

The effect of pre-infection omega-3 fatty acid status on anaemia of infection and morbidity in tuberculosis infected mice

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ABSTRACT

Background: Tuberculosis (TB) is currently a major health problem worldwide, and despite improvement, mortality rates are not ideal. Since the immune response, underlying protection against TB is incompletely understood, long durations of treatment time and poor health outcomes remain a problem. Therefore, host directed non-pharmaceutical interventions, supporting TB treatment, may be a promising approach to improve outcomes. Previous studies have found anti-inflammatory treatment to improve TB outcomes and the anti-inflammatory and pro-resolving properties of omega-3 (n-3) polyunsaturated fatty acids (PUFAs) treatment have been proven beneficial in other inflammatory diseases. Due to the fact that TB is known to be a disease of non-resolving inflammation leading to host tissue damage and poor clinical outcomes, the main aim of this study was to investigate whether sufficient and deficient n-3 PUFA status, after TB infection, had an influence on markers of morbidity, disease progression and anaemia of infection (AI).

Methods: Eighteen 8 to 12-week-old C3HeB/FeJ mice were infected with TB via the intranasal route (high dose acute infection), and 12 mice via the aerosol route (low dose chronic infection) after conditioning for six weeks on an n-3 PUFA sufficient (FAS) or deficient (FAD) diet. Red blood cell (RBC) fatty acid status, whole blood haemoglobin (Hb), and body weight were determined the day before infection and markers of AI (Hb, plasma ferritin, transferrin receptor (TfR) and hepcidin), morbidity (appearance, respiratory rate, and body weight) and disease progression and clinical outcomes (lung bacillary load, organ indexes, and lung cytokine concentrations) were analyzed and compared between the two different groups 35 days after infection upon euthanization.

Results: At euthanasia, intranasal and aerosol groups showed differences in PUFA composition with regards to all n-6 PUFA (all $p < 0.001$) and n-3 PUFAs (all $p < 0.001$, except docosahexaenoic acid (DHA) in aerosol subgroup $p = 0.003$) in RBC. Mice from the aerosol FAD subgroup had lower Hb concentrations prior to infection ($p = 0.003$), and a lower bacterial load ($p = 0.008$) and spleen-body-weight-index ($p = 0.006$) at 35 days post infection. In contrast, in the aerosol infected mice, the decrease in Hb in the n-3 FAS subgroup were more than in the n-3 FAD subgroup during the five weeks of TB infection ($p = 0.027$). No effects were found post-infection in other markers of AI. Inflammatory marker profiles showed a trend ($p=0.061$) towards lower pro-inflammatory cytokine IL-12 in the aerosol n-3 FAD subgroup.

Conclusion: The lower-dose aerosol infection model was generally more sensitive to effects of n-3 PUFA sufficiency and deficiency. Without infection, n-3 PUFA sufficiency may contribute to

improved Hb concentrations compared to n-3 PUFA deficiency. However, n-3 PUFA sufficiency may give rise to a bigger decrease in Hb concentrations after infection. Furthermore, contradicting to what was expected n-3 PUFA deficiency caused slower disease progression compared to an n-3 PUFA sufficient status. Future research should investigate whether an additional n-3 PUFA supplement together with TB medication (rather than only providing sufficient n-3 PUFA intake) may improve markers of AI and whether morbidity may be affected positively or even worsened in this case.

Key terms:

Anaemia of infection (AI), disease progression, morbidity, omega-3 (n-3) polyunsaturated fatty acid (PUFA) status, tuberculosis (TB)

OPSOMMING

Agtergrond: Tuberkulose (TB) is tans 'n groot gesondheidsprobleem wêreldwyd, en ten spyte van verbetering is sterftesyfers steeds nie ideaal nie. Sedert die immuunrespons aangaande onderliggende beskerming teen TB onvolledig verstaan word, bly die behandelingstydperk en swak gesondheidsuitkomste nog steeds 'n probleem. Daarom kan nie-farmaseutiese ingrepe, wat TB-behandeling ondersteun, 'n belowende benadering wees om uitkomste te verbeter. Vorige studies het gevind dat anti-inflammatoriese behandeling TB-uitkomste verbeter, en die anti-inflammatoriese eienskappe van omega-3 (n-3) poli-onversadigde vetsure (POVS) is bewys om 'n positiewe bydrae in die behandeling van ander inflammatoriese siektes te hê. As gevolg van die feit dat TB bekend is daarvoor dat dit 'n siekte is van oordrewe inflammasie, wat lei tot weefselskade en swak kliniese uitkomste, was die hoofdoel van hierdie studie om te ondersoek of voldoende, asook 'n gebrekkige n-3 POVS status, voor en gedurende die verloop van TB-infeksie 'n invloed gehad het op merkers van morbiditeit, siekteprogressie, en anemie as gevolg van infeksie (AI).

Metodes: Agtien agt tot 12-week-oue C3HeB/FeJ-muise is met TB geïnfekteer deur gerbuik te maak van 'n intranasale infeksie roete ('n hoë dosis akute infeksie), en 12 muise is geïnfekteer deur 'n aerosol ('n lae dosis kroniese infeksie) nadat hulle vir ses weke op 'n n-3 POVS voldoende (VSV) of gebrekkige (VSG) dieet gekondisioneer was. Rooibloedsel (RBS)-vetsuurstatus, hemoglobien (Hb) vlakke, en gewig is die dag voor infeksie gemeet. Vyf en dertig dae na infeksie was merkers van AI (Hb, plasma ferritien, transferriënreseptor (TfR) en hepsidien), merkers van morbiditeit (voorkoms, respiratoriese tempo, en gewig) en siekteprogressie (long bakteriële lading, orgaanindekse en long sitokienkonsentrasies) gemeet, ontleed, en vergelyk tussen die twee verskillende groepe.

Resultate: Na genadedood het die intranasale en aerosol-infeksie groepe verskille in POVS-samestelling getoon met betrekking tot alle n-6 POVS (alle $p < 0.001$) en n-3 POVS (alle $p < 0.001$, behalwe dokosaheksaensuur (DHS) in die aerosol-infeksie subgroep $p = 0.003$) in RBS. N-3 VSG muise van die inaseming-infeksie subgroep het aansienlik laer Hb-vlakke voor infeksie getoon ($p = 0.003$), en na infeksie het dieselfde muise 'n laer bakteriële lading ($p = 0.008$) en 'n laer milt-liggaamsgewig-indeks gewys ($p = 0.006$). In kontras het die n-3 VSV muise van die aerosol-infeksie subgroep aansienlik verminderde Hb-vlakke getoon aan die einde van die studie ($p = 0.027$). Geen effekte is na infeksie gevind in die ander merkers van AI nie. Inflammatoriese profiele het 'n neiging ($p=0.061$) tot laer pro-inflammatoriese sitokien IL-12 vlakke in die aerosol-infeksie n-3 VSG subgroep getoon.

Gevolgtrekking: Sonder infeksie kan n-3 POVS genoegsaamheid bydra tot verlaagde Hb-vlakke, maar na aerosol-infeksie kan dit moontlik aanleiding gee tot styging van Hb-vlakke. Verder, teenstrydig met wat verwag is het die navorsers gevind dat n-3 POVS-tekort stadiger siekte progressie veroorsaak in vergelyking met 'n n-3 POVS voldoende status subgroep. Sonder infeksie kan n-3 POVS die afregulering van inflammatoriese prosesse in die liggaam veroorsaak, en sodoende bydra tot hoër Fe-absorpsie en dus hoër Hb-vlakke. Toekomstige navorsing kan ondersoek instel om vas te stel of addisionele n-3 POVS-aanvulling tesame met TB-medikasie (eerder as om net genoegsame POVS te verskaf) merkers van AI kan verbeter, en of morbiditeit positief of selfs negatief kan beïnvloed word.

Sleutel terme:

Anemie as gevolg van infeksie (AI), siekteprogressie, morbiditeit, omega-3 (n-3) poli-onversadigde vetsuur (POVS) status, tuberkulose (TB)

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AI	Anemia of infection
ALA	alpha-linolenic acid
ANCOVA	analysis of covariance
CD	cluster of differentiation
CEN	Centre of Excellence for Nutrition
COX	cyclooxygenase
DAFF	Department of Agriculture, Forestry and Fisheries
DCytB	duodenal cytochrome B
DGLA	dihomo- γ -linolenic acid
DHA	docosahexaenoic acid
DMT1	divalent metal transporter-1
DPA	Docosapentaenoic acid
ELISA	Enzyme-linked immunosorbent assays
EPA	eicosapentaenoic acid
EPO	erythropoietin
FAD	Fatty acid deficient
FAME	fatty acid methyl ester
FAS	Fatty acid sufficient
Fe	iron
Fe ²⁺	ferrous form

LIST OF ABBREVIATIONS (CONTINUED)

Fe ³⁺	dietary non-heme iron
FPN	ferroportin
GC-MS	Gas Chromatography-Mass Spectrometry
GLA	gamma-linolenic acid
Hb	Haemoglobin
HCP1	Heme carrier protein-1
HIV	human immunodeficiency virus
Hox1	Homeobox protein
Hp	hephaestin
ICU	Intensive Care Unit
IDM	Institute of Infectious Disease and Molecular Medicine
IES	inhalation exposure system
IFN	interferon
IFN- γ	Interferon-gamma
IL	Interleukin
IVC	individually ventilated cages
LA	Linolenic acid
LCPUFA	long-chain polyunsaturated fatty acid
LM	Lipid mediator
LOX	lipoxygenase
LTs	Leukotrienes

LIST OF ABBREVIATIONS (CONTINUED)

LXs	Lipoxins
MaR	Maresins
MDR-TB	multi-drug resistant tuberculosis
MTB	<i>Mycobacterium tuberculosis</i>
n-3	n-3
n-6	omega-6
NK	Natural killer
NSAIDs	non-steroidal anti-inflammatory drugs
NWU	North-West University
PBS	Phosphate-buffered saline
PCDDP	Pre-Clinical Drug Development Platform
PD	Protectins
PGs	Prostaglandins
PGE	Prostaglandin E
PTB	Pulmonary Tuberculosis
PUFA	Polyunsaturated fatty acid
RBC	red blood cell
RDA	Recommended Dietary Allowances
ROS	Reactive oxygen species
Rvs	Resolvins
SA	South Africa

LIST OF ABBREVIATIONS (CONTINUED)

SEM	Standard error of the mean
SIRS	systemic inflammatory response syndrome
SOP	Standard Operating Procedure
SPLM	Specialized pro-resolving lipid mediators
SPSS	Statistical Programme for Social Sciences
TB	tuberculosis
T (CM) cells	Central Memory T-cell
T (EM) cells	Effector Memory T-cell
Tf	transferrin
Tf-Fe ²	transferrin-bound iron
TfR	transferrin receptor
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
TGF	transforming growth factor
TNF	tumor necrosis factor
UCT	University of Cape Town
WBCs	White Blood Cells
WHO	World Health Organization

LIST OF SYMBOLS AND UNITS

α	alpha
β	Beta
cm	centimeter
CFH	Cubic feet per hour
CFU	Colony Forming Units
dL	deciliter
°C	degrees Celsius
g	gram
g/kg	gram per kilogram
>	greater than/ above
Hz	Hertz
<	less/ lower than
μ l	microliter
mg	milligram
ml	milliliter
mm	millimeter
min	minutes
nm	nanometers
n	number
OD	optical density
pg:	picogram

LIST OF SYMBOLS AND UNITS (CONTINUED)

ppm parts per million

% percentage

RPM revolutions per minute

sec seconds

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CHAPTER 1 INTRODUCTION

1.1 Background and motivation

Tuberculosis (TB) continues to have a detrimental effect in South Africa (SA) as this country is currently ranked as one of the top 20 countries with i) the highest estimated numbers of incident TB cases, ii) highest estimated numbers of incident TB cases among people living with human immunodeficiency virus (HIV), and iii) highest estimated numbers of incident multi-drug resistance TB (MDR-TB) worldwide (WHO, 2017). Ranking above HIV, TB is responsible for more deaths than any other single bacterial pathogen in SA. In this specific country, about 25 000 deaths are caused by active TB yearly, excluding people who are co-infected with HIV (WHO, 2016). TB infection creates long-term consequences, leaving mortality and cure rates unfavourable (WHO, 2017).

A number of causative agents for TB exist, some of which include *Mycobacterium tuberculosis* (MTB), *M. bovis* or *M. africanum* (Banuls *et al.*, 2015). The current animal study will focus specifically on MTB, as this pathogenic bacterium is known to be the most common cause of human TB (DOH, 2014). *Mycobacterium tuberculosis* is reported to be extraordinarily successful when it comes to infecting and persisting in human beings (Braverman, 2017). This pathogen is transmitted when an uninfected individual inhales droplets from the sputum of an infected individual (Long & Schwartzman, 2014).

Non-resolving inflammation is a key problem during TB infection, as it may cause unwanted consequences in the body, such as excessive lung tissue damage, higher susceptibility to infection and systemic inflammatory response syndrome (SIRS) (Donoghue *et al.*, 2017; Serhan, 2017; Serhan *et al.*, 2007). Research shows that TB takes advantage of the inflammatory process and T-cell response of the host, and because of its complex intracellular survival strategies, causes non-resolving inflammation in the latent and active states. This is a fundamental pathogenic feature of TB, which leads to host tissue destruction, altered eicosanoid and other lipid mediator production, as well as anaemia of infection (AI) (Kaufmann & Dorhoi, 2013). It is therefore imperative to consider new therapeutic interventions that will target host inflammatory processes to address these problems (Hawn *et al.*, 2013; Kaufmann & Dorhoi, 2013).

Anaemia of infection, a type of microcytic anaemia that occurs during a variety of inflammatory disorders (Nemeth & Ganz, 2014), is associated with significant morbidity as well as increased mortality rates in individuals who present with infectious diseases like TB (Minchella *et al.*, 2014;

Oliveira *et al.*, 2014). Anaemia of infection results in low amounts of serum iron (Fe) in the body. This is a problem of considerable magnitude as research has proven that Fe plays an important role during host immune response, as well as having a significant impact on the course of infectious disease (Cherayil, 2011; Johnson & Wessling-Resnick, 2012). It is further notable that the treatment of Fe deficiency during AI can be difficult as “too little,” but also “too much” Fe can cause unwanted results (Jonker & Van Hensbroek, 2014).

Supportive treatment that will limit non-resolving inflammation is needed, as this may help with the resolution of anaemia of infection, contribute towards improving Fe status and mitigate other resulting negative effects. This is a relevant topic to investigate, as a review of the available literature has proven pharmaceutical anti-inflammatory agents to be beneficial during TB with regards to significant decreases in the size and number of lung lesions, decreased bacillary load, as well as reduced treatment time (Behar *et al.*, 2011; Kroesen *et al.*, 2017; Majeed *et al.*, 2015). However, some medication may cause serious unwanted damage and side effects (Newman, 2018). Hawn *et al.* (2013) and Kaufmann & Dorhoi (2013) suggested further investigations of treatment that alter mediators of inflammatory processes are needed and that the extensive treatment time of TB merits these investigations.

An emerging area of research now focuses on the role of lipid mediators as regulators of host defense against TB. Lipid mediators (also termed eicosanoids), which are derived from polyunsaturated fatty acids (PUFAs), have been identified as very important bioactive molecules with the capacity to regulate inflammation and host susceptibility following TB infection (Divangahi *et al.*, 2009). n-6 PUFAs lead to the production of pro-inflammatory lipid mediators, while inflammation-resolving lipid mediators are derived from omega-3 (n-3) PUFAs (Hidaka *et al.*, 2015). According to research, these lipid mediator pathways can be manipulated and thereby influence inflammation (Divangahi *et al.*, 2013; Fullerton *et al.*, 2014). By balancing lipid mediator production during TB, the progression or termination of inflammation can be signalled (Kaufmann & Dorhoi, 2013; Robinson *et al.*, 2015; Serhan, 2017). This approach has been found to be successful in various other inflammatory diseases (Berquin, 2009; Calder, 2014; Calder, 2015; Ma *et al.*, 2015; Pradelli *et al.*, 2012), and may be unique in intervening therapeutically and limiting non-resolving inflammation, thereby addressing inflammatory lung tissue damage, as well as AI during TB.

With the anti-inflammatory effects of n-3 PUFAs being widely reviewed, these long-chain polyunsaturated fatty acids (LCPUFAs) are reported to have multiple mechanisms of action in the inflammatory response pathways, contributing to the resolution thereof, as well as the restoration of immune competence (Calder 2011, Calder, 2013; Fullerton *et al.*, 2014; Serhan *et*

al., 2015; Serhan, 2017; Serhan & Petasis, 2011). Altering a patient's n-3 PUFA status may therefore be a non-pharmaceutical approach to benefit anti-inflammatory and pro-resolving pathways during TB infection, but with fewer side effects. Interestingly, n-3 PUFA status has also been found to have an effect on Fe status (with or without disease). It is reported that a higher n-3 PUFA intake may be associated with a lower risk of Fe depletion (Jamieson *et al.*, 2013). Furthermore, the supplementation of n-3 PUFAs and Fe in combination has been found to exert a safer profile with regard to respiratory morbidity than when supplementing Fe alone (Malan *et al.*, 2016). Therefore, after TB infection, a good n-3 PUFA status may not only limit non-resolving inflammation, but also influence Fe status, contributing towards reduced Fe depletion and the resolution of AI and its resulting negative effects. Currently little is known about the effect of pre-infection n-3 PUFA status on AI and morbidity during TB infection, as no study has ever been performed on this topic, thus it is a relevant area for investigation.

