

The effect of omega-3 fatty acid status on lung inflammation in tuberculosis-infected mice

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Mini dissertation submitted in partial fulfilment of the requirements for the degree *Master of Science* in Dietetics at the North-West University

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Graduation: May 2021
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PREFACE

“Now all glory to God who is able with His mighty power at work within us, to accomplish infinitely more than we might ask or think.” Ephesians 3:20

I owe my deepest gratitude towards the following people who supported me through it all:

Firstly, thank you to my heavenly Father for providing me with an opportunity to be able to further my studies and for giving me the strength and determination I needed to see it through.

My loving husband Chris. Thank you for always supporting me and allowing me to study full time. Your love, motivation and support kept me going. Thank you for being my biggest supporter and taking over all the responsibilities at home to allow me time to finish what I started. You will forever be my person.

To my supervisor, Dr L. Malan. What a phenomenal woman to know, be inspired by and to work with. I am forever grateful to have had you as my mentor. You have taught me more than Google ever could. Thank you for the unconditional support and effort you gave to guide me in producing the best thesis that I can. Thank you for going above and beyond of what is expected of a supervisor.

To my co-supervisor, Mrs A. Nienaber. Thank you for all your effort to help me make sense of it all. I am forever grateful for all your support along the way even when you were busy with your own studies, you always made time.

Mom and Dad. Thank you for being my inspiration, for all the prayers and support and most of all, thank you for always believing in me and pushing me to be the best I can.

A special thanks to Dr Suraj Parihar and Dr Mumin Ozturk from the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town for all your help and hard work in executing the study and assisting with laboratory analyses.

Cecile Cook and Adriaan Jacobs, thank you for all the long hours and assistance in the laboratory.

Marlise Korff, one of the greatest friends a girl could ask for. Thank you for just being you, for always listening and supporting me until the very end. I am forever grateful for having you as a friend.

My best friend and sister, Melandri. Even though you could not understand when I talked about my work, thank you. Thank you for always listening and motivating me to finish when I did not want to. Also thank you for always fixing my documents when I messed it up and drawing my figures for this thesis. You are the best and I love you so much!

Lastly, thank you to the Nutricia Research Foundation for providing me with a scholarship enabling me to study full-time. I am forever grateful for this opportunity.

ABSTRACT

Background:

Tuberculosis (TB) bacilli successfully survive within the host. It causes an immune response that is defensive but also harmful to the host's lung tissue, because of perpetuating inflammation. Non-resolving inflammation in TB persists and can occur in both latent and active TB. Omega-3 polyunsaturated fatty acids (n-3 PUFA) have anti-inflammatory and inflammation-resolving activity due to their conversion to lipid mediators and influence on inflammatory cytokines.

Aim:

This project aimed to determine the effect of n-3 PUFA status prior to infection on lung inflammation and pathology in *Mycobacterium tuberculosis* (*Mtb*)-infected C3HeB/FeJ mice.

Methods:

Uninfected mice (n=20) were conditioned on either n-3 PUFA sufficient (n-3FAS) or deficient (n-3FAD) diets for six weeks and continued these diets post *Mtb* infection for an additional four weeks until euthanasia. Lung bacterial load, lung- and spleen-weight indexes and lung histology (free alveolar space), cytokines and lipid mediators were assessed.

Results:

The low n-3 PUFA status group (n-3FAD) had a trend to present with lower bacterial loads ($p=0.095$) and presented with lower spleen-weight indexes ($p=0.041$), more free alveolar space ($p<0.001$), lower pro-inflammatory lung cytokine concentrations (interleukin -1 β , $p=0.007$; interleukin-6, $p=0.003$; interferon-gamma, $p<0.001$), and a less pro-resolving lipid mediator profile compared with the n-3FAS group (a trend for lower prostaglandin (PG) E₃, $p=0.068$ and protectin D1, $p=0.087$; 18-hydroxyeicosapentaenoic acid, $p=0.006$ and 17-hydroxydocosahexaenoic acid, $p=0.028$; and higher PGE₂, $p=0.032$).

Conclusion:

Contrary to what we hypothesized, if no drugs were administered, a low n-3 PUFA status prior to TB infection resulted in lower lung bacterial loads, and spleen body weight index, with a higher percentage lung free alveolar space and lower pro-inflammatory lung cytokine concentrations despite having a less pro-resolving lipid mediator profile.

Key terms:

Lung inflammation, mouse model, omega-3 (n-3) polyunsaturated fatty acid (PUFA) status, tuberculosis (TB)

LIST OF ABBREVIATIONS

AA	arachidonic acid
ALA	α -linolenic acid
AIDS	acquired immunodeficiency syndrome
ANCOVA	analysis of covariance
CFU	colony-forming units
CEN	Centre of Excellence for Nutrition
CIDRI	Centre of Infectious Diseases Research in Africa
COX	cyclooxygenase
DAFF	Department of Agriculture, Forestry and Fisheries
DHA	docosahexaenoic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assays
EPA	eicosapentaenoic acid
FA	fatty acid
FAD	fatty acid deficient
FAS	fatty acid sufficient
GCMS	gas chromatography mass spectrometry

LIST OF ABBREVIATIONS (CONTINUED)

HDT	host-directed therapy
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
HIV	human immunodeficiency syndrome
ICU	intensive care unit
IDM	infectious diseases and molecular medicine
IFN	interferon
IFN- α	interferon-alpha
IFN- γ	interferon-gamma
IL	interleukin
IVC	individually ventilated cages
LA	linoleic acid
LCPUFA	long-chain polyunsaturated fatty acids
LM	lipid mediator
LOX	lipoxygenase
LTBI	latent tuberculosis infection
LTs	leukotrienes
LXs	lipoxins
MaR	maresins
MDR-TB	multi-drug resistant tuberculosis
<i>Mtb</i>	<i>Mycobacterium Tuberculosis</i>

LIST OF ABBREVIATIONS (CONTINUED)

NSAIDS	non-steroidal anti-inflammatory drugs
n-3	omega-3
n-6	omega-6
n-3FAD	omega-3 fatty acid deficient diet
n-3FAS	omega-3 fatty acid sufficient diet
NWU	North-West University
PBS	phosphate-buffered saline
PBMC	peripheral blood mononuclear cells
PD	protectins
PG	prostaglandin
PTB	pulmonary tuberculosis
PUFA	polyunsaturated fatty acids
RBC	red blood cells
Rvs	resolvins
SA	South Africa
SEM	standard error of the mean
SOP	standard operating procedure
SPM	specialised pro-resolving lipid mediators
SPSS	Statistical Programme for Social Sciences

LIST OF ABBREVIATIONS (CONTINUED)

TB	tuberculosis
Th	T helper
TNF- α	tumour necrosis factor – alpha
Tx	thromboxane
UCT	University of Cape Town
WBC	white blood cells
WHO	World Health Organization

LIST OF SYMBOLS AND UNITS

α	alpha
β	beta
CFU	colony-forming units
dL	decilitre
$^{\circ}\text{C}$	degrees Celsius
g	gram
g/kg	gram per kilogram
>	greater than/ above
Hz	Hertz
<	less/ lower than
μl	microlitre
mg	milligram
ml	millilitre
min	minutes
nm	nanometres
n	number
pg	picogram
%	percentage
RPM	revolutions per minute
sec	seconds

TABLE OF CONTENTS

PREFACE	I
ABSTRACT	III
LIST OF ABBREVIATIONS	IV
LIST OF ABBREVIATIONS (CONTINUED).....	V
LIST OF ABBREVIATIONS (CONTINUED).....	VI
LIST OF ABBREVIATIONS (CONTINUED).....	VII
LIST OF SYMBOLS AND UNITS.....	VIII
CHAPTER 1 INTRODUCTION.....	1
1.1 Background and motivation	1
1.2 Rationale for study	1
1.3 Research aim and objectives.....	3
1.3.1 Aim	3
1.3.2 Objectives.....	3
1.4 Structure of dissertation	4
1.5 Research outputs emanating from this study	4
1.6 Contributions of members of the research team.....	5
1.6.1 MSc student contribution towards study.....	5
BIBLIOGRAPHY	7
CHAPTER 2 LITERATURE REVIEW.....	11
2.1 Global tuberculosis disease burden	11
2.2 Pathogenesis and immune control of TB	13
2.3 Anti-inflammatory treatment in TB and the potential role of n-3 PUFA	16

2.3.1	Host-directed anti-inflammatory treatment	16
2.3.2	Anti-inflammatory properties of n-3 PUFA and lipid mediators	19
2.3.3	Research on the effects of n-3 PUFA status/supplementation and TB outcomes	25
2.4	Other host immune and inflammatory response markers	30
2.4.1	Lung cytokines.....	30
2.4.2	Lung histology	34
2.5	Animals	39
2.5.1	The C3HeB/FeJ species mouse TB model	39
2.6	Literature summary	40
BIBLIOGRAPHY		41
CHAPTER 3 RESEARCH METHODOLOGY		51
3.1	Study design, materials, and methods.....	51
3.1.1	Study design.....	51
3.1.2	The C3HeB/FeJ species mouse model.....	52
3.1.3	Facilities and setting	53
3.1.4	Housing and husbandry	53
3.1.5	Diet compositions	54
3.1.6	Tuberculosis strain and aerosol infection procedure	55
3.2	Data and sample collection.....	55
3.2.1	Euthanasian procedure.....	55
3.2.2	Blood sampling	55
3.2.3	Lung sampling	56

3.3	Laboratory analysis.....	57
3.3.1	Fatty acid analysis	57
3.3.2	Lung cytokine concentrations	58
3.3.3	Lung lipid mediators.....	59
3.3.4	Histological analysis (free alveolar space)	60
3.4	Statistical analysis.....	60
BIBLIOGRAPHY		61
CHAPTER 4 RESULTS		64
4.1	Baseline red blood cell phospholipid polyunsaturated fatty acid status and body weight prior to tuberculosis infection	64
4.2	Endpoint total phospholipid n-6 and n-3 polyunsaturated acid status.....	66
4.3	Progression of tuberculosis infection in mice (lung and spleen bacterial loads, lung, and spleen weight indexes)	68
4.4	Free alveolar space and histology	73
4.5	Markers of inflammation	75
4.5.1	Lung cytokine concentrations	75
4.5.2	Pro-resolving lipid mediators.....	78
4.5.3	Pro-inflammatory lipid mediators.....	78
CHAPTER 5 DISCUSSION		83
5.1	Introduction	83
5.2	Total phospholipid polyunsaturated fatty acid compositions in red blood cells and lung homogenates	84
5.3	Disease progression	86

5.3.1	Lung and spleen indexes and bacillary loads.....	86
5.4	Histology	87
5.5	Lung lipid mediator concentrations	88
5.6	Lung cytokine concentrations	89
5.7	Conclusion	90
	BIBLIOGRAPHY	92
	CHAPTER 6 CONCLUDING REMARKS	99
6.1	Aim and objectives	99
6.1.1	Research aim	99
6.1.2	Research objectives	99
6.2	Main findings arising from this research	99
6.2.1	The effect of n-3 PUFA status on lung and spleen bacterial loads, weight indexes and histology (disease progression)	100
6.2.2	Markers of inflammation	100
6.3	Research study limitations and recommendations.....	101
6.4	Future prospects	102
	BIBLIOGRAPHY	103
	ANNEXURES.....	105
6.5	ANNEXURE A: Ethical approval from the North-Wes University Ethics Committee.....	105
6.6	ANNEXURE B: Proofreading and language editing	107

LIST OF TABLES

Table 1-1.	Research team members and their contributions	5
Table 1-2.	Advisors for this research project	6
Table 2-2.	Different mechanisms of action and significance of HDTs	18
Table 2-7.	In vivo and in vitro studies conducted on the effect of omega-3 long chain polyunsaturated fatty acids on tuberculosis outcomes	27
Table 2-8	Summary of cytokine classification by immune response	32
Table 2-9	Summary of beneficial and damaging roles of cytokines involved in tuberculosis.....	33
Table 3-1.	Fatty acid composition of diets	54
Table 4-1	Baseline red blood cell total phospholipid polyunsaturated fatty acid status six weeks after preconditioning on n-3FAS and n-3FAD diets.....	65
Table 4-2	Endpoint red blood cell total phospholipid n-6 and n-3 polyunsaturated fatty acid status of Mycobacterium tuberculosis-infected mice after providing n-3FAS and n-3FAD diets for three weeks.....	67
Table 4-3	Endpoint lung total phospholipid n-6 and n-3 polyunsaturated fatty acid status of Mycobacterium tuberculosis-infected mice after providing n-3FAS and n-3FAD diets for three weeks	68
Table 4-4	Lung and spleen bacillary loads and weight indexes 4 weeks post infection of Mycobacterium tuberculosis-infected mice after providing n-3FAS and n-3FAD diets for three weeks	70
Table 4-5	Representative haematoxylin stained images of lungs of Mycobacterium tuberculosis-infected mice after providing n-3FAS and n-3FAD diets for three weeks.....	74
Table 4-6	Lung cytokine concentrations 4 weeks post infection of Mycobacterium tuberculosis-infected mice after providing n-3FAS and n-3FAD diets for three weeks.....	77

Table 4-7	Lipid mediator signalling in lung homogenates at 4 weeks post infection of Mycobacterium tuberculosis-infected mice after providing n-3FAS and n-3FAD diets for three weeks	79
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LIST OF FIGURES

Figure 2-1.	The 30 three high burden TB country lists and overlapping areas (Taken and adapted from (WHO, 2019:23))	13
Figure 2-2	Overview of the pathway of conversion of linoleic acid and alpha-linoleic acid LCPUFA (Taken and adapted from Calder (2017:3))	21
Figure 2-3.	Lipid mediator synthesis.....	23
Figure 2-4.	Immunomodulatory effects of SPMs during the inflammatory response (Taken and adapted from Fullerton et al. (2014:15)).....	24
Figure 2-5.	Lipid mediator class switching in exudates in the resolution of acute inflammation (Taken and adapted from Serhan et al. (2015:3)).....	25
Figure 2-6	Histology images of Mycobacterium Tuberculosis infected human lungs (Taken from Lenaerts et al. (2015:290)).....	36
Figure 2-7	Development of lung lesions histology images of a murine model of active tuberculosis over time (Taken from Irwin et al. (2015:593)).....	37
Figure 2-8	Difference between three lesion types with regards to cellular structure organization and collagen containment (Taken from Irwin <i>et al.</i> (2015:594))	38
Figure 3-1:	Experimental study design	52
Figure 3-2	Lung lobe sampling	57
Figure 4-1	Lung- (a) and Spleen- (b) weight-indexes four weeks post-infection after providing n-3FAS and n-3FAD diets for three weeks ^{1,2}	71
Figure 4-2	Lung- (a) and Spleen- (b) bacillary loads 4 weeks post infection after providing n-3FAS and n-3FAD diets for three weeks ^{1,2}	72
Figure 4-3	Free alveolar space 4 weeks post infection after providing n-3FAS and n-3FAD diets for three weeks ^{1,2}	73

Figure 4-4	Lung cytokine concentrations of (a)-IL1a; (b)- IL6; (c)-IL1β; (d)- IFNγ and (e)- IL-17 4 weeks post infection after providing n-3FAS and n-3FAD diets for three weeks ^{1,2}	76
Figure 4-5	Pro-resolving lipid mediators (a)-5-HEPE; (b)- 9-HEPE; (c)-I11-HEPE; (d)- PGE ₃ and (e)- 17-HDHA between n-3FAS and n-3FAD groups 4 weeks post infection after providing n-3FAS and n-3FAD diets for three weeks ^{1,2}	81
Figure 4-6	Pro-inflammatory lipid mediators (a)-8-HETE; (b)- 11-HETE; (c)-PGE2 and (d)- PGD2 between n-3FAS and n-3FAD groups 4 weeks post infection after providing n-3FAS and n-3FAD diets for three weeks ^{1,2}	82

CHAPTER 1 INTRODUCTION

1.1 Background and motivation

Tuberculosis (TB) is initiated by the causative agent *Mycobacterium tuberculosis* (*Mtb*). It affects mainly the lungs (pulmonary tuberculosis) and is a chronic infectious disease that continues to be a major public health problem (Marakalala *et al.*, 2018:1; WHO, 2019:1). Tuberculosis has been referred to as “the white plague”, “consumption”, “wasting disease” or “phthisis”, stressing the multifaceted pathophysiology of the disease (Dorhoi & Kaufmann, 2016b:154). The contagious nature, complicated immunological response, and progression of the disease lead to long-term treatment consequences (Barberis *et al.*, 2017:9). The bacteria are transmitted *via* the inhalation of air droplets, usually when an infected individual cough (Glaziou *et al.*, 2018:272). Depending on whether patients exhibit typical clinical TB symptoms, they are classified with either latent or active TB (Kroesen *et al.*, 2018:2).

Individuals presenting with latent TB infection (LTBI) contain but do not destroy the TB bacilli, therefore acting as a reservoir for active TB (Kaufmann & Dorhoi, 2013:441). According to the WHO (2018:2), nearly 1.7 billion individuals, or 23% of the world’s population, have LTBI and are at risk of developing active TB throughout their lives. This containment of LTBI is mainly due to a controlled inflammatory response (Kaufmann & Dorhoi, 2013:441). Once a disbalance arises between the LTBI and the host immune system, the bacilli become both active and replicative, increasing the bacillary load, thereby leading to active TB and the spread of the disease (Kaufmann & Dorhoi, 2013:441).

According to the latest World Health Organization (WHO) global TB report, an approximate 1.2 million TB deaths occurred among HIV-negative and 251000 among HIV-positive individuals during 2017 (WHO, 2019:45). Moreover, South Africa currently remains amongst the WHO list of the thirty countries with the highest TB burden, accounting for 85% of cases worldwide (WHO, 2019:47).

1.2 Rationale for study

TB bacilli are successful in adjusting to survive within the host. Even though the host’s immune response is protective, it can also become harmful to the infected tissue because of perpetuating inflammation (Chai *et al.*, 2018:1; Hunter, 2018:1; Kroesen *et al.*, 2018:1). Non-resolving inflammation in TB persists and can occur in both LTBI and active TB (Kaufmann & Dorhoi, 2013:441). Both the host and the bacilli closely regulate this inflammatory response (Sasindran & Torrelles, 2011:1), but there are damaging implications in individuals with active TB once the

host's response is both excessive and unproductive, as it generally results in lung damage (Calder, 2017:7; Kroesen *et al.*, 2018:1).

Diagnosis and effective treatment of individuals with TB prevent millions of deaths annually; however, there are still substantial gaps in detection and successful treatment (WHO, 2019:45). Even though the disease burden of TB is declining worldwide by 2% per year across all WHO regions, it is not declining fast enough to reach the primary 2020 milestones of the End TB Strategy in time (WHO, 2019:2). Additionally, the lack of effective vaccines, prolonged treatment, prevalence of HIV co-infection and the presence of multi-drug resistant (MDR) strains aggravate the situation (WHO, 2018:67). The extensive duration of current TB treatment regimens highlights the importance of scrutinizing additional research for host-directed treatment (HDT) that modifies inflammation and may shorten treatment duration (Kaufmann & Dorhoi, 2013:1549; Kim & Yang, 2017:1549; Kolloli & Subbian, 2017:171).

The host immune response, manipulated by the pathogen, causes damage to lung tissue (Dorhoi & Kaufmann, 2016a). Unfortunately, fissures, scarring or pleural adhesions occur in 14 to 100% of patients, leading to impaired lung function and poor quality of life even after TB cure (De La Mora *et al.*, 2015; Manji *et al.*, 2016; Meghji *et al.*, 2016; Nihues *et al.*, 2015; Theegarten *et al.*, 2006). Our immune systems are equipped with various immune cells, categorised into either innate or adaptive immune response cells (Gutiérrez *et al.*, 2019:1), the primary goal being to protect the host from the invading pathogens, to eradicate these pathogens and also to dispose of damaged tissue (Wu *et al.*, 2019:1).

It is well known that certain nutritional deficiencies can negatively impact immune function as the immune system relies on sufficient nutrients for proper functioning (Gutiérrez *et al.*, 2019:1; Wu *et al.*, 2019:1). A few of these nutrients include vitamin D, vitamin E, zinc and n-3 polyunsaturated fatty acids (PUFAs) (Wu *et al.*, 2019:1). Not only is n-3 PUFA energy dense, it also mediates the control of certain cell functions (Wu *et al.*, 2019:7). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the two main n-3 long-chain (LC)PUFAs. Although humans and animals can produce n-3 LCPUFAs from the shorter-chain essential n-3 PUFA, alpha-linolenic acid (ALA), originating from some plants, they are found mostly in seafood, specifically in oily fish (Calder, 2017:7). Daily intake of these n-3 LCPUFAs changes the structure of blood and tissue membrane phospholipid fatty acids where they serve as precursors for the production of pro-resolving lipid mediators, which control the resolution of inflammation and thereby affect immune response (Browning *et al.*, 2012:748; Calder, 2014:2; Calder, 2017:7; Rees *et al.*, 2006:340). As a result, n-3 LCPUFA status has a therapeutic effect in various chronic inflammatory diseases. Given that lung tissue also locally produces lipid mediators in conjunction with recruited immune cells, the composition of lung and immune cell FA is essential for modulatory effects of the local

immune response and inflammation in pulmonary TB (Calder, 2014:10; Kiss *et al.*, 2010:1088; Sanak, 2016:484).

A global dietary intake review estimates that less than 20% of the world's population consumes the recommended ≥ 250 mg/day of n-3 LCPUFA, leading to very low n-3 LCPUFA blood levels ($\leq 4\%$) in North America, Central and South America, Europe, the Middle East, Southeast Asia, and Africa (Stark *et al.* (2016:133). The n-3 LCPUFA status is therefore often low or very low in many populations and communities where there is also a high burden of TB, like South Africa (Berquin *et al.*, 2008:271; Calder, 2010:365; Calder *et al.*, 2018:13; Ford *et al.*, 2016:2; Husson *et al.*, 2016:524; Martin & Stapleton, 2010:540; McMurray *et al.*, 2011:628; Molfino *et al.*, 2017:2).

Keeping the pro-resolving benefits of n-3 LCPUFA and the inflammatory pathology of TB in mind, it would be expected that a sufficient n-3 LCPUFA status may be beneficial in TB patients because of the inflammatory damage caused by the disease. The effect of supplementation with n-3 LCPUFA before TB infection compared with having a sufficient status has been investigated previously in animal models (Bonilla *et al.*, 2010:399, McFarland *et al.*, 2008:2123; Paul *et al.*, 1997:623). However, there is no data comparing sufficient and poor n-3 LCPUFA status. This investigation is important, because low n-3 LCPUFA status exists in populations where TB is prevalent, making this scenario important to investigate.

1.3 Research aim and objectives

1.3.1 Aim

The aim of this project was to determine the effect of n-3 LCPUFA status prior to infection on lung inflammation and pathology in *Mtb*-infected C3HeB/FeJ mice.

1.3.2 Objectives

To compare the effects of a low versus a sufficient n-3 PUFA status in *Mtb*-infected C3HeB/FeJ mice on:

1. clinical outcomes, i.e. lung and spleen bacterial load and weight indexes, and
2. lung inflammation using lung lipid mediators and cytokine concentrations and percentage free alveolar space.

1.4 Structure of dissertation

This mini-dissertation is presented in chapter format, comprising five chapters, written according to the guidelines for postgraduate students at the North-West University (NWU). At the end of each chapter a reference list is provided, according to the NWU Harvard referencing style.

Chapter 1 serves as the introductory chapter and provides the background and rationale for the research question. This chapter also states the study aim and objectives and describes the structure of the dissertation, the envisaged research outputs and lastly, the contributions of the members of the research team.

Chapter 2 provides a literature review, outlining the global disease burden of TB, as well as the specific pathogenesis and immune control of the TB bacilli. Furthermore, current anti-inflammatory treatment regimens in TB and the potential role of n-3 PUFA and its status is also discussed in the literature review. Lastly, animal models in TB research are reviewed, focussing specifically on the C3HeB/FeJ mouse model.

Chapter 3 serves as the research methodology chapter and presents all methods used during this study. This includes animals and housing, the aerosol infection method and diet compositions, as well as sample collections and laboratory analyses of these. In addition, the statistical analysis used to achieve the study objectives is discussed.

The results of the study obtained from the methods used in Chapter 3 are discussed in Chapter 4, in accordance with the study objectives.

The results from Chapter 4 are discussed in Chapter 5. In this chapter, the effect of n-3 PUFA status on lung inflammation and pathology in TB-infected mice is discussed.

Chapter 6 serves as the closing chapter. This chapter consists of a summary of all the main findings of this study and how the objectives were reached. It includes limitations of the study, practical recommendations, and suggestions for future research. This chapter concludes the mini-dissertation.

1.5 Research outputs emanating from this study

The results of this MSc project will form part of an article submitted to *Mucosal Immunology* and will be presented at a national scientific conference. The fundamental purpose of this research is to translate the findings to the human situation. This animal research study assisted in the understanding of the underlying mechanisms that could not be measured in humans. It contributes to the understanding of how n-3 PUFA status affects lung inflammation and pathology in TB-infected mice and the main findings will help in the planning of a human clinical trial.

1.6 Contributions of members of the research team

The contributions of the research team are presented in Table 1-1. Supervisors during the course of the research project are presented in Table 1-2. No members of the research team had any conflicts of interest.

1.6.1 MSc student contribution towards study

Table 1-1. Research team members and their contributions

Team member	Qualification and professional registration	Role and responsibilities	Institution
Prof L Malan	<i>PhD Nutrition</i> Medical Biological Scientist, HPCSA	Principal investigator/ Project head Supervisor for MSc student	CEN, NWU
Dr A Nienaber	<i>MSc Dietetics</i> Dietitian, HPCSA	Co-supervisor for MSc student Project coordinator	CEN, NWU
Mrs S King	<i>BSc, Dietetics</i> Dietitian, HPCSA	MSc student: execution of the study, sample collection, bacillary load analysis, histology and LM analysis in own and main study, statistical analysis, reporting of findings and writing of the dissertation.	CEN, NWU
Dr S Parihar	PhD, Immunology	Expert in mouse models in tuberculosis and laboratory analysis	CIDRI-Africa, IDM, UCT

CEN: Centre of Excellence for Nutrition; NWU: North-West University; IDM: Institute of Infectious Diseases and Molecular Medicine, Division of Immunology; UCT: University of Cape Town; CIDRI-Africa: Wellcome Centre of Infectious Diseases Research in Africa

Table 1-2. Advisors for this research project

Supervisors	Role and responsibilities	Institution
Mr Rodney Lucas	Laboratory Animal technologist	UCT, Centre for Animal Research Excellence, South Africa
Mrs D Beukes-Maasdorp	Manager and supervisor, Centre for Human Metabolomics, Biochemistry, NWU, Potchefstroom Campus.	Centre for Human Metabolomics, Biochemistry, NWU

CEN: Centre of Excellence for Nutrition; NWU: North-West University; IDM: Institute of Infectious Diseases and Molecular Medicine, Division of Immunology; UCT: University of Cape Town; CIDRI-Africa: Wellcome Centre of Infectious Diseases Research in Africa

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CHAPTER 2 LITERATURE REVIEW

2.1 Global tuberculosis disease burden

Tuberculosis (TB) continues to be a major public health problem with approximately 10 million new incident cases reported to the World Health Organization (WHO) globally during 2018. Therefore, for most countries, the end of the TB epidemic is still not a reality (WHO, 2019:1). Tuberculosis is a highly infectious disease caused by the slow-growing acid-fast bacillus *Mycobacterium tuberculosis* (*Mtb*), *Mycobacterium Bovis*, *Mycobacterium Africanum*, *Mycobacterium Microti* and *Mycobacterium Canetti* (DOH, 2014:8; WHO, 2019:1). Of these, *Mtb* is the most common cause of TB and is spread between individuals via the airborne route by droplet nuclei (DOH, 2014:8).

