before submitting your tables.

For an illustrated table quality checklist, visit the Table Checklist (38).

Figures

Submit each figure in a separate file. Image files (TIF, EPS) and Microsoft PowerPoint (PPT,) are acceptable figure files. Figures prepared as Word, PNG, or JPEG will not be accepted.

Compile figure titles and legends on one or more pages in the manuscript's .doc file rather than on the figure itself, but include figure keys on figures, within the bounds of the graphs or on X-axes, not in legends. Figure titles should concisely describe the species or participants and treatments but are not required to call out panels. Each legend should contain enough detail, including an explanation of the results of statistical tests shown to ensure that the figure is interpretable without reference to the text. For figures with 2 or more panels, describe each panel in the legend, beginning with the panel letter. Minimize repetition of methodology, but specific assay conditions can be given.

Submit all panels of a multipanel figure on a single page, aligning the panels horizontally and/or vertically with one another. Minimize white space within and between panels. Label each panel, A, B, C, D, etc., without the word, “figure,” or the figure number, in the upper-left corner of the panel.

Label axes clearly with variables and where appropriate, units of measure. Show significant differences using symbols or letters. Remove outer boxes from figures and figure panels.

Size all text on figures proportionately and large enough to be legible after reduction to 1 column width of <8.5 cm or, in rare cases, 2 column widths. Preferred text size is 7 points.

- 1 column: 18p0 / 3 inches / 7.6 cm
- 1.5 column: 27p0 / 4.5 inches / 11.5 cm
- Maximum width (to span 2 columns): 34p0 / 5.7 inches / 14.4 cm
- Maximum height: 53p0 / 8.8 inches / 22.4 cm

Because of the expense of color printing, which is in part subsidized by *JN*, please submit no more than two color figures per manuscript. Only those figures that are essential to display in color will be printed in color. Avoid unnecessary color for histograms, line drawings, etc. When color is deemed unnecessary, the editors may ask authors to resupply figures in black and white or in gray tone. When possible, prepare multipanel figures that group all color images into a single figure. Color reproduction costs will be charged to the author. Color may be used in Supplementary data.

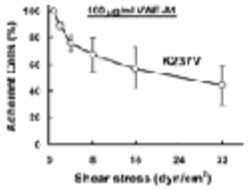
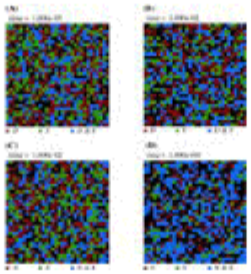
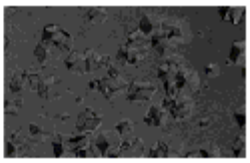
Image resolution

If a figure is very small in the manuscript submission system–generated PDF file, the resolution of the figure file was likely not high enough. Upload a higher resolution figure before approving the PDF. Files must conform to the following minimum resolution specifications:

Line art. 1000 dpi

Combination halftones. 600 dpi (grayscale or color images and type)

Halftones. 300 dpi (grayscale or color with no type or lettering)

Line art	Combination Halftones (grayscale or color images and type)	Halftones (grayscale or color with no type or lettering)
		
1000 dpi	**600 dpi**	**300 dpi**

For an illustrated figure quality checklist, visit the Figure Quality Checklist (40). For video on preparing digital images for publication, visit the Preparing Digital Images for Publications series (41).

Supplementary Data

Unusually lengthy descriptions of experimental procedures, extensive data, extra figures, etc. may be published as online-only attachments to published articles. For example, if several primers were used, they should be listed in a supplemental table. Supplementary data should be integral to the manuscript but impractical to include in the printed article. References to the availability of supplemental data on authors' websites will not be included in published articles.

Please note the following:

1. For revised submissions, do not use yellow highlighting to show changes made to your Supplementary data. Remember that Supplementary data are not copyedited before publication and will be published exactly as you upload the supplemental file(s).
2. Supplemental text, tables, and figures can be single spaced and should not include line numbers, but should otherwise be in JN format.
3. Label the top of all Supplementary data pages with the header, "Supplementary data."
4. Name and label each unit of supplemental material as appropriate (e.g., Supplemental Table 1, Supplemental Table 2, Supplemental Figure 1, Supplemental References, etc.)
5. Call out all Supplementary data parenthetically in the manuscript text; e.g.: (Supplemental Fig. 1).
6. Place supplemental figure legends immediately under the supplemental figure.
7. If the citations in the Supplementary data do not appear elsewhere in the printed paper, they should NOT be added to the paper's References section. Instead, prepare a Supplemental References section [beginning with (1), (2), etc.] and include it at the end of supplemental materials.