1.2 Conceptual framework, aim and objectives

This animal study investigated whether n-3 PUFA status had an effect on markers of AI, morbidity, and disease progression after TB infection. For this investigation, research has demonstrated a mouse model (C3HeB/FeJ) to provide the best option with regards to practical aspects and similarities to human lung lesions related to clinical TB outcomes (Rivera & Ganz, 2009). n-3 PUFA intake can be manipulated in a mouse model under controlled conditions, as it mimics the human situation where n-3 PUFA intake in some individuals might not be ideal (Richter *et al.*, 2014; Stark *et al.*, 2016). The current experiment forms part of a larger study, which will further investigate whether n-3 PUFA in combination with Fe supplementation would serve as an effective supportive treatment for improving inflammatory and morbidity outcomes of TB infection.

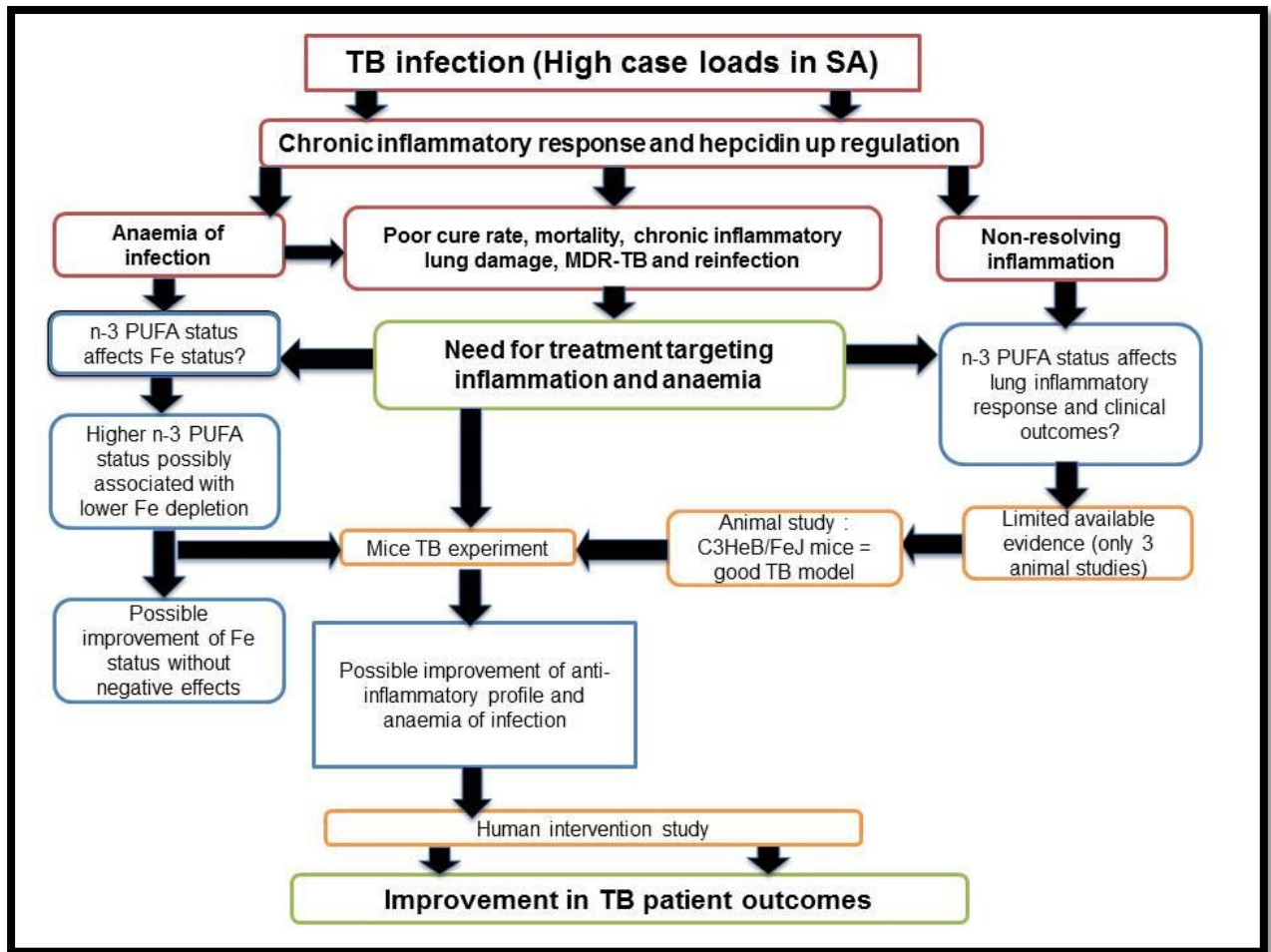


Figure 1-1: The Conceptual framework of the topic under investigation.

Fe: Iron; MDR-TB: multi-drug resistant tuberculosis; n-3: n-3; PUFA: polyunsaturated fatty acid; SA: South Africa; TB: tuberculosis

1.2.1 Research aim

The aim of this experimental animal study was to determine the effect of pre-infection n-3 PUFA status on AI and morbidity in TB infected mice.

1.2.2 Research objectives

The objectives of this study were to determine whether there was a difference between subgroups with a pre-infection n-3 PUFA sufficient or deficient status after TB infection, with regard to markers of:

1. AI (Fe status parameters i.e. serum ferritin, transferrin receptor (TfR), hepcidin, and haemoglobin (Hb));

2. morbidity (monitoring appearance and respiratory rate and measuring food intake and body weight change)
3. disease progression (lung bacillary load, organ indexes, and inflammatory marker profiles)

1.3 Structure of dissertation

This chapter style mini-dissertation is a compilation of five chapters, specifically written according to the postgraduate guidelines of the North-West University (NWU). Bibliography are provided at the end of each chapter, written in Harvard style as required by the mandatory referencing style of the NWU.

The current introductory chapter (Chapter 1) provides context to the research question being asked. This chapter states the aim and objectives, provides a description of the dissertation's structure, envisaged research outputs, as well as the contributions of the different research team members and research advisors.

Chapter 2 consists of a literature review, specifically focusing on the resulting negative effects of TB, a dietary approach against AI and morbidity after TB infection, as well as animal models used as human substitutes in the study of TB.

Chapter 3 provides the methodologies used, i.e. animals and housing, infection and study execution procedures, sample collection and analysis, as well as statistical analyses performed to obtain the results necessary to answer the research question.

Chapter 4 describes the results obtained from the methods that were followed in Chapter 3.

Chapter 5 gives a broad discussion regarding the results. In this chapter, the effect of pre-infection n-3 PUFA status on all study objectives are discussed.

Chapter 6 is the concluding chapter. This chapter provides a summary of the main findings emanating from this study, some limitations, practical recommendations, and recommendations for future studies. This chapter completes the mini-dissertation.

1.4 Research outputs emanating from this study

The results of this MSc project will be submitted to *the Journal of infectious diseases* and presented at a national scientific conference. This animal study will contribute to the understanding of underlying mechanisms that cannot be measured in humans. The long-term

goal of this research is to translate findings to the human situation. This pre-clinical investigation may be the first step towards elucidating the role of n-3 PUFA status prior to and during active TB in possibly reducing AI and morbidity during TB infection.

1.5 Contributions of members of the research team

The contributions of the researchers that were part of this research project are presented in Table 1-1. Advisors for this research project are presented in Table 1-2.

1.5.1 MSc student's contribution towards study

The MSc student was involved in the planning and organisation of the MSc sub-study. She was responsible for the monitoring (i.e. feeding; weighing; TB morbidity sheet scores) and data collection of the mice, and helped with the infection procedures (intranasal and aerosol infection with H3Rv TB strain), as well as the euthanasia and biological sample harvesting procedures of the mice (i.e. blood and organ collection and blood processing). Furthermore, the student performed plating of infected lungs, CFU counts and assisted in Fe parameter and cytokine Enzyme-linked immunosorbent assays (ELISA) analyses. She was also in charge of the statistical analysis of the sub-study and reporting of all findings. Lastly, she contributed to the execution of the larger study, including infection, monitoring and data collection procedures.

Table 1-1: List of members and their contribution to this research project

Team member	Qualification	Professional registration	Role and responsibility
Dr. L Malan	<i>Ph.D., Nutrition</i>	Medical Biological Scientist, HPCSA	Principal investigator/ Project head Co-supervisor for MSc student
Mrs. A Nienaber	<i>MSc, Dietetics</i>	Dietitian, HPCSA	Student supervisor Project coordinator
Prof. J Baumgartner	<i>Ph.D., Nutrition</i>	None	Study supervisor
Mr. K Venter	<i>M.B.A, BSc (Hons), IAT Levels 3 & 2 Diplomas in Animal Science & Technology</i>	ART0012/97	Laboratory animal technologist.

Continuation of Table 1-1: List of members and their contribution to this research project			
Team member	Qualification	Professional registration	Role and responsibility
Miss M Britz	<i>BSc, Dietetics</i>	Dietitian, HPCSA	MSc student Involved in the planning and organisation of sub-study. Mice monitoring, data collection, infection and execution procedures. Responsible for statistical analysis and reporting of findings. Writing up of mini-dissertation.

Table 1-2: Advisors for this research project

Advisors	Role and responsibilities	Institution
Prof D Loots	Advisory expert in Tuberculosis laboratory analyses	Laboratory of Infectious Disease Metabolomics at the Center for Human Metabolomics, NWU
Dr. S Parihar	Advisory expert in mice models of Tuberculosis studies and laboratory analyses	Institute of Infectious Diseases and Molecular Medicine (IDM), Division of Immunology, University of Cape Town, Cape Town

CEN: Centre of Excellence for Nutrition; NWU: North-West University

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CHAPTER 2 LITERATURE REVIEW

The current Chapter is intended to harness the reader with some background knowledge regarding Tuberculosis (TB) infection and the role that n-3 polyunsaturated fatty acids (PUFAs) may play in the battle against this pathogen. Section 2.1 provides a broad discussion about the negative effects that TB infection may have in the body, as well as the action from the immune system in response to this invading pathogen. Section 2.2 gives detailed information about lipid mediators (LM) as a dietary approach against anaemia of infection (AI) and morbidity after TB infection. Lastly, Section 2.3 explains different animal models used as human substitutes when studying TB, as well as the specific model that was chosen for this animal experimental study.

2.1 Tuberculosis and its resulting negative effects

2.1.1 South Africa's battle against tuberculosis

Active TB accounts for the death of 1.7 million people per year (WHO, 2017), and being one of the world's largest global killers, the African continent in particular suffers from this infection. The World Health Organization (WHO) classifies TB as a regional emergency in Africa, with South Africa (SA) being reported as one of the top 20 countries with the highest estimated numbers of TB cases worldwide (WHO, 2017). Tuberculosis has negative health and economic effects in underdeveloped countries such as SA. Furthermore, a lack of effective vaccines, long periods of treatment, and co-infections, such as *human immunodeficiency virus* (HIV), may worsen this situation (Vilaplana *et al.*, 2013).

The spread of tuberculosis is via airborne sputum droplets containing bacilli (Thirunavakarasu & Santhanam, 2017). This bacterial infection is, therefore, highly prevalent within a context of overcrowding and intimate living quarters, especially in disadvantaged population groups (Sullivan, 2017). In SA, TB is commonly found amongst healthcare workers, residents in assisted living facilities, skilled nursing homes or hospitals, minors and people who present with altered host-cellular immunity (Mahan *et al.*, 2012). Ageing, malnutrition or vitamin deficiencies, cancer, immunosuppressive therapy, HIV, end-stage renal disease, diabetes, and tobacco users are all examples of high-risk patients for developing active TB (Jeong & Lee, 2008; Sullivan, 2017; WHO, 2016), and of these, HIV together with TB is a substantial problem in SA. According to the WHO, HIV positive individuals are 26 to 31 times more susceptible to TB development compared to those not infected with HIV (WHO, 2012). In fact, throughout the African continent, TB mortality is highest amongst patients suffering from HIV due to their suppressed immune status (DOH, 2014, WHO, 2016).

Tuberculosis has multiple infectious sites as well as a wide range of clinical disease manifestations. For this reason, researchers refer to this infection as a very complicated disease (Cayabyab *et al.*, 2012). Active TB, if left untreated, can result in extremely high morbidity and mortality rates (WHO, 2017); nonetheless, early diagnosis and appropriate treatment could lower mortality rates. In 2016, there were 6.3 million incident TB cases reported globally. From these incidents, the most recent data from treatment outcomes indicated a success rate of 83 percent (%) (WHO, 2017), meaning millions of people who present with active TB are successfully treated each year. Nonetheless, according to the WHO, there is still a large gap for improvement (WHO, 2017). Being one of the top high burden TB countries, South Africa is included as one of the global multi-drug resistant TB (MDR-TB) burden countries targeted for specialised support to programmatic management of MDR-TB, and for WHO monitoring and assessment throughout the period of 2016 to 2020, as shown in Figure 2-1 (WHO, 2016).

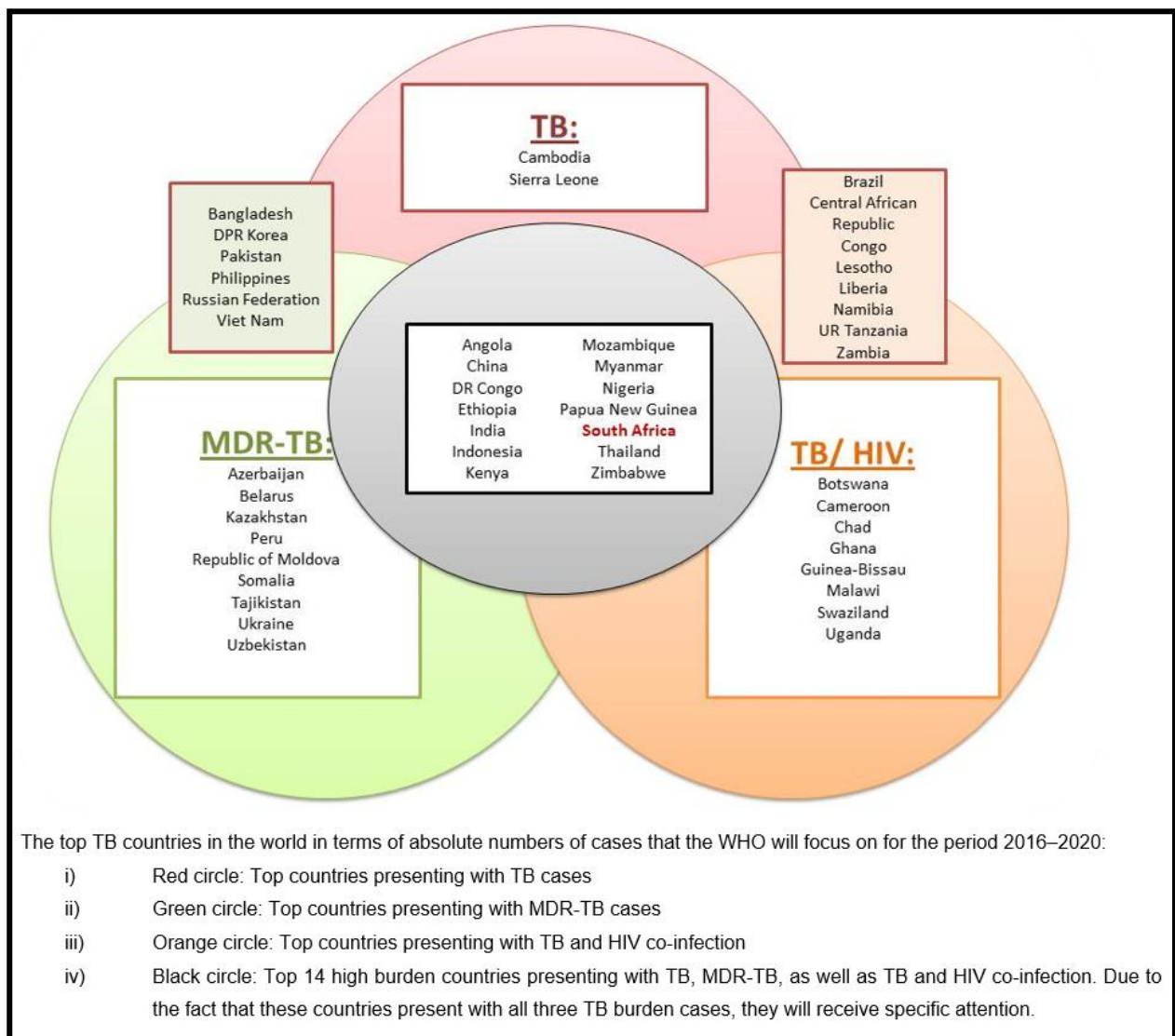


Figure 2-1: The World Health Organization high tuberculosis burden countries

Image adapted from WHO (2016)

HIV: *human immunodeficiency virus*; MDR-TB: multi-drug resistant TB; TB: Tuberculosis

Working alongside the WHO, researchers and medical teams not only in SA but globally, are persistent in the fight against the TB epidemic. The driving goal is to develop tools to improve treatment and reduce morbidity and mortality rates amongst TB infected individuals. In order to gain a better understanding of the target of treatment, it may be useful to explore the TB infection's pathogenesis, focusing on how the host (humans) and the pathogen (TB infection) interact with each other, and how the host protects itself against this pathogen.