South Africa (SA) remains on the WHO's list of the 30 high TB burden countries which accounts for 87% of the world's cases (WHO, 2019:3). Although TB is curable, it is one of the communicable diseases that still contributes significantly to the global disease burden as it is one of the top ten causes of mortality (Akeju *et al.*, 2017:259; WHO, 2019:1). While there are proper diagnosis and treatment available to most individuals developing TB, it is evident that TB is the primary cause of death from a sole infectious agent, ranking above human immunodeficiency syndrome / acquired immunodeficiency syndrome (HIV/AIDS) (WHO, 2019:1). The latter is also true in SA, with high TB death rates in individuals living with HIV (Mzabe & Loveday, 2019:167).

Throughout 2018 there were an estimated 1.2 million deaths amongst HIV-negative individuals and a further 251 000 deaths amongst HIV-positive individuals compared to the 300 000 HIV-positive deaths in 2017 (WHO, 2019:1). Human immunodeficiency syndrome, together with secondary opportunistic infections, are currently a tremendous burden in SA, with TB being the most common opportunistic infection (Mahtab & Coetzee, 2017:428). Since HIV-positive individuals present with suppressed immunities, it is estimated that 50-60% of these patients infected with TB will go on to develop active disease (DOH, 2014:8). In addition, drug-resistant TB contributed to the global public health problem as 3.5% of the cases reported in 2017 and 18% of previously treated cases presented with multi-drug resistant TB (MDR-TB) (WHO, 2018:1; WHO, 2019:2). The 2018 WHO Global TB report also stated that the treatment success of individuals presenting with MDR-TB worldwide, was a meagre 55%.

Diagnosis and effective treatment of individuals with TB prevent millions of deaths annually; however, there are still substantial and constant gaps in detection and treatment. Most of the identified gaps were in the African region where the burden of HIV and TB co-infection is the greatest (WHO, 2018:2). Tuberculosis has harmful health and economic effects, specifically in

underdeveloped countries. Additionally, the lack of effective vaccines, prolonged treatment, prevalence of HIV co-infection and the presence of MDR strains aggravate the situation (Vilaplana *et al.*, 2013:199).

Even though the disease burden of TB is declining across all WHO regions and in the majority of countries by 2% per year worldwide, it is not declining fast enough to reach the primary 2020 milestones of the End TB Strategy in time (WHO, 2018:2). The WHO recently defined three new high burden country lists, i.e., one for TB, one for MDR-TB and one for HIV associated TB. These lists each consist of 30 countries and accounts for approximately 90% of the global TB burden. These three lists are visually represented in Figure 2-1 where an overlap is shown across all three lists. South Africa is one of the countries that appear on all three lists (WHO, 2019:24). Nevertheless, as incidence and mortality rates rapidly decline in the African regions (4.1% and 5.6% respectively), South Africa is on the right track in achieving the 2020 END TB milestone (WHO, 2019:3).

With the latter in mind, the most effective TB treatment to decrease mortality rates, medical expenses and improve the quality of life of TB survivors, is imperative. To achieve better outcomes and effective treatment of TB disease, the disease pathophysiology as well as the underlying mechanisms must be well understood.

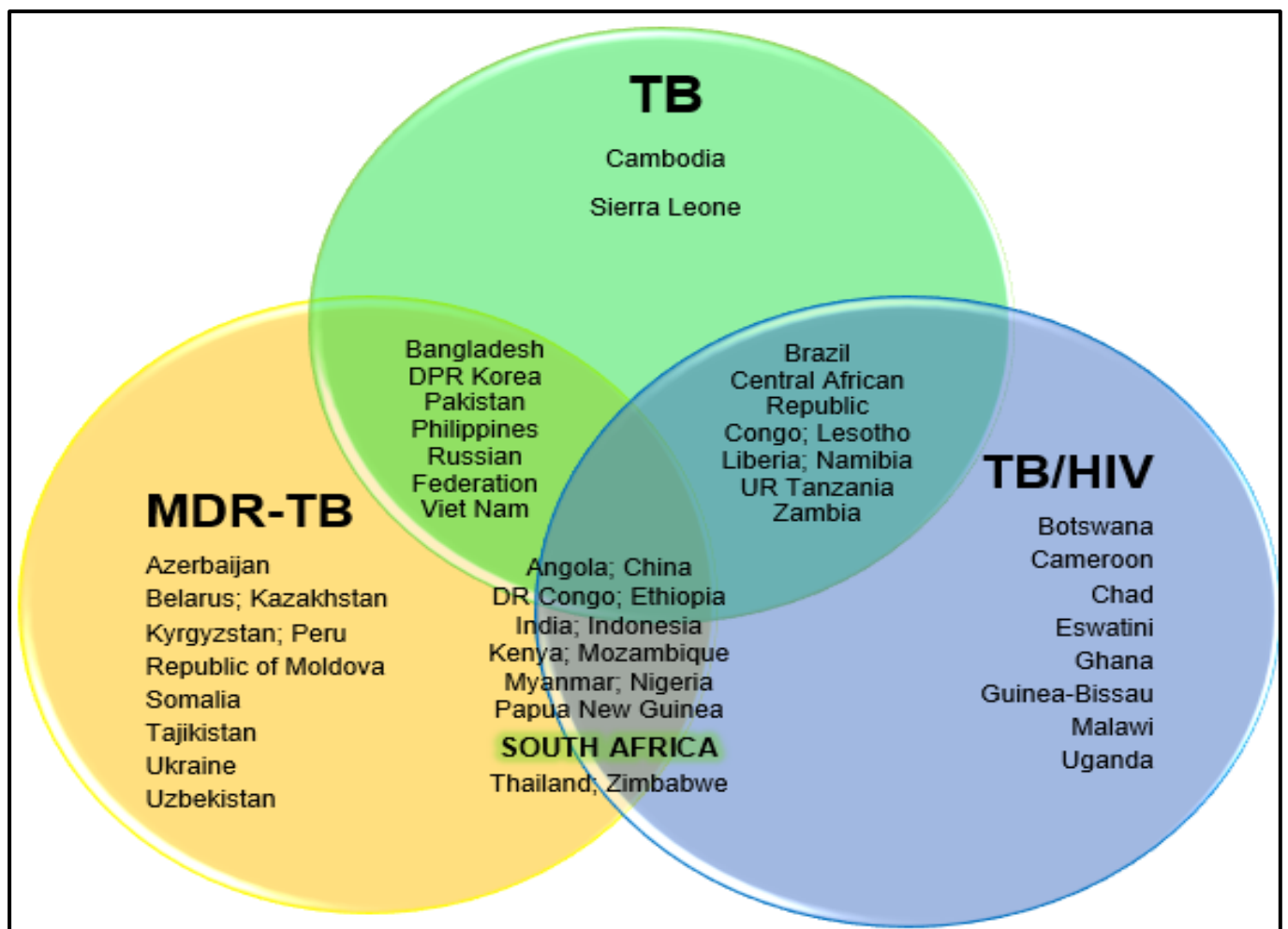


Figure 2-1. The 30 three high burden TB country lists and overlapping areas (Taken and adapted from (WHO, 2019:23))

2.2 Pathogenesis and immune control of TB

As outlined above, *Mtb* is a slow-growing acid-fast bacillus transferred between individuals in droplet nuclei via the airborne route (DOH, 2014:8). *Mycobacteria* is extremely contagious and small enough to spread to the alveolar spaces in the lungs, where the bacteria will replicate and initiate an inflammatory response. These droplets transporting live *Mtb* can stay airborne in any dark, poorly ventilated, and enclosed space for up to four hours, but direct sunlight rapidly eradicates the bacilli (DOH, 2014:8).

Tuberculosis is mainly a disease of the lung, known as pulmonary TB, however, the systemic spread of *Mtb* to other organs such as the bone, lymph nodes and meninges can lead to other forms of extra-pulmonary or disseminated TB (Cooper, 2009:393; DOH, 2014:13; O'Garra *et al.*, 2013:476; WHO, 2019:1). The development of active TB disease is the outcome of ecological,

host and pathogen interactions, and well-known risk factors include HIV coinfection, immunodeficiency, diabetes mellitus, overpopulation, malnutrition, and poverty (O'Garra *et al.*, 2013:477).

After an individual has been exposed to TB, the bacteria can be latent, commonly referred to as inactive TB or primary infection. During this period the infected person is often asymptomatic, with no clinical sign of disease and cannot spread TB to others (O'Garra *et al.*, 2013:478). It is believed that the latent stage is preserved by an active immune response in the host which is initiated by the *Mtb* bacilli infection (O'Garra *et al.*, 2013:478). Nonetheless, 5 - 10% of people infected with latent TB are at risk for developing active TB throughout their lifetime, but, as mentioned earlier, this number can increase to 50% in those with severely suppressed immunities such as HIV coinfection (DOH, 2014:8; O'Garra *et al.*, 2013:477; Sinclair *et al.*, 2011:7; WHO, 2019:3).

According to O'Garra *et al.* (2013:477); Robinson *et al.* (2015:47) exposed individuals can be classified into three groups: 1) Asymptomatic individuals, due to the bacilli failing to create an infection; 2) Active TB individuals with strong cell-mediated immunities without developing TB and lastly; 3) a minor group of individuals also with active TB, but who goes on to develop clinical TB. The common phases in the pathology of TB infection include spontaneous suppression, asymptomatic latent TB and quick developing primary TB or reactivation of TB diseases when inactive bacilli start to replicate (Apt & Kramnik, 2009:195; DOH, 2014:10).

The bacilli are extremely inflammatory and can recruit immune cells into the lower respiratory tract (Robinson *et al.*, 2015:13). Experimental models revealed that the initial and most important host response to *Mtb* infection contains an inflow of phagocytic cells including predominantly local alveolar macrophages and recruited neutrophils (Kiran *et al.*, 2016:169; O'Garra *et al.*, 2013:487). Because *Mtb* infection forms rapidly within the lung, the adaptive response is too slow to identify that infection has ensued, and by the time the specific T cells reach the site of infection, phagocytes control the lung lesion and restrict the ability of the T cell to persist or function (Cooper, 2009:393; Robinson *et al.*, 2015:47). Even though macrophages use a variety of approaches to combat *Mtb*, the bacilli developed extensive matching resistance mechanisms, to help prevent destruction and survive to multiply within the macrophages (Denisenko *et al.*, 2019:1).

Four phases, based on experimental models, are well defined in the pathogenesis of Pulmonary TB (van Crevel *et al.*, 2002:294; Zuniga *et al.*, 2012:2).

- 1) Inhalation of *Mtb*.
- 2) Inflammatory cell recruitment.
- 3) Control of Mycobacteria proliferation; and
- 4) Post-primary TB.

Following inhalation and establishment of *Mtb* in the airways and lung parenchyma, the bacilli gain access to alveoli and undergo a few events, involving phagocytosis by phagocytic antigen-presenting cells including the macrophages within the alveolar spaces and the uptake by neutrophils as well as dendritic cells (DCs) (Ahmad, 2011:3; Bozzano *et al.*, 2014:3; Kiran *et al.*, 2016:169; O'Garra *et al.*, 2013:487; Smith, 2003:467; Walzl *et al.*, 2011:344; Zuniga *et al.*, 2012:2). Frequently, instant bacterial killing by diverse macrophage bactericidal mechanisms, including the generation of toxic reactive oxygen intermediates and reactive nitrogen intermediates, follows the inhalation of *Mtb* (Zuniga *et al.*, 2012:2). The effectiveness of the above-mentioned mechanism to kill the bacilli is reliant on the pathogenic features of the inhaled *Mtb* strain, the inherent microbicidal volume of the alveolar macrophages and the inflammatory microenvironment at the infection site (Denisenko *et al.*, 2019:3; Zuniga *et al.*, 2012:2).

The *Mtb* bacilli are identified by host receptors, including Toll-Like receptors (TLRs) which are expressed on both immune cells (macrophages, DCs and specific T cells), and non-immune cells (fibroblasts and epithelial cells) (Hawn *et al.*, 2013:609). The contact of *Mtb* with the TLR starts an intracellular signalling cascade that ends in a pro-inflammatory response, which is advantageous to the host. Conversely, the bacilli also have advanced strategies to activate signals that diminish or control the innate immune response which is again advantageous to the pathogen (Ahmad, 2011:3).

After phase 1 engulfment, the bacilli that survived can now grow and replicate inside the alveolar macrophages and DCs, which stimulates the production of immune mediators such as; tumour necrosis factor-alpha (TNF- α), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), interleukin-1 alpha (IL-1 α), interleukin-1 beta (IL-1 β) and chemokines, chemokine [C-C motif] ligand 2 [CCL2], CCL3, CCL5, chemokine [C-X-C motif] ligand 8 [CXCL8] and CXCL10. This triggers the macrophages to induce quick killing of the bacilli, but also initiates lung damage, thereby enabling transmission (DOH, 2014:13; Hawn *et al.*, 2013:609; Jordao *et al.*, 2008:1379; Kaufmann & Dorhoi, 2013:441; Kiran *et al.*, 2016:169; Zuniga *et al.*, 2012:2). These cytokines mediate both inflammatory and immune reactions. Classical cytokines produced during *Mtb* infection, includes IL-2, IFN- γ , IL-6, IL1 α/β , IL-12 and TNF- α (Zuniga *et al.*, 2012:5). Although the monocytes, neutrophils and lymphocytes travel to the main site of infection, they are incapable to efficiently kill the bacilli (Ahmad, 2011:4).

Phase 3 involves the inhibition of *Mtb* replication with efficient cell-to-cell interaction and the formation of a granuloma. The granuloma essentially forms a wall of cells containing bacteria to prevent spreading. Due to the prolonged cytokine stimulation, macrophages separate into epithelioid cells and become giant merged cells (Zuniga *et al.*, 2012:2). Additional distinctive features of progressive TB disease, apart from the granuloma development, are the formation of lesions with significant necrosis and cavitation (Kiran *et al.*, 2016:169).

The structural design of the granuloma is recognised by the accumulation of T cells and infected macrophages, which engulf and contain the *Mtb*, to inhibit their transmission (Zuniga *et al.*, 2012:2). This granuloma or structured aggregate of developed macrophages is enclosed by fibroblasts, combined with neutrophils, DCs, natural killer cells, B cells, CD4⁺ and CD8⁺ T cells and is hypoxic (Ahmad, 2011:4; Bozzano *et al.*, 2014:3). The CD4⁺ T cells producing IFN- γ detects the infected macrophages with antigens from *Mtb* and eradicate them (Ahmad, 2011:4).

The abovementioned mechanisms lead to the progression of a contained primary TB infection which may ultimately become a dormant infection, and an active series of cellular initiation and suppression averts the reproduction and transmission of the *Mtb* bacilli (Zuniga *et al.*, 2012:2). The final phase, TB reactivation, occurs as a result of *Mtb* perseverance related to a suppressed immunity, where the dormant disease may be reactivated, initiating damage to the bronchi and preparing for the transmission of the *Mtb* to other parts of the lungs (Zuniga *et al.*, 2012:2).

These four phases of *Mtb* infection are characterised by the capability of the host's innate and adaptive immune systems to either eliminate or regulate *Mtb* (Walzl *et al.*, 2011:344). Defensive immunity against *Mtb* is complex and not entirely understood but depends on a widespread range of both innate and adaptive immune responses (O'Garra *et al.*, 2013:476; Walzl *et al.*, 2011:345). Improved understanding of TB pathogenesis will aid in the development of adjunctive treatment, which will undeniably be beneficial to individuals and curb the burden of *Mtb* globally (Bozzano *et al.*, 2014:8; Smith, 2003:489; van Crevel *et al.*, 2002:304; Zuniga *et al.*, 2012:11).

2.3 Anti-inflammatory treatment in TB and the potential role of n-3 PUFA

2.3.1 Host-directed anti-inflammatory treatment

Current barriers involved in the successful therapy of TB is not only limited to the time-consuming period of treatment, but also include the interaction with HIV drugs, possible drug toxicity or intolerance and the increasing incidences of drug resistance (Hawn *et al.*, 2013:608; Kroesen *et al.*, 2017:1; Stek *et al.*, 2018:2; Zumla *et al.*, 2013:389; Zumla *et al.*, 2014:327). This causes

concerns with regards to adherence (Hawn *et al.*, 2013:608; Zumla *et al.*, 2014:328). The extensive duration of current TB treatment protocols highlights the importance that additional research for treatments that modify inflammatory mediators and may shorten treatment duration should be scrutinized (Kaufmann & Dorhoi, 2013:445; Kim & Yang, 2017:1549; Kolloli & Subbian, 2017:171).

Hawn *et al.* (2013:608) reviewed the existing literature and revealed that supplementing existing anti-TB treatment with immune-enhancing host modulators may accelerate the eradication of *Mtb* infection, and reduce treatment duration, lung tissue injury caused by the disease and the risk of relapsing. To achieve the latter, a range of immunotherapies and host-directed therapies (HDTs) are currently being developed. Host-directed therapies, a new adjunct TB treatment, is a term used to describe treatment possibilities with small particles or biological agents (Palucci & Delogu, 2018:173). These compounds enhance the host's protective immunity and or control excessive inflammation through manipulating the body's reaction to the TB bacilli, instead of targeting the bacilli itself (Stek *et al.*, 2018:2). Host-directed therapies consist of two key strategies: 1) clearing pathogens by improving the antimicrobial actions and 2) prevent irreversible lung tissue damage by regulating excessive inflammation (Kim & Yang, 2017:1553).

As mentioned before HDTs can be anticipated to lessen the microbial burden and improve the host inflammatory response while only being required in small quantities for a short period, and it may be used together with current TB drugs to improve overall outcomes (Kim & Yang, 2017:1553). Thus, the aim is not to replace any current TB treatment but rather to enhance recovery by adjunctive therapy (Kaufmann *et al.*, 2017:36).

Several HDT compounds targeting the host in reducing inflammation have been studied with effective outcomes, including Vitamin D, aspirin, ibuprofen and corticosteroids (Byrne *et al.*, 2007:315; Kroesen *et al.*, 2017:772; Palucci & Delogu, 2018:176; Vilaplana *et al.*, 2013:203). Vitamin D plays a vital role in controlling and eliminating the *Mtb* infection through 25-hydroxyvitamin D receptor (VDR) activation, found on all immune cells (Salahuddin *et al.*, 2013:2). According to Palucci and Delogu (2018:176), children and adults presenting with a vitamin D₃ (cholecalciferol) deficiency, are at increased risk for developing TB. The interest in vitamin D as an HDT arose from historical concepts that showed that vitamin D, either as sunlight exposure or cod liver oil, provided beneficial outcomes, however, studies report different results (Hawn *et al.*, 2013:612; Martineau *et al.*, 2007:794; Tiberi *et al.*, 2018:12). While some studies showed activation of the host immune response, improved clinical as well as radiographic outcomes, and enhanced sputum conversion time, others failed to prove this (Martineau *et al.*, 2011:2; Salahuddin *et al.*, 2013:10).

With regards to non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, low-dose aspirin and diclofenac, various preclinical research studies showed a substantial reduction in the size and quantity of lung lesions, a decline in the bacillary load and improved survival compared to the control group when administering aspirin or ibuprofen (Byrne *et al.*, 2007:315; Kroesen *et al.*, 2017:772; Vilaplana *et al.*, 2013:199). See Table 2-1 for a summary of the mechanisms of action of certain HDTs discussed above.

Table 2-1. Different mechanisms of action and significance of HDTs*

HDT compound	Mechanisms	Clinical significance	Study reference
Vitamin D3	Promotes the production of reactive oxygen and nitrogen intermediates, cAMP and DEFB4	Activates innate immune response	Liu <i>et al.</i> (2009); Liu <i>et al.</i> (2007); Verway <i>et al.</i> (2013)
	Increasing the expression of ATGs (ATG5 & Beclin-1)	Initiates autophagy of diseased cells	Campbell and Spector (2012)
	Suppresses NF-κB signalling pathways Expression of MMPs pro-inflammatory cytokines and chemokines	Increases inflammatory resolution during treatment	Coussens <i>et al.</i> (2009); Coussens <i>et al.</i> (2012); Song <i>et al.</i> (2013)
	Suppressing MHC class II molecules and reduces CD4 T cell initiation Suppresses production of cytotoxic cells and augment differentiation of regulatory T cells	Decreases inflammation and lung tissue damage triggered by aggravated production of cytotoxic particles	(Baeke <i>et al.</i> , 2011; Harishankar <i>et al.</i> , 2016; Imazeki <i>et al.</i> , 2006; Jeffery <i>et al.</i> , 2009)
Ibuprofen (NSAID)	Obstructs COX1 and COX2 pathways and suppresses prostaglandin E2 production and increases TNF production in macrophages	Controls TNF-α production and decreases inflammatory response	Vilaplana <i>et al.</i> (2013)

HDT compound	Mechanisms	Clinical significance	Study reference
Aspirin (NSAID)	Enhances production of Leukotrienes A ₄ (LXA ₄)	Activates anti-mycobacterial activities mediated by Vitamin- D	*
Statins (Simvastatin)	Downregulation of the production of pro-inflammatory cytokines	Suppresses tissue damage and inflammation	*

* Kolloli and Subbian (2017:4)

cAMP: cathelicidin antimicrobial peptide, DEFB4: beta-defensin-4, ATG: autophagy-related proteins, NF-κB: nuclear factor-kappa, MMPs: matrix metalloproteinases, MHC: major histocompatibility complex, COX: cyclooxygenase, TNF: tumour necrosis factor

2.3.2 Anti-inflammatory properties of n-3 PUFA and lipid mediators

Serhan (2017:1274), reported strong evidence of the active biochemical pathways involved in inflammation resolution. It is also well known that chronic inflammation is a fundamental characteristic of many illnesses (Serhan, 2014:1). To achieve homeostasis of tissues and a defensive host response, resolution of the acute inflammatory response is crucial (Serhan *et al.*, 2015:1). Omega-3 (n-3) lipids and these emulsions, are known to influence biochemical signalling pathways, consequently altering the immune reactions (Mayer & Seeger, 2008:121). Furthermore, n-3 long-chain polyunsaturated fatty acids (LCPUFA) are acknowledged for the fact that they can alter leukocyte activity, modify lipid mediator (LM) production, and ultimately regulate the release of cytokines (Mayer & Seeger, 2008:121)

Lipids play an important role in our daily nutrition intake by means of providing essential fatty acids, being a concentrated form of energy and serving as the building blocks for cell membranes (Calder *et al.*, 2018:1). The omega 6 (n-6) PUFA linoleic acid (LA) and the n-3 PUFA α-linolenic acid (ALA) are essential nutrients and cannot be produced in the human body (Calder *et al.*, 2018:2). These fatty acids are converted to longer chain metabolites which have important biological functions in the body. Linoleic acid is the metabolic predecessor of arachidonic acid (AA) while ALA is the metabolic predecessor for eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) (Calder *et al.*, 2018:2). See Figure 2-2 for a summary of the elongation and desaturation of LA and ALA to the longer-chain (LC) n-6 and n-3 PUFA metabolites. It is clear

from this metabolic pathway that there is competition among the n-6 and n-3 LCPUFA families for the enzymes involved in these metabolic pathways (Calder, 2017:2).

Both n-3 and n-6 LCPUFA are key components of cell membrane phospholipids with relative amounts determined on availability from an individual's diet (Calder, 2017:2). The dietary intake of n-3 LCPUFA, mainly EPA and DHA, in the form of cold water fatty fish and their fish oils (FO) or FO supplements, has commonly been suggested to decrease inflammation in chronic inflammatory diseases including asthma, atherosclerosis, diabetes, cardiovascular disease, rheumatoid arthritis and neurodegenerative diseases such as Alzheimer disease (Calder, 2013; Husson *et al.*, 2016:524; Serhan & Petasis, 2011:5922). Widespread research recommends that the anti-inflammatory and pro-resolving characteristics of n-3 LCPUFA may also be favourable in reducing inflammation in infectious diseases such as TB (Husson *et al.*, 2016:524). Although inflammation is an important process, it is also known that an extreme or excessive inflammatory response can cause more tissue damage than initially generated by the pathogen (Calder, 2017:7; Husson *et al.*, 2016:524).

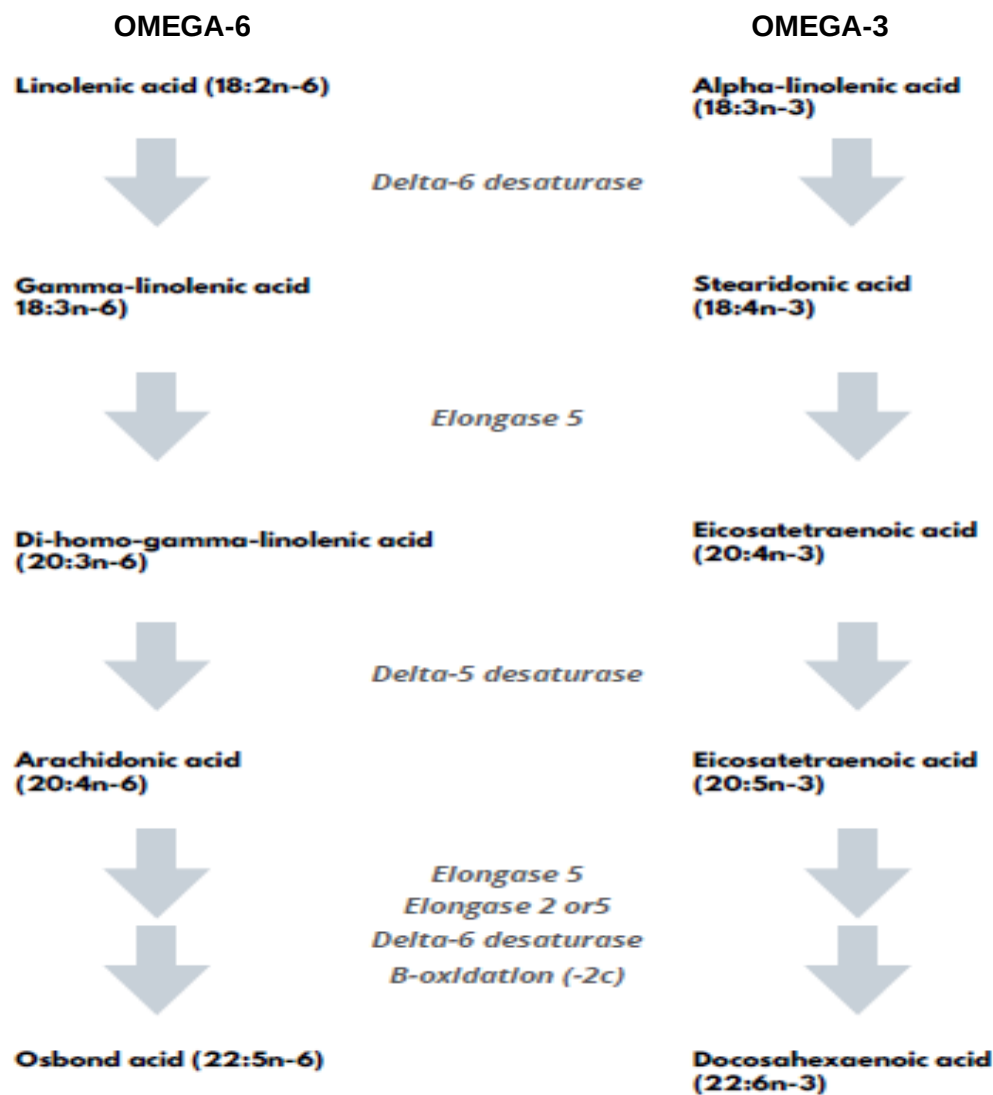


Figure 2-2 Overview of the pathway of conversion of linoleic acid and alpha-linoleic acid LCPUFA (Taken and adapted from Calder (2017:3))

Diets high in n-3 LCPUFA down-regulate the functions of immune cells by serving as predecessors of anti-inflammatory LMs (McMurray *et al.*, 2011:628). Lipid mediators are local acting bioactive signalling molecules, formed by the enzymatic production (cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome p450 (CYP)) of LCPUFA such as AA, EPA and DHA (Dennis & Norris, 2015:1; Mayer & Seeger, 2008:122). Lipid mediators, also called eicosanoids are a group of localised hormones. In contrast to classical hormones, local hormones are not synthesised in endocrine glands and transported to the target site but are produced directly within the tissue. Pro-inflammatory LMs are mostly produced from AA, which is found in cell membranes. Arachidonic acid gives rise to three major classes of pro-inflammatory LMs, namely, prostaglandins (PGs), thromboxanes (TBXs) and leukotrienes (LTs). More recently a new group of LMs were discovered, namely, the n-3 LCPUFA derived specialised pro-resolving LMs (SPM) including resolvins (Rvs), produced from EPA and protectins (PDs) and maresins produced from

DHA, which act to resolve inflammation (Calder, 2017:5; Fullerton *et al.*, 2014:13). The effects of Rvs, protectins and maresins have widely been studied in both cell culture and animal model experiments showing these LMs have both anti-inflammatory as well as inflammation resolving properties (Calder, 2017:6).