8. Format the footnote to the manuscript title with specific information about the Supplementary data available. For example: Supplemental Table 1 and supplemental methods are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents available on the Journal homepage.

Submit Supplementary data video files in the native format. To avoid excessive delays in downloading the files, limit videos to no more than 5 MB in size and 60 s in length. Authors are encouraged to use QuickTime's "compress" option when preparing files to help control file size.

Charges to Authors

Authors will be billed for the following items:

Color reproduction costs

Authors are encouraged to use color figures (generally a maximum of 2 per paper) when they will enhance the presentation of the data. Authors will be charged the subsidized rate of £294 / $400 / €356 per figure, although this fee will be waived for corresponding authors who are ASN members, when color is deemed essential by the editor. During the manuscript submission process, all authors must indicate that color figures are included and that they agree to pay the subsidized color reproduction fee of £294 / $400 / €356 per figure.

Page charges

Corresponding authors who are members of ASN will be billed:

- £74/$100/€90 for each of the first 7 pages and
- £100/$135/€121 for each additional page

Corresponding authors who are not members of ASN will be billed:

- £103/$140/€125 for each of the first 7 pages and
- £121/$165/€146 for each additional page

This charge does not apply to reports of ASN meetings, official ASN statements, letters to the Editor, book reviews, or articles invited by the Editor-in-Chief, and page charges may be waived by the Society for acceptable reasons such as the lack of funding from grant or institutional sources as verified by an institutional official. Requests for waivers will not affect review of manuscript or delay publication.

Requesting a fee waiver

Waivers should be requested when the acceptance letter is received or when author proofs are returned. Requests for waivers must be submitted by the corresponding author on institutional letterhead and cosigned by an appropriate institutional official to verify that no institutional or grant funds are available for the payment of page charges. Authors and their institutions shall determine who is best-suited to provide this verification. Please include the journal name,

manuscript title, and the manuscript ID number with your request. Waiver requests should be sent to jnsubmit@nutrition.org.

Supplement fees

For more information on supplement fees, please contact Gina Farago (gina.farago@oup.com).

References in the *JN* ITAs

1. *The Journal of Nutrition* [Internet]. Bethesda (MD): American Society for Nutrition; c2013–2014. JN's Guide for Authors' Use in Estimating Length; 2013 Sep 3 [cited 2014 Jan 17] [1 p.]. Available from: https://nutrition.org/wp-content/uploads/2018/01/word_counts.pdf.
2. *The Journal of Nutrition* [Internet]. Bethesda (MD): American Society for Nutrition; c2014. General Information on Supplement Publications; 2012 Aug 13 [updated 2016 Mar 17; cited 2012 Aug 13]; [about 3 screens]. Available from https://nutrition.org/publications/jn-supplements/.
3. *The Journal of Nutrition* [Internet]. Bethesda (MD): American Society for Nutrition; c2006-2014. *JN* Cover Illustration Permission and Description Form; 2006 Aug [updated 2016 May 12; cited 2014 Jan 17]; [1 p.]. Available from https://nutrition.org/wp-content/uploads/2018/01/coverpermission.pdf.
4. Council of Science Editors, Style Manual Subcommittee. Scientific Style and Format: the CSE manual for authors, editors, and publishers. 8th ed. Chicago: The University of Chicago Press; 2014.
5. American Society for Nutrition [Internet]. Bethesda (MD): The Society; c2009–2014. ASN Journals' Conflict of Interest Guidelines; 2009 Dec 29 [cited 2014 Jan 17]; [about 4 screens]. Available from http://www.nutrition.org/publications/guidelines-and-policies/conflict-of-interest/.
6. CONSORT Group. CONSORT: Transparent Reporting of Trials [Internet]. Ottawa (ON): CONSORT Group; 1996 [updated 2010; cited 2015 Apr 29]. Available from http://www.consort-statement.org/.
7. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JPA, Clarke M, Devereaux PJ, Kleijnen J, Moher D. PRISMA: Transparent Reporting of Systematic Reviews and Meta-Analyses [Internet]. Ottawa (ON): Ottawa Hospital Research Institute; 2009 [updated 2009; cited 2015 Apr 29]. Available from http://www.prisma-statement.org/.
8. National Research Council. Guide for the Care and Use of Laboratory Animals. 8th Edition. Washington, DC: National Academies Press; 2011.
9. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. ARRIVE guidelines [Internet]. London (England): National Centre for the Replacement Refinement & Reduction of Animals in Research; 2010 [last update unknown; cited 2015 Apr 29]. Available from: http://www.nc3rs.org.uk/arrive-guidelines.
10. Baker DH. Problems and pitfalls in animal experiments designed to establish dietary requirements for essential nutrients. *J Nutr* 1986;116:2339–49.
11. American Institute of Nutrition. Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. *J Nutr* 1977;107:1340–48.
12. American Institute of Nutrition. Second report of the ad hoc committee on standards for nutritional studies. *J Nutr* 1980;110:1726.