2.1.2 Tuberculosis pathogenesis and host immune response

Mycobacterium tuberculosis (MTB), *M. bovis* and *M. africanum* are the causative agents of human TB (Banuls *et al.*, 2015). Of these three, MTB is the most common and is transmitted when a person inhales droplets from the sputum of an infected individual. It is known that the highly infective bacteria-laden droplets responsible for infection can float in the air (indoor, dark spaces) for up to four hours (Kaufmann & Dorhoi, 2013; Mahan *et al.*, 2012), however, contact with direct sunlight will cause the destruction of the tubercle bacilli.

Mycobacterium TB can spread to different parts of the body, including the circulatory system, central nervous system, lymphatic system, genitourinary system, bones and joints (DOH, 2014), but because the lungs are the main sites of entry, individuals most commonly present with pulmonary TB (PTB) (Kaufmann & Dorhoi, 2013). According to research, the bacteria reside in the upper lobes of the lungs (as this is an oxygen-rich environment) and in general affect resident alveolar macrophages in pulmonary alveolar sacs (Sohaskey & Voskuil, 2015). It is here where the MTB bacterium spends an essential part of its life cycle, and infection will then rely upon the microbial capacity of the macrophages and additionally, bacterial virulence (Kaufmann & Dorhoi, 2013).

Following infection, the immune system may clear the MTB bacteria in certain individuals and the person will not become infected. However, if the immune system fails to eliminate the bacteria, it may exist in a quiescent (inactive) stage for prolonged periods. At this stage, the immune system can control the pathogen through an immune response and inflammation (the aggregation of fluid, plasma proteins, and white blood cells (WBCs)) to limit host damage, as reviewed by Croasdell (2016). However, if the bacteria replicate and escape immune control, it will cause active TB (Walzl *et al.*, 2011); this can occur anytime following infection. Clinical outcomes of MTB include, i) no clinical or laboratory evidence of infection, ii) infection without active disease, or iii) active disease. Figure 2-2 is a visual representation of the progression of TB inside the host. It is found that in an otherwise healthy population, there is a 5 to 15% risk that latent TB will progress to active TB (Sinclair *et al.*, 2011, WHO, 2016). Again, this percentage will be higher in individuals presenting with altered host cellular immunity (Jeong & Lee, 2008; Sullivan, 2017; WHO, 2016).

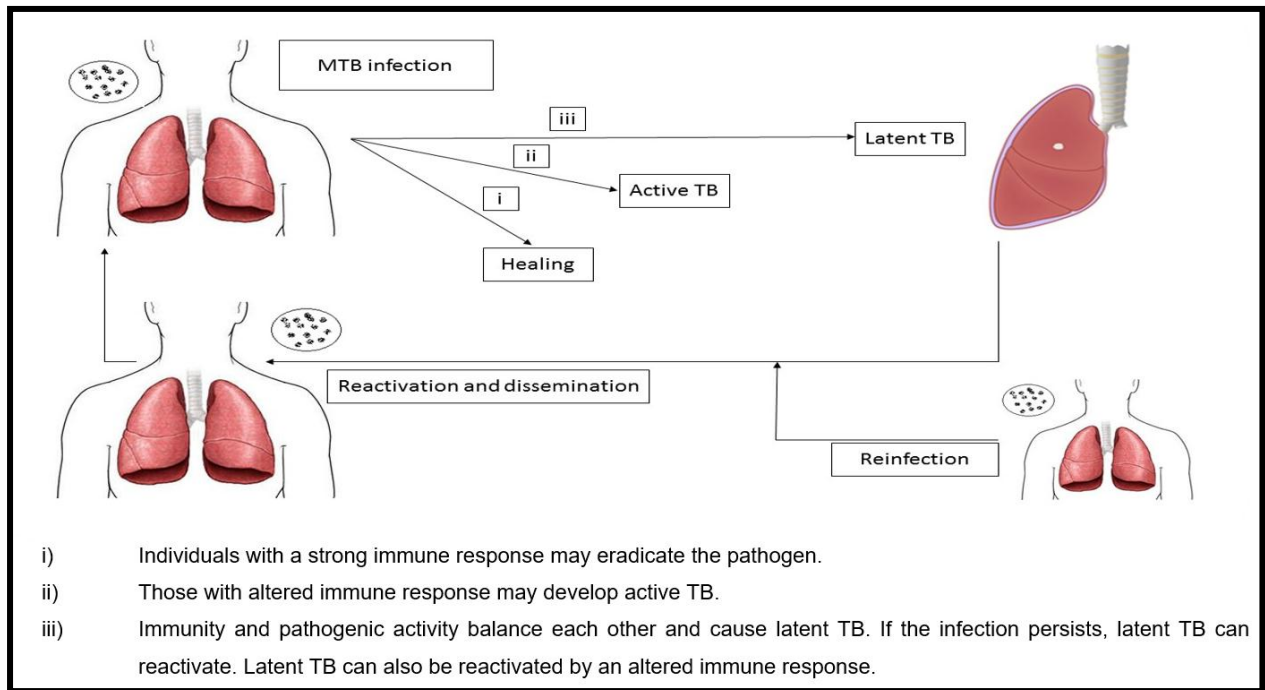


Figure 2-2: The progression of TB inside the host

Image adapted from Majeed *et al.* (2015)

MTB: *Mycobacterium tuberculosis*; TB: Tuberculosis

The capability of host immune response to control or eradicate the infection will determine the phase of MTB (Figure 2-3). The immune response is separated into two types, namely the innate and adaptive response. The innate immune system (first phase) is a complex system that encompasses of physical barriers (obstructions to infection e.g. epithelia of skin, gastrointestinal, respiratory, genitourinary tracts), antibody-mediated immunity and cellular components (i.e. neutrophils, macrophages, dendritic cells, and innate lymphoid cells) (Walzl *et al.*, 2011). Due to its fast activation, this type of immune response provides the first line of defense against infective pathogens (Khan *et al.*, 2016). Unsuitable activation of this type of immune response can result in inflammatory states (Lowe *et al.*, 2012). If these inflammatory states persist and are not terminated, it will ultimately lead to tissue damage and metabolic changes will occur (Chen *et al.*, 2018).

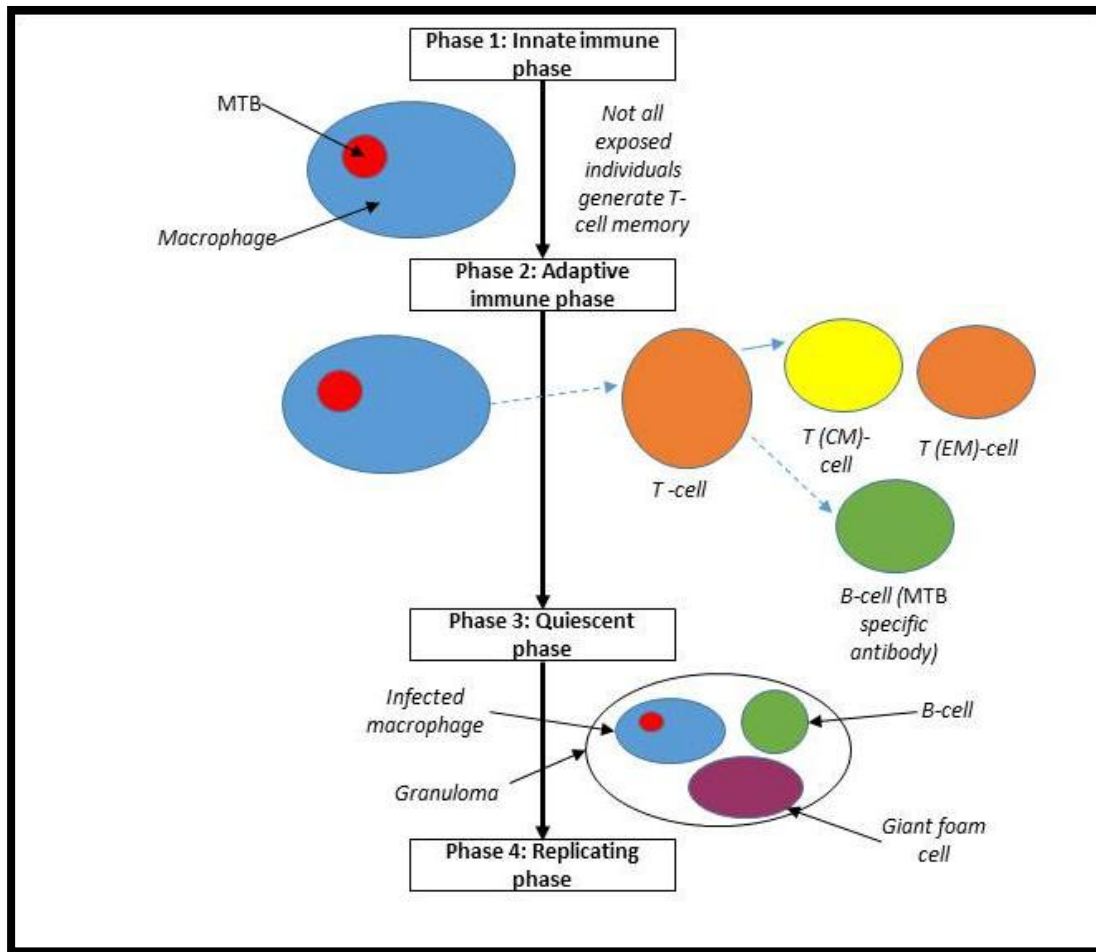


Figure 2-3: Immune responses to MTB exposure and infection

Image adapted from Walzl *et al.* (2011)

MTB: *Mycobacterium tuberculosis*; T (CM) cell: Central Memory T-cell; T (EM) cell: Effector Memory T-cell

During the innate immune response phase, various biochemical mediators are produced in response to the invading pathogen (in this case TB infection). Phagocytic macrophages (a type of WBC found at the site of infection) and neutrophils (neutrophilic WBCs) are essential cells associated with innate immunity (Navegantes *et al.*, 2017). Neutrophils target the bacteria by secreting hydrolytic as well as oxidising agents in order to protect the immune system (Kroesen *et al.*, 2017). A neutrophil suppresses a bacillus, whereafter it has a chance to eradicate the pathogen; the neutrophil will then perish (via apoptosis or necrosis) prior to being consumed by a mononuclear phagocyte (most commonly a macrophage). Influenced by the manner of neutrophil death and cytokine release (Section 2.1.3) before death, macrophage response can either be pro-inflammatory or inflammation resolving. These initial events can regulate the general host outcome from infection, as presented in Figure 2-4 (from left to right) (Lowe *et al.*, 2012).

infection and cause the MTB exposed individual to enter the quiescent phase (third phase) (Figure 2-3) (Khan *et al.*, 2016; Urdahl *et al.*, 2011; Walzl *et al.*, 2011).

In the quiescent phase (also referred to as latent or inactive TB), the immune system fails to eliminate the bacteria, and it can stay inside the granuloma for decades (Silva Miranda *et al.*, 2012; Walzl *et al.*, 2011). Granulomas, generally produced in response to infection, have a central area containing infected macrophages, epithelioid cells, multinucleated giant cells and foam cells (T-cells) (Silva Miranda *et al.*, 2012). All these cells are surrounded by a rim of lymphocytes (mainly a cluster of differentiation (CD) 4⁺ and CD8⁺ T-cells, B-cells, as well as fibroblasts). Macrophages are separated from healthy tissues through granuloma formation but are kept in close contact with T-cells (Egen *et al.*, 2008), and it is for this reason that the risk for progression from latent TB to active TB is low (5 to 15%) in an otherwise healthy population. However, environmental or genetic factors may cause the reactivation of the MTB bacteria (Jeong & Lee, 2008; Sullivan, 2017; WHO, 2016). When the bacterium is reactivated, it will provoke the death of the infected macrophages (Silva Miranda *et al.*, 2012), the granulomas are then disrupted and necrotic zones develop in its centre. Eventually, the granuloma structure will disintegrate, causing the replicating phase (fourth phase) (Figure 2-3). The bacterium then escapes immune control, spreads to other parts of the lung, and forms more lesions. This causes extreme abnormalities of the immune system to be induced and pro-inflammatory markers increase. Tuberculosis (a disease that is characterised by non-resolving inflammation) takes advantage of the inflammatory process and causes the body to continually synthesise pro-inflammatory mediators (Dorhoi *et al.*, 2011). Ultimately, inflammation will not resolve, leading to altered immune function, cell damage, disrupted T-Helper cell balance, and altered memory T-cell and antibody production (Kaufmann & Dorhoi, 2013; Lowe *et al.*, 2012; Walzl *et al.*, 2011).

Due to the fact that TB causes an immune response, modulates inflammatory processes and alters lipid mediator production (Kaufmann & Dorhoi, 2013). This infection causes non-resolving inflammation in the latent as well as active states (Dorhoi & Kaufmann, 2014). Even though acute inflammation plays an important role in host defense against the TB pathogen, non-resolving inflammation may cause undesirable damage in the body (i.e. local tissue damage, skeletal loss, wasting, and systemic inflammatory response syndrome (SIRS)) (Calder, 2003; Kroesen *et al.*, 2017; Serhan *et al.*, 2007). Chronic inflammation (inflammation extended for a long period i.e. weeks, months or years) will ultimately lead to decreased oxygen-carrying capacity from red blood cells (RBCs), as well as AI (Cherayil, 2010; Jonker & Van Hensbroek, 2014).

2.1.3 Cytokine production as part of the inflammatory response to tuberculosis

Cytokines can be described as low molecular weight regulatory proteins secreted by WBCs, as well as a variety of other cells in the body, in response to a number of stimuli (mainly by activated lymphocytes, macrophages, dendritic cells, endothelial cells, and connective tissue cells) (Arango Duque & Descoteaux, 2014). The term “cytokine” describe proteins such as interleukins (ILs) (produced by one leukocyte and act on another leukocyte), lymphokines (produced by lymphocytes), monokines (produced by monocytes), chemokines (cytokines with chemotactic activities), interferons (involved in antiviral responses), and colony stimulating factors (supports the growth of blood cells) (Nedoszytko *et al.*, 2014). Hundreds of cytokines have already been recognised in the past (Cameron & Kelvin, 2013).

When homeostasis is disturbed, for example by TB infection (as described in Section 2.1.2), cytokines are released (in the innate as well as the adaptive immune response) to mediate inflammatory and immune reactions (Domingo-Gonzalez *et al.*, 2016). Cytokines are greatly involved in the pathogenesis of human inflammatory or autoimmune disease and can affect the immune response to infection in positive and negative ways (Moudgil & Choubey, 2011; Domingo-Gonzalez *et al.*, 2016). Table 2-1 describes these positive and negative roles of specific cytokines in the context of TB infection. Cytokines involved in acute inflammation differ from those that play a role in chronic inflammation. Interleukin-1 alpha (IL-1 α), IL-1 beta (β), IL-6, tumor necrosis factor (TNF)- α and chemokines are involved in acute inflammation, whilst IL-12, TNF- β , and interferon-gamma (IFN- γ) play a significant role in chronic inflammation (Dinarello, 1991; George *et al.*, 2015; Manca *et al.*, 2001; Ordway *et al.*, 2007); conversely, Interleukin-17 is involved in both acute and chronic inflammation. Furthermore, these cytokines can be pro-inflammatory (leading to excessive inflammation) or anti-inflammatory (serve to promote healing) (Etna *et al.*, 2014; George *et al.*, 2015), for example, during TB infection, TNF- α , IL-1, IL-6, IL-17, and IL-22 are pro-inflammatory, whilst transforming growth factor beta (TGF- β) and IL-10 are anti-inflammatory.

With cytokine analysis, it can be problematic to generalise the roles of individual cytokines because cytokine signalling can lead to elevated or diminished expression of membrane proteins, and/or cause effector molecules to be secreted (Janeway *et al.*, 2005). Furthermore, cytokine combinations can act together or opposed, relying upon the state of the target cells as well as the combinations, quantities, and the sequential order of cytokine production (Janeway *et al.*, 2005).