Lipid mediators are responsible for guiding both pro- and anti-inflammatory reactions during infection or diseases (Mayer & Seeger, 2008:122). They play a key role in inflammatory reactions triggered by infection or tissue damage, as well as the consequential resolution or inhibition of those reactions (Dietzold *et al.*, 2015:1). The LMs exert varying effects depending on the organ they are produced in. Furthermore, each LM shows distinctive features, thereby distinguishing its role in inflammation from that of other LMs produced from the same LCPUFA (Bennet & Gilroy, 2016:1). Synthesis of LMs originate from fatty acids present in cell membranes, most importantly AA, a 20-carbon fatty acid with 4 double bonds making it an unsaturated fatty acid. Prostaglandin H_2 is synthesised from AA through the phospholipase A_2 enzyme which cleaves membrane phospholipids to release A_2 , serving as the starting point for the synthesis of other prostaglandins and thromboxanes. Arachidonic acid, in addition, gives rise to the production of leukotriene A_4 (LT) through lipoxygenases, from which additional leukotrienes are then formed (Dennis & Norris, 2015:3). See figure 2-3 for an overview of LM synthesis. The LMs and intermediates marked in red are those measured during this research study.

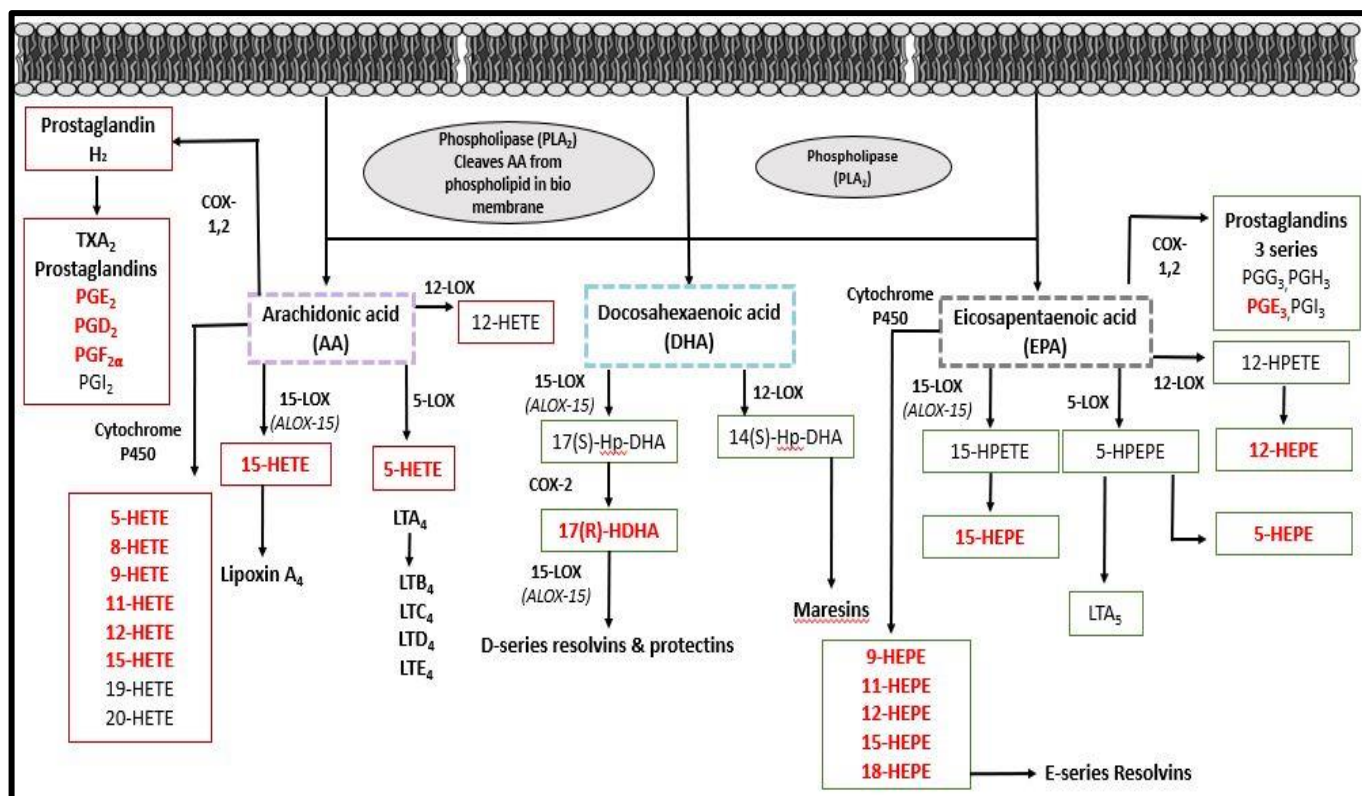


Figure 2-3. Lipid mediator synthesis

AA: arachidonic acid; COX: Cyclooxygenase; HEPE: hydroxyeicosapentaenoic; HETE: hydroxyeicosatetraenoic acid; Hp-DHA: hydroxyperoxydocosahexenoic acid; HPEPE: hydroxyperoxyeicosapentaenoic acid; HPETE: hydroxyperoxyeicosatetraenoic acid; LOX: lipoxygenase pathways; LT: leukotriene; PG: prostaglandin; PLA₂: phospholipase A₂; TX: thromboxane

As the inflammatory process develops, particular stop signals at suitable time points, including LXs, Rvs and PGs, prepare the way for monocytes to relocate and differentiate into phagocytosing macrophages which eradicates dead cells and then leaves the infection site (Serhan *et al.*, 2007:327). These SPMs function to stop polymorph nuclear cell tissue infiltration; lessen collateral phagocyte tissue destruction; reduce the time that the resolution of inflammation takes; augment macrophage phagocytosis; balance pro-inflammatory mediators; enhance microbial eradication and clearance; and increase tissue restoration (Serhan, 2017:1277). Figure 2-4 provides a summary of the immunomodulatory functions of SPMs on resolution of inflammation.

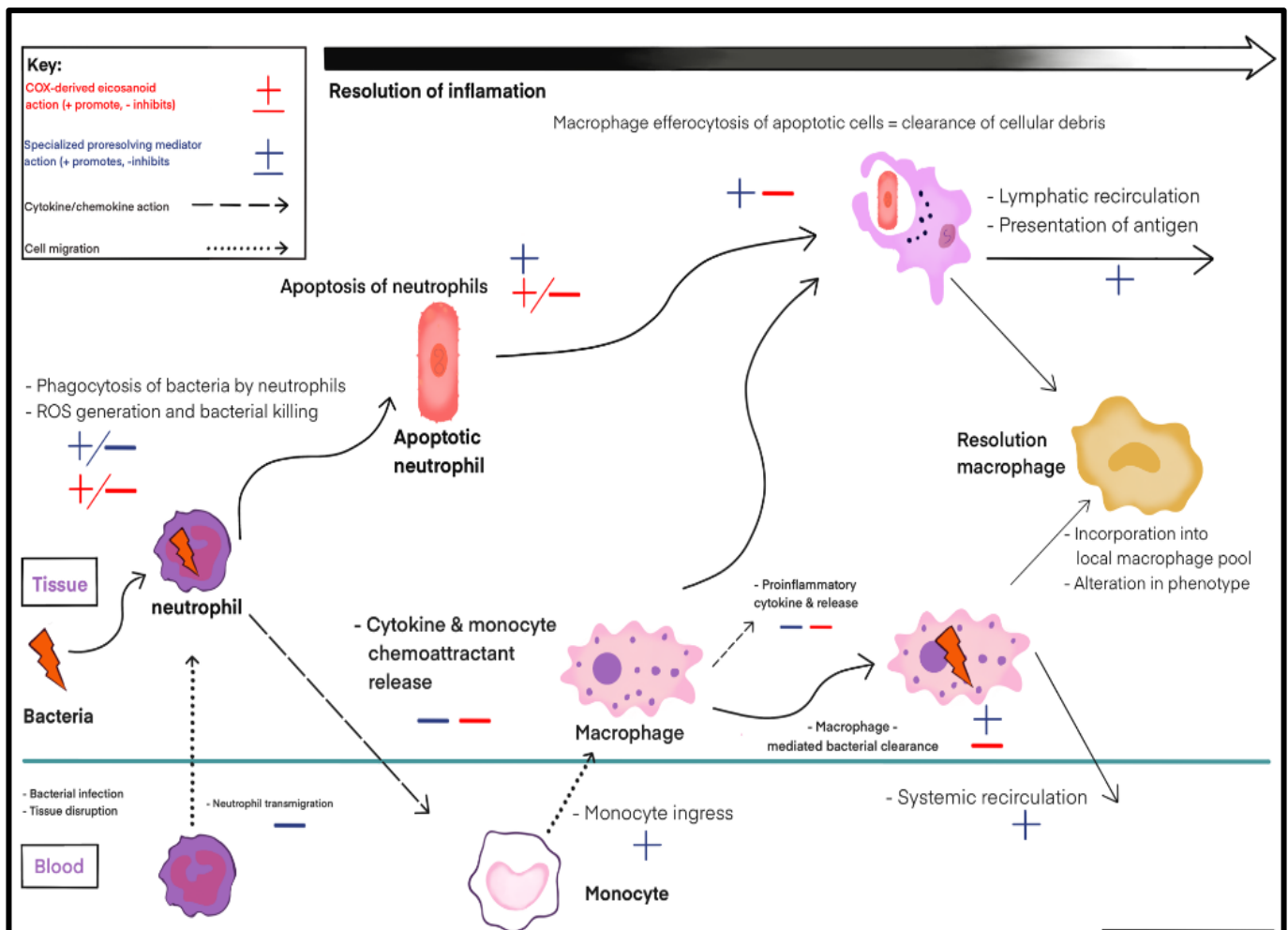


Figure 2-4. Immunomodulatory effects of SPMs during the inflammatory response
(Taken and adapted from Fullerton et al. (2014:15))

COX: Cyclooxygenase; ROS: Reactive oxygen species; SPM: Specialised pro-inflammatory lipid mediators

Throughout the inflammatory response, syntheses of LMs increases over time as seen in Figure 2-5. Initially, mostly pro-inflammatory mediators, including PGs and LTs, are produced followed by what is called “lipid mediator class switching”, which leads to the production of pro-resolving mediators (LXs, Rvs and PDs), hence prompting the resolution of inflammation (Serhan & Petasis, 2011:4). Specialised proinflammatory lipid mediators are produced during the resolution phase of inflammation to control initial occurrences in acute inflammation including oedema and leukocyte trafficking (Serhan *et al.*, 2015:3). In instances where these pro-resolving LMs are not produced in a timely manner, the inflammatory process persists, escalating into a chronic inflammatory state which in due course promotes fibrosis (Serhan & Petasis, 2011:4).

The discovery of Rvs, PDs and maresins is of importance since these powerful LMs provided the primary molecular basis for the numerous health benefits ascribed to n-3 LCPUFA, including inflammation resolving properties (Serhan & Petasis, 2011:5925). In addition to the production

of SPMs, n-3 LCPUFA also changes lipid cell membrane structures, substituting a small portion of AA in cell membranes and in that way decreasing n-6 LCPUFA as a substrate for generating inflammatory LMs. In this way, n-3 LCPUFA help to resolve inflammation and restore homeostasis (Dietzold *et al.*, 2015:2; Fullerton *et al.*, 2014:13; Serhan & Petasis, 2011:5924). Therefore, balanced LM production is of extreme significance in TB control through signalling for either the development or cessation of inflammation (Kaufmann & Dorhoi, 2013:443).

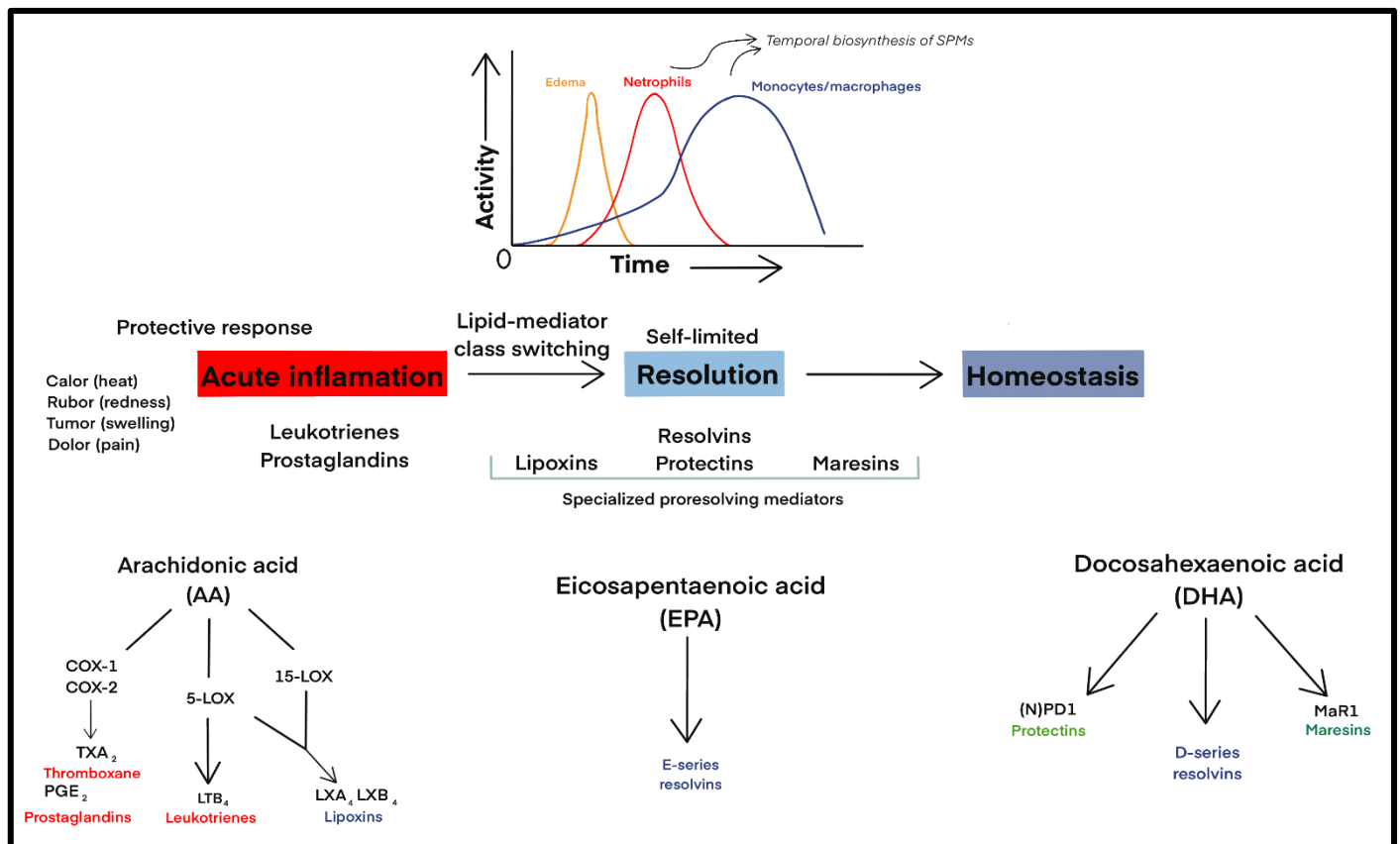


Figure 2-5. Lipid mediator class switching in exudates in the resolution of acute inflammation (Taken and adapted from Serhan *et al.* (2015:3))

COX: Cyclooxygenase; LOX: lipoxygenase; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; LTB₄: leukotriene B₄; LX: lipoxins; MaR1: maresins; PD1: protectin D1; PGE₂: prostaglandin E₂; TXA₂: thromboxane A₂.

2.3.3 Research on the effects of n-3 PUFA status/supplementation and TB outcomes

Numerous clinical trials show that n-3 LCPUFA supplementation is advantageous in reducing inflammation and improving disease outcomes such as morbidity and mortality in a variety of inflammatory chronic diseases, including diabetes mellitus, rheumatoid arthritis, inflammatory

bowel disease and neurodegenerative disease (Berquin *et al.*, 2008:271; Calder, 2010:365; Calder *et al.*, 2018:13; Husson *et al.*, 2016:524; Martin & Stapleton, 2010:540; Mayer & Seeger, 2008:126; McMurray *et al.*, 2011:628; Molino *et al.*, 2017:2). However, with regards to antibacterial immunity, the same anti-inflammatory features that are advantageous in these conditions may be harmful (McMurray *et al.*, 2011:628). This stems from two separate animal studies suggesting that higher n-3 LCPUFA levels in tissues may impair TB resistance. These studies involved guinea pigs infected with virulent *Mtb* and fed with n-3 PUFA enriched diets (McFarland *et al.*, 2008:2123; Paul *et al.*, 1997:623) and transgenic fat-1 mice with endogenous production of n-3 PUFA (Bonilla *et al.*, 2010:399), which exhibited augmented bacillary loads and distinct disease development (McMurray *et al.*, 2011:628). In another study, examining if outcomes would vary when supplementing n-3 and n-6 PUFAs either before or after infection, no pronounced effects were observed (Jordao *et al.* (2008:1384). The authors reported that although EPA supplementation meaningfully increased bacilli growth, AA supplementation produced a strong bactericidal effect (Jordao *et al.* (2008:1384).

The study mentioned earlier by Bonilla *et al.* (2010:404), raised awareness that n-3 LCPUFAs showed immune-suppressive effects and cautioned that its supplementation could lead to augmented susceptibility and decreased capability of the host to maintain the infection. However, in this study, the emphasis was on the effects on n-3 LCPUFA supplementation preceding *Mtb* infection, instead of supplemental treatment for active TB, such as those studies investigating other anti-inflammatory drugs (e.g. Aspirin or Ibuprofen) which did show improved clinical outcomes (Bonilla *et al.*, 2010:404; Marzo *et al.*, 2014:59; Vilaplana *et al.*, 2013:201). To the knowledge of the researchers, no research has yet been published on the effect of low compared to sufficient n-3 PUFA status before TB infection on lung inflammation.

Keeping the benefits of n-3 PUFA and the pathology of TB in mind, it would be expected that a sufficient n-3 LCPUFA status may be beneficial in TB patients due to the inflammatory response of the disease and the lungs being the primary site of infection. However, there is a lack of research. Furthermore, despite the availability of animal models, none of the models comprises of all characteristics of human TB (Bonilla *et al.*, 2010:399; Jordao *et al.*, 2008:1379; McFarland *et al.*, 2008:2126; Singh & Gupta, 2018:460). See Table 2-2 for in vivo and in- vitro studies conducted on the effect of omega -3 long chain polyunsaturated fatty acids on TB outcomes

Table 2-2. 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 <i>et al.</i> (2010a)	Murine macrophage-like cells line J774.A1 (ATCC TIB-67) infected with virulent H37Rv <i>Mtb.</i>	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 <i>et al.</i> (2010b)	8 to 10-week-old Wild type (control group) and <i>fat-1</i> transgenic mice infected with virulent H37Rv <i>Mtb</i> via the aerosol route.	<i>Fat-1</i> 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 spleen of <i>fat-1</i> mice at weeks 4 and 8 (P< 0.05) and week 12 (P<0.01). Higher bacterial loads in the spleen of <i>fat-1</i> mice at week 12 (P<0.005). Lower lung inflammatory response in <i>fat-1</i> mice compared with the control group.
		<i>Ex vivo</i> experiment on peritoneal cells of either Wild type or <i>fat-1</i> mice, infected with virulent H37Rv <i>Mtb.</i>	N/A	N/A	Higher bacterial loads in <i>fat-1</i> 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-1 β (P<0.05), and monocyte chemotactic protein-1 (P<0.05), were down-regulated in <i>fat-1</i> mice.

	Reference	TB model	Intervention		Outcomes
			Treatment	Duration	
2)	Jordao <i>et al.</i> (2008)	6 to 8-week-old female, BALB/c mice infected intranasally with 10 ³ CFU of bacilli.	Fat-free diet supplemented with either: (1) 10% safflower oil (n-6, LA content 7.5%) or (2) 10% Ropufa (n-3, EPA content 1.5% and DHA 1.1%) or (3) Control diet.	Diets introduced 2 weeks prior to infection. Mice were euthanised at day 21 and 63 post-infection.	n-3 LCPUFAS-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).
	Jordao <i>et al.</i> (2008)	Mouse macrophage cell line J774A.1 infected with <i>Mtb</i> .	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 <i>et al.</i> (1994)	H37Rv-infected guinea pig macrophages.	Controls: 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).

	Reference	TB model	Intervention		Outcomes
			Treatment	Duration	
4)	McFarland <i>et al.</i> (2008)	Weanling male and female outbred Hartley strain guinea pigs infected with virulent <i>Mtb</i> H37Rv via the respiratory route for 6 weeks until necropsy.	Diets contained as main lipid source either: (1) corn oil (n-6) (5.8% LA content) or (2) Menhaden fish oil (n-3) (fish oil: corn oil = 3:1 and 1.6% LA content 1.6%).	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 ($5.73 \pm 0.11 \log_{10}$ CFU) compared with the n-6 PUFA-fed group ($5.19 \pm 0.15 \log_{10}$ CFU) at 3 weeks ($P = 0.01$). Higher bacterial load in lungs in n-3 PUFA-fed group ($5.7 \pm 0.025 \log_{10}$ CFU) compared with the n-6 PUFA-fed group ($4.4 \pm 0.16 \log_{10}$ CFU) at 6 weeks ($P = 0.001$). No difference in histological evaluations between groups.
5)	Paul <i>et al.</i> (1997)	Male weanling guinea pigs infected with 180 CFU of H37Rv <i>Mtb</i> for 7 weeks until necropsy.	Animals divided into 3 groups: (1) n-6 PUFA group or (2) n-3 LCPUFAS group (n=18) (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 LCPUFAS-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.
6)	Nienaber <i>et al.</i> (2020)	Mycobacterium tuberculosis aerosol infected C3heB/FeJ male mice 28 days until euthanasia.	Animals divided into 4 groups: (1) Control and uninfected or (2) Iron group or (3) EPA/DHA group or (4) Iron and EPA/DHA group	Mice were fed respective diets for 3 weeks, whereafter they were euthanized 28 days post-infection.	The EPA/DHA group presented with lower lung bacterial loads compared to control group, however this effect was weakened in the group supplemented with Iron and EPA/DHA.

*Taken and adapted from Nienaber (2020:74)

AA: Arachidonic acid; BSA: Bovine Serum Acid; CFU: colony-forming units; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; IL: interleukin; LA: linoleic acid; LCPUFAS: long-chain polyunsaturated fatty acids; LTB₄: leukotriene B₄; MCP-1: monocyte chemoattractant protein-1; *Mtb*: *Mycobacterium tuberculosis*; n-3: omega 3; n-6: omega-6; PGE₂: prostaglandin; PUFA: polyunsaturated; TE: total energy

2.4 Other host immune and inflammatory response markers

2.4.1 Lung cytokines

Cytokines are described as small soluble proteins, produced by specific cell types that act primarily in a paracrine fashion. They produce signals that prompt changes in neighbouring cells, consequently altering the behaviour of those cells (Domingo-Gonzalez *et al.*, 2016:1; Romero-Adrian *et al.*, 2015:16). Other ways cytokines can function include autocrine action (acting on cells that secreted them) and endocrine action (acting on distant cells) (Zhang & An, 2007:1). The term cytokine encompasses proteins including, the TNF family, the ILs and the chemokines (Domingo-Gonzalez *et al.*, 2016:1).

Interactions between *Mtb* and the host immune system are complex and associated with releasing a range of cytokines, secreted in response to an infection by various cell types (Etna *et al.*, 2014:1). Domingo-Gonzalez *et al.* (2016:1), highlights the importance that not one cytokine involved in complex diseases such as TB can be labelled as either good or bad. Rather, they produce both defensive and pathological outcomes reliant on the context of disease. As discussed in Section 1.2, the production of cytokines starts after the initial engulfment of the bacilli, which in turn leads to the recruitment and activation of inflammatory cells (Domingo-Gonzalez *et al.*, 2016:2; Kaufmann & Dorhoi, 2013:441; Kiran *et al.*, 2016:169). The latter mediates both inflammatory and immune reactions. Traditional cytokines produced during *Mtb* infection, includes IL-2, IFN- γ , IL-6, IL1 α/β , IL-12 and TNF- α (Zuniga *et al.*, 2012:5).

Cytokines are classified as either being pro-inflammatory (excessive inflammation) or anti-inflammatory (restorative) and the balance amongst these two groups control the success of the immune reactions and the extent of tissue damage (Romero-Adrian *et al.*, 2015:18). Furthermore, these cytokines can be involved in either acute or chronic inflammation through a multifaceted and often conflicting system of interactions (Turner *et al.*, 2014:2563). It is, therefore, possible to categorize cytokines according to the immune response (see Table 2-2). As mentioned before, cytokines are produced by different cell types, however, helper T cells (Th) and macrophages are the main producers (Zhang & An, 2007:2). T helper cells are separated into T helper type 1 (Th1) and T helper type 2 (Th2) cells in accordance with the pattern whereby cytokines are secreted (Flesch & Kaufmann, 1993:318). T helper type 1 cells produce IL-2 and IFN- γ , whereas Th2 cells secrete IL-4, IL-5, IL-6, and IL-10. It is, however, impossible to establish the source of the specific cytokine during analysis hence studies do not refer to Th1 or Th2 cytokines (Flesch & Kaufmann, 1993:318).