13. American Institute of Nutrition. Guidelines for describing diets for experimental animals. *J Nutr* 1987;117:16–7.
14. American Institute of Nutrition. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993;123:1939–51.
15. International Committee of Medical Journal Editors [Internet]. Philadelphia: Annals of Internal Medicine; c2011–2013. Preparing for Submission; 2013 [cited 2014 Jan 1]; [about 9 screens]. Available from: http://www.icmje.org/recommendations/browse/manuscript-preparation/preparing-for-submission.html.
16. List of Serials Indexed for Online Users [Internet]. Bethesda (MD): U.S. National Library of Medicine. 2004 [cited 2014 Jan 17]. Available from http://www.nlm.nih.gov/tsd/serials/terms_cond.html.
17. Young DS. Implementation of SI units for clinical laboratory data, style specifications and conversion tables. *Ann Intern Med* 1987;106:114–29. Reprinted, *J Nutr* 1990;120:20–35.
18. Sparknotes: units, scientific notation, and significant figures [Internet]. New York (NY): SparkNotes LLC; 2015 [update unknown; cited 2015 Apr 29]. Available from http://www.sparknotes.com/chemistry/fundamentals/units/s ection3.rhtml.
19. Significant Digits[Internet]. Guelph (ON): University of Guelph, Department of Physics; [cited 2016 Dec 19]. Available from: https://www.physics.uoguelph.ca/tutorials/sig_fig/SI G_dig.htm.
20. *The Journal of Nutrition* [Internet]. Bethesda (MD): American Society for Nutrition; c2011. Table 1: Common abbreviations and units that may be used without definition; 2011 Sep 13 [cited 2011 Sep 13]. [3 p.]. Available from https://nutrition.org/wp-content/uploads/2018/01/jn-abbreviation-tables.pdf.
21. *The Journal of Nutrition* [Internet]. Bethesda (MD): American Society for Nutrition; c2010. Table 2 SI prefixes and Table 3 Common abbreviations for units of measure; 2010 Nov 5 [cited 2014 Jan 17]; [1 p.]. Available from https://nutrition.org/wp-content/uploads/2018/01/jn-abbreviation-tables-2-and-3.pdf.
22. UniGene [Internet]. Bethesda (MD): National Center for Biotechnology Information, U.S. National Library of Medicine; [date unknown]: [cited 2014 Jan 17]. Available from http://www.ncbi.nlm.nih.gov/unigene.
23. HUGO Gene Nomenclature Committee [Internet]. Cambridge, UK: National Human Genome Research Institute; [date unknown]: [cited 2014 Jan 17]. Available from http://www.genenames.org/.
24. Mouse Nomenclature Home Page [Internet]. Farmington (CT): Mouse Genome Nomenclature Committee; c1996. [cited 2014 Jan 17]. Available from http://www.informatics.jax.org/mgihome/nomen/.
25. Rat Nomenclature Database [Internet]. Milwaukee (WI): Bioinformatics Program, HMGC; 2013. [cited 2014 Jan 17]. Available from http://rgd.mcw.edu/nomen/nomen.shtml.
26. The Bovine Genome Database [Internet]. Columbia (MO): The University of Missouri: c2010. [cited 2014 Jan 17]. Available from http://bovinegenome.org/.
27. Chicken Gene Nomenclature Consortium [Internet]. Tucson (AZ): Biotechnology Computing Facility at the University of Arizona; c 2013. [cited 2014 Jan 17]. Available from: http://birdgenenames.org/cgnc/.
28. Xenbase [Internet]. Calgary: Xenopus Gene Nomenclature Committee; 2005. [cited 2014 Jan 17]. Available from http://www.xenbase.org/gene/static/geneNomenclature.jsp.
29. The Zebrafish Model Organism Database [Internet]. Eugene (OR): The University of Oregon; c1994. [cited 2014 Jan 17]. Available from http://zfin.org/.