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

Cytokine*	Positive role in TB*	Negative role in TB*	Examples of human TB studies who tested the specific cytokines	Examples of TB studies who tested the specific cytokines in mouse models
Tumor necrosis factor-alpha (TNF- α)	• Essential for survival following MTB infection. • Initiation of innate cytokine and chemokine response and phagocyte activation.	• Mediator of tissue damage.	Fallahi-Sichani <i>et al.</i> , 2012 Gleeson <i>et al.</i> , 2016 Gonzalez <i>et al.</i> , 2018 Keane <i>et al.</i> , 2000 Zeng <i>et al.</i> , 2011	Hölscher <i>et al.</i> , 2005 Hölscher <i>et al.</i> , 2008 Juffermans <i>et al.</i> , 2000 Lockhart <i>et al.</i> , 2006 Maiga <i>et al.</i> , 2015 McNab <i>et al.</i> , 2014 Ordway <i>et al.</i> , 2007 Wilson <i>et al.</i> , 2010
Interferon-gamma (IFN- γ)	• Essential for survival following TB infection. • Coordinates and maintains mononuclear inflammation. • Expressed by antigen specific T-cells.	• Potentially pathogenic	Abu-Taleb <i>et al.</i> , 2011 Diel <i>et al.</i> , 2011 Dong & Yang, 2015 Gonzalez <i>et al.</i> , 2018 Matthews <i>et al.</i> , 2012 Özbek <i>et al.</i> , 2005 Stefan <i>et al.</i> , 2010 Vankayalapati <i>et al.</i> , 2000 Wilkinson <i>et al.</i> , 1999 Zeng <i>et al.</i> , 2011	Behrends <i>et al.</i> , 2013 Dhiman <i>et al.</i> , 2012 Green <i>et al.</i> , 2012 Hölscher <i>et al.</i> , 2005 Juffermans <i>et al.</i> , 2000 Lockhart <i>et al.</i> , 2006 Manca <i>et al.</i> , 2001 Moguche <i>et al.</i> , 2015 Monin <i>et al.</i> , 2015 Ordway <i>et al.</i> , 2007 Sakai <i>et al.</i> , 2014 Schneider <i>et al.</i> , 2010 Wilson <i>et al.</i> , 2010
Interferon-alpha/beta (IFN- α /IFN- β)	• Required for initial recruitment of phagocytes to the lung.	• Overexpression of IFN-α/IFN-β results in recruitment of permissive phagocytes and regulation of T-cell accumulation and function.	Berry <i>et al.</i> , 2010 George <i>et al.</i> , 2015	Antonelli <i>et al.</i> , 2010 Desvignes <i>et al.</i> , 2012 McNab <i>et al.</i> , 2014

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

Cytokine*	Positive role in TB*	Negative role in TB*	Examples of human TB studies who tested the specific cytokines	Examples of TB studies who tested the specific cytokines in mouse models
Interleukin-6 (IL-6)	• Potentiates early immunity – nonessential unless a high dose infection.	-	George <i>et al.</i> , 2015 Nolan <i>et al.</i> , 2013 Oh <i>et al.</i> , 2018	Lockhart <i>et al.</i> , 2006 Manca <i>et al.</i> , 2001 Ordway <i>et al.</i> , 2007 Sodenkamp <i>et al.</i> , 2012
Interleukin-1 α (IL-1 α)/ Interleukin-1 β (IL-1 β)	• Essential for survival following TB infection. • Induction of IL-17. • Promotes prostaglandin E2 (PGE2) to limit IFN-α	-	Gleeson <i>et al.</i> , 2016 Gonzalez <i>et al.</i> , 2018 Wilkinson <i>et al.</i> , 1999	Guler <i>et al.</i> , 2011 Juffermans <i>et al.</i> , 2000 Lockhart <i>et al.</i> , 2006 Maiga <i>et al.</i> , 2015 McNab <i>et al.</i> , 2014
Interleukin-18 (IL-18)	• May augment IFN-γ – non-essential. • Regulator of neutrophil/monocyte accumulation of neutrophil and monocyte accumulation, optimal induction of IFN-γ by T-cells.	-	Gleeson <i>et al.</i> , 2016 Vankayalapati <i>et al.</i> , 2000	Schneider <i>et al.</i> , 2010
Interleukin-12 (IL-12)	• IL-12p40 and IL-12p35 essential for survival following TB infection. • Mediate early T-cell activation, polarization, and survival.	• Overexpression of IL-12p70 is toxic during TB infection.	Altare <i>et al.</i> , 2001 George <i>et al.</i> , 2015 Özbek <i>et al.</i> , 2005 Vankayalapati <i>et al.</i> , 2000	Behrends <i>et al.</i> , 2013 Hölscher <i>et al.</i> , 2005 Hölscher <i>et al.</i> , 2008 Lockhart <i>et al.</i> , 2006 Manca <i>et al.</i> , 2001 McNab <i>et al.</i> , 2014 Monin <i>et al.</i> , 2015 Ordway <i>et al.</i> , 2007

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

Cytokine*	Positive role in TB*	Negative role in TB*	Examples of human TB studies who tested the specific cytokines	Examples of TB studies who tested the specific cytokines in mouse models
Interleukin-23 (IL-23)	- Required for IL-17 and IL-22 expression during MTB infection. - Non-essential in low dose challenge required for long-term control.	Mediates increased pathology during chronic challenge	Bandaru <i>et al.</i> , 2014	Behrends <i>et al.</i> , 2013 Hölscher <i>et al.</i> , 2008 Monin <i>et al.</i> , 2015 Wozniak <i>et al.</i> , 2006
Interleukin-27 (IL-27)	May control inflammation and reduce pathology.	Regulates protective immunity to TB infection by limiting the migration and survival of T-cells at the inflamed site.	Larousserie <i>et al.</i> , 2004	Hölscher <i>et al.</i> , 2005 McNab <i>et al.</i> , 2014 Wozniak <i>et al.</i> , 2006
Interleukin-35 (IL-35)	Regulate the availability of subunits of IL-12, IL-27.	Potential immunoregulatory role.	Dong & Yang, 2015 Kong <i>et al.</i> , 2016	Zhang <i>et al.</i> , 2016 (Not TB specific)
Interleukin-17 (IL-17)	- Essential for survival following infection with some strains of MTB. - Induction and maintenance of chemokine gradients for T-cell migration.	Drives pathology via S100A8/A9 and neutrophils.	Bandaru <i>et al.</i> , 2014 Dong & Yang, 2015 George <i>et al.</i> , 2015 Matthews <i>et al.</i> , 2012 Singh <i>et al.</i> , 2018	Behrends <i>et al.</i> , 2013 Khader <i>et al.</i> , 2007 Lockhart <i>et al.</i> , 2006 Wilson <i>et al.</i> , 2010
Interleukin-22 (IL-22)	Induces antimicrobial peptides and promotes epithelial repair, inhibits intracellular growth of MTB in macrophages.	-	Dhiman <i>et al.</i> , 2013 Matthews <i>et al.</i> , 2012 Singh <i>et al.</i> , 2018 Zeng <i>et al.</i> , 2011	Behrends <i>et al.</i> , 2013 Dhiman <i>et al.</i> , 2012 Khader <i>et al.</i> , 2007 Wilson <i>et al.</i> , 2010

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

IFN- α : Interferon-alpha; IFN- β : Interferon-beta; IFN- γ : Interferon-gamma IL: Interleukin; MTB: *Mycobacterium tuberculosis*

2.1.4 The aetiology of anaemia of infection

Anaemia of infection (also called anaemia of inflammation, normocytic normochromic anaemia, or anaemia of chronic disease), is associated with inflammatory diseases, acute or chronic infections and malignancies, and may cause adverse effects in the body (Nemeth & Ganz, 2014). Anaemia of infection is the most common type of anaemia presented in TB patients (Lee *et al.*, 2006). During inflammation, multiple players form part of the pathophysiology of AI, including immune cells (which maintain the development of AI through co-existing mechanisms), the liver (responsible for hepcidin production; the principle mediator during AI), the spleen (site of iron (Fe) retention in macrophages), kidneys (inflammatory mediators (i.e. TNF and IL-1) inhibit erythropoietin (EPO) production) and erythron (reduces the erythropoietic drive during normal or increased EPO concentrations) (D'Angelo, 2013; Nairz *et al.*, 2016). Anaemia of infection ultimately leads to blocked Fe release, reduced Fe absorption, and internalisation and deprivation of ferroportin in erythrocytes and macrophages (D'Angelo, 2013).

Table 2-2 shows the difference between AI and Fe deficiency anaemia, and it is important not to mistake one for the other. In order to assess markers of AI, one can make use of the Fe parameters mentioned in Table 2-2. The current animal experimental study chose to report serum ferritin, transferrin receptor (TfR), hepcidin and haemoglobin (Hb).

Table 2-2: The difference between anaemia of infection and iron deficiency anaemia*

Biochemical parameters	Anaemia of infection	Iron deficiency anaemia
Red blood cells	Decreased	Decreased
White blood cells	Normal to increased	Decreased to normal
Serum iron	Decreased	Decreased
Ferritin	Normal to increased	Decreased
Transferrin	Decreased to normal	Increased
Transferrin saturation	Decreased	Decreased
Hepcidin	Increased	Decreased
Haemoglobin	Decreased	Decreased
Transferrin receptor	Normal	Increased
Cytokines	Increased	Normal
Mean corpuscular volume	Decreased to normal	Decreased
Red blood cell distribution width	Normal to increased	Increased

* Oliveira *et al.* (2014); Weiss & Goodnough (2005)

2.1.5 Iron physiology during deficiency and overload

Iron absorption generally occurs in the duodenum and jejunum (Mahan *et al.*, 2012). Ferroportin (a Fe exporter) allows transportation of Fe across the duodenum enterocytes into the blood stream (Deshpande *et al.*, 2018). After absorption of Fe, it is transported in the form of plasma Fe, or it binds to transferrin (Brown & Justus, 1958). Figure 2-5 summarises the pathways for cellular Fe transport. Dietary Fe (also referred to as heme Fe) can be found in the Hb, myoglobin, and some enzymes of animal food sources, while non-heme Fe is mostly found in plant foods (Ems & Huecker, 2017).

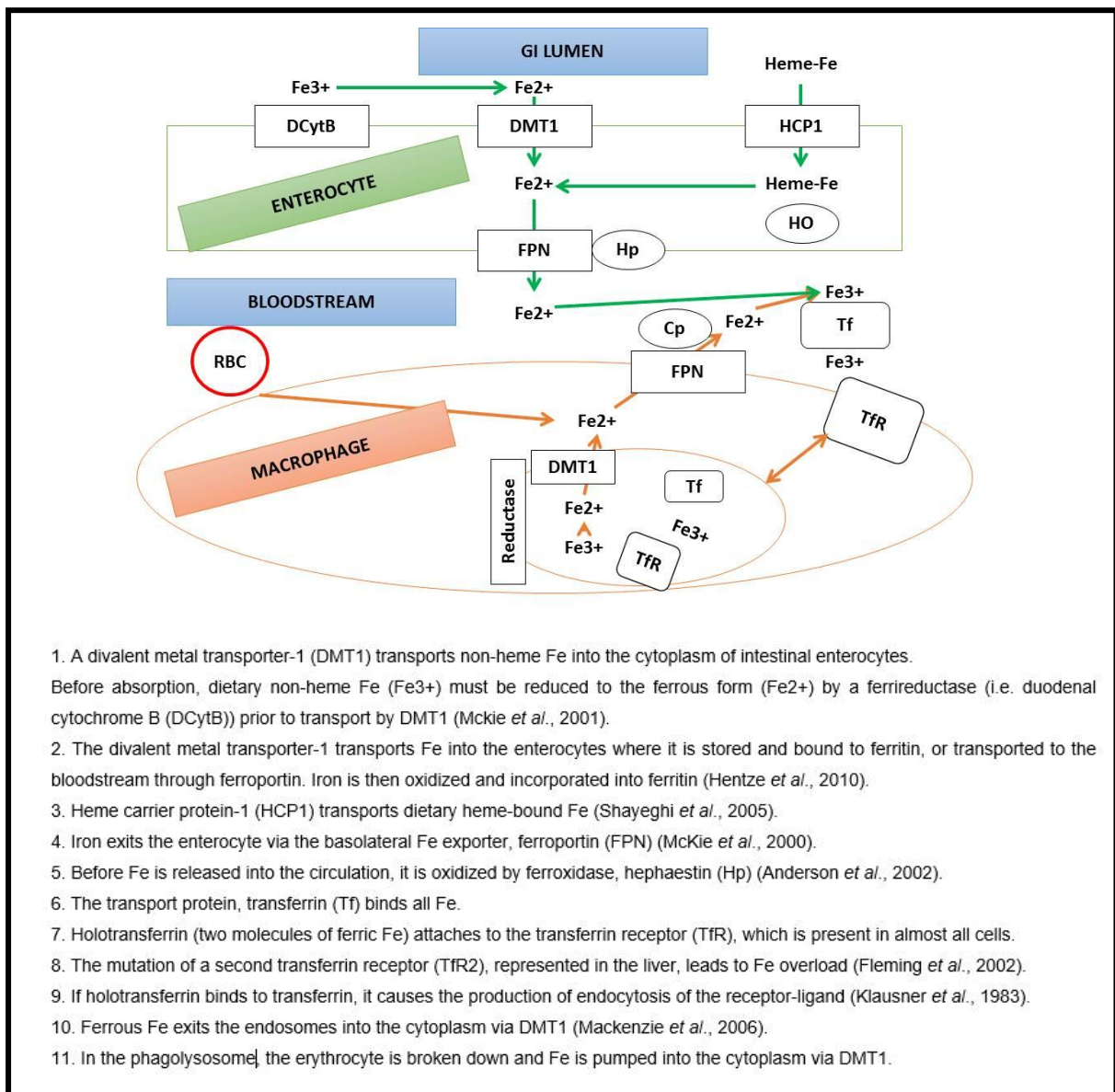


Figure 2-5: Pathways for cellular iron transport

Image adapted from Rivera (2006)

Hepcidin (hepatic bactericidal protein - a small peptide hormone) and ferroportin are the two controlling systems of Fe metabolism. Hepcidin regulates the activity of ferroportin (Zhang & Rouault, 2018). Although mainly produced in the liver, small quantities of hepcidin can be found in the heart and nervous system (Miseta *et al.*, 2015). Production in the liver is responsive to liver Fe, inflammation, hypoxia, and anaemia (Section 2.1.6). During Fe deficiency, hepcidin will be down-regulated (resulting in low hepcidin production) (Hentze *et al.*, 2010). This causes ferroportin to release Fe from hepatocytes, macrophages, duodenal enterocytes, and other cells. Figure 2-6 is a schematic representation of Fe homeostasis during Fe deficiency. Figure 2-7 conversely, represents Fe homeostasis during Fe overload. High amounts of Fe will lead to the up-regulation of hepcidin (resulting in increased hepcidin production), which ultimately cause ferroportin to degrade. Iron will then remain within hepatocytes, macrophages, duodenal enterocytes and other cells (Hentze *et al.*, 2010).

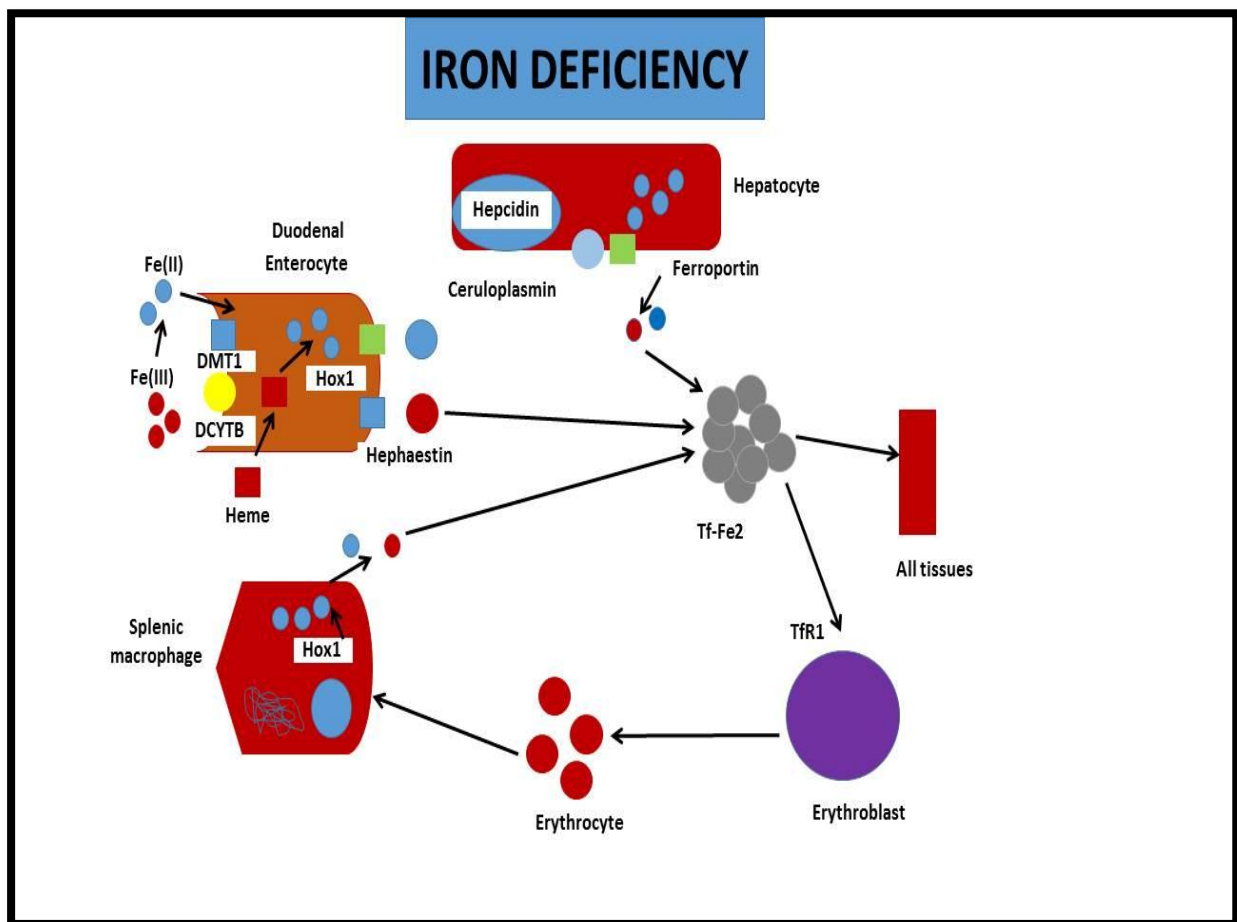


Figure 2-6: Regulation of iron homeostasis in iron deficiency

Image adapted from Hentze *et al.* (2010)