Immunity against *Mtb* infection is reliant on the activity of Th1 cells releasing IFN- γ , IL-12 and TNF- α (Romero-Adrian *et al.*, 2015:16). Interferon-gamma is responsible for the activation of macrophages and IL-1 β acts on macrophages in both murine models and humans by inducing macrophages to instantly killing the *Mtb* bacilli, whereas IL-6 is vital in the host resistance and protection against the *Mtb* bacilli (Romero-Adrian *et al.*, 2015:16; Verbon *et al.*, 1999:110). Furthermore, IL-12, IL-17, IFN- γ and TNF- α are all part of the granuloma formation during *Mtb* infection (Romero-Adrian *et al.*, 2015:17).

Wu *et al.* (2017:68), conducted a study to determine the cytokine profiles associated with patients presenting with either active or latent TB compared to non-infected patients. They found that the following cytokines were produced in abundance across all three groups: IL-1 β , IL-2, IL-6, IL-10, IL-17, G-CSF, IFN- γ , IP-10, MIP-1a and TNF- α . However, IL-2, IL-10, IFN- γ , IP-10 and TNF- α was substantially higher within the latent TB group compared to the active TB group (Wu *et al.*, 2017:68). It was determined that IL-2 and IL-10 can be used as biomarkers in differentiating active from latent TB and non-infected groups and could further assist in improving TB diagnosis (Wu *et al.*, 2017:68). Each cytokine, grounded on its biological activities and interactions with the immune system, will have significant outcomes on *Mtb* infection regulation or suppression (Romero-Adrian *et al.*, 2015).

See Table 2-3 for a summary of the different cytokines released in response to *Mtb* infection and the beneficial and or damaging roles they play in the disease (Domingo-Gonzalez *et al.*, 2016:57; Etna *et al.*, 2014:23; Romero-Adrian *et al.*, 2015; Zhang & An, 2007).

Table 2-3 Summary of cytokine classification by immune response*

Immune response	Cytokine family	Cytokine members
Adaptive immunity	Common γ chain receptor ligands	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21
	Common β chain (CD131) receptor ligands	IL-3, IL-5, GM-CSF
	Shared IL-2 β chain (CD122)	IL-2, IL-15
	Shared receptors	IL-13 (IL-13R–IL-4R complex)
		TSLP (TSLPR–IL-7R complex)
Pro-inflammatory signaling	IL-1; IL-6; TNF- α ; IL-17; Type I IFN; Type II IFN; Type III IFN	IL-1 α , IL-1 β , IL-1ra, IL-18, IL-33, IL-36 α , IL-36 β , IL-36 γ , IL-36Ra, IL-37 and IL-1Hy2 IL-6, IL-11, IL-31, CNTF, CT-1, LIF, OPN, OSM; TNF- α , TNF- β , BAFF, APRIL ; IL-17A-F, IL-25 (IL-17E); IFN- α , IFN- β , IFN- ω , IFN- κ , ; IFN- γ IFN λ 1 (IL-29), IFN λ 2 (IL-28A), IFN λ 3 (IL-28B)
Anti-inflammatory signaling	IL-12; IL-10; IL-4; IL-11	IL-12, IL-23, IL-27, IL-35 IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29

*Turner *et al.* (2014:2564)

CNTF: ciliary neurotrophic factor; CT-1: cardiotrophin-1, GM-CSF: granulocyte macrophage colony stimulating factor; IFN: interferon; LIF: leukaemia inhibitory factor; OPN: osteopontin; OSM: oncostatin M; TNF- α : tumour necrosis factor α ; TSLP: thymic stromal lymphopoietin. **Cytokines highlighted in blue are involved in TB infection**

Table 2-4 Summary of beneficial and damaging roles of cytokines involved in tuberculosis

Cytokine	Targets	Beneficial/positive role	Damaging/negative role
TNF-α	Proinflammatory	• Important for survival following <i>Mtb</i> infection. • Initiate cytokine and chemokine response and activation of phagocytes.	Facilitates tissue damage.
IFN-γ	Activation of macrophages and bacterial killing	• Important for survival following <i>Mtb</i> infection. • Coordinates and maintains mononuclear inflammation. • Expressed by antigen-specific T cells. • Macrophage activation (controls intracellular bacilli's)	Possibly pathogenic.
IL-2	Proinflammatory	• Stimulates the growth of B, T and NK cells. • Essential in the formation of granulomas and cellular immunity • Used as immunotherapy in MDR-TB (leads so fewer lung lesions)	Not always used as a diagnostic marker for TB as only low amounts are released.
IL-6	Proinflammatory	• Potentiates initial immunity/acute phase response – nonessential unless a high dose infection. • Required for host resistance to <i>Mtb</i> infection.	
IL-1α IL-1β	Proinflammatory	• Important for survival/host resistance following <i>Mtb</i> infection. • Directly kills <i>Mtb</i> within human/ mouse macrophages • Induction of IL-17 and promotes prostaglandin E2 (PGE2) to limit IFN-α	
IL-12	Th1 polarization	• Controls <i>Mtb</i> infection in both mouse/ human models • Facilitate early T-cell activation, polarization, and survival.	Overexpression of IL-12p70 is toxic during <i>Mtb</i> infection.
IL-17	Proinflammatory	• Vital for survival following infection with certain <i>Mtb</i> strains. • Induction and maintenance of chemokine gradients for T-cell migration.	Drives pathology via S100A8/A9 and neutrophils.

Domingo-Gonzalez et al. (2016:57), Romero-Adrian et al. (2015:16), Etna et al. (2014:2)

IFN- α : Interferon-alpha; IFN- β : Interferon-beta; IFN- γ : Interferon-gamma IL: Interleukin; *Mtb*: *Mycobacterium tuberculosis*; TNF- α : Tumor necrosis factor-alpha

2.4.2 Lung histology

There are a number of types of pulmonary TB lesions in humans which are all diverse, showing contrasts with regard to cell structures and organization, degree of containment and the extent of necrotic tissue (Driver *et al.*, 2012:3181; Gupta *et al.*, 2016:1; Irwin *et al.*, 2015:591). Regardless of the heterogeneity, most of the TB infected lesions in humans present with a fibrotic enclosed necrotic core (Lenaerts *et al.*, 2015:291). In order to translate animal study results to the human population, animal models of active TB should imitate the pathophysiological conditions thought to occur in humans presenting with TB lung lesions (Irwin *et al.*, 2015:591; Lenaerts *et al.*, 2015:291). These pathophysiological conditions include the development of necrotic tissues (caseous necrosis) enclosed within a collagen border, which affects nutrient and treatment supply to the bacillus (Dartois, 2014:8; Irwin *et al.*, 2015:591; Lenaerts *et al.*, 2015:291). Additionally, other pathophysiology conditions include: 1) hypoxia which impacts the metabolic activity of *Mtb* bacilli, 2) intracellular and extracellular bacilli populations affecting drug efficiency, as well as 3) liquefaction and development of cavities (Grosset, 2003:834; Irwin *et al.*, 2015:591; Lenaerts *et al.*, 2015:291).

A murine model of active TB will be discussed later in this literature review. However, no animal model can imitate the whole TB disease profile. Nevertheless, animal models are without question critical in improving our understanding of TB pathophysiology (Kramnik & Beamer, 2016:221). Figure 2-6 shows the major types of pulmonary lesions found in both active and latent TB disease in human beings (Lenaerts *et al.*, 2015:290). In the above-mentioned figure, hallmark characteristics of human TB such as noticeable inflammation with an inflow of lymphocytes and monocytes, later converting into granulomas with caseous necrosis enclosed with a fibrotic border can be seen (Lenaerts *et al.*, 2015:289).

Irwin *et al.* (2015:593), conducted an inclusive histological analysis of pulmonary lesions following infection in the C3HeB/FeJ mouse strain. Three heterogeneous lesion Types, as seen in human pulmonary TB lesions, which varied in cellular structure and organization, following 5 weeks of aerosol infection, were identified. These lesion types were labelled as type I, II and III lesions (Irwin *et al.*, 2015:593). These types of lesions are illustrated in Fig 2-7. The Type I lesions best resembled those found in granulomas of human TB, because they were compact and condensed caseous necrotic lesions (Driver *et al.*, 2012:3182; Irwin *et al.*, 2015:593). These lesions developed 34-45 days after infection with low dose aerosols and resulted in a focal accretion of foamy macrophages combined with neutrophils often next to the bronchus. The number of neutrophils rapidly increased as the morphology of the lesion progressed leading to a dense core now filled with neutrophils enclosed with foamy macrophages as illustrated in Figure 2-8B. By

week 7-10 after infection, granulomas presented more structurally organized, associated with a dense neutrophilic core, with or without necrosis (Figure 2-6C). The fibrotic border is seen in Figure 2-6F (Irwin *et al.*, 2015:593).

Although these Type I lesions are more like those of human TB lesions, it is important to note that the cellular structure varies (Irwin *et al.*, 2015:597). The Type II lesions are like Type I. However, they lack the fibrotic border and cellular structure and develop more rapidly, adversely impacting survival outcomes (Irwin *et al.*, 2015:597). It differs in the sense that within humans, the granuloma core is thought to contain normal and foamy macrophages together with lymphocytes whereas Type I lesions in the mouse strain is primarily derived from neutrophilic infiltration (Irwin *et al.*, 2015:597). Essentially, Type I lesions in C3HeB/FeJ mice comprise of plenteous extracellular bacilli inside the necrotic core whereas Type III lesions showed intracellular bacilli within non-necrotic lesions (Irwin *et al.*, 2015:600).

Given that the C3HeB/FeJ mouse strain closely resembles the hallmark characteristics in human TB, it makes it possible to examine the effect of n-3 LCPUFA within lung tissues. This enables us to see how it affects the long-term lung damage compared to control groups.

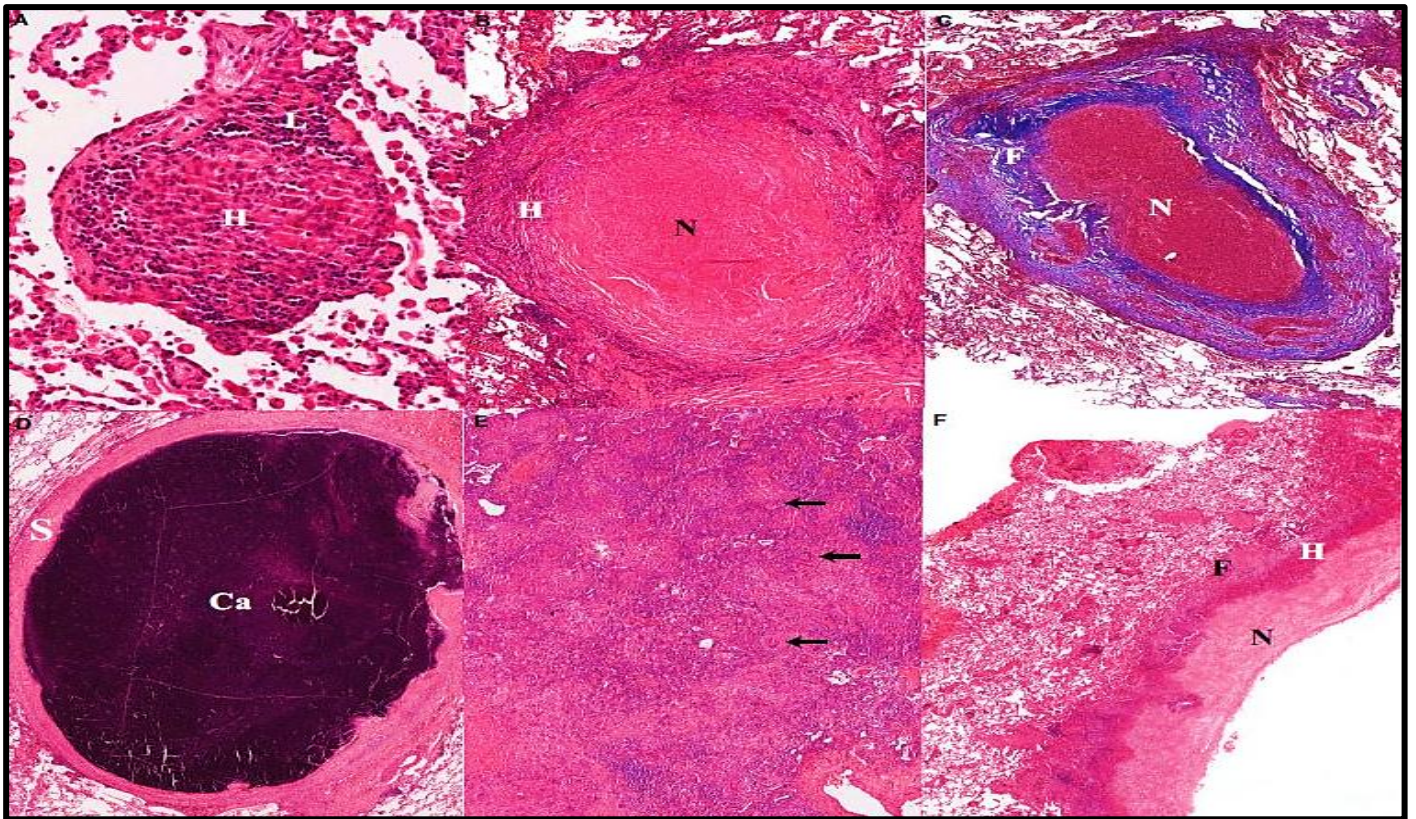


**Figure 2-6 Histology images of Mycobacterium Tuberculosis infected human lungs
(Taken from Lenaerts et al. (2015:290))**

(A). Initial non-necrotizing granuloma with lymphocyte border (L) bluish due to the visible nuclei and small cytoplasm; the granuloma core consists macrophages **(H)**. **(B).** necrotizing granuloma: the necrotic core appearing as unformed pink material **(N)**, enclosed by a fibrotic border and cellular infiltrates **(F)** visibly defining the granuloma from the nearby lung alveoli. **(C).** Stained necrotic granuloma showing the collagenous regions in blue. The dense fibrotic border **(F)** comprises of fibroblasts, collagen, new vessels, and dispersed chronic inflammatory cells. Note the noticeable vascularization of the fibrotic border. **(D).** Calcified granuloma with sclerotic border **(S)** accompanied with absence of mononuclear infiltrates and no visible infectious activity; Ca: area of calcification/hardening. **(E).** tuberculous pneumonia: merging aggregates of epithelioid histiocytes mixed with Langhans' and multinuclear giant cells (arrows). The more bluish parts are lymphoid aggregates. The latter are the same cells comprising a granuloma, however in the absence of a fibrotic border they spread to fill alveolar space. **(F).** Wall of cavitating tuberculous granuloma representing a trilaminar structure with central necrosis **(N)**, histiocyte aggregates **(H)** and peripheral fibrosis **(F)**. The fibrotic border has mononuclear infiltrates which shows a still active and developing TB lesion.

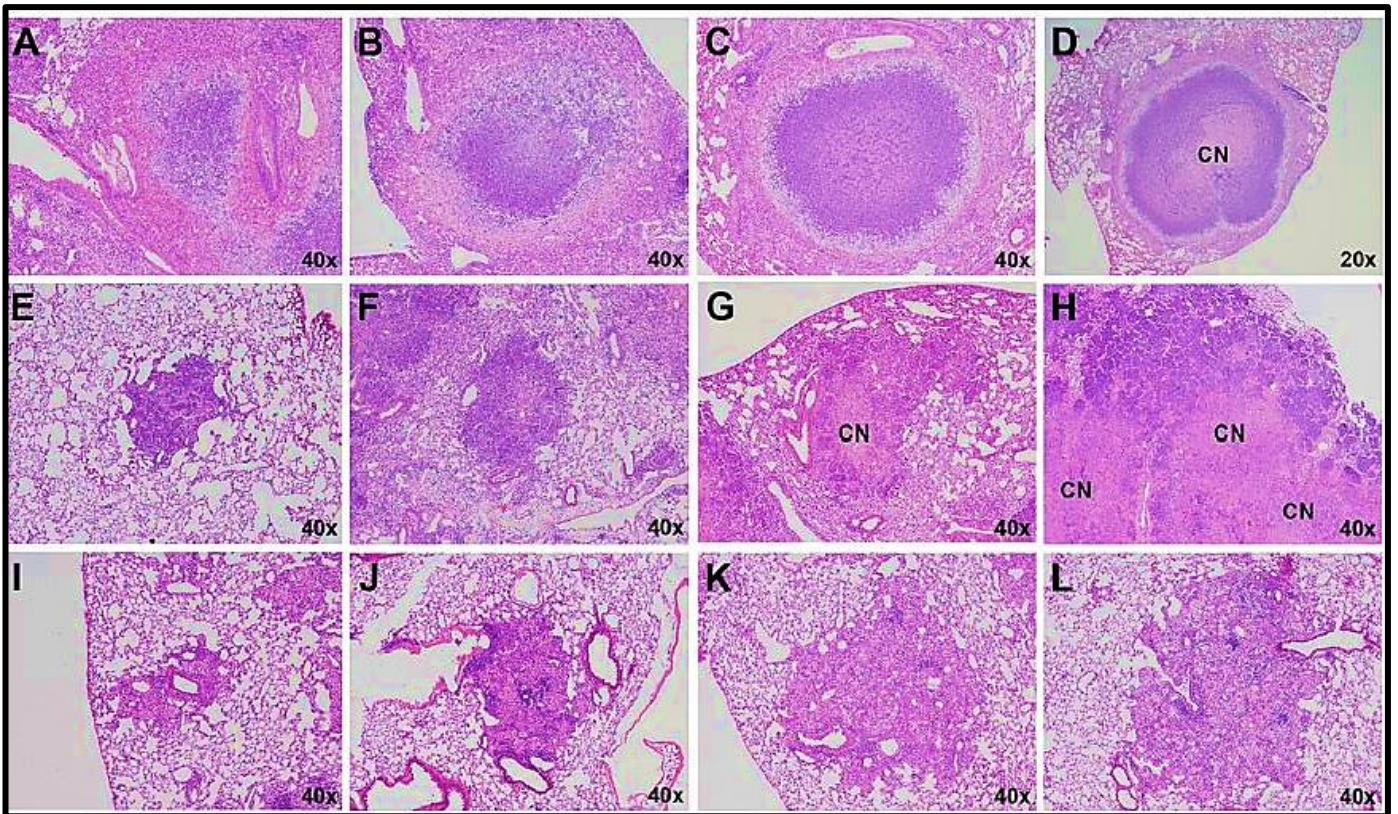


Figure 2-7 Development of lung lesions histology images of a murine model of active tuberculosis over time (Taken from Irwin et al. (2015:593))

Development of Type I, II and III lesions over time. Each slide represents a single H & E stained lesion derived from the study group animals euthanized at defined time points. Development of Type I lesions from individual animals at 45 (A), 50 (B), 55 (C) and 61 (D) days following aerosol infection. Progression of Type II lesions from individual animals at 20 (E), 35 (F), 38 (G) and 40 (H) days following aerosol infection. Progression of Type III lesions from individual animals at 30 (I), 35 (J), 61 (K) and 75 (L) days following aerosol infection. CN: caseous necrosis.

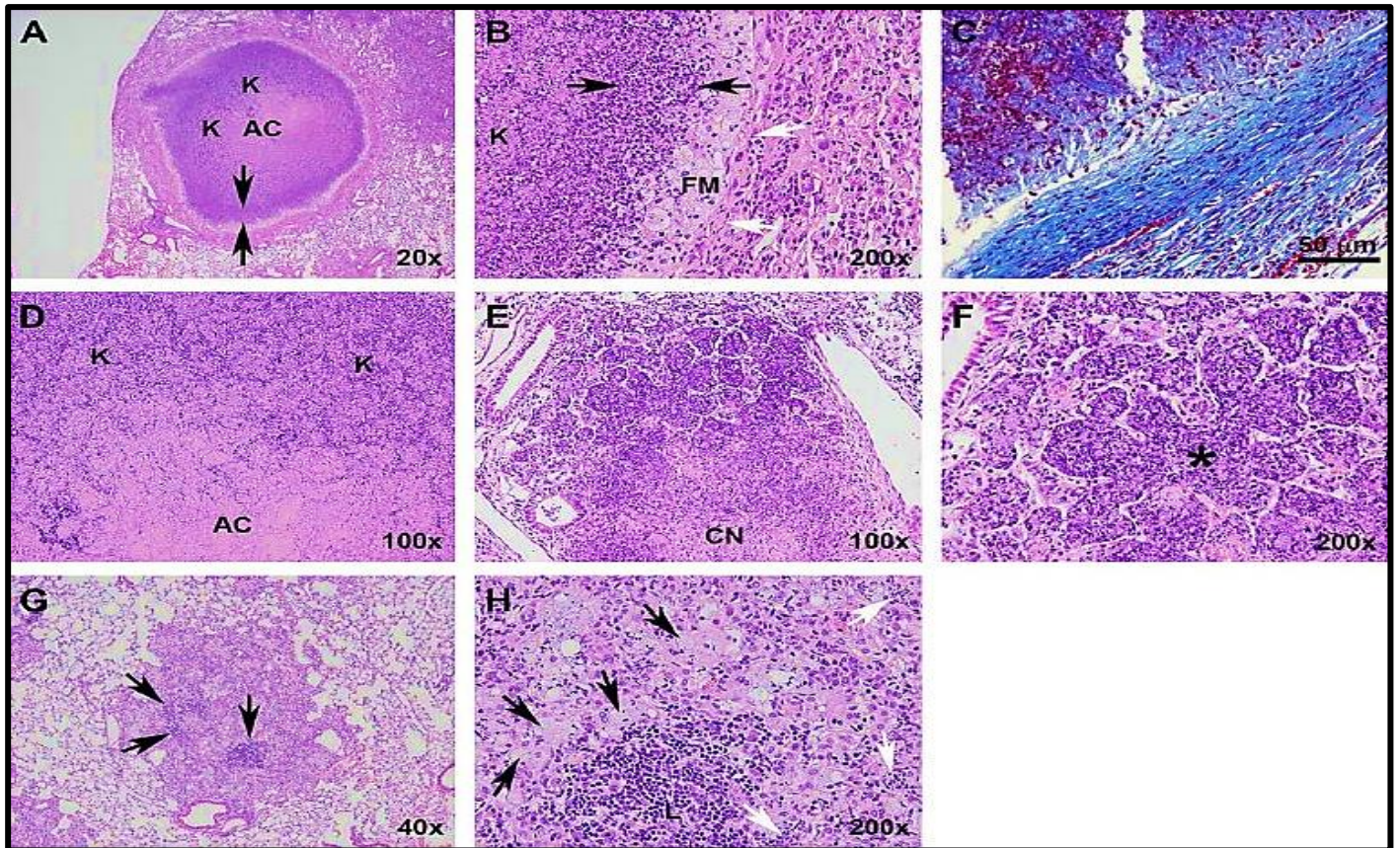


Figure 2-8 Difference between three lesion types with regards to cellular structure organization and collagen containment (Taken from Irwin *et al.* (2015:594))

(A) Type I granuloma showing the acellular caseum, distinct band of darkly stained intact neutrophils (arrows outline margins), primarily neutrophil-derived zone of karyorrhectic debris, lightly stained foamy macrophage border, and collagen containment. **(B)** Higher-magnification image of A showing an area of intact neutrophils (black arrows), foamy macrophage border and collagen containment (white arrows). **(C)** Masson's Trichrome stain for collagen along the peripheral margin of a Type I lesion. **(D)** Interior region of a Type I granuloma, showing the acellular caseum and karyorrhectic debris. **(E)** Type II lesion with a caseous necrotic interior, and extensive consolidation of peripheral alveoli. **(F)** Pulmonary acinus (*) completely obstructed with neutrophils and cellular fragments. **(G)** Type III lesion with distinct lymphocytic clusters (arrows). **(H)** Higher-magnification image of G showing foamy macrophages (black arrows) and a lymphocytic cluster (**L**). Small, isolated pockets containing small numbers of neutrophils (white arrows) are occasionally present, especially at later time points as foamy macrophages undergo necrosis.

AC: acellular caseum; K: karyorrhectic debris; FM: foamy macrophages; CN: central necrosis. H&E staining unless otherwise noted.

2.5 Animals

2.5.1 The C3HeB/FeJ species mouse TB model

A diverse range of pre-clinical models such as mice, rats, rabbits, guinea pigs and non-human primates, has been used for assessing new treatments and vaccines for controlling TB (Singh & Gupta, 2018:456). Even though these models are examined closely for their legitimacy, the most effective and most used for TB research include the mouse, the rabbit, guinea pigs and non-human primates (Apt & Kramnik, 2009:196; Dharmadhikari & Nardell, 2008:503; Kaufmann & Dorhoi, 2013:107; Singh & Gupta, 2018:456).

Despite the accessibility of various pre-clinical animal models, none of the models outlines all characteristics of human TB, with each model displaying different advantages and disadvantages limiting their effectiveness for pre-clinical drug testing (Driver *et al.*, 2012:3182; Singh & Gupta, 2018:456). However, the impact of these various pre-clinical models in the study of TB is substantial and animal models have been the guiding principle in improving our knowledge about this disease (Singh & Gupta, 2018:456).

The similarities amongst human and animal physiology offer a valuable context to understand human systems (Singh & Gupta, 2018:457). Guinea pigs are viewed as the animal model imitating human TB pathology the closest, developing comparable necrotic granulomas and with high susceptibility to TB infection, however, they are markedly costlier compared to mice models and they require diets high in vitamin C (Kaufmann & Dorhoi, 2013:109; Singh & Gupta, 2018:459).

For various practical reasons, including, cost-effectiveness, easy handling, less space necessity, the convenience of inbred, outbred and transgenic mouse strains as well as the presence of immunological tools, the mouse, specifically the C3HeB/FeJ species, is an ideal animal model utilised in TB research (O'Garra *et al.*, 2013:485; Singh & Gupta, 2018:456). Using the mice model instead of higher-order animals also adheres to the “replacement” principle of the four R principles.

In the search for better experimental animal models, to study human TB, the C3HeB/FeJ mouse strain with a recessive allele, best resembles hallmark characteristics found in human TB. More specific, this mouse strain mimics the necrotic process which leads to dissolving lesions and resembles those of active TB in the human population (Vilaplana *et al.*, 2013:199). The capability to cause necrosis in lung lesions is a significant characteristic of TB pathogenesis that preserves *Mtb* in the human population (Kramnik & Beamer, 2016:221).

The C3HeB/FeJ mouse strain, frequently discussed in research papers as Kramnik mice, have been widely used as a TB mouse model (Driver *et al.*, 2012:3182; Gupta *et al.*, 2013:601; Irwin *et al.*, 2015:600; Lanoix *et al.*, 2015:604; Marzo *et al.*, 2014:56; Rosenthal *et al.*, 2012:4332; Vilaplana *et al.*, 2013:200). Using the C3HeB/FeJ mice model therefore also adheres to the “refinement” principle of the four R principles. This susceptible mouse strain is a powerful tool for discovering mechanisms underlying the pathogenesis of TB as well as assisting in the revealing of significant factors that contribute to the progression of active TB (O'Garra *et al.*, 2013:485). By using this specific mouse model, the effectiveness of TB treatment will be more characteristic of what is expected in *Mtb* exposed humans (Lanoix *et al.*, 2015:604).