30. FlyBase: A Database of Drosophila Genes & Genomes [Internet]. Bethesda (MD): The Genetics Society of America; 1993–2016. [updated 2016 Oct 18; cited 2014 Jan 17]. Available from http://flybase.org/.
31. International Union of Biochemistry and Molecular Biology. Enzyme Nomenclature, Recommendations 1992. Orlando (FL): Academic Press; 1992.
32. American Institute of Nutrition. Nomenclature policy: abbreviated designations of amino acids. *J Nutr* 1987;117:15.
33. American Institute of Nutrition. Nomenclature policy: generic descriptions and trivial names for vitamins and related compounds. *J Nutr* 1990;120:12–9.
34. Roig M. (St. Johns University). Avoiding plagiarism, self-plagiarism, and other questionable writing practices: guide to ethical writing [Internet]. Rockville (MD): US Department of Health & Human Services, Office of Research Integrity [updated 2013 May 15; cited 2014 Jan 17]. Available from http://ori.hhs.gov/avoiding-plagiarism- self-plagiarism-and-other-questionable-writing-practices- guide-ethical-writing.
35. The COPE Council, Editor. Code of Conduct for Journal Publishers [Internet]. [place unknown]: Committee on Publication Ethics; 1999 [updated 2011; cited 2017 May 10]. Available from: http://publicationethics.org/resources/code-conduct.
36. International Committee of Medical Journal Editors, editor. Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals: Scientific Misconduct, Expressions of Concern, and Retraction [Internet]. Philadelphia (PA): Annals of Internal Medicine / American College of Physicians; 1978 [2015 Dec; cited 2017 May 10]. Available from: http://www.icmje.org/recommendations/browse/publishing- and-editorial-issues/scientific-misconduct-expressions-of- concern-and-retraction.html.
37. *The Journal of Nutrition* [Internet]. Bethesda (MD): American Society for Nutrition; c 2009. Permission to Re- Publish Copyrighted Content in *The Journal of Nutrition*: Information about *The Journal*; 2009 Oct 2 [cited 2009 Oct 2]; [1 p.]; Available from https://nutrition.org/wp-content/uploads/2018/01/ifora_republish.pdf.
38. Lockhart, P and McCormack S. Table Checklist [Internet]. Waterbury (VT): Dartmouth Journal Services; 2011 Dec [updated 2015 May 20; cited 2015 Oct 14]. Available from https://nutrition.org/wp-content/uploads/2017/11/ASNTableChecklist.pdf.
39. Lockhart, P and Vanderberg M. Figure quality checklist [Internet]. Waterbury (VT): Dartmouth Journal Services; 2011 Jun [updated 2015 Jun 26; cited 2015 Oct 14]. Available from https://nutrition.org/wp-content/uploads/2017/11/ASNFigureChecklist.pdf.
40. Vanderberg, M. Preparing Digital Images for Publication [Internet]. Waterbury (VT): Dartmouth Journal Services; c2011 [updated 2012 Sep 27; cited 2011 Jun 23]. Available from https://nutrition.org/publications/preparing-digital-images-publication/.

Journal of Nutrition Production office
The Journal of Nutrition
Oxford University Press
2001 Evans Road, Suite 112
Cary, NC 27513
tel: (919) 677-0977 ext. 5289
email: jn@oup.com

Editorial office
The Journal of Nutrition
Children's Nutrition Research Center
Baylor College of Medicine

1100 Bates Street
Houston, TX 77030
tel: (713) 798-7169, fax: (713) 798-7171
email: jnutr@bcm.edu

Business office and ASN headquarters
American Society for Nutrition
9211 Corporate Blvd., Suite 300
Rockville, MD 20850
tel: (240) 428-3650, fax: (240) 404-6798
email: journal@nutrition.org

Quick Formatting List

1. Title page, including
   a. Title
   b. Author names and affiliations
   c. Corresponding author's contact information
   d. Last names listed as they should be indexed in PubMed
   e. Running title
   f. Footnotes to the title concerning
      i. Source of financial support
      ii. Conflict of interest statement
      iii. Online Supporting Material[1]
      iv. Abbreviation footnote[1]
2. Structured abstract with clinical trial registration[1,2]
3. Keywords for publication
4. Continuous line numbers
5. Page numbers
6. Text double spaced
7. Acknowledgments with an author contribution statement
8. Reference List
9. Response to reviewers[3]
10. Changes from the previous version highlighted in yellow[3]
11. Text source file must be a Word 6.0 or later document including
    a. tables[1]
    b. figure legends[1]
12. Each figure must be uploaded separately[3]

1. [1]If applicable
2. [2]Registration is required for all clinical trials that began after July 1, 2010.
3. [3]Revisions only