DCytB: duodenal cytochrome B; DMT1: divalent metal transporter-1; Fe: Iron; Hox1: Homeobox protein; Tf-Fe2: transferrin-bound iron; TfR1: Transferrin receptor-1

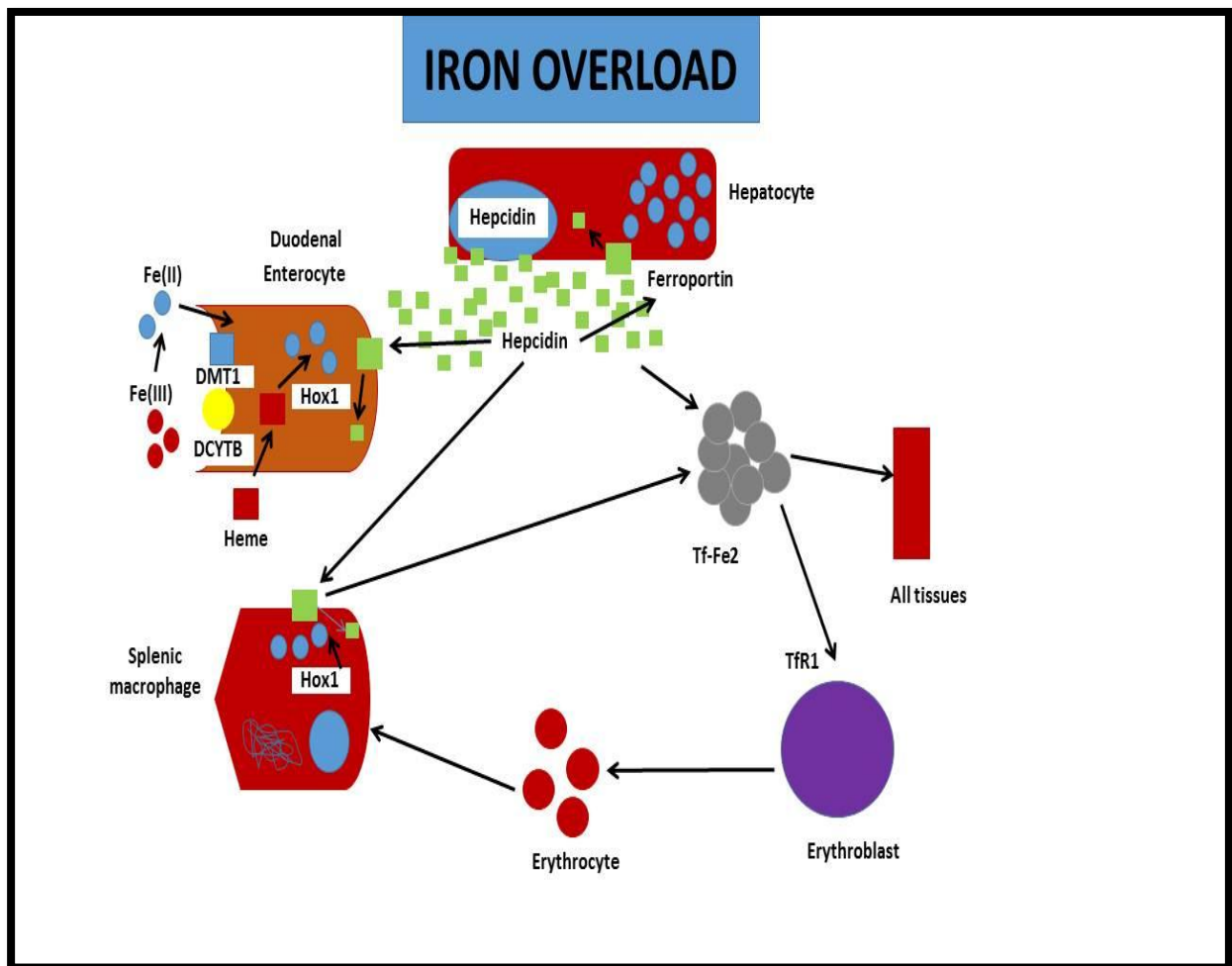


Figure 2-7: Regulation of iron homeostasis in iron overload

Image adapted from Hentze *et al.* (2010)

DCyTB: duodenal cytochrome B; DMT1: divalent metal transporter-1; Fe: Iron; Hox1: Homeobox protein; Tf-Fe2: transferrin-bound iron; TfR1: Transferrin receptor-1

2.1.6 The role of hepcidin during chronic inflammation

The negative Fe regulatory hormone hepcidin acts as the principal mediator during AI (D'Angelo, 2013). When chronic inflammation is present in the body, cytokines (Section 2.1.3) are released that increases hepcidin production. Hepcidin then mainly acts on the mucosa cell and inhibits macrophage Fe release, as well as intestinal Fe absorption (by blocking ferroportin channels) (D'Angelo, 2013). Increased hepcidin production can consequently lead to low gut Fe absorption; resulting in low amounts of serum Fe (Munoz *et al.*, 2009). This decrease in serum Fe causes low Hb production, ultimately leading to anaemia. It is important to keep in mind that certain cytokines (e.g. IL-6) can up-regulate the expression of hepcidin (regardless of Fe

status), which may also lead to a decreased Fe bioavailability during chronic inflammation (Dunn *et al.*, 2007).

2.1.7 Iron deficiency and anaemia of infection associated with tuberculosis

Iron deficiency is a common complication of TB, with an estimation of 32 to 86% of TB patients suffering from it (Isanaka *et al.*, 2012; Lee *et al.*, 2006; Sahiratmadja *et al.*, 2007). Even though it has not been specifically investigated, a large number of these Fe deficiency cases during TB infection are ascribed to AI (Minchella *et al.*, 2014). However, despite more than 50 years of investigation, studies have not been able to conclusively explain the exact mechanism behind this (Nemeth & Ganz; 2014). Blood loss because of haemoptysis (coughing up of blood), bone marrow involvement with tubercular granuloma in disseminated TB, nutritional deficiency, AI as a consequence of a decreased erythrocyte survival, hypoferremia initiated by the shift of Fe from a transferrin-bound available state to a ferritin-incorporated storage state, suppression of erythropoiesis by direct effects of cytokines on the marrow, effects of inflammation on erythropoietin production, and anaemia as a consequence of chronic inflammation, are all theories that have been suggested (Baynes *et al.*, 1986; Lombard & Mansvelt, 1993; Nemeth & Ganz, 2014). Given the consequences of AI, including evidence indicating a negative connection between anaemia, Fe redistribution and TB susceptibility (McDermid *et al.*, 2013; Wisaksane *et al.*, 2013), as well as reports linking AI to poor clinical outcomes and significant increases in morbidity and mortality rates (Isanaka *et al.*, 2012; Minchella *et al.*, 2014; Nagu *et al.*, 2014; Oliveira *et al.*, 2014; Shimazaki *et al.*, 2013), further research of anaemia management in TB patients is clearly needed.

As pointed out, AI (a common complication of TB) affects Fe status. However, from the viewpoint of the host and the pathogen, it is possible to link Fe status and TB pathogenesis. Iron status plays an important role in host immune response to infections as it influences both the innate and adaptive immune response (Cherayil, 2011). Iron does not only act as a co-factor for enzymes to maintain their functionality, but is necessary for the bactericidal activity of macrophages and the correct functioning of T-cells (Devi *et al.*, 2003). When an individual is infected with MTB, this infection takes advantage of the inflammatory process (Section 2.1.2), while thriving on Fe and using it as an essential nutrient to grow. In response to this, host immune recognition of MTB causes the production of a pro-inflammatory reaction that inhibits Fe access for the bacteria (Boelaert *et al.*, 2007). As shown in Figure 2-8, MTB is located in a initial phagosome, which developed to the late phagosome and phagolysosome phases are obstructed and its acidification is restricted. Macrophage Fe sources consist of heme-bound Fe from RBCs and Fe bound to transferrin and lactoferrin. Iron can be incorporated into proteins

(including the Fe-storage protein ferritin) when released into the cytoplasm. As it occurs in Fe overload (Figure 2-7) or in the presence of inflammation (Section 2.1.6), hepcidin regulates the activity of ferroportin to restrict pathogen access to Fe in order for it to grow. Ultimately, MTB responds by generating siderophores, which are molecules that bind Fe more strongly than host Fe-storage proteins. Lipophilic and cell-bound mycobactins, as well as free carboxymycobactins (two types of siderophores), are synthesised to seize Fe from several host Fe sources (including transferrin, lactoferrin, and heme). The seized Fe is then transported across the MTB cell wall (Jones & Niederweis, 2011; Tullius *et al.*, 2011), which may cause an increase of Fe availability in the mycobacterial phagosome (a process necessary for MTB growth and virulence) (De Voss *et al.*, 2000; Reddy *et al.*, 2013; Wagner *et al.*, 2005).

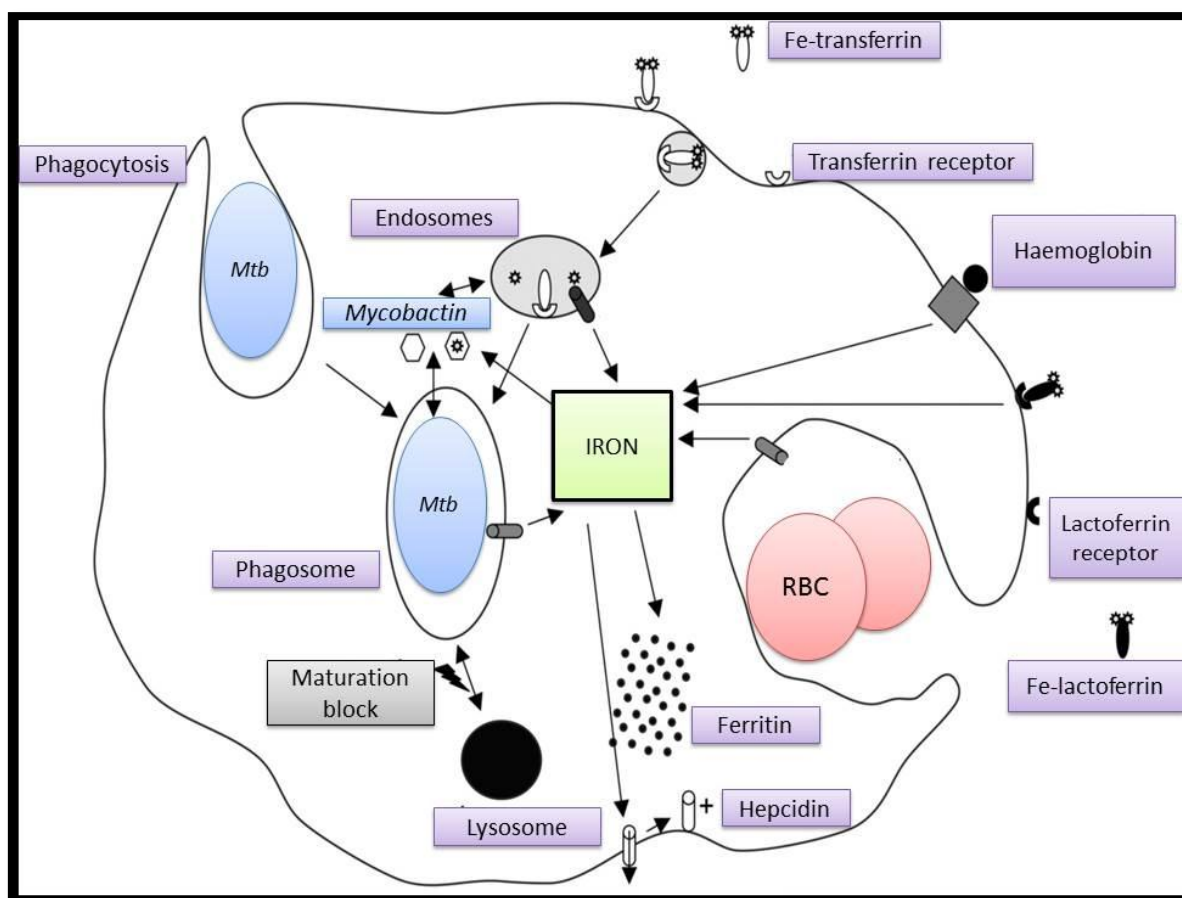


Figure 2-8: Schematic representation of an MTB-infected macrophage and its Fe metabolism

Image adapted from Boelaert *et al.* (2007)

Fe: Iron; MTB: *Mycobacterium tuberculosis*; RBC: Red Blood Cell

2.1.8 Treatment of iron deficiency during tuberculosis infection

The treatment of Fe deficiency during TB is difficult, as “too little” Fe has negative effects on host defense, as well as clinical outcomes, while “too much” Fe (for example the provision of Fe to ensure competent immune functioning) will lead to an increased Fe availability for the MTB pathogen to grow and replicate (Jonker & Van Hensbroek, 2014). Cronje *et al.* (2005) suggested that an extreme amounts of Fe treatment can stimulate MTB growth, its replication and progression to clinical disease and death from TB. Furthermore, an increased mortality rate was observed in mouse models of infection when pre-treated with high amounts of Fe (1% = 10,000 ppm) (reviewed by Rivera (2006)). Javadi *et al.* (2004) also reported that a high dose of Fe (1 ml of Fe dextran (5mg/ml)) after the onset of inflammation, led to an increased mortality rate in mice. The right amount of Fe intake and availability at the right time may thus provide sufficient immune protection against TB pathogens. However, because data for the treatment of Fe deficiency during infections are scarce (Jonker and Van Hensbroek, 2014), further investigations are needed. It may be beneficial to investigate other ways to provide sufficient immune protection against inflammation and AI during TB.

2.2 A dietary approach against anaemia of infection and morbidity in tuberculosis

2.2.1 Lipid mediators as anti-inflammatory agents

As described in the literature above, TB causes non-resolving inflammation in the latent and active states, which ultimately leads to AI, unwanted tissue damage and other undesirable clinical outcomes. However, previous research shows there are mechanisms for the resolution of inflammation, which allows reduced pro-inflammatory responses, resulting in a return to homeostasis. Furthermore, as reviewed by Croasdell (2016), anti-inflammatory and pro-resolving molecules can suppress pro-inflammatory cells and signals, representing a phenotypic shift in immune cell function towards repair and homeostasis. With this in mind, studies show that dietary factors may have an influence on the immune system during the resolution of inflammation (Ricordi *et al.*, 2015).

Lipids serve as structural membrane components, a source of energy, as well as regulators of the host immune response (Calder, 2015; Dennis & Norris, 2015). In the body, all cells have lipid bilayers (cell membranes) that consist mainly of phospholipids (Raphael & Sordillo, 2013). Phospholipids and their metabolites are involved in a number of critical cellular control systems, for example, signal transduction, as well as lipid mediator (LM) (also known as eicosanoids) production (Raphael and Sordillo, 2013). Phospholipids commonly have two polyunsaturated

fatty acids (PUFAs) linked to them. The type of PUFA that is linked to the phospholipid will have an effect on the structural and functional aspects of the cell membrane, as the released PUFA can act as a signalling molecule, precursor of ligands for transcription factors, or as precursor for the biosynthesis of LM (Calder, 2010). The immune response is influenced greatly by phagocytic and cell signalling capacity of the immune cells, which in turn relies upon the PUFA composition of the phospholipid bilayer.

Specialised pro-resolving mediators (SPLM) are a class of LM formed in cells by the enzymatic oxygenation of PUFAs (Fullerton, 2014). They are produced via the cyclooxygenase (COX), lipoxygenase (LOX), and/ or cytochrome P450 pathways (Calder, 2014), and synthesised by various cell types, including macrophages, platelets, neutrophils, epithelial cells, endothelial cells and fibroblasts (reviewed by Croasdell (2016)). They are also recognised in the human lungs, tonsils, synovial liquid, adipose tissue, the placenta and human milk, and play a critical role in the active resolution of inflammation (Arnardottir *et al.*, 2016). These mediators are endogenously delivered by means of transcellular synthesis (Serhan, 2005, Serhan *et al.*, 2008, Serhan, 2017), and act through cells of the innate immune system (among other cell types) to interpose their anti-inflammatory and pro-resolving effects by suppressing pro-inflammatory actions and promoting resolution pathways (Fullerton, 2014; Russell & Schwarze, 2014; Serhan, 2017; Serhan & Petasis, 2011). Figure 2-9 summarises the immunomodulatory actions of SPLMs on inflammatory resolution processes.