2.6 Literature summary

As seen from the literature review, TB remains a major global burden, especially in countries such as South-Africa. It is further evident that there is room for adjunct TB treatment as mortality rates are still high. It is important to consider new treatment interventions by supplementing existing anti-TB treatment with immune-enhancing host modulators to accelerate the eradication of *Mtb* infection and reduce treatment duration and lung tissue injury. Several studies reported positive TB outcomes by supplementing with anti-inflammatory treatments, which favours further investigation into whether n-3 status may elicit similar results. For this study, the C3HeB/FeJ mouse model was used, that it closely resembles the characteristics found in human TB. The following chapters will elaborate more into our findings making this a novel study.

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CHAPTER 3 RESEARCH METHODOLOGY

In this chapter, the methodologies of the study are described. The outline of chapter 3 is as follows: Section 3.1 provides detailed information regarding the study design, methods, and materials. Section 3.2 and 3.3 describe all sample collections and laboratory analyses that were performed during the study. All analytical protocols were strictly followed in accordance with standard operating procedures (SOPs), which are attached at the end of this thesis. Lastly, Section 3.4 provides a description of statistical methods that were used to meet the study objectives.

Ethical approval was obtained from the AnimCare Ethics committee of the Faculty of Health Sciences of the North-West University (NWU), Potchefstroom (Ethics number: NWU-00567-19-S5 and the Animal ethics committee (015/040) of the Faculty of Health Sciences, UCT.

3.1 Study design, materials, and methods

3.1.1 Study design

This study examined the effect of n-3 PUFA status prior to TB infection on lung inflammation regarding histopathology, cytokine concentrations and lipid mediator signalling. Twenty, male C3HeB/FeJ mice, 4-6 weeks old were randomised into two arms and conditioned for six weeks to be either n-3 PUFA sufficient (n-3FAS group) or deficient (n-3FAD group). They were then infected with *Mtb* at 10-12 weeks of age, continued on the same diets and euthanised at 28 days after infection. The study was performed in two experiments, with an n-3FAS and n-3FAD group of n=5 in each consecutive experiment. Figure 1 shows the experimental design of the total group.

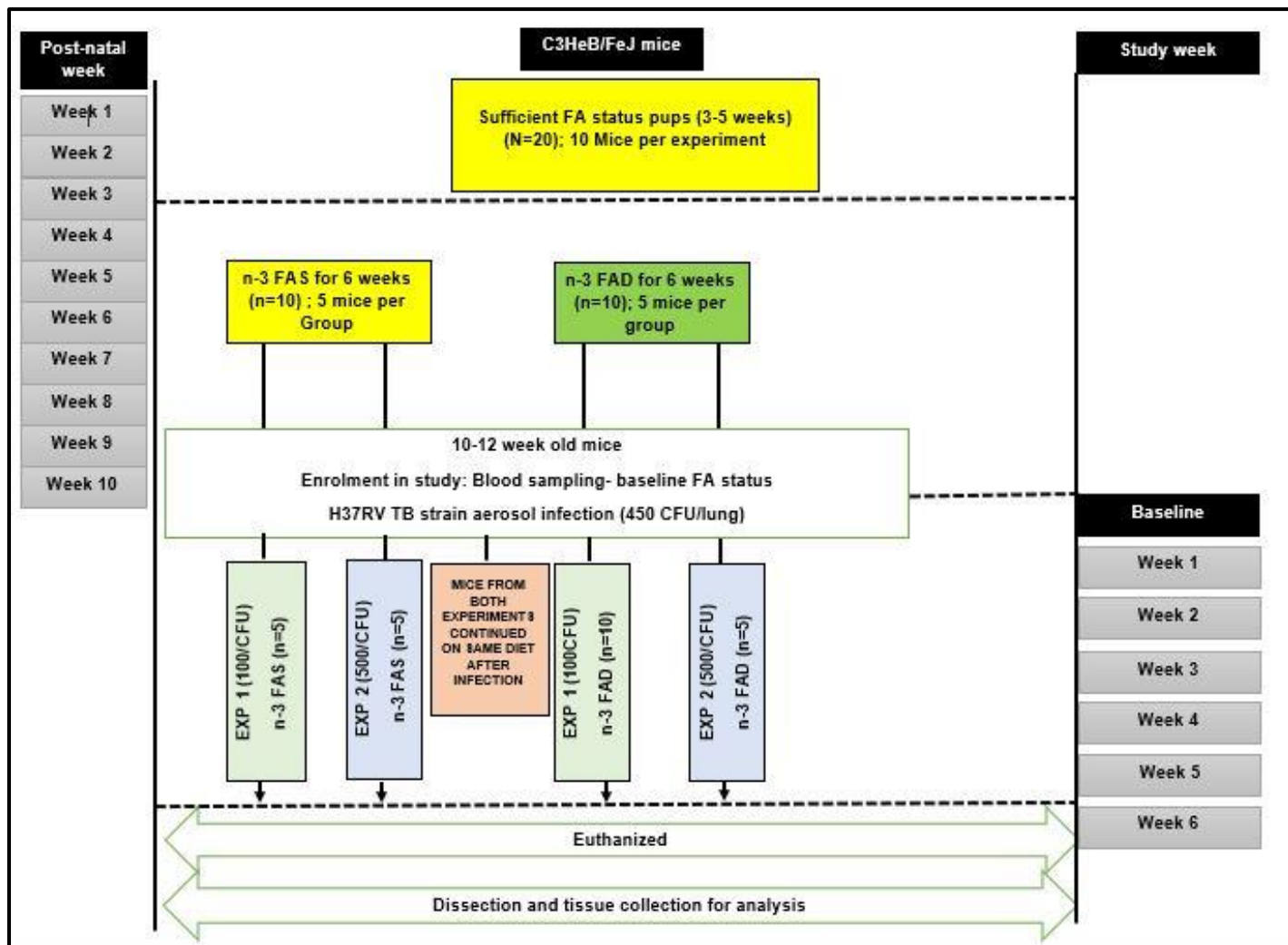


Figure 3-1: Experimental study design

The study was performed in two experiments, with an n-3FAS and n-3FAD group of n=5 in each group across each consecutive experiment. After euthanasia, the mice continued on their respective diets. The two experiments were executed separately. All mice per experiment were infected within the same batch. One day following infection, four mice were euthanized to confirm the infection dose, which was 100 and 500 colony-forming units (CFU)/mouse for experiment 1 and 2 respectively.

FA: fatty acid; n-3: omega-3; PUFA: polyunsaturated fatty acid

Groups: n-3FAS = Mice n-3 PUFA-sufficient diet; n-3FAD = Mice receiving an n-3 PUFA-deficient diet.

3.1.2 The C3HeB/FeJ species mouse model

The animal model chosen for this research project was the C3HeB/FeJ mouse model. The C3HeB/FeJ mouse species, also known as Kramnik mice, have been widely used as a TB mouse model (Driver *et al.*, 2012:3182; Gupta *et al.*, 2013:601; Irwin *et al.*, 2015:600; Lanoix *et al.*, 2015:604; Marzo *et al.*, 2014:56; Rosenthal *et al.*, 2012:4332; Vilaplana *et al.*, 2013:200). It was critical that the animal model imitated the pathophysiological conditions thought to occur inside

pulmonary TB lesions, with the ultimate goal of translating our research findings into the human situation (Driver *et al.*, 2012:3182; Irwin *et al.*, 2015:591; Marzo *et al.*, 2014:56; Vilaplana *et al.*, 2013:200). Common inbred mouse strains have been examined for infection resistance with the *Mtb* H37Rv strain, and the mice were categorised as being either highly resistant or highly susceptible (Medina & North, 1998:270). The C3HeB/FeJ mouse strain that was used in this research project was amongst those that were highly susceptible to *Mtb* infection. Tuberculosis remains a chronic disease, primarily of the lungs, regardless of the infected route (aerosol, intranasal or intra-venous) (Medina & North, 1998:273).

3.1.3 Facilities and setting

The experimental animal study was conducted in a Department of Agriculture, Forestry and Fisheries (DAFF) accredited biosafety level 3 (BSL3) animal facility at the Institute of Infectious Disease and Molecular Medicine (IDM), Division of Immunology, University of Cape Town (UCT), South Africa (SA). The BSL3 unit where the study was conducted is equipped with 150 cages, an autoclave, a stainless-steel counter, 3 biosafety cabinets and a Glas-Col Middlebrook Inhalation Exposure System. All sample collections were done under the supervision of trained animal technologists of the IDM UCT Animal Facility and sample collection and preparation were also conducted in a class II biosafety cabinet in the safety unit prior to transport for analysis elsewhere.

3.1.4 Housing and husbandry

The mice were housed under a 12h/12h light/dark cycle (lights on at 06:00) at 22 ± 2 °C and $55 \pm 10\%$ relative humidity. The cages were standard type II long mouse individually ventilated cages (IVC) (391 x 199 x 160 mm [WxDxH]) with corn-cob bedding. There were three to four mice per cage. All mice had *ad libitum* access to purified laboratory diets and demineralised water (18 MΩ). The mice also had environmental enrichment in the cages. The mice were weighed once per week in a class II biosafety cabinet and monitored daily by utilising study-specific monitoring sheets. Humane endpoints are described in detail in the monitoring sheet. Animals would have been subjected to humane endpoint euthanasia if they had lost 20% of their body weight from baseline of the study period. Strict safety precautions were followed with regard to personal protective clothing, including safety glasses and N95 masks.

3.1.5 Diet compositions

Purified experimental diets were commercially obtained from Dyets Inc. (Bethlehem, USA). All diets were custom prepared in pellet form and stored at -20°C until use. Diets were thawed in batches, by placing them in a refrigerator (4°C). Once the bags had been opened the diets were placed in an airtight container. Mice were provided with the AIN-93G purified diets using the laboratory rodent formulation (Reeves *et al.*, 1993). The AIN-93G diet, which was the n-3 PUFA-sufficient diet (n-3FAS) diet in this study, offers optimal amounts of linoleic acid and alpha-linolenic acid (precursors for n-6 LCPUFA and n-3 LCPUFA respectively) to ensure optimal tissue saturation of arachidonic (AA) and docosahexaenoic acid (DHA).

Both diets were iso-caloric and contained 10% fat with modifications of fat source as described in Table 3-1. Gas chromatography mass spectrometry (GCMS) analysis conducted at the manufacturer was used to confirm the fatty acid composition of the diets (Table 3-1) (Beard *et al.*, 2006; Grill *et al.*, 2001; Romero *et al.*, 2010). Mice were randomly assigned to an n-3 PUFA-deficient (n-3FAD) (n=10) or PUFA-sufficient (n-3FAS) diet (n=10) and continued these diets for six weeks prior to infection in order to establish either sufficient or low n-3 PUFA status.

Table 3-1. Fatty acid composition of diets¹

Group	Group description	Fat source	LA g/100g	ALA g/100g	AA g/100g	EPA g/100g	DHA g/100g
n-3FAS	Mice receiving an n-3 PUFA-sufficient diet.	The basal AIN-93G formulation contained soybean oil at 70 g/kg diet and hydrogenated coconut oil at 30 g/kg (Baumgartner <i>et al.</i> , 2012; Fitsanakis <i>et al.</i> , 2009; Grill <i>et al.</i> , 2001).	3.54	0.436	< 0.007	< 0.007	< 0.007
n-3FAD	Mice receiving an n-3 PUFA-deficient diet.	The FAD diets contained hydrogenated coconut oil at 81 g/kg diet and safflower oil at 19 g/kg diet (Harauma <i>et al.</i> , 2010; Moriguchi <i>et al.</i> , 2013; Moriguchi <i>et al.</i> , 2001; Moriguchi & Salem Jr, 2003).	1.3	0.01	< 0.007	< 0.007	< 0.007

¹Based on gas chromatography mass spectrometry (GCMS) analysis of diets.

ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LA: linoleic acid; n-3FAD, omega-3 fatty acid deficient; n-3FAS, omega-3 fatty acid sufficient.

3.1.6 Tuberculosis strain and aerosol infection procedure

Mice were infected with ~100 CFU/lung to establish a chronic infection, based on previous C3HeB/FeJ mouse TB research studies (Jordao *et al.*, 2008:1380; Medina & North, 1998:273; Vilaplana *et al.*, 2013:200). A virulent H37Rv *Mtb* strain was used for the infection. It was cultivated, harvested, and suspended at the IDM where the study was conducted. To ensure virulence for *in vivo* studies, strain H37Rv was passaged once in mice and stored as frozen aliquots. Prior to infection, stock solutions of *Mtb* were thawed and vortexed with 3 mm borosilicate glass beads (Sigma) to break up bacterial clumps. This stock was used to prepare fresh subcultures for infection. Pulmonary infection was performed using an inhalation exposure system (model A4224, Glas-Col Middlebrook). To infect mice with a natural dose of 100 CFU/lung, animals were exposed for 40 min to an aerosol generated by nebulising approximately 6ml of a suspension containing 2×10^6 live bacteria by means of an inhalation exposure system (IES) (Guler *et al.*, 2011:1340). To confirm the infection dose, mice were euthanised one day post infection and lung cultures, determined as described below.

3.2 Data and sample collection

Tail vein blood was collected before infection at baseline. After infection mice were weighed once per week and monitored daily by utilising TB- and study-specific monitoring sheets. Mice were euthanised on day 28 after infection and blood and lung samples were collected.

3.2.1 Euthanasian procedure

Euthanasia procedures were performed in accordance with the South African National Guidelines and UCT practice for laboratory animal standard operating procedure for euthanasia, at the P3 safety unit, by halothane exposure followed by cervical dislocation (Parihar *et al.*, 2013). This euthanasia method is approved by the American Veterinary medical association (AVMA) and is used by the Animal facility of UCT as a standard operating procedure.

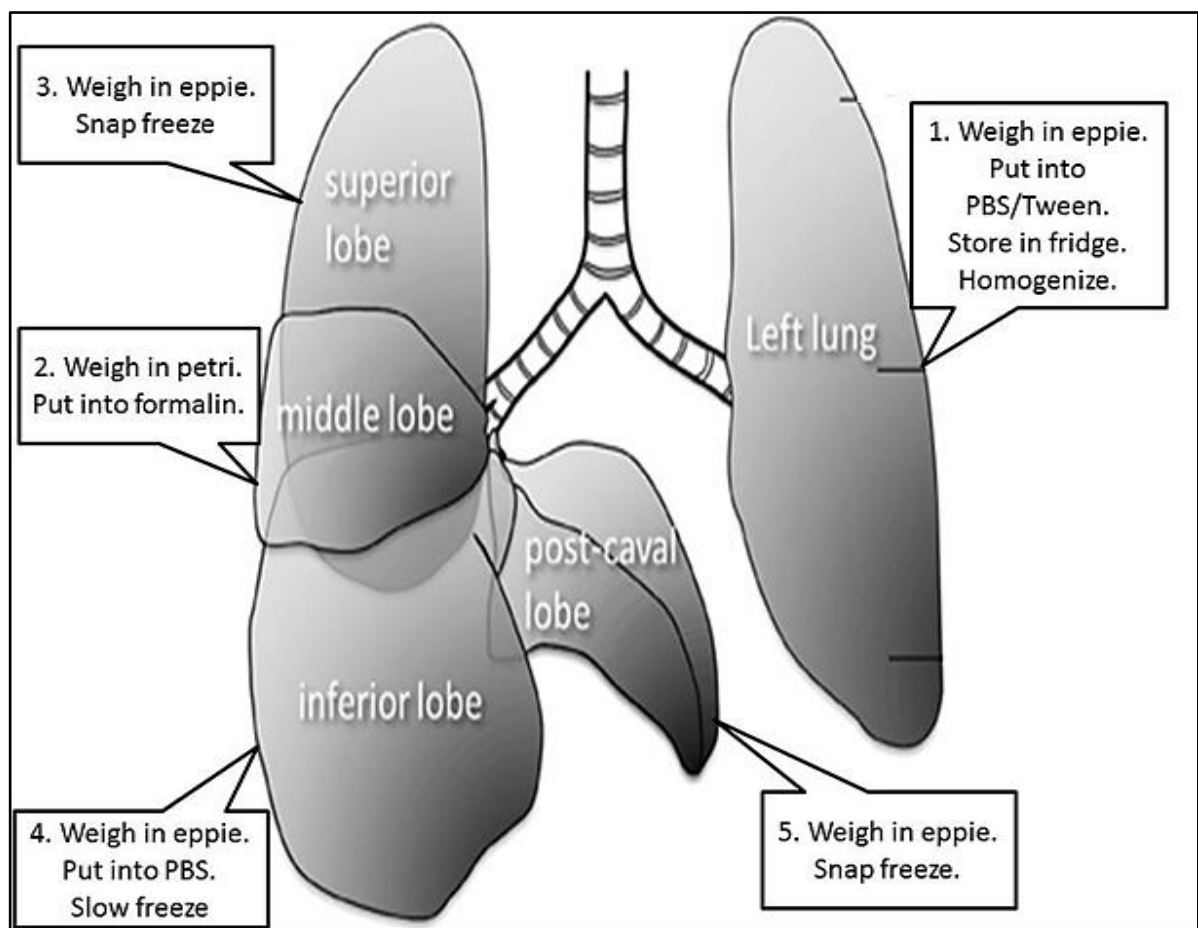
3.2.2 Blood sampling

Blood samples were collected for analysis of red blood cell (RBC) total phospholipid fatty acid composition. The baseline blood sample was taken from the lateral tail vein using the “tail nick” method. The mice were either warmed under a warming lamp or gently wrapped in a warm cloth to dilate the blood vessel prior to taking the sample. They were then placed in a tube restrainer

and a maximum of 200 µl blood sample was collected. At euthanasia trunk blood was collected by heart puncture. The blood was collected in EDTA-coated Minivette® tubes (Minivette® POCT, 1000 µl, Sarsted) and centrifuged. The RBC were washed twice with saline before storage at -80 °C.

3.2.3 Lung sampling

Lung tissue was collected for the analysis of total phospholipid fatty acid composition, lipid mediators, histopathology, and cytokine concentrations. The lungs were removed aseptically by making an incision through the thoracic cavity with surgical scissors to present the thoracic wall (so that the lungs were readily visible on either side of the heart). With small-toothed forceps, the bronchial tree close to the heart was gripped and the lung lobes were cut out. The left lung lobe



was placed into 1 ml phosphate-buffered saline (PBS), stored in the fridge at 4 °C, and afterwards homogenised for the analysis of the bacillary load and lung cytokines. The right superior lung lobe was snap frozen in liquid nitrogen and stored at -80 °C for lipid mediator analysis. The right middle lobes for histology were submerged in formalin (10%). See Figure 3-3 for a visual illustration of the lung lobes that were used for analysis.

Figure 3-2 Lung lobe sampling

The left lung lobe was placed into 1 ml phosphate-buffered saline (PBS), stored in the fridge at 4 °C, and afterwards homogenised for the analysis of the bacillary load and lung cytokines. The right superior lung lobe was snap frozen in liquid nitrogen and stored at -80 °C for lipid mediator analysis. The right middle lobes for histology were submerged in formalin (10%)

3.3 Laboratory analysis

3.3.1 Fatty acid analysis

A sample volume of 50 µl for baseline RBC, and 300 µl for endpoint RBC or lung homogenates were analysed for total phospholipid fatty acid composition within 6 months. Fatty acids were analysed by using gas chromatography tandem mass spectrometry (GCMSMS) on an Agilent Technologies 7890A Gas Chromatograph system equipped with an Agilent Technologies 5975C VL mass selective detector (Agilent Technologies, Santa Clara, USA). Total phospholipids were extracted with chloroform: methanol (2:1 vol:vol; containing 0.01% butylated hydroxytoluene) by using a modification of the method of Foch *et al.* (Folch *et al.*, 1956). Lipid extracts were concentrated, and the neutral lipids were separated from the phospholipids by using thin-layer chromatography (silica gel 60 plates, 10 x 20 cm; Merck) and eluted with diethyl ether: petroleum ether: acetic acid (30:90:1 vol: vol:vol). The lipid band that contained phospholipids was removed from the thin-layer chromatography plate and transmethylated with methanol: sulphuric acid (95:5 vol:vol) at 70°C for 2 h to yield fatty acid methyl esters (FAMES). The resulting FAMES were extracted with water and hexane. The organic layer was evaporated, redissolved in hexane, and analysed by using quadruple gas chromatography–electron impact–mass spectrometry on an Agilent Technologies 7890A Gas Chromatograph system equipped with an Agilent Technologies 5975C VL mass selective detector (Agilent Technologies). The gas chromatography separation of FAMES was carried out on a BPX 70 capillary column (60 m x 0.25 mm x 0.25 mm; SGE Analytic Sciences, Melbourne, Australia) by using helium as the carrier gas at a flow rate of 1.3 mL/min. The gas chromatography injector was held at a temperature of 280 °C, and the mass spectrometry source was maintained at temperatures of 230°C. The injection volume of the sample solution was 1 µL by using a split ratio of 1:1. The oven temperature was programmed from 130 °C to 240 °C, rose from 130 °C to 200 °C at 2°C/min, was held at 200 °C for 4 min, and then rose at 5 °C/min to 220 °C. After the temperature was held isothermal at 220 °C for 5 min, it was increased by 10°C/min to 240 °C, where it was retained for 5min. The total analysis time was 53 min. Mass spectrometry with 70 eV electron ionisation was carried out in full-scan acquisition mode, and all mass spectra were acquired over the m/z range of 50–550. Quantification of FAME was done by using the selected-ion extraction method on the basis of the response of 2 diagnostic ions. FAME peaks were identified and calibrated against a standard

reference mixture of 33 FAMES (Nu-Chek Prep, Waterville, USA) and two single FAME standards (Larodan Fine Chemicals AB, Solna, Sweden). Data analysis was performed by using Masshunter mass spectrometry data software (Agilent G1701EA version E.02.00.493). Relative percentages of fatty acids were calculated by taking the concentration of a given fatty acid derivative as a percentage of the total concentration of all fatty acids identified in the sample. Quality of data was assured with a separate calibration for each fatty acid, monitoring of internal standard (1,2 – diheptadecanoyl – sn – glycerol – 3 phosphorylcholine, Matreya, Pennsylvania, USA) and Levey Jennings graphs for a pooled plasma control analysed with each batch.

3.3.2 Lung cytokine concentrations

Lung cytokines were measured with the 16 plex Quansys Biosciences Q-Plex™ Mouse Cytokine Screen (Quansys Bioscience, Logan, UT, USA) measuring IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-1, IFN γ , TNF α , MIP-1 α , GMCSF and RANTES. Following preparation, all reagents were equilibrated to room temperature (20-25 °C) before use. An 8-point calibrator curve (7 points plus 1 blank) was prepared in polypropylene tubes. The undiluted prepared calibrator served as the high point of the standard curve, and the sample diluent served as the negative.

The lung homogenates were centrifuged and the supernatant diluted x3 times (20 μ l sample + 40 μ l diluent) with enough sample diluent to have 50 μ l per well in polypropylene tubes. Afterwards, 50 μ l per well of the calibration curve and samples was added by pre-wetting the pipette tips three times, drawing up the liquid into the pipette, and then dispensing it back into the original vessel, aspirating 50 μ l and dispensing into the Q-Plex Array 96-well plate. All samples and calibration curves were loaded onto the plate within 10 min. The plate was then covered with a plate seal and shaken on a plate shaker that was set to 500 revolutions per min (RPM) for one hour at room temperature. After that, the plate was washed three times. Next, 50 μ l Detection Mix was added per well, covered with a new plate seal, and returned to the plate shaker (set to 500 RPM) for one hour at room temperature. The plate was washed three times once again, and 50 μ l of Stretavidin-HRP1X was added per well. The mixture was returned to the plate shaker (set to 500 RPM) for 15 min at room temperature. Thereafter the plate was washed six times, and 50 μ l was added to the previously prepared substrate. The plate was placed in the Q-View Imager Pro, Q-View Software opened, and a project was created. The exposure time was set to 300 sec and the images were captured (Brant & Fabisiak, 2009:227; Guler *et al.*, 2015; Jordao *et al.*, 2008:1380; Lim *et al.*, 2009:122; Malan *et al.*, 2016; Newton & Dixon, 2012; Oldenburg *et al.*, 2000:506).

3.3.3 Lung lipid mediators

Lipid mediators were extracted and analysed with liquid chromatography tandem mass spectrometry (LCMSMS). Lipid mediators were extracted from tissue (~50 mg homogenised lung lobe) with solid phase extraction (SPE) using Strata-X (Phenomenex, Torrance, United States of America). Samples were diluted with water and adjusted to 15% methanol (v/v), to a final volume of 4.6 ml. Internal standards, PGB2-d4 and 12-HETE-d8 (500 pg of each), were added to each sample. To remove any precipitated proteins, the samples were incubated on ice for 30 min and then centrifuged at 3000 rpm for 8 min. The resulting clear supernatants were then acidified with 1 N hydrochloric acid to pH 3.0 and immediately applied to SPE cartridges that had been preconditioned with 2 ml methanol followed by 4 ml water. The cartridges were then washed with 10ml 15% (v/v) methanol, and 4 ml hexane in this order. Lastly, the lipid mediators were eluted with 6ml methyl formate. A vacuum manifold (Phenomenex) was used to perform the SPE and the vacuum was carefully regulated so that single drops could be seen from each cartridge. The methyl formate was evaporated under a fine stream of nitrogen and the residue was dissolved in 20 μ l acetonitrile, flushed with nitrogen and stored at most for four days at -20°C before LCMSMS analysis (Malan *et al.*, 2016:18).

Samples were analysed with an Agilent Technologies 6410 MSMS coupled to an Infinity 1260 HPLC pump (Santa Clara, United States of America). The mass spectrometer has a mass range of 15–1650 m/z, minimum multiple reaction monitoring (MRM) dwell time of 5 ms with 10 MRMs and a maximum of 99 MRM transitions per time segment. The instrument was operated in negative electrospray ionisation mode. At least two transitions were monitored for each compound, using a dynamic MRM method. The gas temperature and flow were set at 350°C and 12 l/min respectively, and the capillary voltage at 4000V. Chromatographic analysis was performed on a C18 column (Poroshell, 2.7 μ , 100x2.1mm²; Agilent Technologies) with a flow rate of 0.4 ml/min and column temperature of 50°C . Sample injections were performed with an Agilent G1367B auto sampler. The sample chamber temperature was set at 5°C and the injection volume at 15 μ l, subsequent to mixing 5 μ l of sample (in acetonitrile) with 10 μ l water in the auto sampler, just before injection. The analyses were performed using an acetonitrile-based system with a flow program, by mixing two solvents (A and B) with programmed ratio changes for optimal separation of compounds. Solvent A was water/glacial acetic acid, 99:1(v/v) and solvent B 100% acetonitrile. Data were then quantified with Masshunter B05 02, using external calibration for each compound and two internal standards to correct for losses and matrix effects during sample preparation and analysis (Malan *et al.*, 2016:18).

3.3.4 Histological analysis (free alveolar space)

All histological analysis was done according to the protocol for Nikon Microscope quantification of pulmonary histopathology, focusing on the quantification of the free alveolar space. Lungs were fixed in 4% neutral buffered formalin. Imbedded sections of the right lung were sliced and stained with hematoxylin and eosin (H&E). Three sections at 10 microns apart were analysed. An ocular meter (Nikon NIS-Elements, Nikon Corporation, Tokyo, Japan) was used to determine the free alveolar space in the lungs and/or granulomatous area and alveolar space as a percentage of the total lung.