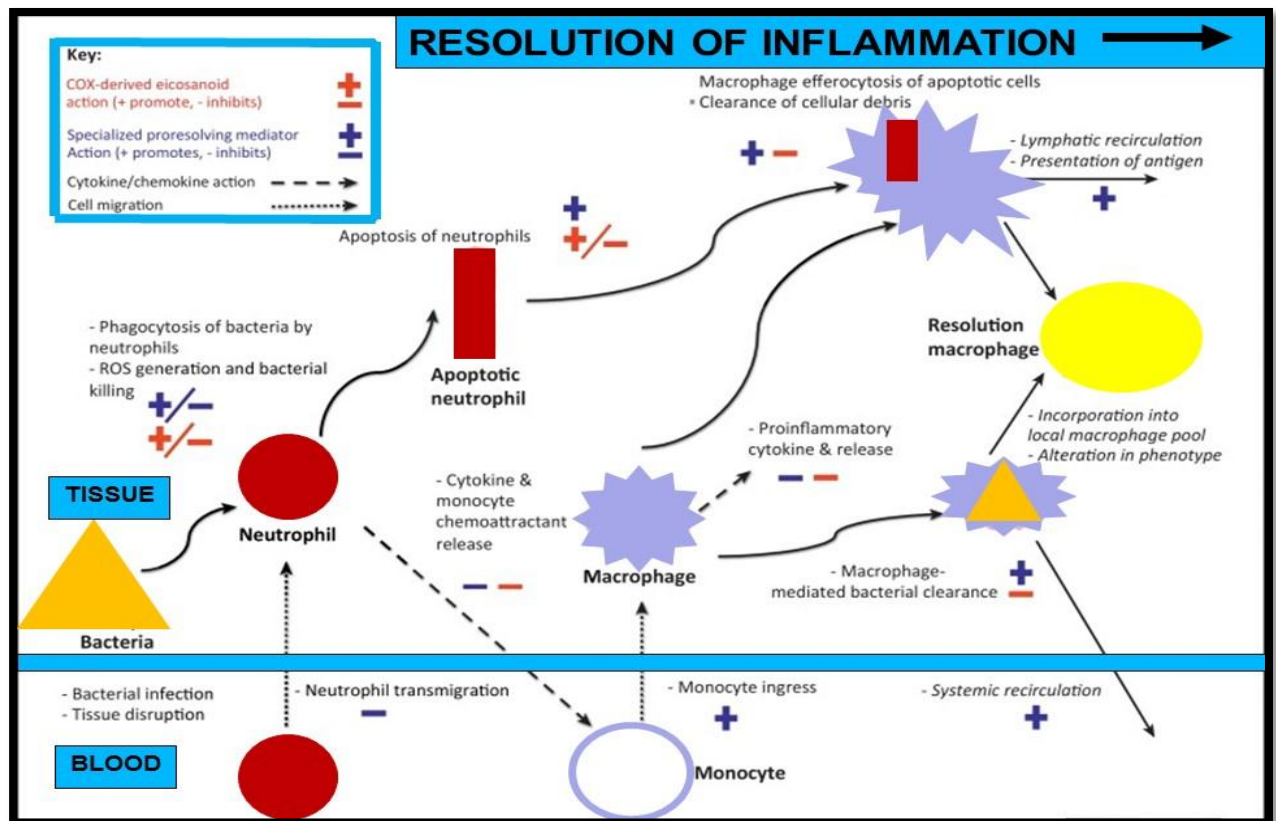


Figure 2-9: The immunomodulatory actions of specialised pro-resolving mediators on inflammatory resolution processes

Image adapted from Fullerton *et al.* (2014)

Cox: Cyclooxygenase; ROS: Reactive oxygen species

Inflammatory LM derive mainly from n-6 PUFAs, whilst anti-inflammatory LM is from n-3 PUFAs. As seen in Figure 2-10, LM are further divided into families of prostaglandins (PGs), leukotrienes (LTs) and lipoxins (LXs) (n-6 series), as well as protectins (PD), resolvins (Rvs) and maresins (MaR) (n-3 series). By influencing several aspects of immune and inflammatory reactions, LM rise as key regulators in the crosstalk amongst innate and adaptive immunity (Fullerton *et al.*, 2014; Serhan & Petasis, 2011).

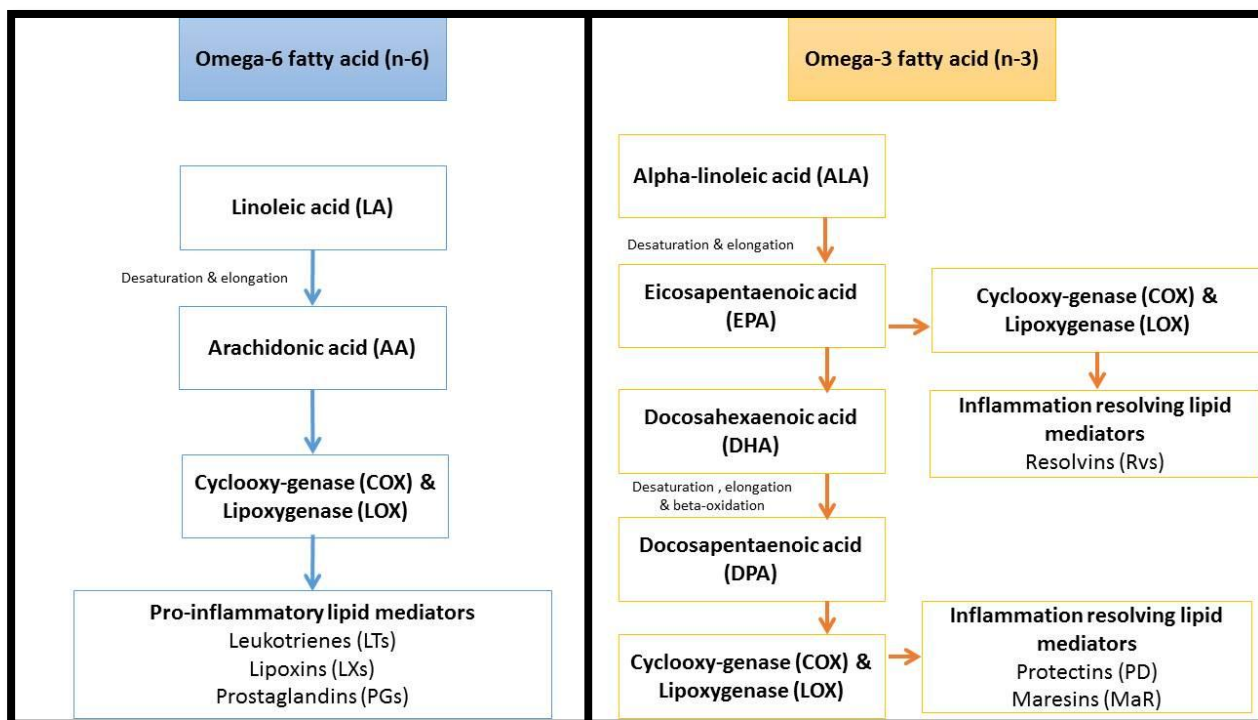


Figure 2-10: A summary of the metabolic pathways of selected n-6 and n-3 polyunsaturated fatty acids to their respective LMs used in this Mini-dissertation

Image adapted from Hidaka *et al.* (2015)

Alpha-linolenic acid (ALA, n-3) and linoleic acid (LA, n-6) PUFAs are referred to as essential fatty acids because humans and other animals cannot produce them (Mahan *et al.*, 2012). However, humans and animals are able to desaturate (the addition of a double bond) and elongate (the addition of a 2-carbon unit) LA to arachidonic acid (AA) (n-6 series) and ALA to long-chain polyunsaturated fatty acids (LCPUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (n-3 series) (Figure 2-10) (Hidaka *et al.*, 2015). Alpha-linolenic acid, from the n-3 PUFA series (the best sources of ALA is of plant/nuts/seed origin), and LA, from the n-6 series (soy, corn, nuts, seeds, and vegetable oils), are the two most commonly consumed essential fatty acids in the diet (Calder, 2014). However, very low n-3 PUFA status in proportion to n-6 PUFA status is observed in low-income countries, as well as in the Western-type diets of developed countries, with the ratio being 15 to 25:1 (n-6:n-3) (Simopoulos, 2011). It is further suggested that more individuals present with higher n-6 PUFA content in their cell membranes compared to n-3 PUFA content (Scafoli *et al.*, 2017). Increased intake of n-6 PUFA will result in higher AA content in the phospholipid membrane, whereas higher intake of n-3 PUFA will lead to increased EPA and DHA content.

N-3 PUFA status can generally be expressed as a proportion to other fatty acids, for example, n-6:n-3 ratio. One should keep in mind that n-3 PUFA status may differ from individual to individual, one reason being that sources of dietary fatty fish differ with regard to n-3 PUFA content (even those from the same species), according to the area, season, food intake, type of water, stage in life cycle, and whether the fish was wild or cultivated (Calder, 2014). Furthermore, the human body has different PUFA content in different lipid pools. For this reason, EPA and DHA content in cell membranes differ (Calder, 2014). When a person consumes n-3 PUFA, DHA is reported to be readily incorporated into the cell membranes of the central nervous system and other organs (i.e. the eyes, testes, and breast milk) (Hooper *et al.*, 2017; Iizuka-Hishikawa *et al.*, 2017; Lauritzen *et al.*, 2016). Likewise, n-3 PUFA consumption will increase n-3 PUFA content in the different lipid pools, blood cells (e.g. RBCs) and tissues (depending on dosage and time of consumption). In this way, increased PUFA consumption will increase PUFA content in the different pools. However, when a person decreases his/her n-3 PUFA intake to a normal amount, EPA and DHA status will decrease and return to baseline levels (Yaqoob *et al.*, 2000).

In the human population, the correct amount of n-3 PUFA intake per day can become a little complicated, as no Recommended Dietary Allowances (RDA) have been established for n-3 PUFAs, and no biochemical marker or specific dietary intake are available to indicate the deficiency of this nutrient (Innis & Friesen, 2008). However, global recommendations for EPA and DHA intake exist. According to the WHO, n-3 PUFA consumption in the general adult population should be 1-2% of total energy intake. A number of countries (including Australia, Europe, France, Austria, Germany, Switzerland, Belgium, United Kingdom etc.) have recommended EPA and DHA intake for their own countries (Flock *et al.*, 2013; Harris *et al.*, 2009). National authorities from these (and other) countries suggest a daily consumption range of about 250 – 500 milligram (mg) EPA and DHA, or a minimum of two servings of fatty fish (for example mackerel, salmon, trout, herring, tuna and sardines) per week for a general healthy adult population (Flock *et al.*, 2013; Kuratko *et al.*, 2014; Smuts & Wolmarans, 2013).

In order to assess n-3 PUFA status one can make use of blood markers. One such valuable blood marker is the n-3 index, the interpretation of which could be the sum of EPA and DHA content in RBCs (Luxwolda *et al.*, 2011). The n-3 PUFA index as a blood marker for PUFA status is very scarce, nonetheless, plasma and RBC membrane phospholipid fatty acid compositions are n-3 PUFA status markers (Fekete *et al.*, 2009; Hooper *et al.*, 2017). The current animal experimental study chose to report fatty acid status in RBC membrane phospholipids. According to Hooper *et al.* (2017), the determination of RBC fatty acid

composition to assess fatty acid status is a long-term marker of fatty acid intake, considering, considering that fatty acids are stable in the membranes of RBCs for up to three months (Hooper *et al.*, 2017). Red blood cell fatty acid concentrations can also reflect concentrations present in tissues (Harris *et al.*, 2004).

2.2.2 N-3 polyunsaturated fatty acid status and chronic inflammation

It is evident that n-3 PUFAs are unique, both structurally and from a dietary point of view. n-3 LCPUFAs that are produced when ALA is consumed have various functions that play an important role during chronic inflammation (Bonilla *et al.*, 2010a; Calder, 2015; Mahan *et al.*, 2012; Serhan & Petasis, 2011; Serhan, 2017), as EPA and DHA can cause pro-resolving LM to be produced (Hidaka *et al.*, 2015). Reportedly, n-3 PUFAs are incorporated into the lipid bilayer, thereby leading to an increased production of anti-inflammatory and pro-resolving LM. In return, these fatty acids also replace AA (the principal n-6 fatty acid precursor for the production of pro-inflammatory LM) in the lipid membrane, which leads to a reduction in n-6 PUFA as a substrate for producing inflammatory mediators (Calder, 2015; Serhan & Petasis, 2011). It seems the major anti-inflammatory effects of EPA and DHA might take effect by the arrangement of Rvs, PD, and MaR, which actively regulate and activate the inflammation resolving process, as seen in Figure 2-11(Calder, 2013; Serhan, 2017; Serhan & Petasis, 2011).

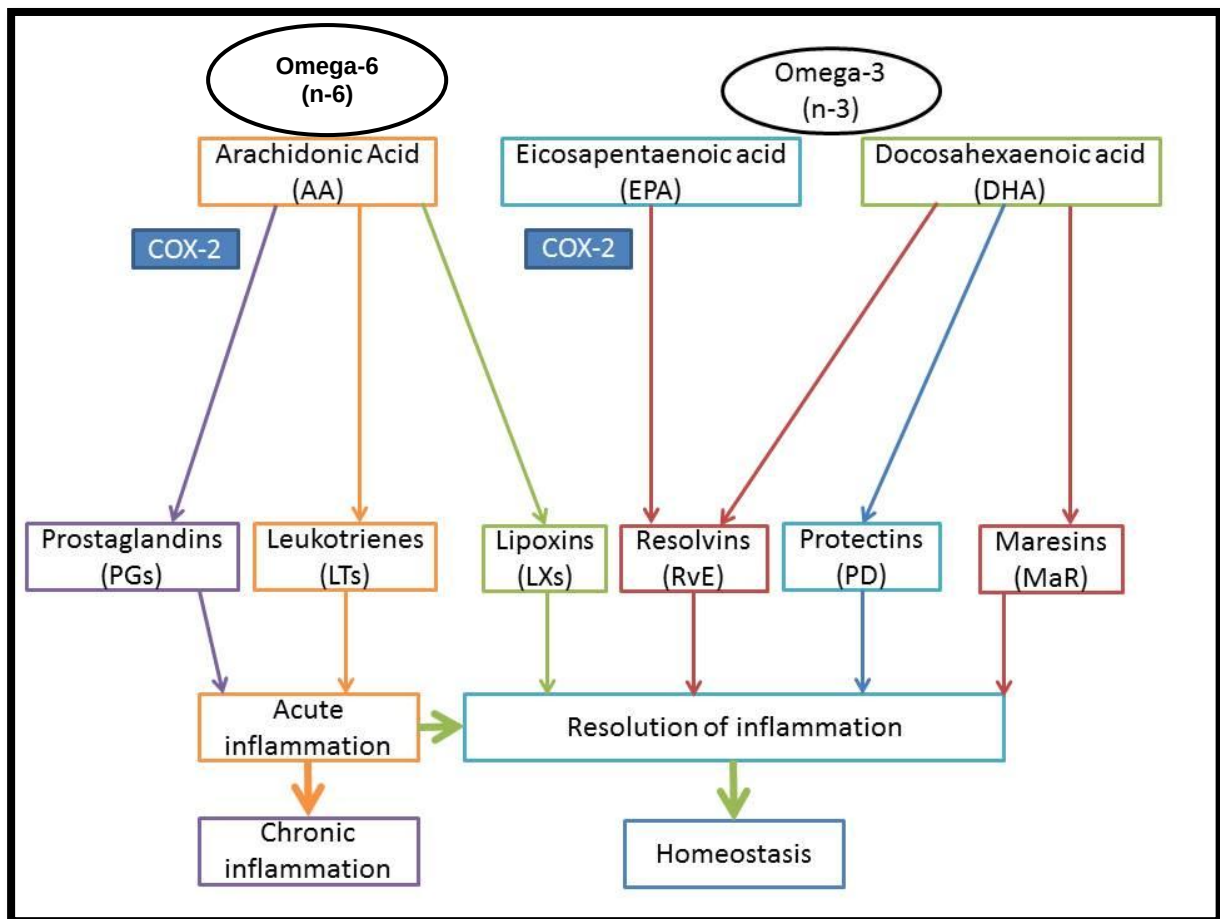


Figure 2-11: Biosynthetic cascades and actions of selected lipid mediators derived from arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid

Image adapted from Serhan & Petasis (2011)

During inflammation, the synthesis of n-6 and/or n-3 PUFA derived LM increases with time (Serhan & Petasis, 2011). Early SPLMs (PG and LT) are synthesised within seconds and minutes. These mediators control oedema and post-capillary events at the site of infection subsequent to the recruitment of neutrophils (Figure 2-12) (Serhan & Petasis, 2011). As time passes (may be hours or days), leucocyte recruitment, monocytes and macrophages prompt a growing amount of mediators. During initial infection, the body mainly produces pro-inflammatory mediators from n-6 AA (LTs and PGs), and afterwards starts to produce anti-inflammatory mediators from n-3 EPA and DHA (LXs, Rvs, PD, and MaR) for the resolution of inflammation. The release of, the mobilisation of EPA and DHA from the circulation, as well as the synthesis of SPLMs from these substrates, also follows a time course that supports initial inflammatory response as part of the host defence, followed by the resolution thereof and the return to homeostasis (Serhan, 2017; Serhan & Petasis, 2011). Therefore, the resolution of

inflammation could be described as an active process driven by the balance between pro- and anti-inflammatory lipid-derived mediators to maintain homeostasis (Gilroy & De Maeyer, 2015). Interestingly, the SPLM Rv (n-3 series) is reported to not only decrease pro-inflammatory cytokine production as part of the inflammation resolving process, but also enhance bacterial clearance, promote the resolution of infective lung injury, and improve pathological changes and survival in acute lung injury (El Kebir *et al.*, 2012; Seki *et al.*, 2010; Wang *et al.*, 2011).

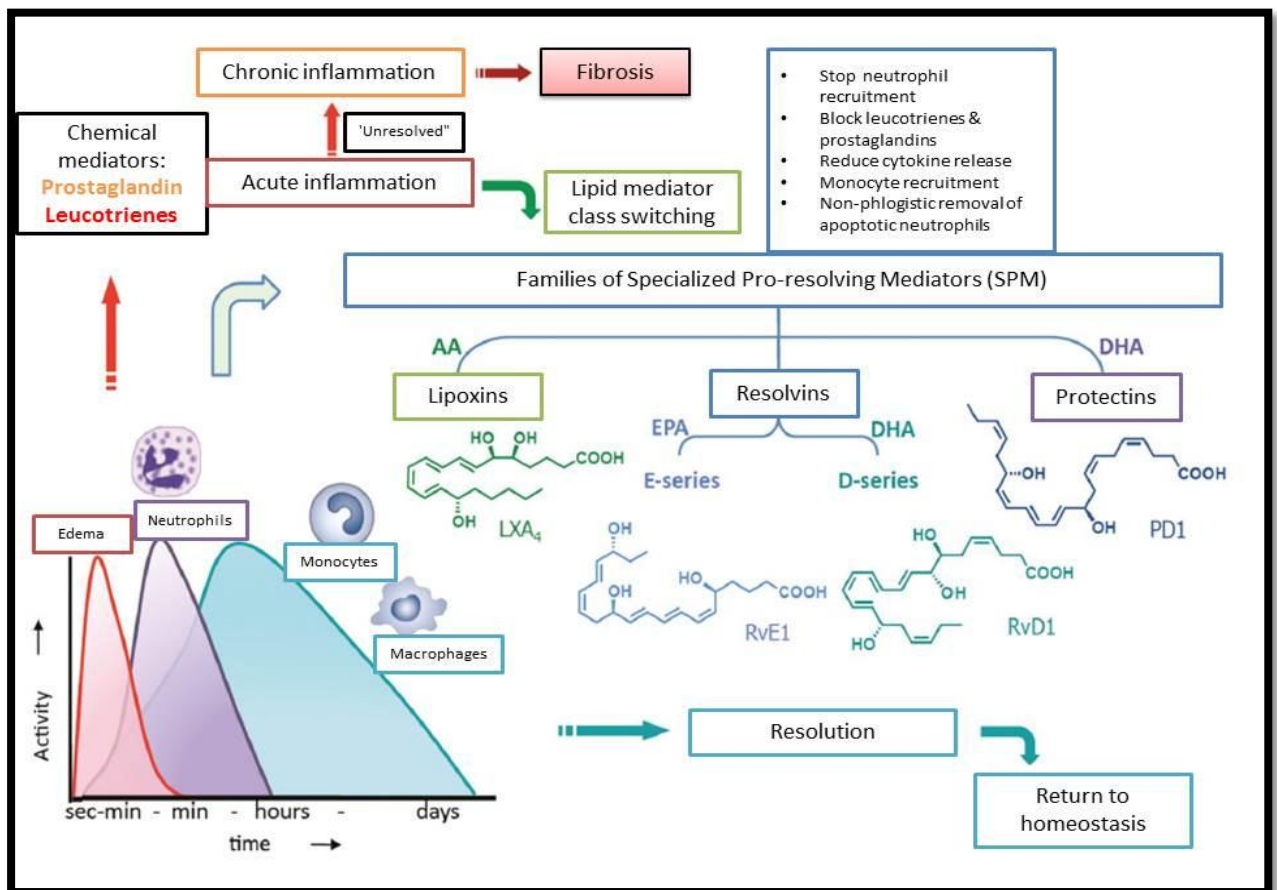


Figure 2-12: Inflammatory response and resolution time course: roles of pro-resolving lipid mediators

Image adapted from Serhan & Petasis (2011)

AA: Arachidonic Acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid

From the above information, it can be hypothesized that the maintenance of an anti-inflammatory and pro-resolving fatty acid profile could be beneficial and this is where n-3 PUFA status may fit. Sufficient n-3 PUFA intake and availability may not only be beneficial in reducing inflammation, but also in protecting the host against TB infection, and the restoration of immune competence (Fullerton *et al.*, 2014; Serhan & Petasis, 2011). It is important to remember that an

increased intake of n-3 PUFAs should be used along with therapy, and not as a replacement for drug therapies (Calder *et al.*, 2009; Serhan, 2017).

2.2.3 The role of n-3 polyunsaturated fatty acids in the clinical setting

The effects of n-3 PUFA have been investigated in various conditions, and include, but are not limited to cancer, Intensive Care Unit (ICU) and surgical patients, neurodegenerative diseases, rheumatoid arthritis, inflammatory bowel disease, asthma, type 2 diabetes, obesity, metabolic syndrome, acute respiratory distress syndrome, childhood allergic disease, and cardiovascular disease (Berquin *et al.*, 2009; Calder, 2015; Ma *et al.*, 2015; Mayer & Seeger, 2008; Murphy *et al.*, 2012; Pradelli *et al.*, 2012; Serhan, 2017). The researchers of these studies concluded that n-3 PUFA intake could positively affect clinical outcomes, including the reduction in cardiovascular disease risk, as well as increasing body weight in cancer patients. N-3 PUFA has also been described as having a therapeutic role in inflammatory diseases, in the sense that these fatty acids can successfully reduce markers of inflammation and positively affect clinical outcomes, such as morbidity, length of hospital and ICU stay (Berquin *et al.*, 2009; Calder, 2014; Ma *et al.*, 2015; Mayer & Seeger, 2008; Murphy *et al.*, 2012; Pradelli *et al.*, 2012).

2.2.4 Anti-inflammatory medication in tuberculosis treatment

Researchers examined the role of anti-inflammatory medication in the treatment of TB. Study results proved these medications (referred to as non-steroidal anti-inflammatory drugs (NSAIDs)) to be beneficial with regard to the size and number of lung lesions, inflammatory response, bacillary load and survival (Kroesen *et al.*, 2017; Marzo *et al.*, 2014; Vilaplana *et al.*, 2013). Table 2-5 shows a list of NSAIDs that have proven to be beneficial during TB treatment.

Table 2-3: Non-steroidal anti-inflammatory drugs acting as anti-tubercular non-antibiotics (including *in vitro* studies).*

Drug	Study
Ibuprofen	Antibacterial activity of the anti-inflammatory compound ibuprofen (Elvers <i>et al.</i> , 1995).
Ibuprofen and Carprofen	Antitubercular specific activity of ibuprofen and the other 2-arylpropanoic acids using the HT-SPOTi whole-cell phenotypic assay (Guzman <i>et al.</i> , 2013).
Aspirin	Salicylate reduces susceptibility of <i>Mycobacterium tuberculosis</i> to multiple antituberculosis drugs (Schaller <i>et al.</i> , 2002).

Continuation of Table 2-3: Non-steroidal anti-inflammatory drugs acting as anti-tubercular non-antibiotics (including *in vitro* studies).*

Drug	Study
Diclofenac	Potential management of resistant microbial infections with a novel non-antibiotic: the anti-inflammatory drug diclofenac sodium (Dutta <i>et al.</i> , 2007).
	<i>In vitro</i> and <i>in vivo</i> antimycobacterial activity of antiinflammatory drug, diclofenac sodium (Dutta <i>et al.</i> , 2004).
	Synthesis, <i>in vitro</i> and <i>in vivo</i> antimycobacterial activities of diclofenac acid hydrazones and amides (Sriram <i>et al.</i> , 2006).
Celecoxib	Inhibition of bacterial multidrug resistance by celecoxib, a cyclooxygenase-2 inhibitor (Kalle <i>et al.</i> , 2011).
	Design and synthesis of novel anti-tuberculosis agents from the celecoxib pharmacophore (Salunke <i>et al.</i> , 2015).
Oxyphenbutazole	Non-steroidal anti-inflammatory drug sensitizes <i>Mycobacterium tuberculosis</i> to endogenous and exogenous antimicrobials (Gold <i>et al.</i> , 2012).
NSAIDs bound to metal-complexes	Anti-inflammatory drugs interacting with Zn (II) metal ion based on thiocyanate and azide ligands: synthesis, spectroscopic studies, DFT calculations and antibacterial assays (Chiniforoshan <i>et al.</i> , 2014).
	Organotin meclofenamic complexes: synthesis, crystal structures and antiproliferative activity of the first complexes of meclofenamic acid – novel anti-tuberculosis agents (Kovala-Demertzi <i>et al.</i> , 2009).

*Kroesen *et al.* (2017)

According to Kroesen *et al.* (2017), NSAIDs block COX enzymes. By inhibiting these enzymes, NSAIDs inhibit all different series of PG (pro-inflammatory lipid mediators including i) Prostaglandin E (PGE)-1 produced from dihomo- γ -linolenic acid (DGLA); ii) PGE-2 produced from AA; iii) and PGE-3 produced from EPA) synthesis and promote a switch to EPA as well as DGLA for oxidation, ultimately leading to a reduction in inflammation, as illustrated in Figure 2-13.

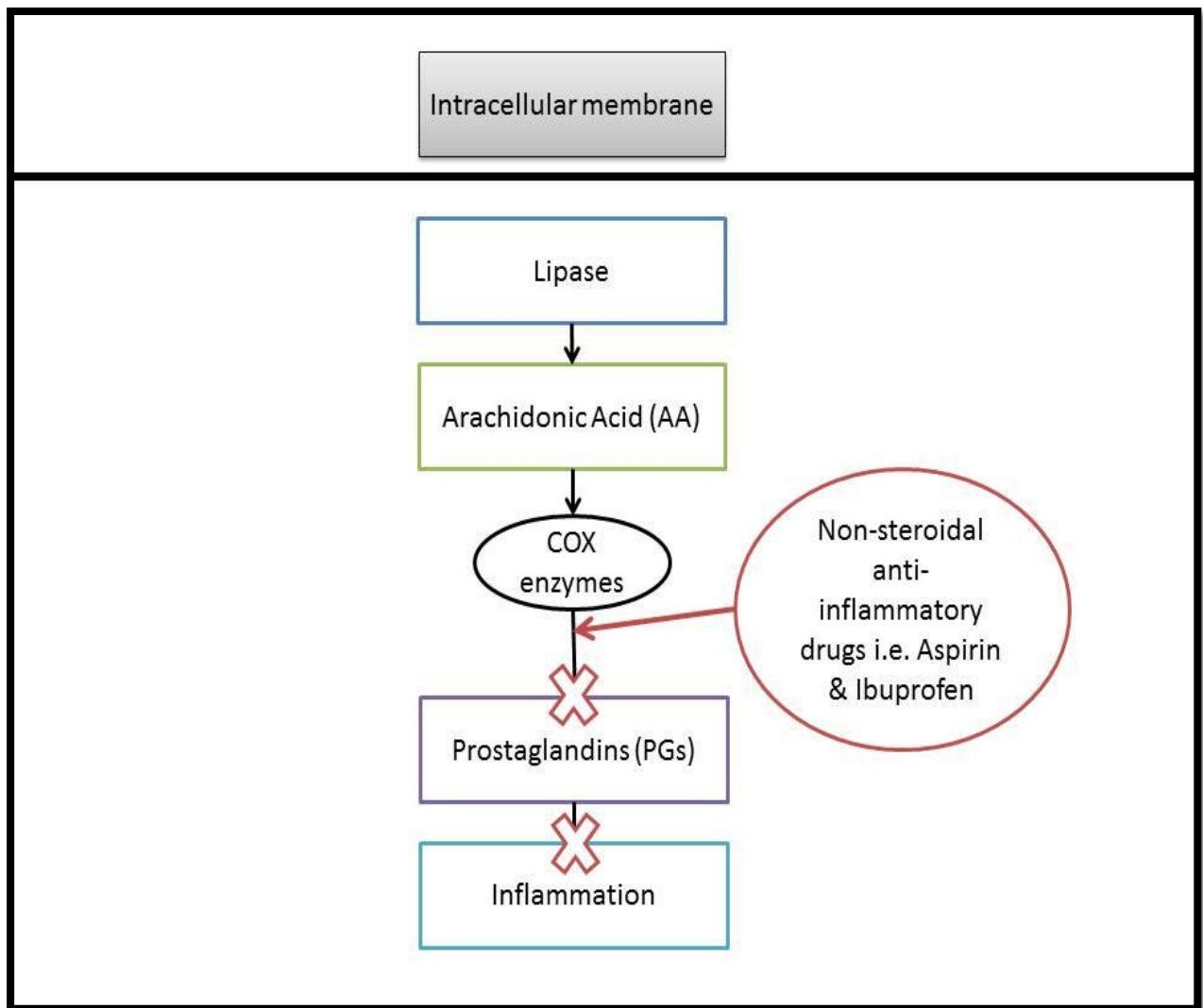


Figure 2-13: Non-steroidal anti-inflammatory drugs inhibit cyclooxygenase enzymes and cause a reduction in inflammation

Image adapted from Malan (2014)

2.2.5 The role of n-3 polyunsaturated fatty acids in tuberculosis

With the positive findings regarding anti-inflammatory medication in TB treatment, the anti-inflammatory effects of n-3 PUFA may also have a promising effect on the inflammatory response to TB and thereby improve clinical outcomes, without the well-known unwanted side effects of anti-inflammatory medication. Altering this status could be a non-pharmaceutical approach in which sufficient n-3 PUFA status may benefit anti-inflammatory pathways during TB infection. Studies to support this statement are however scarce; only a few animal studies have investigated the effect of n-3 PUFA supplementation on TB (Bazinet *et al.*, 2004; Bonilla *et al.*,

2010a; Bonilla *et al.*, 2010b; Jordao *et al.*, 2008; McFarland *et al.*, 2008; Paul *et al.*, 1997). Two of these studies concluded that besides their health benefits, n-3 PUFA enriched diets may be beneficial in response to TB with regard to resistance, disease progression and bacillary load (Jordao *et al.* 2008; Paul *et al.*, 1997). Jordao *et al.* (2008) reported that n-3 PUFA enriched diets (EPA content 1.5% and DHA 1.1%) led to a reduction in mycobacterial loads in the lungs and the spleen of TB infected mice. Paul *et al.* (1997) investigated the influence of n-3 PUFAs and n-6 PUFAs on animal experimental TB models (guinea pigs), and concluded that supplementing animals with EPA and DHA (a 26 cal % fat diet containing 1.4 cal% EPA and 0.9 cal% DHA) prior to TB infection positively affected resistance to TB due to its anti-inflammatory properties.

In contrast to these positive findings, Bonilla *et al.* (2010a) and Bonilla *et al.* (2010b) reported that the supplementation of n-3 PUFAs could have immunosuppressive effects, resulting in an increased susceptibility and reduced ability of the host to control the infection. Furthermore, McFarland *et al.* (2008) reported that n-3 PUFA-fed guinea pigs (9.55g/100g body weight EPA and 10.38g/100g body weight DHA) presented with decreased resistance to TB infection compared to those fed n-6 PUFA. A reason for these negative findings may be due to the fact that the researchers supplemented n-3 PUFA (or provided an n-3 PUFA enriched diet) prior to TB infection, whilst focusing only on the immune suppressive effects of the n-3 PUFA, rather than investigating the effects of n-3 PUFA status on TB progression. Nevertheless, the current animal experimental study is the first to investigate the effects of n-3 PUFA status on TB outcomes.

2.2.5.1 The amount of n-3 polyunsaturated fatty acid availability may influence host defense against tuberculosis

Previous evidence indicates that an n-3 PUFA deficient individual can markedly increase their EPA and DHA intake through dietary change or the intake of good quality supplements (Calder, 2014). The level of action that n-3 PUFAs may have on immune function could be influenced by the intake and availability thereof, as this will affect the immune cell membrane composition of PUFA, thereby influencing inflammatory and immune outcomes (Fritsche, 2006; Malan *et al.*, 2014). However, with a decreased intake of n-3 PUFAs, the immune cell status of DHA and EPA will diminish, as described in Section 2.2.1. During a human experimental study, individuals received n-3 PUFAs (2.1g EPA and 1.1g DHA/day) for a period of 12 weeks (Yaqoob *et al.*, 2000). Eicosapentaenoic acid and DHA reached a peak in mononuclear cells after four weeks of treatment. Researchers stopped giving supplementations to participants

after 12 weeks of treatment, and blood samples were taken eight weeks post-treatment. As a result, the amount of EPA and DHA present in mononuclear cells had returned to its original amount (Yaqoob *et al.*, 2000).

In the clinical setting, contrasting results have been observed when supplementing n-3 PUFAs along with therapy. Some researchers suggest that too high amounts of n-3 PUFA intake and availability may impair host immune response, whilst moderate amounts may provide sufficient immune protection (Calder, 2007; Fullerton *et al.*, 2014; Pradelli *et al.*, 2012). With this in mind, two studies found that high amounts of n-3 treatment caused suppression of lymphocytic response to mitogen stimulation, increased circulating concentrations of lipoperoxides, natural killer (NK) cell activity and delayed-type hypersensitivity reactions (>1g EPA and DHA/day) (Field *et al.*, 2002; Robinson *et al.*, 2001), while moderate amounts of n-3 PUFA treatment (<1g EPA and DHA/day) seemed to enhance immune functions (Calder, 2007; Fullerton *et al.*, 2014). However, due to contradicting results from a meta-analysis that reported the efficacy of n-3 PUFA enrichment in surgical and ICU patients (>1g EPA and DHA/day) (Pradelli *et al.*, 2012), more research may be needed to investigate the correct amount of n-3 PUFA needed for supplementation in the clinical setting.