3.4 Statistical analysis

The data from this project were analysed by using IBM Statistical Program for Social Sciences (SPSS) Statistics software (version 25). Graphpad prism was also used to visually represent the data. Power analysis to determine the suitable number of animals needed was based on previous animal studies that used C3HeB/FeJ mice, with similar research outcomes (Bouté *et al.*, 2017; Driver *et al.*, 2012; Irwin *et al.*, 2015; Marzo *et al.*, 2014; Vilaplana *et al.*, 2013). Corresponding to these studies, five mice per group were decided on as sufficient to examine the research outcomes. The experiment was repeated, giving a total of ten mice per group. The normality of the data was tested using, histograms and the Shapiro-Wilk test. Homogeneity of variance was examined by the Levene's test. The data were examined for the presence of outliers by using box plots. Results of normally distributed (parametric) data were expressed as means $\pm$ standard error of the mean (SEM). A p-value of < 0.05 was considered statistically significant. Independent T-tests were used to compare the effect of *Mtb* infection in n-3 LCPUFA-low status mice compared with mice with sufficient n-3 LCPUFA status before infection with regard to markers of lung inflammatory response, i.e. lung lipid mediators, histology and cytokines.

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CHAPTER 4 RESULTS

In this chapter, the main findings of the research project will be provided. The results will be discussed within the two experiments (experiment 1= E1; experiment 2= E2) separately and as a total group.

4.1 Baseline red blood cell phospholipid polyunsaturated fatty acid status and body weight prior to tuberculosis infection

Before infection, mice were conditioned for six weeks on either omega-3 polyunsaturated fatty acid (n-3 PUFA) sufficient (n-3 FAS) (n=10) or -deficient (n-3FAD) (n=10) diets, whereafter the red blood cell (RBC) phospholipid fatty acid composition of a sub-sample (n=10) of mice was analysed in order to determine whether dietary fat modifications reflected in the cell membrane compositions (Table 4-1). After the preconditioning phase, the n-3FAD mice presented with a lower linoleic acid (LA) ($p < 0.001$) and higher arachidonic acid (AA) ($p = 0.026$), osbond acid, total omega-6 long chain polyunsaturated fatty acid (n-6 LCPUFA) and n-6:n-3 LCPUFA ratio; and with lower EPA, DHA and total n-3 LCPUFA compared to the n-3FAS mice (all $p < 0.001$).

Table 4-1 Baseline red blood cell total phospholipid polyunsaturated fatty acid status six weeks after preconditioning on n-3FAS and n-3FAD diets ^{1,2}

	N-3FAS (n=5)	n-3FAD (n=5)	p-value
n-6 PUFA			
18:2n-6 (Linoleic acid)	10.63 ± 0.42	7.74 ± 0.16	<0.001
20:4n-6 (Arachidonic acid)	17.94 ± 0.46	19.56 ± 0.37	0.026
22:5n-6 (Osbond acid)	1.12 ± 0.06	3.81 ± 0.25	<0.001
Total n-6 LCPUFA	22.79 ± 0.33	28.33 ± 0.47	<0.001
n-3 PUFA			
18:3n-3 (Alpha-linolenic acid)	0.03 ± 0.02	0.00 ± 0.00	0.195
20:5n-3 (Eicosapentaenoic acid)	0.20 ± 0.01	0.04 ± 0.01	<0.001
22:6n-3 (Docosahexaenoic acid)	7.89 ± 0.32	3.97 ± 0.26	<0.001
Total n-3 LCPUFA	8.73 ± 0.24	4.18 ± 0.25	<0.001
Ratio			
Total n-6: n-3 LCPUFA ratio	2.62 ± 0.05	6.87 ± 0.42	0.001

¹ Data are expressed as mean ± SEM

² Independent T-tests were used to determine differences between n-3FAS and n-3FAD groups
FAD: fatty acid deficient group; FAS: fatty acid sufficient group; n-3: omega-3; LCPUFA: long chain polyunsaturated fatty acid.

4.2 Endpoint total phospholipid n-6 and n-3 polyunsaturated acid status

Fatty acid status was measured in RBC and lung homogenates. The endpoint n-6 and n-3 PUFA status in RBC and lungs in the n-3FAS and n-3FAD groups are described in Table 4-2 (experiment 1, 2 and total group) and Table 4.3 (experiment 2). Endpoint n-6 and n-3 PUFA status of lungs were only done for experiment 2 of this study. Three weeks post TB infection at euthanasia, the n-3FAD group presented with lower LA and higher AA, osbond acid and total n-6 LCPUFA RBC composition compared with the n-3FAS groups in both experiments and the total group (all $p < 0.001$). Furthermore, the n-3FAD group in both experiments and the total group had a lowered Alpha-linolenic acid (ALA), Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA) and total n-3 LCPUFA, composition (all $p < 0.001$), except DHA from experiment 1, $p = 0.003$). Lastly, a higher total n-6: n-3 LCPUFA ratio was observed in the n-3FAD groups compared with the n-3FAS groups (all $p < 0.040$). These findings show that the three-week dietary intervention following infection succeeded in modifying the fatty acid status of the mice accordingly.

Table 4-2 Endpoint red blood cell total phospholipid n-6 and n-3 polyunsaturated fatty acid status of *Mycobacterium tuberculosis*-infected mice after providing n-3FAS and n-3FAD diets for three weeks ^{1,2}

	EXPERIMENT 1 (n =9)			EXPERIMENT 2 (n =10)			TOTAL GROUP (n =19)		
	n-3FAS (n=5)	n-3FAD (n=4)	p-value	n-3FAS (n=5)	n-3FAD (n=5)	p-value	n-3FAS (n=10)	n-3FAD (n=9)	p-value
n-6 PUFA									
18:2n-6 (Linoleic acid)	10.87 ± 0.11	8.74 ± 0.12	<0.001	9.98 ± 0.20	7.55 ± 0.31	<0.001	10.42 ± 0.18	8.08 ± 0.27	<0.001
20:4n-6 (Arachidonic acid)	18.12 ± 0.16	20.41 ± 0.08	<0.001	17.71 ± 0.27	20.45 ± 0.25	<0.001	17.92 ± 0.16	20.43 ± 0.14	<0.001
22:5n-6 (Osbond acid)	1.15 ± 0.04	4.13 ± 0.46	<0.001	0.58 ± 0.02	2.76 ± 0.18	<0.001	0.87 ± 0.10	3.37± 0.32	<0.001
Total n-6 LCPUFA	23.19 ± 0.51	29.09 ± 0.60	<0.001	20.17 ± 0.33	25.47 ± 0.39	<0.001	21.68 ± 0.53	27.08 ± 0.71	<0.001
n-3 PUFA									
18:3n-3 (Alpha-linolenic acid)	0.09 ± 0.00	0.01 ± 0.00	<0.001	0.05 ± 0.00	0.00 ± 0.00	<0.001	0.07 ± 0.01	0.01 ± 0.00	<0.001
20:5n-3 (Eicosapentaenoic acid)	0.24 ± 0.00	0.04 ± 0.01	<0.001	0.13 ± 0.00	0.02 ± 0.00	<0.001	0.19 ± 0.02	0.03 ± 0.01	<0.001
22:6n-3 (Docosahexaenoic acid)	8.36 ± 0.13	3.78 ± 0.55	0.003	6.09 ± 0.21	1.72 ± 0.15	<0.001	7.22 ± 0.39	2.63 ± 0.43	<0.001
Total n-3 LCPUFA	9.41 ± 0.20	4.00 ± 0.58	<0.001	6.63 ± 0.20	1.82 ± 0.15	<0.001	8.02 ± 0.48	2.79 ± 0.46	<0.001
Ratio									
Total n-6: n-3 LCPUFA ratio	2.47 ± 0.05	7.87 ± 1.40	0.030	3.05 ± 0.05	14.38 ± 1.19	0.001	2.76 ± 0.10	11.49 ± 1.42	<0.001

¹ Data are expressed as mean ± SEM

² Independent T-tests were used to determine differences between n-3FAS and n-3FAD groups

FAD: fatty acid deficient group; FAS: fatty acid sufficient group; n-3: omega-3; LCPUFA: long chain polyunsaturated fatty acids

Table 4-3 **Endpoint lung total phospholipid n-6 and n-3 polyunsaturated fatty acid status of *Mycobacterium tuberculosis*-infected mice after providing n-3FAS and n-3FAD diets for three weeks ^{1,2}**

	EXPERIMENT 2 (n =9)		
	n-3FAS (n=5)	n-3FAD (n=4)	p-value
n-6 PUFA			
18:2n-6 (Linoleic acid)	7.48 ± 0.14	5.06 ± 0.27	<0.001
20:4n-6 (Arachidonic acid)	14.40 ± 0.15	15.19 ± 0.58	0.188
22:5n-6 (Osbond acid)	1.13 ± 0.05	5.17 ± 0.27	<0.001
Total n-6 LCPUFA	21.24 ± 0.19	26.58 ± 0.65	<0.001
n-3 PUFA			
18:3n-3 (Alpha-linolenic acid)	0.09 ± 0.00	0.01 ± 0.00	<0.001
20:5n-3 (Eicosapentaenoic acid)	0.17 ± 0.01	0.23 ± 0.15	0.734
22:6n-3 (Docosahexaenoic acid)	8.08 ± 0.15	2.39 ± 0.26	<0.001
Total n-3 LCPUFA	10.03 ± 0.14	2.90 ± 0.244	<0.001
Ratio			
Total n-6: n-3 LCPUFA ratio	2.12 ± 0.03	9.29 ± 0.65	0.002

¹ Data are expressed as mean ± SEM

² Independent T-tests were used to determine differences between n-3FAS and n-3FAD groups

FAD: fatty acid deficient group; FAS: fatty acid sufficient group; n-3: omega-3; LCPUFA: long chain polyunsaturated fatty acids

4.3 Progression of tuberculosis infection in mice (lung and spleen bacterial loads, lung, and spleen weight indexes)

One day following infection, four mice were euthanized to confirm the infection dose, which was 100 and 500 colony-forming units (CFU)/mouse for experiment 1 and 2 respectively. The total lung and spleen CFU $\log_{10}$, as well as the lung and spleen weight indexes after three weeks of dietary intervention (four weeks post-infection) from the two experiments individually and together as a total group, are summarised in table 4-4.

Four weeks after *Mycobacterium Tuberculosis* (*Mtb*) infection the spleen-weight indexes were lower overall in the n-3FAD groups across experiment 1, experiment 2 and for the total group ($p = 0.006$, $p = 0.041$ and $p = 0.004$, respectively) compared with the n-3FAS groups (Figure 4-1 b). However, there were no differences with regard to the lung weight indexes, between the n-3FAS and n-3FAD groups across both experiments and for the total group (Figure 4-1 a). The n-3FAD mice from experiment 1 presented with a lowered total lung CFU count ($p = 0.002$) compared with the n-3FAS group. Bacilliary loads in lung and spleen tissue were higher in the n-3FAS groups across both experiment 1 and experiment 2 with a significant difference between groups from experiment 1 ($p < 0.005$) with regards to lung bacilliary loads (Figure 4-2 a and b).

Table 4-4 Lung and spleen bacillary loads and weight indexes 4 weeks post infection of Mycobacterium tuberculosis-infected mice after providing n-3FAS and n-3FAD diets for three weeks ^{1,2}

	EXPERIMENT 1 (n =10)			EXPERIMENT 2 (n =10)			TOTAL GROUP (n =20)		
	n-3FAS (n=5)	n-3FAD (n=5)	p-value	n-3FAS (n=5)	n-3FAD (n=5)	p-value	n-3FAS (n=10)	n-3FAD (n=10)	p-value
Total Lung CFU Log₁₀	6.03 ± 0.04	5.44 ± 0.13	0.002	6.76 ± 0.03	6.62 ± 0.07	0.095*	6.39 ± 0.13	6.03 ± 0.21	0.154
Spleen CFU Log₁₀[*]	3.81 ± 0.24	3.44 ± 0.24	0.315	3.86 ± 0.20	3.56 ± 0.31	0.440	3.83 ± 0.15	3.51 ± 0.19	0.187
Lung-weight-index	0.69 ± 0.01	0.70 ± 0.02	0.395	0.93 ± 0.04	0.81 ± 0.04	0.073*	0.81± 0.04	0.76 ± 0.03	0.331
Spleen-weight-index	0.58 ± 0.01	0.51± 0.02	0.006	0.67 ± 0.01	0.56 ± 0.04	0.041	0.62 ± 0.02	0.53 ± 0.02	0.004

¹ Independent T-tests were used to determine differences between the n-3FAS and n-3FAD groups

² Data are expressed as mean ± SEM

*Tendency/Trend towards significance

CFU: colony-forming unit; FAD: fatty acid deficient group; FAS: fatty acid sufficient group; LCPUFA: long chain polyunsaturated fatty acids; n-3: omega-3; n-6: omega-6

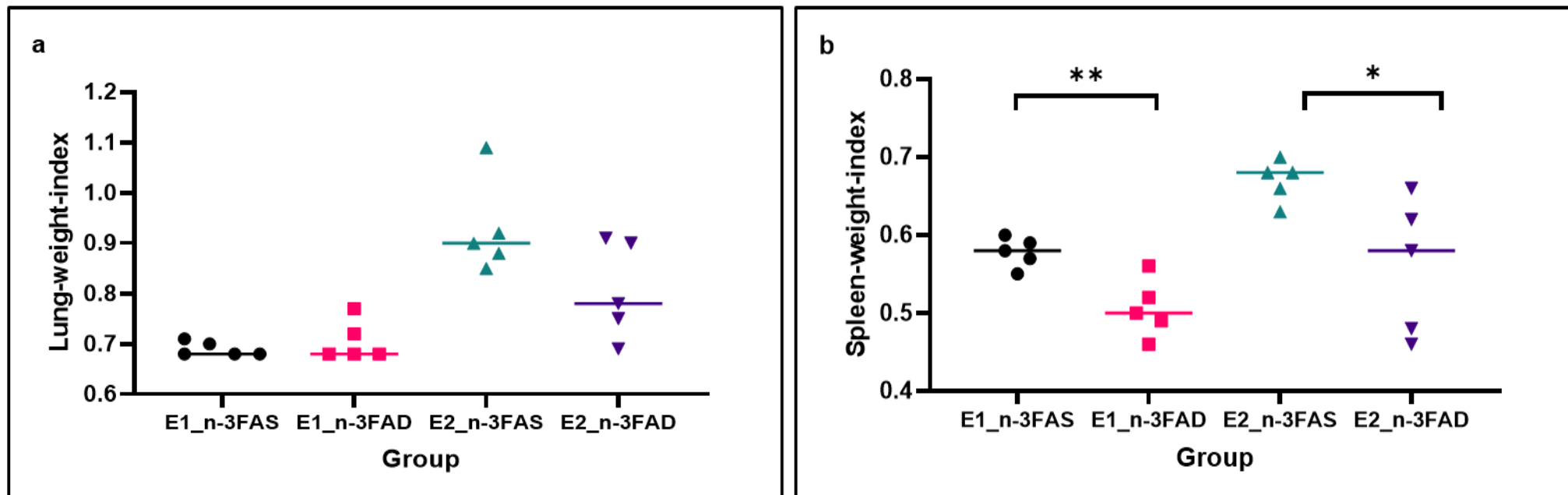


Figure 4-1 Lung- (a) and Spleen- (b) weight-indexes four weeks post-infection after providing n-3FAS and n-3FAD diets for three weeks^{1,2}

¹ Independent T-tests were used to determine differences between the n-3FAS and n-3FAD groups

² Data are expressed as mean ± SEM

E1: Experiment 1; E2: Experiment 2; n-3FAD: omega-3 fatty acid deficient; n-3FAS: fatty acid sufficient

** P < 0.005 ; * p < 0.05

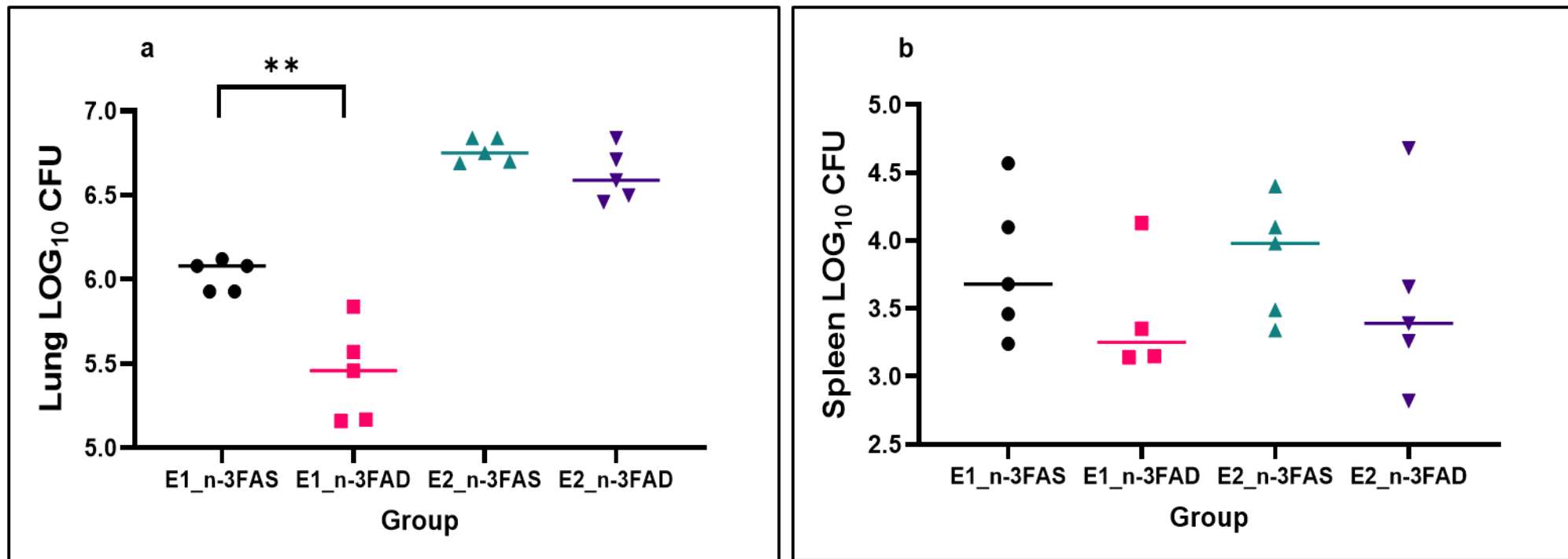


Figure 4-2 Lung- (a) and Spleen- (b) bacillary loads 4 weeks post infection after providing n-3FAS and n-3FAD diets for three weeks

^{1,2}

¹ Independent T-tests were used to determine differences between the n-3FAS and n-3FAD groups

² Data are expressed as mean ± SEM

E1: Experiment 1; E2: Experiment 2; n-3FAD: omega-3 fatty acid deficient; n-3FAS: fatty acid sufficient

** P < 0.005

4.4 Free alveolar space and histology

Figure 4-3 visually represents the free alveolar spaces of the sufficient and low n-3 PUFA status groups from both experiments. Throughout both experiments and for the total group the n-3FAD groups presented with a higher percentage of free alveolar space compared with the n-3FAS groups. Within experiment 1 and the total group, there were more free alveolar space in the n-3FAD than the n-3FAS group ($p = 0.001$ and $p = 0.021$, respectively), whereas experiment 2 showed the same trend towards significance ($p = 0.087$). Table 4-5 displays the images of representative haematoxylin staining of lungs of the n-3FAS and n-3FAD groups for both experiments.

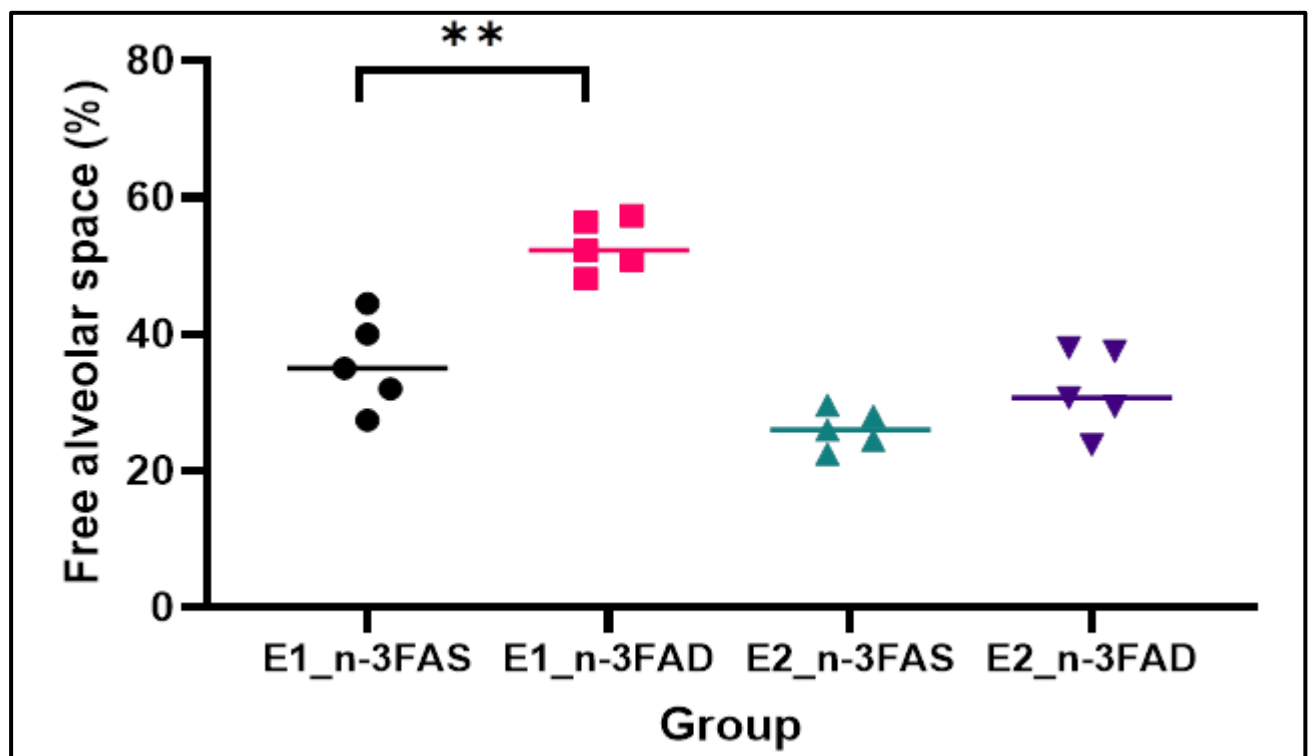


Figure 4-3 Free alveolar space 4 weeks post infection after providing n-3FAS and n-3FAD diets for three weeks^{1,2}

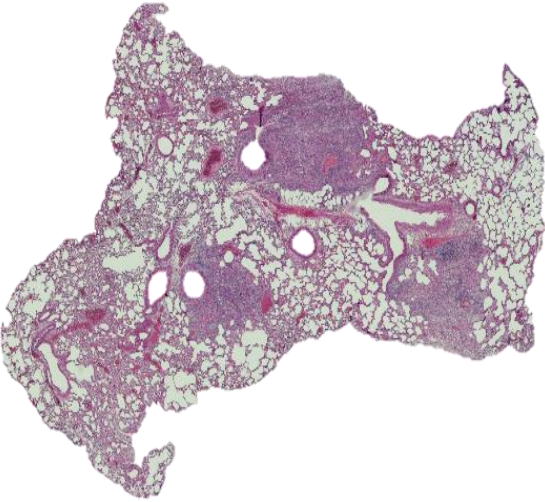
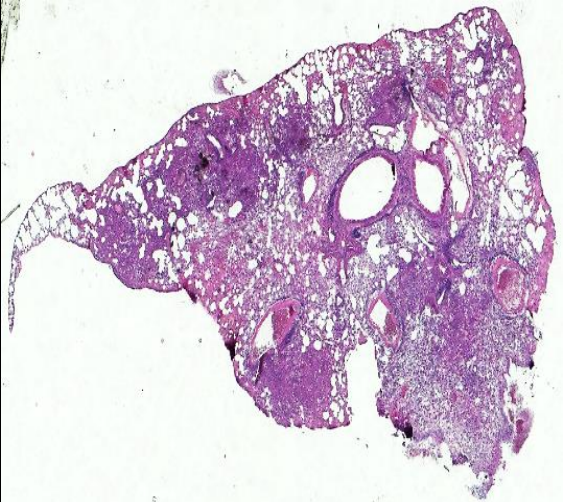
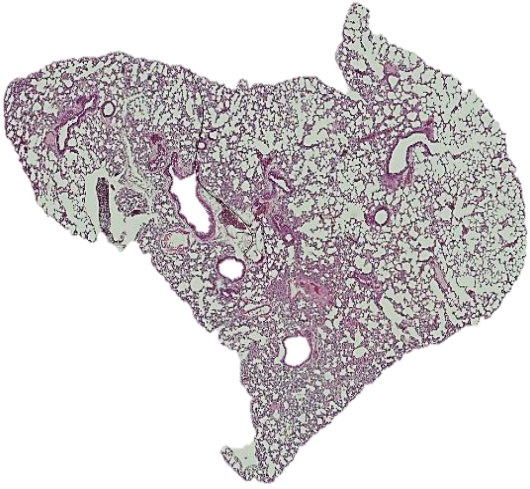
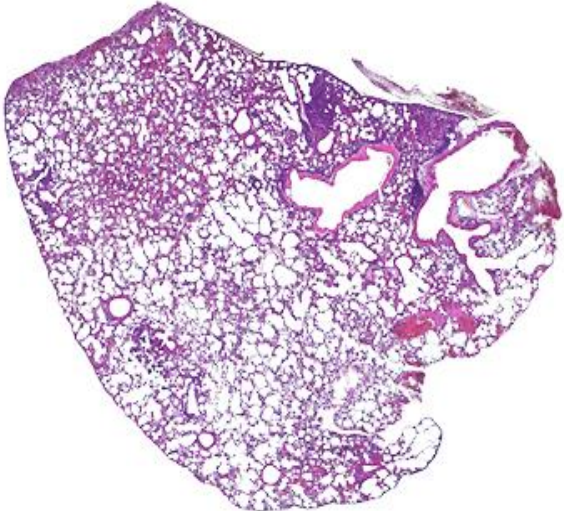
¹ Independent T-tests were used to determine differences between the n-3FAS and n-3FAD groups

² Data are expressed as mean ± SEM

E1: Experiment 1; E2: Experiment 2; n-3FAD: omega-3 fatty acid deficient; n-3FAS: fatty acid sufficient

** $P < 0.005$; * $p < 0.05$

Table 4-5 **Representative haematoxylin stained images of lungs of Mycobacterium tuberculosis-infected mice after providing n-3FAS and n-3FAD diets for three weeks**

HISTOLOGICAL ANALYSIS IMAGES		
Group	Experiment 1	Experiment 2
n-3FAS	 Free Alveolar space: 34 %	 Free Alveolar space: 23 %
n-3FAD	 Free Alveolar space: 60.5 %	 Free Alveolar space: 38 %

n-3FAS: omega 3-3 fatty acid sufficient; n-3FAD: omega-3 fatty acid deficient

4.5 Markers of inflammation

4.5.1 Lung cytokine concentrations

Mice from experiment 2 generally presented with higher lung cytokine concentrations than experiment 1. In the total group, IL-1 α , IL-6 and IFN- γ were lower in the n-3FAD than the n-3FAS group ($p = 0.049$, $p = 0.044$ and $p = 0.035$ respectively), and IL-1 β showed a similar trend ($p = 0.099$). In experiment 1, the n-3FAD mouse groups presented with lower IL-1 α , IL-1 β , IL-17, IFN- γ and GMCSF concentrations compared to the n-3FAS groups ($p \leq 0.001$, $p = 0.005$, $p = 0.048$, $p = 0.019$, $p = 0.030$ respectively). Table 4-6 summarises the findings of these lung cytokines for both experiments separately and for the total group. Figure 4-6 shows the results for IL-1 α ; IL-1 β ; IL-6; IFN- γ and IL-17.

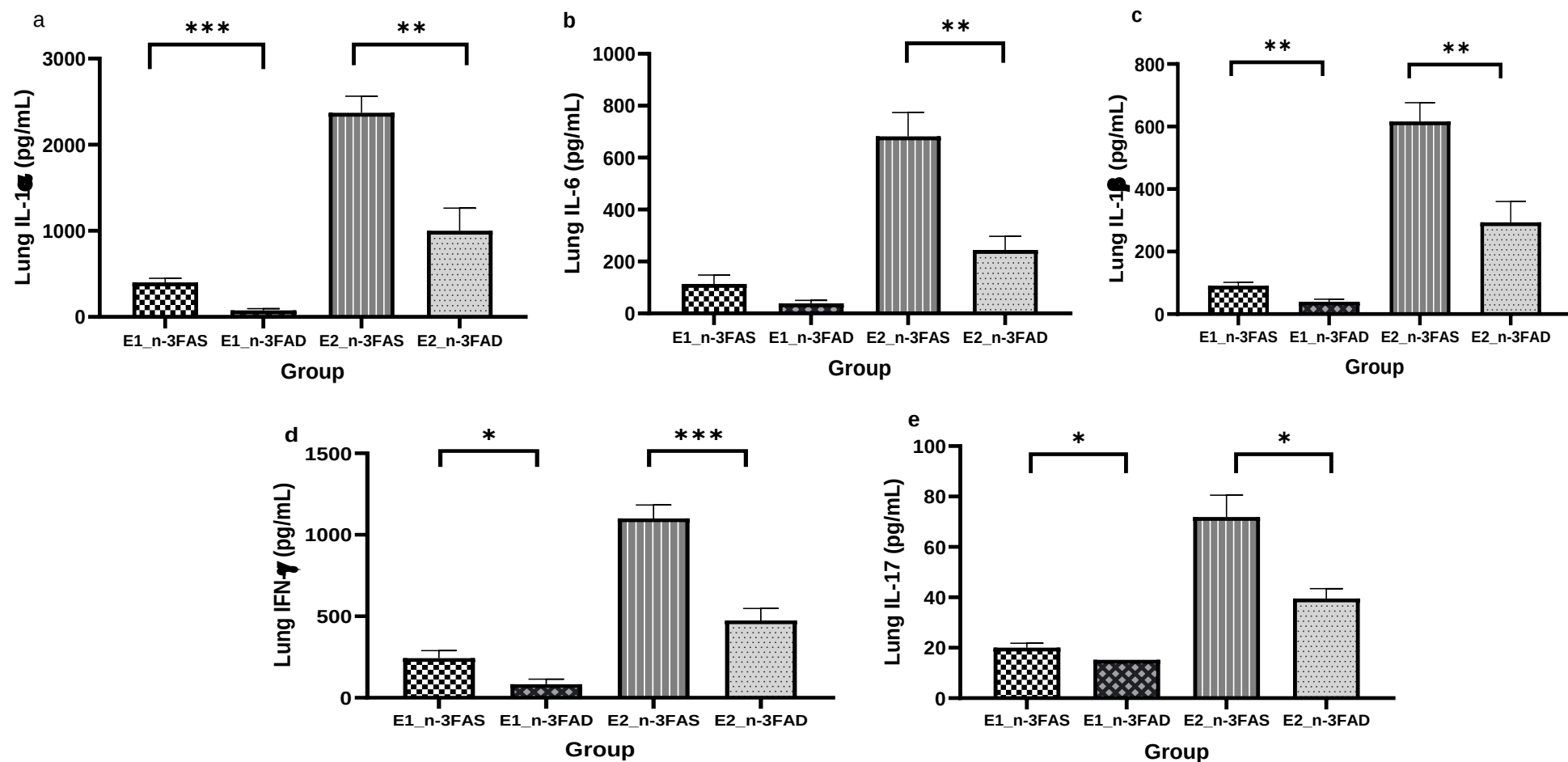


Figure 4-4 Lung cytokine concentrations of (a)-IL1 α ; (b)- IL6; (c)-IL1 β ; (d)- IFN γ and (e)- IL-17 4 weeks post infection after providing n-3FAS and n-3FAD diets for three weeks^{1,2}

¹ Independent T-tests were used to determine differences between the n-3FAS and n-3FAD groups

² Data are expressed as mean $\pm$ SEM

E1: Experiment 1; E2: Experiment 2; n-3FAD: omega-3 fatty acid deficient; n-3FAS: fatty acid sufficient

*** P < 0.001 ; ** P < 0.005 ; * p < 0.05

Table 4-6 Lung cytokine concentrations 4 weeks post infection of Mycobacterium tuberculosis-infected mice after providing n-3FAS and n-3FAD diets for three weeks ^{1,2}

INTERLEUKIN pg/ml	EXPERIMENT 1 (n =10)			EXPERIMENT 2 (n =10)			TOTAL GROUP (n =20)		
	n-3FAS (n=5)	n-3FAD (n=5)	p-value	n-3FAS (n=5)	n-3FAD (n=5)	p-value	n-3FAS (n=10)	n-3FAD (n=10)	p-value
IL- 1 α	401.10 $\pm$ 48.48	76.94 $\pm$ 18.59	< 0.001	2370.36 $\pm$ 194.7	1002.36 $\pm$ 260.62	0.004	1385.73 $\pm$ 341.51	539.65 $\pm$ 197.38	0.049
IL -1 β	90.83 $\pm$ 10.80	39.34 $\pm$ 8.33	0.005	616.47 $\pm$ 59.54	293.51 $\pm$ 67.08	0.007	353.65 $\pm$ 92.13	166.42 $\pm$ 53.01	0.099*
IL -6	113.94 $\pm$ 33.82	39.05 $\pm$ 11.59	0.069*	682.26 $\pm$ 91.28	244.18 $\pm$ 53.08	0.003	398.10 $\pm$ 105.25	141.62 $\pm$ 42.72	0.044
IL -12p70	24.48 $\pm$ 2.17	24.11 $\pm$ 4.05	0.939*	50.24 $\pm$ 10.18	44.96 $\pm$ 8.49	0.700	37.36 $\pm$ 6.52	34.53 $\pm$ 5.63	0.747
IL -17	20.05 $\pm$ 1.72	15.21 $\pm$ 0.04	0.048	71.87 $\pm$ 8.71	39.53 $\pm$ 3.95	0.010	45.96 $\pm$ 9.60	27.37 $\pm$ 4.46	0.103
IFN γ	243.55 $\pm$ 45.34	83.50 $\pm$ 30.74	0.019	1100.51 $\pm$ 82.58	474.70 $\pm$ 73.48	< 0.001	672.02 $\pm$ 149.57	279.10 $\pm$ 75.24	0.035
TNF- α	23.38 $\pm$ 14.85	5.20 $\pm$ 0.68	0.288	38.11 $\pm$ 3.31	30.06 $\pm$ 5.16	0.226	30.74 $\pm$ 7.58	17.62 $\pm$ 4.81	0.162
GMCSF	13.79 $\pm$ 1.83	7.59 $\pm$ 1.49	0.030	30.75 $\pm$ 3.33	20.57 $\pm$ 2.68	0.044	22.27 $\pm$ 3.35	14.08 $\pm$ 2.60	0.069*

¹Data are expressed as mean $\pm$ SEM

² Independent T-tests were used to determine differences between n-3FAS and n-3FAD groups

*Tendency/Trend towards significance

FAD: fatty acid deficient; FAS: fatty acid sufficient; GMCSF: Granulocyte-macrophage colony stimulating factor; IL: Interleukin; n-3: omega-3; n-6: omega-6; pg: picogram; ml: millilitre ; TNF- α : Tumour necrosis factor alpha; IFN γ : Interferon gamma

4.5.2 Pro-resolving lipid mediators

Table 4-7 summarises the findings of lipid mediator (LMs) signalling for both experiments individually and for the total group. In mice fed with n-3FAD diets across experiment 1, experiment 2 and the total group, an increase in the less inflammatory prostaglandin E_3 (PGE_3) was observed compared with the n-3FAS groups ($p = 0.052$; $p = 0.068$ and $p = 0.009$, respectively). Moreover, the n-3-FAD group also presented with lowered lung concentrations of the EPA-derived intermediates 5-, 9-, 11-, 12-, 15 and 18- hydroxyeicosapentaenoic acid (HEPE) compared with n-3FAS groups (Total group $p < 0.050$, Table 4-7). The n-3FAD diet group in experiment 1 presented with a higher lung concentration of PGE_2 and lower concentrations of 9-HEPE ($p = 0.001$ and $p = 0.002$, respectively) compared with the n-3FAS diet group.

There was a trend in experiment 1 for 18-, and 5-HEPE ($p = 0.060$ and 0.064 , respectively), and in experiment 2 for 12-HEPE, PGE_3 and PD_1 ($p = 0.078$, $p = 0.068$ and 0.087 , respectively) to be lower in the n-3FAD group.

4.5.3 Pro-inflammatory lipid mediators

With regards to the AA derived pro-inflammatory LMs, PGE_2 , PGD_2 and intermediates 8-, 9- and 11- hydroxyeicosatetraenoic acids (HETE), the n-3FAD groups resulted in significantly higher lung concentrations especially across experiment 2, except with regards to 8- and 9- HETE (See Figure 4-6: $p = 0.032$; $p = 0.005$; and $p = 0.017$ respectively). Across both experiment 2 and the total group results, higher lung concentrations of the abovementioned pro-inflammatory LMs were seen in the n-3FAD groups compared to the n-3FAS groups. However, the latter was not observed in experiment 1, where the n-3FAD group resulted in lower lung concentrations of the pro-inflammatory LMs, except for 8- and 9-HETE. See Table 4-7 for a summary of the findings. Figure 4-6 visually illustrates 8-HETE, 11-HETE, PGE_2 and PGD_2 between n-3FAS and n-3FAD groups 4 weeks post infection after providing n-3FAS and n-3FAD diets for three weeks.

Table 4-7 Lipid mediator signalling in lung homogenates at 4 weeks post infection of Mycobacterium tuberculosis-infected mice after providing n-3FAS and n-3FAD diets for three weeks^{1,2}

	EXPERIMENT 1 (n =10)			EXPERIMENT 2 (n =10)			TOTAL GROUP (n =20)		
	n-3FAS (n=5)	n-3FAD (n=5)	p-value	n-3FAS (n=5)	n-3FAD (n=5)	p-value	n-3FAS (n=10)	n-3FAD (n=10)	p-value
PGF₂α	179.92 ± 30.87	228.83 ± 24.84	0.252	86.71 ± 10.32	135.57 ± 14.54	0.025	133.31 ± 21.84	182.02 ± 20.63	0.121
TBxB₂	3796.37 ± 1127.92	4265.22 ± 961.76	0.760	3866.21 ± 823.98	4998.04 ± 652.37	0.313	3831.29 ± 658.58	4631.63 ± 561.29	0.367
PGE₃	51.75 ± 17.74	10.11 ± 4.35	0.052	39.55 ± 14.23	9.31 ± 1.69	0.068*	45.65 ± 10.91	9.71 ± 2.20	0.009
PGE₂	9121.08 ± 2511.09	7762.16 ± 1562.50	0.658	7082.19 ± 1185.97	12046.79 ±1500.43	0.032	8100.64 ± 1352.59	9904.47 ± 1246.10	0.340
PGD₂	201.91 ± 28.01	196.35 ± 31.63	0.899	59.64 ± 7.22	128.07 ± 16.57	0.005	130.78 ± 27.35	162.21 ± 2032	0.368
PD₁	7.95 ± 2.01	2.25 ± 1.07	0.037	3.03 ± 0.98	1.11 ± 0.08	0.087*	5.49 ± 1.34	1.68 ± 0.54	0.022
@18HEPE	9.60 ± 3.51	0.50 ± 0.00	0.060*	10.59 ± 1.81	1.31 ± 0.38	0.006	10.10 ± 1.87	0.90 ± 0.22	0.001
@15HEPE	51.96 ± 14.48	2.32 ± 1.50	0.026	16.66 ± 2.23	1.61 ± 0.10	0.002	34.31 ± 9.07	1.96 ± 0.72	0.006
@11HEPE	74.29 ± 21.79	9.32 ± 2.84	0.040	36.74 ± 6.39	10.43 ± 1.92	0.004	55.52 ± 12.40	9.88 ± 1.63	0.005
@12HEPE	1965.37 ± 489.52	293.88 ± 10.02	0.027	1411.76 ± 518.28	191.27 ± 26.81	0.078*	1688.57 ± 348.50	242.80 ±21.72	0.002
@9HEPE	33.99 ± 10.85	9.88 ± 8.88	0.124	28.34 ± 3.42	1.34 ±0.34	0.001	31.16 ± 5.44	5.61 ±4.43	0.002

	EXPERIMENT 1 (n =10)			EXPERIMENT 2 (n =10)			TOTAL GROUP (n =20)		
	n-3FAS (n=5)	n-3FAD (n=5)	p-value	n-3FAS (n=5)	n-3FAD (n=5)	p-value	n-3FAS (n=10)	n-3FAD (n=10)	p-value
@5HEPE	6.10 ± 2.29	0.30 ± 0.00	0.064*	3.45 ± 0.57	1.59 ± 0.12	0.030	4.77 ± 1.20	0.94 ± 0.22	0.006
@15HETE	3444.36 ± 741.63	3520.38 ± 1295.93	0.961	1856.54 ± 177.80	2549.77 ± 271.78	0.065*	2650.45 ± 446.41	3035.08 ± 644.82	0.630
@17HDHA	518.67 ± 131.22	133.31 ± 39.65	0.023	195.14 ± 38.77	90.47 ± 5.98	0.028	356.91 ± 84.07	111.89 ± 20.21	0.018
@11HETE	4763.04 ± 1026.18	4358.93 ± 1086.25	0.794	1964.63 ± 167.19	3236.82 ± 389.38	0.017	3363.84 ± 676.57	3797.87 ± 575.22	0.631
@12HETE	6066.07 ± 1233.52	7755.59 ± 702.95	0.268	5191.37 ± 2039.90	7730.35 ± 1369.15	0.332	5628.72 ± 1133.18	7742.97 ± 725.53	0.134
@8HETE	259.47 ± 51.51	397.59 ± 108.99	0.285	179.15 ± 62.80	260.06 ± 40.05	0.309	219.31 ± 40.56	328.83 ± 59.34	0.145
@9HETE	817.66 ± 176.62	642.14 ± 279.57	0.610	779.20 ± 191.33	989.57 ± 93.08	0.352	798.43 ± 122.92	815.86 ± 150.49	0.930
@5HETE	320.19 ± 78.14	383.70 ± 57.92	0.532	394.40 ± 113.06	433.00 ± 124.80	0.824	357.30 ± 65.96	408.35 ± 65.38	0.589

¹ Independent T-tests were used to determine differences between n-3FAS and n-3FAD groups

² Data are expressed as mean ± SEM

*Tendency/Trend towards significance

FAD: fatty acid deficient group; FAS: fatty acid sufficient group; HDHA: hydroxydocosahexaenoic acid; HEPE: hydroxyeicosapentaenoic; HETE: hydroxyeicosatetraenoic PD₁: protectin D₁; PG: prostaglandin; TBX: thromboxane

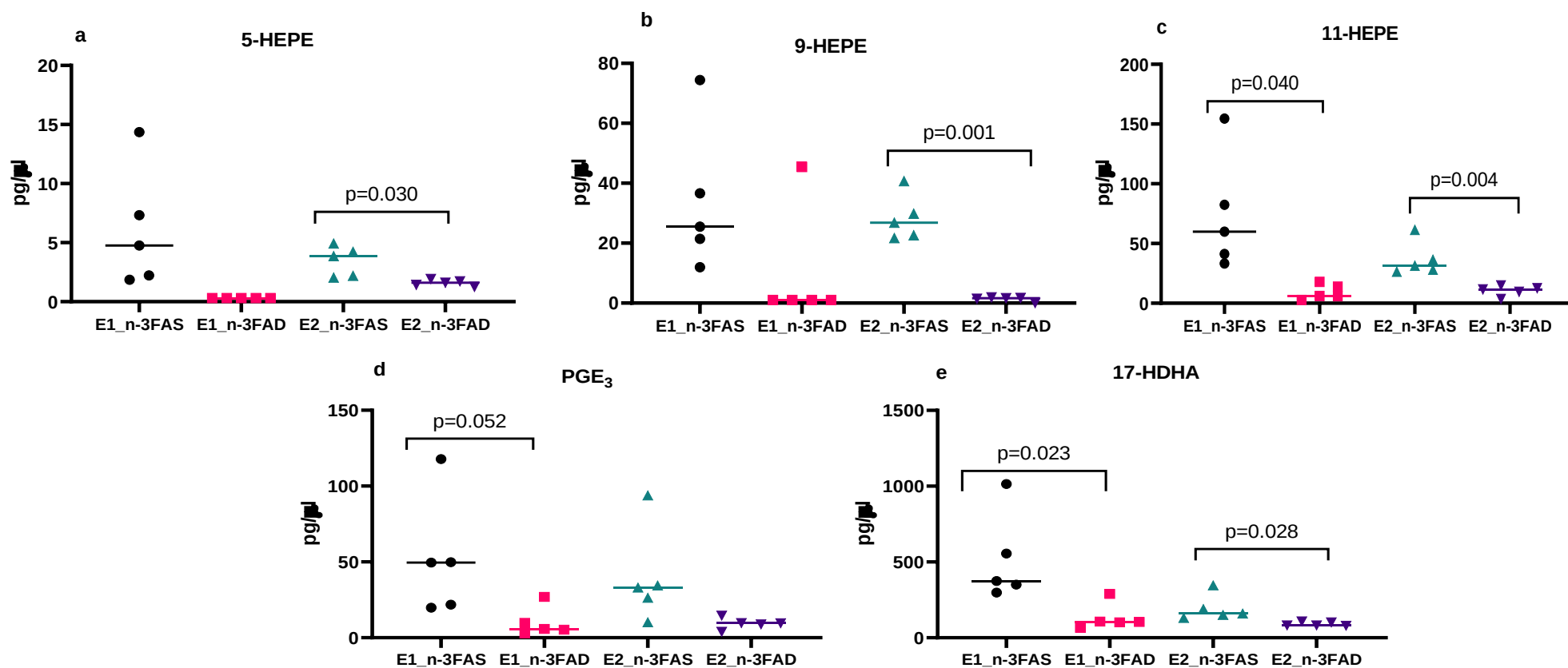


Figure 4-5 Pro-resolving lipid mediators (a)-5-HEPE; (b)- 9-HEPE; (c)-11-HEPE; (d)- PGE₃ and (e)- 17-HDHA between n-3FAS and n-3FAD groups 4 weeks post infection after providing n-3FAS and n-3FAD diets for three weeks^{1,2}

¹ Independent T-tests were used to determine differences between the n-3FAS and n-3FAD groups

² Data are expressed as mean ± SEM

E1: Experiment 1; E2: Experiment 2; n-3FAD: omega-3 fatty acid deficient; n-3FAS: fatty acid sufficient

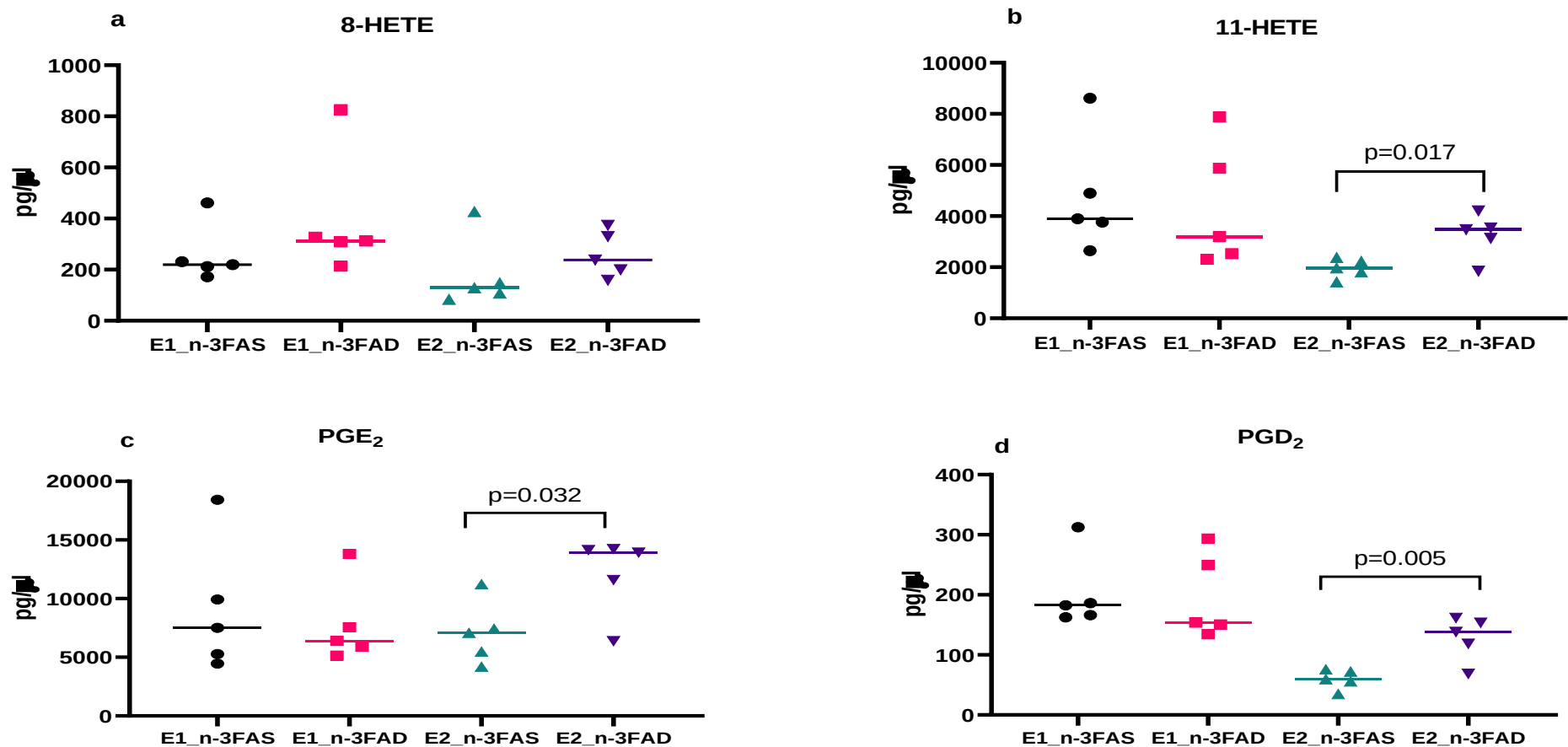


Figure 4-6 Pro-inflammatory lipid mediators (a)-8-HETE; (b)- 11-HETE; (c)-PGE₂ and (d)- PGD₂ between n-3FAS and n-3FAD groups 4 weeks post infection after providing n-3FAS and n-3FAD diets for three weeks^{1,2}

¹ Independent T-tests were used to determine differences between the n-3FAS and n-3FAD groups

² Data are expressed as mean ± SEM

E1: Experiment 1; E2: Experiment 2; n-3FAD: omega-3 fatty acid deficient; n-3FAS: fatty acid sufficient

CHAPTER 5 DISCUSSION

5.1 Introduction

In this chapter, the effect of a low and sufficient omega-3 (n-3) fatty acid (FA) status on lung inflammation in *Mycobacterium tuberculosis* (*Mtb*)-infected mice is discussed. This chapter will start with an introduction followed by section 5.1 which explains the changes in the total phospholipid FA compositions of red blood cells (RBC) between the groups fed FA sufficient (n-3 FAS) and FA deficient (n-3 FAD) diets for a preconditioning phase of six weeks before infection (baseline), until endpoint (three weeks post-infection). Sections 5.2 to 5.5 provide a comprehensive discussion on the effects of the diet interventions on clinical and inflammatory outcomes, including disease progression (bacillary loads and organ weight indexes (Objective 1), lung histology, lung cytokine concentrations and lung lipid mediator (LMs) concentrations (Objective 2).

This research study aimed to determine the effect of low versus sufficient n-3 FA status on lung inflammation in *Mtb*-infected mice. This study is unique in that it is the first to our knowledge to describe the effect of a low compared to a sufficient n-3 FA status on lung inflammation in an animal tuberculosis (TB) model that presents with similar hallmark characteristics found in humans infected with TB. This animal model, the C3HeB/FeJ mouse species, commonly known as Kramnik mice, have been widely used in research studies as a TB mouse model (Driver *et al.*, 2012:3182; Gupta *et al.*, 2013:601; Irwin *et al.*, 2015:600; Lanoix *et al.*, 2015:604; Marzo *et al.*, 2014:56; Rosenthal *et al.*, 2012:4332; Vilaplana *et al.*, 2013:200). In order to translate our research findings to the human situation, it was critical that the selected animal model bears resemblances to the pathophysiological conditions which occur in human pulmonary TB lesions (Driver *et al.*, 2012:3182; Irwin *et al.*, 2015:591; Marzo *et al.*, 2014:56; Vilaplana *et al.*, 2013:200).

In patients presenting with an active and chronic TB infection, the excessive, non-resolving inflammatory response is harmful as this ultimately results in lung tissue damage (Calder, 2017:1; Husson *et al.*, 2016:524; Kroesen *et al.*, 2018:1). Hawn *et al.* (2013:608) reviewed the existing literature and revealed that supplementing existing anti-TB treatment with immune-enhancing host modulators may accelerate the eradication of *Mtb* infection, and reduce treatment duration, lung tissue injury caused by the disease as well as the risk of relapsing. The ability of long-chain omega-3 polyunsaturated fatty acids (n-3 LCPUFA) to inhibit excessive inflammatory responses has long been acknowledged leading to various clinical studies in various inflammatory diseases (Li *et al.*, 2019; Sijben & Calder, 2007:237). The balance between n-3 and omega-6 (n-6) PUFA may be significant in determining the nature and severity of inflammatory reactions (Sijben & Calder, 2007:237).

Keeping the pro-resolving benefits of n-3 LCPUFA and the inflammatory pathology of TB in mind, it would be expected that a sufficient n-3 LCPUFA status may be beneficial in TB patients because of its ability to enhance their capacity to resolve inflammation and limit the inflammatory damage caused by the disease. Therefore, the effect of n-3 LCPUFA supplementation before TB infection compared with having a sufficient status has been investigated previously in animal models (Bonilla *et al.*, 2010b:399; McFarland *et al.*, 2008:2123; Paul *et al.*, 1997:623). These pre-clinical studies found that n-3 LCPUFA supplementation before infection, leads to increased susceptibility to TB infection as well as increased bacterial growth (Bonilla *et al.*, 2010a:6; Bonilla *et al.*, 2010b:399; Jordao *et al.*, 2008:1379; McFarland *et al.*, 2008:2123; Paul *et al.*, 1997:619). However, there are no data comparing a low with sufficient n-3 LCPUFA status before TB infection. Based on the existing evidence we postulated that a low compared with sufficient n-3 LCPUFA status may be detrimental to TB patients because of limited resolving capacity.

5.2 Total phospholipid polyunsaturated fatty acid compositions in red blood cells and lung homogenates

Twenty 4-6 weeks old C3HeB/FeJ male mice were included in this study and conditioned to be either n-3 PUFA sufficient (n-3 FAS group) or have a low n-3 PUFA status (n-3 FAD group) for six weeks prior to infection. This study was performed in two consecutive experiments with both an n-3 FAS and n-3 FAD group (n = 5 each) in each experiment.

The motivation behind the inclusion of a low n-3 PUFA status group was to be able to translate the results into the human situation. A global dietary intake review estimated that less than 20% of the world's population consumed the recommended ≥ 250 mg/day of n-3 LCPUFA, leading to very low n-3 LCPUFA blood levels ($\leq 4\%$) in low-income countries, among others (Simopoulos, 2011:203; Stark *et al.*, 2016:133). The n-3 LCPUFA status is therefore often low or very low in many populations and communities where there is also a high burden of TB, like South Africa (Berquin *et al.*, 2008:271; Calder, 2010:365; Calder *et al.*, 2018:13; Ford *et al.*, 2016:2; Husson *et al.*, 2016:524; Martin & Stapleton, 2010:540; McMurray *et al.*, 2011:628; Molfino *et al.*, 2017:2).

By altering the FA composition of the inflammatory response cells, the production of LMs and cytokines are also affected as being either more pro-inflammatory or anti-inflammatory (Calder, 2010:1). It is well known that the FA composition of the phospholipid bilayer of cells is changed depending on the intake of FAs (Calder, 2010:357; Calder, 2017:1; Molfino *et al.*, 2017:4). The inflammatory process is influenced by LCPUFA through a range of mechanisms which are mostly coupled with changes in the FA cell membrane composition (Calder, 2010:357). Bearing this in

mind, the current study's goal was to alter the FA composition of the mice cell membranes with a nutritional approach.

The mice were provided with custom prepared purified experimental diets (AIN-93 diet with or without FA modifications). Both diets (n-3 FAS and n-3 FAD) have been used in previous experimental animal studies investigating n-3 PUFA deficiency as well as sufficiency in rodents (Baumgartner *et al.*, 2012:1464; Harauma *et al.*, 2010:663; Moriguchi *et al.*, 2013:350; Moriguchi *et al.*, 2001:421; Moriguchi & Salem, 2003:301; Reeves *et al.*, 1993:1942).

To determine whether dietary fat modifications reflected in the cell membranes, the baseline RBC total phospholipid fatty acid composition was analysed in a sub-sample (n = 10) of the mice. As reported in previous studies, RBCs have a long lifespan, showing long term (up to three months in humans) stable FA status and is, therefore, a good indicator of FA status in other tissues (Hooper *et al.*, 2018:82; Revskij *et al.*, 2019:2; Risé *et al.*, 2007:363; Sun *et al.*, 2007:74). As anticipated, after the conditioning period before TB infection, the n-3 FAD group presented with a lower n-3 PUFA composition (alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and total n-3 LCPUFA), and a higher n-6 PUFA composition (AA, Osbond acid, and total n-6 LCPUFAS), except in the case of linoleic acid (LA) in comparison with the n-3 FAS group. The total n-6: n-3 LCPUFA ratio was also significantly higher in the n-3 FAD group, compared to the n-3 FAS group before *Mtb* infection. The infection baseline RBC n-3 PUFA composition results are comparable to those found in other studies where mice were also fed with either an n-3 FAS or n-3 FAD diet (Harauma *et al.*, 2010:663; Moriguchi *et al.*, 2013:350; Moriguchi *et al.*, 2001:421; Moriguchi & Salem, 2003:301). These results show the efficacy of the preconditioning period in successfully modifying the n-3 FA status to being low or sufficient.

Four weeks post-infection, at euthanasia, endpoint FA status was measured in both RBCs and lung homogenates. Similar to the baseline results, the n-3 FAD group still presented with lower LA, and higher AA, osbond acid and total n-6 LCPUFA RBC composition compared to the n-3 FAS group in both experiments and the total group. The n-3 FAD group presented with the highest AA, osbond acid, total n-6 LCPUFAS and n-6: n-3 LCPUFA ratio in both RBCs and lung tissue. These results indicate that the dietary interventions effectively changed the FA composition both in the RBCs and lung tissue in a manner consistent with the dietary FA intake. As the n-3 FAD group presented with the highest n-6 PUFA content at both baseline and endpoint measures, we postulated that this group would exhibit a less pro-resolving and more pro-inflammatory LM profile (Calder, 2017:2; Serhan & Petasis, 2011:3). The opposite was also predicted in the n-3 FAS group, that there would be a higher production of pro-resolving LMs than in the low n-3 FA status groups.

5.3 Disease progression

5.3.1 Lung and spleen indexes and bacillary loads

At four weeks post *Mtb* infection, the n-3 FAD groups from both experiments as well as the total group presented with significantly lower spleen-weight indexes when compared to the n-3 FAS groups. However, no differences were observed concerning the lung-weight indexes. Splenomegaly (enlarged spleen) was previously documented to occur in TB case studies due to the excessive or uncontrolled inflammatory response caused by the *Mtb* bacilli as a result of disseminated disease (Gupta *et al.*, 2008:23; Imani Fooladi *et al.*, 2009:274; Kumar *et al.*, 2017:94; Pottakkat *et al.*, 2010:96). A study done by Paul *et al.* (1997:619) used guinea pigs to determine the effect of dietary n-3 or n-6 PUFAs on the progression of experimental TB. In agreement with our results, they found that disease progression was worse in the group fed high amounts of n-3 PUFAs compared to the group fed mainly n-6 PUFAs. It was also noticed that the guinea pigs in the n-3 PUFA group presented with significantly higher spleen weights compared to the n-6 PUFA group and the reference group at 7 weeks post-infection (Paul *et al.*, 1997:621). The spleen colony forming units (CFU) results agreed with the spleen-weight index results.

The n-3 FAD mice from experiment 1 presented with a lower total lung CFU count compared with the n-3 FAS group. Total lung bacillary loads were also lower in the n-3 FAD groups from both experiment 2 and the total group, though these results were not significant, with a trend towards significance in experiment 2.

Similar findings were observed in two other studies showing that *Mtb* infected guinea pigs fed an n-3 PUFA rich diet had decreased intracellular killing of the TB bacilli and demonstrated enhanced disease progression (Mayatepek *et al.*, 1994:106; McFarland *et al.*, 2008:2123). On the other hand, the low n-3 PUFA status mice presented with lower bacterial loads compared with the sufficient groups. While these findings were not statistically significant, except in experiment 1, it suggests that a lower n-3 PUFA status contributed to better TB outcomes. A previous experimental study reported that mice deficient in n-3 PUFA presented with reduced susceptibility to TB compared to fat-1 transgenic mice, which are capable of endogenous production of n-3 PUFAs (Bonilla *et al.*, 2010b:405). Such results support the idea that a deficiency of n-3 PUFA can protect against TB. However, bearing in mind the immune-modulatory and other important functions of n-3 PUFAs, encouraging individuals being at risk for TB infection to consume a diet low in n-3 PUFA as a precautionary measure would be unrealistic. Whether changing to a diet low or deficient in n-3 PUFA after TB infection may be beneficial cannot be answered by Bonilla's

or the current study design (Bonilla *et al.*, 2010b:405). However, it raises the question of whether higher n-3 PUFA intake should be encouraged in a TB-endemic population.

The higher bacterial loads in the sufficient n-3 PUFA status groups in our study could be because the mice in the n-3 FAS group had a lower n-6 PUFA composition and n-6: n-3 PUFA ratio in their cell membranes. Two experimental animal studies indicated that n-3 PUFAs reduced activation and anti-mycobacterial cell responses (Bonilla *et al.*, 2010b:399; McFarland *et al.*, 2008:2123). Bonilla *et al.* (2010a:2) also reported a higher bacterial load in their DHA treated group. This agrees with the higher lung and spleen bacterial loads in our n-3 FAS groups, that also presented with a higher DHA membrane composition, across both experiments and for the total group compared with the n-3 FAD groups (Chapter 4, Table 4-4).

Contrasting the above, Jordao *et al.* (2008) reported lower bacterial loads in both lungs and spleens of BALB/c *Mtb* infected mice, fed diets rich in n-3 PUFA compared to mice fed a fat-free diet. It should be kept in mind that this study supplemented n-3 PUFA before infection, whereas our study only focused on a sufficient versus low n-3 PUFA status of the mice, without supplementation. In our study, the mice had a balanced n-3 and n-6 PUFA intake which made the n-3 FAS group sufficient in lung n-3 PUFA and n-6 PUFA, and specifically in the important pro-inflammatory LM precursor, AA and the pro-resolving precursors EPA and DHA (Table 4.3) (Reeves *et al.*, 1993:1942). Nonetheless, our findings confirmed those that showed that a low n-3 PUFA status before, during and after infection positively contribute to improved TB outcomes and lower bacillary loads.

5.4 Histology

We hypothesised that n-3 PUFA sufficiency would lead to improved lung histopathology and more free alveolar space due to inflammation being resolved more effectively (Gupta *et al.*, 2016:5; Kumar *et al.*, 2019:5). Lung inflammation is a key element in the formation of lesions, cavities and a granulomatous inflammation milieu is accompanied by either necrotising or non-necrotising granulomas (reference). However, throughout both experiments and for the total group, the n-3 FAD (low n-3 PUFA status) groups presented with a higher percentage of free alveolar space compared with the n-3 FAS groups. The changes in free alveolar space agree with the lower bacterial burden observed in the n-3 FAD groups. Supporting these results, Bonilla *et al.* (2010b:407) showed a decrease in the lung inflammatory response in *fat-1* mice compared with the n-3 PUFA deficient control group. This result was ascribed to the possible poor formation of mature lesions needed to contain bacterial growth. On the other hand, McFarland *et al.* (2008:2125) reported that despite considerable differences in lung bacterial loads between the n-3 PUFA and n-6 PUFA fed guinea pigs, no difference in histological evaluations were observed

between groups. Nevertheless, in our study, the histology findings agreed with the bacterial load findings, and alignment of the inflammatory marker results will be discussed next.

5.5 Lung lipid mediator concentrations

No studies, according to our knowledge, examined the effects of a low versus a sufficient n-3 PUFA status in *Mtb*-infected mice on lung inflammation using lung LMs. In our study, the pro-resolving LMs were generally higher in the n-3 PUFA sufficient than in the poor status mice, as was expected. These included the EPA-derived intermediates 5-, 9-, 11-, 12-, 15 and 18-hydroxyeicosapentaenoic acid (HEPE) as well as DHA-derived 17HDHA and PD1. With regards to the AA derived pro-inflammatory LMs, the results were more diverse. Even though generally higher in the n-3 PUFA deficient groups, only 11 HETE reached significance in experiment 2.

The LM profile of the n-3 FAD (low status) group agreed with its FA status, presenting with lower n-3 PUFA derived LMs. These findings indicate that nutritional n-3 PUFA consumption modifies the lung LMs production in the n-3 PUFA sufficient groups to a more pro-resolving LMs profile with little to no effect with regards to pro-inflammatory LMs. However, compared to the n-3 FAS groups, mice from the n-3 FAD groups generally had lower lung inflammation (as seen from histology results) and decreased pro-inflammatory lung cytokine concentrations (IL-1 α , IL-1 β , IL-17, IFN- γ). This could not be clarified by the FA status of these groups or their LMs profile which justifies the need for additional investigation into these results. The possible explanation for the lower pro-inflammatory lung cytokine concentrations observed in the n-3 FAD groups, could be related to the time points at which the measurements were taken.

Rangel Moreno *et al.* (2002:265) reported that increased prostaglandin E₂ (PGE₂) levels have been associated with substantial immunosuppressive effects which inhibits activation of macrophages. This is detrimental as *Mtb* infection is controlled by the activation of macrophages. However, the role of PGE₂ is not entirely clear as lower levels thereof have been found in early TB infection and increased levels later in TB infection, indicating that the role of PGE₂ changes as the disease progresses (Mayer-Barber & Sher, 2015:270; Rangel Moreno *et al.*, 2002:257). In this study the n-3 FAD groups presented with higher PGE₂ concentrations four weeks post infection, suggesting that the amount of interstitial macrophages would have been increased leading to improved bacterial control and the apparently improved clinical picture observed in the n-3 FAD group (Rangel Moreno *et al.*, 2002:265). However, immune cells were not measured as part of the objectives of this study. Further investigations of immune cells could possibly provide a better explanation into these findings. Vinhaes *et al.* (2019:2) demonstrated that controlled bacterial growth was observed with a balance between LMs and inflammatory cytokines. However, this balance was interrupted when a suppression of PGE₂ occurred which resulted in

Mtb replication and worse disease outcomes (Sorgi *et al.*, 2020:8; Vinhaes *et al.*, 2019:2). The latter could be true for the n-3 FAS mice groups, who presented with lower PGE₂ concentrations and higher bacterial loads. Supporting this, Chen *et al.* (2008:2797) demonstrated that *Mtb in vitro* models displayed decreased levels of PGE₂ inducing lipoxin (LX) A₄ production in infected macrophages, resulting in necrosis and bacterial spreading. Bafica *et al.* (2005:1601) showed that *Mtb* infected mice deficient in 5-LOX (producing less LXs), displayed an increase in IL-12 and IFN- γ concentrations with lower bacterial loads. This finding showed improved bacterial regulation suggesting the detrimental effect of LXs on host response to *Mtb* (Bafica *et al.*, 2005:1601).

Although pro-resolving LMs have recently shown to be associated positively with improvement in clinical outcomes in TB in humans, in our study we found the opposite (Colas *et al.*, 2019:13028; Ruiz *et al.*, 2019:8). The timing of measurements may be a potential reason for these conflicting findings as our results were measured only 4 weeks post-infection. Furthermore, an initial strong inflammatory response is vital for improved bacterial control and host defence and could possibly have been stronger in the n-3 FAD groups due to the lack of pro-resolving (anti-inflammatory) capacity (Kroesen *et al.*, 2018:10). This could have contributed to rapidly and more effectively eradicating the TB bacilli and lessening bacillary loads sooner after infection than in the n-3 FA sufficient groups.

5.6 Lung cytokine concentrations

Cytokines are inflammatory markers in the form of small soluble proteins produced by specific cell types that can alter the characteristics of a cell itself or those of another cell (Domingo-Gonzalez *et al.*, 2016:1; Romero-Adrian *et al.*, 2015:16). Interactions between *Mtb* and the host's immune system are complex and are characterised by releasing a range of cytokines by various cell types, secreted in response to an infection (Etna *et al.*, 2014:1).

Production of cytokines starts after the initial engulfment of the bacilli, leading to the recruitment and activation of inflammatory cells (Domingo-Gonzalez *et al.*, 2016:2; Kaufmann & Dorhoi, 2013:441; Kiran *et al.*, 2016:169). Immune system response in TB infected individuals depends on the activity from T-Helper cells, responsible for secreting the inflammatory cytokines interleukin (IL)-12, interferon (IFN)- γ and tumour necrosis factor-alpha (TNF- α) resulting in macrophage activation (Romero-Adrian *et al.*, 2015:18).

Mice from experiment 2 generally presented with higher lung cytokine concentrations than experiment 1 due to a higher CFU count. In the total group, IL-1 α , IL-6 and IFN- γ were lower in the n-3 FAD than the n-3 FAS group and IL-1 β showed a similar trend. In experiment 1, the n-

3FAD mouse groups presented with lower IL-1 α , IL-1 β , IL-17, IFN- γ and GM-CSF concentrations compared to the n-3 FAS groups. Overall, all cytokines measured were lower in the n-3 FAD groups from both experiments individually and for the combined group. As IL-12, IFN- γ and TNF- α contribute to granuloma formation (controlling bacterial replication) during *Mtb* infection, it would be expected that a lack of these cytokines would contribute to increased bacterial loads (Romero-Adrian *et al.*, 2015:16). As such, Cooper *et al.* (1997:40) & Flynn *et al.* (1995:2515) reported an increase in bacterial growth in mice lacking IL-12, these mice eventually succumbed to infection. In our study, a lower concentration of IL-12 was observed among the n-3 FAD groups (experiment 1, 2 and total group), contradicting this reasoning as the n-3 FAD groups presented with lower bacillary loads compared to the n-3 FAS groups.

We speculated that the n-3 FAD group would present with higher lung cytokine concentrations compared with the n-3 FAS group. However, the n-3 FAD (low status) group presented with lower concentrations of these cytokines. These results are contradictory with the FA status results and the less pro-resolving LMs profile observed. These findings could partially be explained by the lower bacterial loads in the n-3 FAD groups that led to a lesser inflammatory response and thus a lower production of pro-inflammatory markers. Furthermore, the lower concentrations of certain inflammatory cytokines in the low n-3 PUFA status group could be related to the timing of cytokine measurement. In this study, cytokine concentrations were only measured 4 weeks after infection.

Contrasting to our results Bonilla *et al.* (2010a:6) reported that DHA-treated cells (*in vitro* cell culture models) produced fewer pro-inflammatory cytokines at 48 hours post-infection (TNF- α and IL-6). In agreement, TNF- α , IL-6, & IL-1 β were down-regulated in *fat-1* mice (Bhattacharya *et al.*, 2006:928; Bonilla *et al.*, 2010b:399). Jordao *et al.* (2008:2124) reported that EPA reduced TNF- α secretion, whereas AA enhanced TNF- α secretion. These results contradict our findings, however again it should be kept in mind that these studies supplemented n-3 LCPUFA before infection, whereas our study only focused on a sufficient versus low n-3 PUFA status of the mice, without supplementation. Furthermore, these studies also measured cytokine concentrations at different time intervals compared to our study.

5.7 Conclusion

Contrary to what we hypothesized, if no TB drugs were administered, a low n-3 PUFA status prior to TB infection resulted in decreased lung bacterial loads, decreased spleen body weight index and increased lung free alveolar space (decreased inflammation). This could be due to the fact that a low n-3 PUFA status decreased the production of pro-resolving LMs, which may have increased and prolonged the initial pro-inflammatory response, which rapidly and effectively

eradicated the TB bacilli soon after infection, corresponding with the lower bacterial load in this group. In our study, results were measured 4 weeks post-infection. Thus, the late inflammatory response would be lower due to reduced initial infection rates as evidenced by the lower CFU levels and cytokine concentrations in the low status group.

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CHAPTER 6 CONCLUDING REMARKS

This concluding chapter indicates how the objectives of the study have been reached. In addition, the limitations of the study, practical suggestions and potential future research are discussed. The aim and objectives are repeated here for ease of reference.

6.1 Aim and objectives

6.1.1 Research aim

The aim of this project was to determine the effect of omega-3 polyunsaturated fatty acids (n-3 PUFA) status prior to infection on lung inflammation and pathology in *Mycobacteria (Mtb)*-infected C3HeB/FeJ mice.

6.1.2 Research objectives

To compare the effects of a low versus a sufficient omega-3 polyunsaturated fatty acid (n-3 PUFA) status in *Mtb*-infected C3HeB/FeJ mice on:

1. clinical outcomes, i.e. lung and spleen bacterial load and weight indexes, and
2. lung inflammation using lung lipid mediator (LM), cytokine concentrations and percentage free alveolar space.

6.2 Main findings arising from this research

The host immune response is manipulated by the tuberculosis (TB) pathogen (Dorhoi & Kaufmann, 2016:2). Furthermore, it is well known that poor nutrition has a detrimental effect on immune function, and that treatment outcomes of *Mtb* infected individuals may be improved by enhancing their nutritional status (Feleke *et al.*, 2019:2; Wu *et al.*, 2019:1). An example of key nutrients includes n-3 PUFA which is responsible for controlling important cell functions (Wu *et al.*, 2019:1). A daily intake of sufficient n-3 PUFA modifies the phospholipid fatty acid structures of blood and tissue cell membranes which serves as precursors for the production of pro-resolving LMs, which in turn control the resolution of inflammation. In many settings where TB is prevalent, individuals may also present with a low n-3 PUFA status. We, therefore, investigated the effects of a low compared with a sufficient n-3 PUFA status on lung inflammation and pathology in TB infected mice.

6.2.1 The effect of n-3 PUFA status on lung and spleen bacterial loads, weight indexes and histology (disease progression)

One of the major findings of this research was that a low n-3 PUFA status before, during and after TB infection led to improved TB outcomes and decreased disease progression. This was shown by lower bacterial loads as well as lower spleen-weight indexes when compared with the mice from the n-3 PUFA sufficient group (n-3FAS). In support of our findings, several other studies showed enhanced disease progression (impaired host defence) when animals were fed with an n-3 PUFA rich diet before TB infection (Bonilla *et al.*, 2010a:2; Bonilla *et al.*, 2010b:405; McFarland *et al.*, 2008:2123). However, it would be unrealistic to encourage a diet low in n-3 PUFA in TB population groups, keeping in mind the important physiological functions of n-3 PUFA. The higher bacterial loads of the n-3FAS groups could possibly be attributed to the fact that they had a lower omega-6 polyunsaturated (n-6 PUFA) cell membrane composition, resulting in a lower pro-inflammatory capacity at initial infection. Contrasting our findings, Jordao *et al.* (2008), reported lower bacterial loads in mice groups fed a rich n-3 PUFA diet. However, it should be kept in mind that our research focussed on status, without supplementation whereas other studies investigated supplementation of n-3 PUFA before infection (Bonilla *et al.*, 2010a; Bonilla *et al.*, 2010; McFarland *et al.*, 2008; Paul *et al.*, 1997).

In addition, the quantification of lung histology showed that a low n-3 PUFA status resulted in a higher percentage of free alveolar space, indicating lower inflammation, compared with the n-3FAS group. This finding agrees with the lower bacterial loads of the same group.

6.2.2 Markers of inflammation

We further aimed to investigate the effects of a low versus a sufficient n-3 PUFA status in *Mtb*-infected male C3HeB/FeJ mice on lung inflammation by assessing effects on lung LMs and cytokine concentrations. We found that the n-3FAS mice groups presented with higher pro-resolving LMs concentrations compared with the low status mice. The LMs profiles of the low status mice agreed with their FA status, showing a decrease in n-3 PUFA derived LMs. However, these mice showed lower lung inflammation (together with generally lower pro-inflammatory lung cytokine concentrations). This finding could not be explained by the FA status or the LMs profile of the group, justifying the need for further investigation. It has been reported that an increase in the pro-inflammatory prostaglandin E₂ (PGE₂) is associated with immunosuppressive effects, ultimately inhibiting the activation of macrophages (Rangel Moreno *et al.*, 2002). Our findings showed that the low-status mice presented with a higher concentration of PGE₂ at 4 weeks post infection, suggesting a higher number of interstitial macrophages resulting in improved bacterial

control (as seen from the apparent improved clinical picture). A balance between LM and cytokine concentrations is needed for controlled bacterial growth (Vinhaes *et al.*, 2019). This balance is interrupted when lower levels of PGE₂ occurs, ultimately worsening disease outcomes (Vinhaes *et al.*, 2019), as was seen in the n-3FAS mice groups which presented with lower PGE₂ levels as well as higher bacterial load.

Although higher pro-resolving LMs has been associated with improved TB outcomes in humans, our finding suggests otherwise (Colas *et al.*, 2019; Ruiz *et al.*, 2019). We speculated that the n-3FAS mice group would present with lower lung cytokine concentrations compared with the low status group. However, this was not the case, as the low status mice showed generally lower pro-inflammatory lung cytokine concentrations, seemingly contradicting the poor n-3 PUFA status and resulting less pro-resolving LMs profile observed. It is possible that lower bacterial loads in the low status mice groups resulted in a less inflammatory environment causing a decrease in the production of pro-inflammatory cytokines. Furthermore, a plausible explanation for these apparently conflicting results could also be the timing of measurements as our results were measured four weeks post infection.

6.3 Research study limitations and recommendations

Our study had the following limitations:

- i. Lipid mediators (LM) and lung cytokine concentrations were only measured in lung tissue. Further insights might have become apparent if analyses were done in plasma also to assess effects on systemic inflammation.
- ii. Certain lung cytokine concentrations were below detection limits (IL-10 and IL-4), which might have improved our insight if we had detectable values.
- iii. Analyses were only done at one time point (endpoint four weeks post infection and after three weeks of dietary intervention). Adding more time points would have provided more extensive and valuable insights into this study's findings.
- iv. This study did not include standard TB treatment as part of the objectives. Including these, could have yielded different results.

6.4 Future prospects

- i. The following step would be to investigate the effects of n-3 PUFA as well as supplementing n-3 PUFA together with the standard TB drug treatments to determine possible drug-nutrient interactions.
- ii. Female and male C3HeB/FeJ mice can be included in future research to investigate gender specific TB outcomes.
- iii. From the literature review, it was observed that research has mostly centred on pro-inflammatory LM and TB outcomes. Therefore, additional investigation is needed with regards to n-3 PUFA status and pro-resolving LMs in TB infected individuals to determine what an ideal n-3 PUFA status in the TB population should be.
- iv. Lastly, to be able to translate our findings to the human population, it would be ideal to repeat this study design in TB patients, starting with a safety study.

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ANNEXURES

ANNEXURE A: Ethical approval from the North-West University Ethics Committee



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**North-West University Animal Care, Health and
Safety Research Ethics Committee (NWU-
AnimCareREC)**

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal
studies)

23 August 2019

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 23/08/2019, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general 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 effect of omega-3 fatty acid status on lung inflammation in tuberculosis infected mice

Principal Investigator/Study Supervisor/Researcher: Dr L Malan

Student: S King- 23404949

Ethics number:

N	W	U	-	0	0	5	6	7	-	1	9	-	A	1
Institution				Study Number					Year		Status			

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

Application Type: Category 0

Commencement date: 23/08/2019

Expiry date: 31/08/2020

Risk:

Category 0

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is due at the end of August annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:
 - Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and
 - 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.
- The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.

- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves 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 NWU-AnimCareREC or that information has been false or misrepresented;*
 - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
 - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

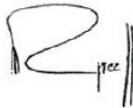
NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,



Digitally signed by
Christiaan B Brink
Date: 2019.08.23
11:56:10 +02'00'

Prof Tiaan Brink
Chairperson NWU-AnimCareREC



Digitally signed by
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Current details: (13009230) G:\My Drive\9.1.5.1.1 Ethics\2019\NWU-00567-19-S5\9.1.5.4.2_Approval_Letter_NWUAnimCareREC.docm 23 August 2019

File reference: 9.1.5.4.2 NWU-00567-19-S5

ANNEXURE B: Proofreading and language editing

H67 Kobra Road

Bynestpoort

Cullinan

8 December 2020

To whom it may concern,

I herewith confirm that I have proofread and edited S. King's mini-dissertation on the effect of omega-3 fatty acid status on lung inflammation in tuberculosis-infected mice as partial fulfilment of the requirements for the degree Master of Science in Dietetics at the North-West University.

I hope it meets approval.

Regards

Alte-Corné Scheepers

BA (HED)(Hons)