2.2.6 The combined effects of n-3 polyunsaturated fatty acids and iron on the immune response against tuberculosis

It is clear from the above literature that both n-3 PUFA and Fe effect TB. As these two nutrients could play a significant independent role during the immune response against TB, additional benefits may come from a combination of n-3 PUFA and Fe treatment. As already pointed out, n-3 PUFAs are precursors of anti-inflammatory and pro-resolving immune modulators, while Fe plays a vital role in host immune response and has a significant impact on the course of the infectious disease (Cherayil, 2011; Jonker & Van Hensbroek, 2014). Some mechanistic interactions between Fe and n-3 PUFAs might involve oxidative stress and cellular response (Malan *et al.*, 2014). It is also suggested that the availability of each nutrient may influence the other's mechanistic performance, as described in Section 2.2.6.1 and Section 2.2.6.2. Furthermore, a deficiency of a combination of these nutrients might have additive effects on immune function. Interestingly, some researchers reported that the intake of n-3 PUFA during inflammatory diseases could cause a decrease in the pro-inflammatory cytokine IL-6 (Coghill *et al.*, 2018; Li *et al.*, 2014). An n-3 x Fe interaction during inflammation (for example, inflammation caused by TB infection) is accomplished, because IL-6 can up-regulate the expression of hepcidin (regardless of Fe status), leading to a decreased Fe bioavailability during chronic inflammation (Section 2.1.6). N-3 PUFA, with its anti-inflammatory effects, thus causes a

decrease in inflammation, as well as IL-6 (a cytokine that potentiates early immunity against a high dose of TB infection).

Several studies have determined the independent effects of n-3 PUFAs and Fe intake and availability in the host. However, studies that have investigated the combined effects of these nutrients on immune response are scarce. A review of the available literature provided one animal and one human study, which investigated immune outcomes related to Fe and n-3 PUFA interactions (Malan *et al.*, 2016; Rorvik *et al.*, 2003). Rorvik *et al.* (2003) reported that a diet of high DHA and EPA amounts (5.9% EPA/DHA) combined with low Fe (45mg/kg¹) increased survival during a natural outbreak of cold-water vibriosis and of furunculosis in farmed Atlantic salmon. Malan *et al.* (2016) investigated whether n-3 PUFAs and Fe, alone and in combination (50mg Fe, and a mixture of 420mg DHA and 80mg EPA per day), reduced respiratory morbidity caused by Fe supplementation in Fe-deficient schoolchildren in South Africa. The authors concluded that the supplementation of n-3 PUFAs and Fe in combination exerted a safer profile regarding respiratory morbidity than when supplementing Fe alone.

2.2.6.1 Iron status affects n-3 polyunsaturated fatty acid status and metabolism

According to Smuts *et al.* (1995), n-3 PUFA status can be affected when an individual presents with Fe deficiency, because this causes an abnormal shift in cellular membrane fatty acid composition. With this in mind, researchers have investigated the effect of higher or lower Fe intake on fatty acid composition in animal and human studies (not TB-specific) (Table 2-4). Some of these studies reported that n-3 PUFA status were decreased in Fe deficient subjects (Cannane & McAdoo, 1987; Krajcovicova-Kudlackova *et al.*, 2004; Stangl & Kirchgessner, 1998; Zhou *et al.*, 2011), while other studies could not confirm this effect (LeBlanc *et al.*, 2009b; Serini *et al.*, 2017; Tichelaar *et al.*, 1997) (Table 2-4).

Interestingly, findings indicate Fe acts as a co-factor for enzymes that are involved in PUFA metabolism, as Fe forms part of the catalytic centre of the COX and LOX enzymes (Gilbert *et al.*, 2011; Kuhn *et al.*, 2005). Apart from each affecting the other, a deficiency in Fe may additionally alter lipid-associated immune response, and will likewise diminish the generation of LM (Gilbert *et al.*, 2011). Table 2-4 includes studies that show the effect of Fe on n-3 PUFA status.

Table 2-4: Studies showing the effect of iron on n-3 fatty acid status and metabolism

Author, year	Animal/human study	Effect of iron on n-3 fatty acid status
Cunnane & McAdoo, 1987	Animal study	A mild impairment in essential fatty acid metabolism in moderately iron-deficient rats.
Krajcovicova-Kudlackova <i>et al.</i> , 2004	Human study	Low serum iron inhibit essential fatty acid synthesis.
LeBlanc <i>et al.</i> , 2009b	Animal study	Moderate iron deficiency alters essential fatty acid and eicosanoid metabolism in adult guinea pig offspring.
Ocheo <i>et al.</i> , 2016	Human study	Iron deficiency is associated with an alteration in n-3 fatty acid content.
Serini <i>et al.</i> , 2017	Human study	Iron overload at the hepatic level prevents the endogenous synthesis of n-3 PUFA.
Stangl & Kirchgessner, 1998	Animal study	Moderate iron deficiency alters essential fatty acid composition in rats. Essential fatty acids were decreased in iron-deficient subjects.
Tichelaar <i>et al.</i> , 1997	Animal study	The mechanism whereby iron deficiency affects growth is related to abnormal fatty acid shifts that disturb the balance of essential fatty acids in membranes. DHA and DGLA were increased in iron-deficient rats. Iron deficiency was associated with reduced ALA and increased DPA and DHA.
Zhou <i>et al.</i> , 2011	Human study	Low serum iron inhibit essential fatty acid synthesis. There is a moderate positive, but highly significant association between ferritin and n-3 fatty acids.

ALA: alpha-linolenic acid; DHA: docosahexaenoic acid; DPA: Docosapentaenoic acid;

DGLA: dihomog-γ-linolenic acid; PUFA: polyunsaturated fatty acid

2.2.6.2 N-3 polyunsaturated fatty acid intake affects iron status

The effect of n-3 PUFA intake on Fe status (Table 2-5) has also been investigated. Some studies (again not TB-specific) demonstrated that diets high in PUFAs reduced Fe retention and led to the development of Fe deficiency (Lukaski *et al.*, 2001; Miret *et al.*, 2003; Rao & Larkin, 1984; Shotton & Droke, 2004; Van Dokkum *et al.*, 1983). Other studies suggested that a higher

n-3 PUFA status was positively associated with Fe status (Clauss *et al.*, 2008; Jamieson *et al.*, 2013; Mutanen *et al.*, 2016; Ochoa *et al.*, 2016). If this statement is true, then n-3 PUFA status may not only affect Fe status, but also limit non-resolving inflammation, contributing towards reduced Fe depletion, and causing the resolution of anaemia (and its resulting negative effects) after TB infection. Table 2-5 includes studies that show the effect of n-3 PUFA intake on Fe status.

Table 2-5: Studies demonstrating the effects of n-3 fatty acid intakes on iron status

Author, year	Animal/human study	Effect of n-3 fatty acid intake on iron status
Clauss <i>et al.</i> , 2008;	Animal study	Animals consuming diets high in ALA (n-3) were less likely to suffer from Fe-storage diseases and inflammatory diseases.
Jamieson <i>et al.</i> , 2013	Human study	A higher n-3 fatty acid status was associated with a lower risk of iron depletion
Lukaski <i>et al.</i> , 2001	Human study	Serum ferritin decreased during consumption of essential fatty acids. Higher PUFA intake caused a lower iron status.
Mutanen <i>et al.</i> , 2016	Human study	Essential fatty acid intake had a positive association with serum ferritin. Low iron status probably attenuates the conversion of LA to AA.
Miret <i>et al.</i> , 2003	Animal study	Reduced non-heme iron stores in rats that consumed fish oil. Increased lipid peroxidation caused higher iron absorption.
Ochoa <i>et al.</i> , 2016	Human study	DHA is reported to stimulate Fe metabolism.
Rodriguez <i>et al.</i> , 1996	Animal study	Increased lipid peroxidation caused higher iron absorption.
Shotton & Droke, 2004	Animal study	Diets high in polyunsaturated fatty acids decreased iron absorption and led to the development of iron deficiency in rats.
Van Dokkum <i>et al.</i> , 1983	Human study	Higher PUFA intake caused a lower iron status.

AA: Arachidonic acid; LA: Linolenic acid; PUFA: Polyunsaturated fatty acid

Tables 2-4 and 2-5 show that various studies have investigated the interactions between Fe and n-3 PUFAs. These studies provide evidence that Fe intake and availability influences n-3 PUFA status and metabolism, and vice versa. A few studies also provided evidence that a combination of these nutrients might have positive effects on immune function (Section 2.2.5). However, while it is not clear what role these nutrients' interactions may play in the immune response to TB, it is where our larger animal study fits in. It may be very useful to determine whether a combination of n-3 PUFA and Fe improves host Fe metabolism and reduces chronic inflammatory processes during active TB. For the current research project of this dissertation, we investigated the effect of n-3 PUFA status on AI in TB mice.

2.2.7 Other important nutrients for tuberculosis treatment

Apart from n-3 PUFAs, other nutrients have been studied for TB treatment. Vitamin D supplements, for example, have been used for decades in the treatment of TB, and in 1903, Niels Ryburg won a Nobel prize in Medicine for treating TB of the skin with light radiation (vitamin D production) (Grzybowski & Pietrzak, 2012). It is clear that researchers are particularly interested in this vitamin as purportedly, vitamin D deficiency is a strong risk factor for the development of active TB (Gao *et al.*, 2010). Furthermore, one cohort study that assessed vitamin A and D deficiency in the development of TB in HIV patients, found that vitamin A deficiency were also very strongly predictive of TB risk (Tenforde *et al.*, 2017). The authors reported that individuals who were vitamin A deficient were above ten times more likely to develop active TB. Furthermore, because micronutrient deficiency is considered to be the most frequent cause of secondary immunodeficiency and infection-related morbidity (i.e. TB), other important nutrients that can alter progression to active TB and may be of benefit includes zinc, vitamin E, vitamin C, copper and selenium (Gupta *et al.*, 2009). However, routine nutritional supplementation during active TB may not provide any clinical benefits. A recent systematic review on nutritional supplements for people that are treated for active TB concluded that supplementation may contribute to body weight gain, but is not proven to improve clinical outcomes during the course of TB infection (Grobler *et al.*, 2016). It is further suggested that routine supplementation higher than the RDA does not provide any clinical benefits, as no evidence exists to confirm this.

2.2.8 Assessing markers of morbidity and disease progression in tuberculosis infection

Reportedly, clinical outcomes, such as incidence and duration of illness, may serve as measurements for infectious morbidity (Birch *et al.*, 2010; Minns *et al.*, 2010). No single immune function parameter has been found to draw conclusions regarding the modulation of the

immune system as a whole. Therefore, the best measurement to use is clinical outcomes of the infection itself (Albers *et al.*, 2005). Clinical endpoints (for example morbidity and mortality) as a result of infections like TB, provide the best indication of the host's capacity to develop coping mechanisms against the pathogen and in this manner reflect the general balance between pathogen exposure and the incorporated host defense. Furthermore, in order to give useful insight into mechanisms of immune function in relation to clinical outcomes, gene expression of oxidative stress-associated genes and inflammatory genes, as well as immune function markers that are directly impacted by Fe and n-3 PUFA-derived immune modulators (DHA and EPA), can be used (Albers *et al.*, 2005; Calder, 2012). Markers of morbidity and markers of disease progression measured in TB infected subjects include body weight (animals and humans) (Berquin *et al.*, 2009; Bouté *et al.*, 2017; Guler *et al.*, 2015), appearance and respiratory rate (animals and humans) (Fairall *et al.*, 2005; Papadopoulos *et al.*, 2006), bacillary loads (animals) (Marzo *et al.*, 2014), lung cytokines (animals and humans) (Altare *et al.*, 2001; Jordao *et al.*, 2008), and organ indexes (animals) (Gupta *et al.*, 2008). All of these markers will contribute to the understanding of underlying mechanisms of immune function in relation to clinical outcomes.

2.3 Animal models as human substitutes

2.3.1 Animal models used in the study of tuberculosis

Due to limitations in the investigation of mechanisms in humans, animal models are used as human substitutes to study the immune response against TB (Zhan *et al.*, 2017). Although the spectra of human and animal TB are not equal, there are similarities between human and animal lesions (Singh & Gupta, 2018). Furthermore, in order to understand the outcomes necessary for pathogen transmission and evolutionary success in terms of MTB, experimental animal models can be very useful (Kramnik & Beamer, 2016). Models of animals previously used in the study of TB include mice, guinea pigs, rabbits, non-human primates, bovine calves, zebrafish, rats, ferrets, mini pigs, fruit flies, nematodes, planarians, and even amoebas (Kramnik & Beamer, 2016). Figure 2-14 provides a visual representation of some of the animal models, and how they translate the individual stages of the immunological life cycle during TB infection.

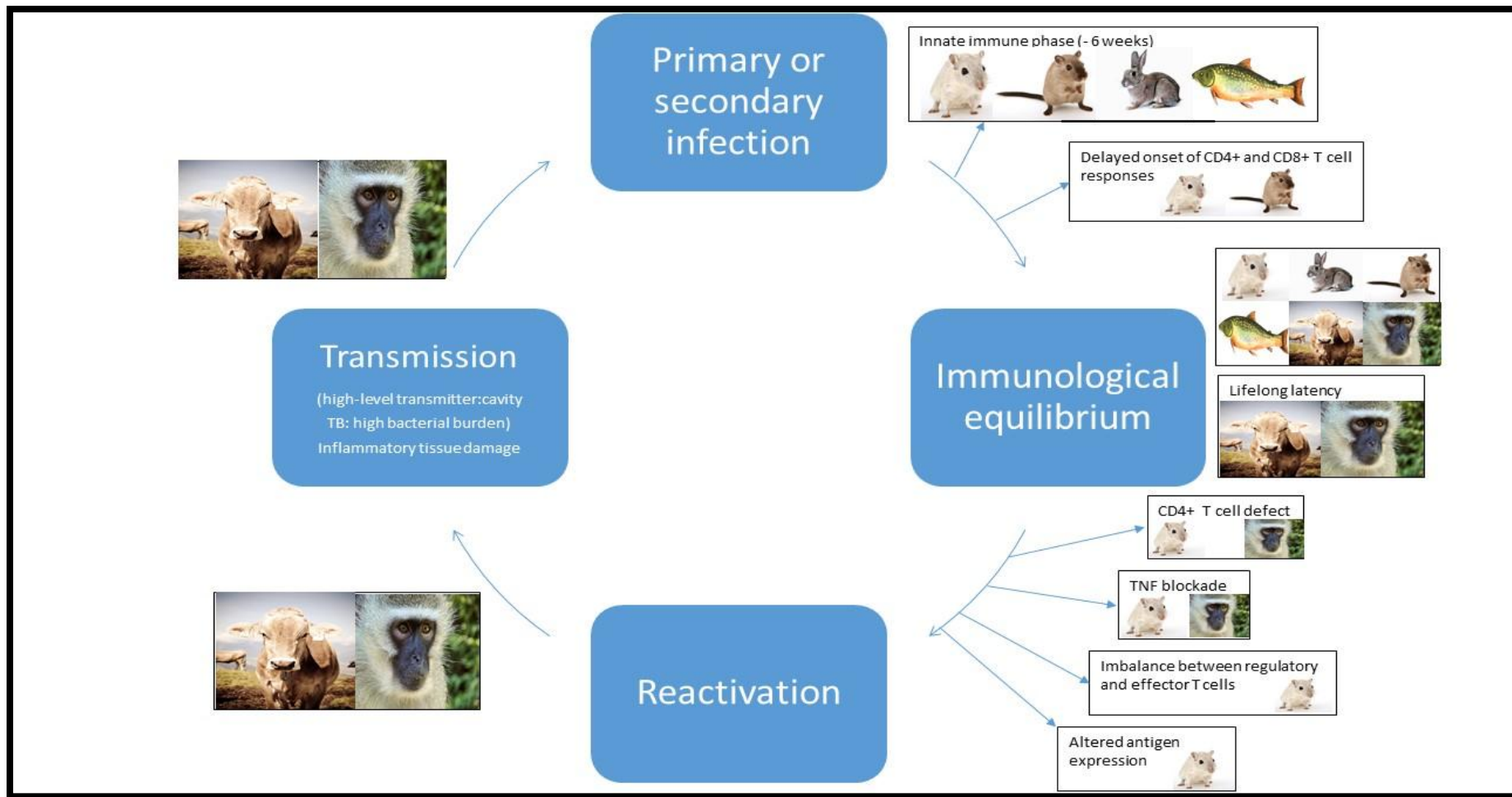


Figure 2-14: Animal models and how they translate the individual stages of the immunological life cycle during tuberculosis infection

Image adapted from Ernst. (2012)

CD: cluster of differentiation; TB: tuberculosis; TNF: tumor necrosis factor.

The perfect animal model is one that conforms to scientific and ethical criteria (Ferdowsian & Beck, 2011). However, when working with animal TB models, it is important to keep in mind that not all the results can be extrapolated to human models successfully (Evans *et al.*, 2010; Fuchs *et al.*, 2009). From the viewpoint of ethics, experimental animals with lung infections caused by bacteria (i.e. TB) are the most vigorous controversial models to be used (Ferdowsian & Beck, 2011). Furthermore, several pathogens cause the death of non-human mammals, by means of lung infections. Thus, to reproduce human infection and lung damage effectively, animal models need to be optimised. It is important to note that anatomical, physiological, genetic, and molecular similarities and differences exist in different species (Apt & Kramnik, 2009). With this in mind, the advantages and disadvantages of different animal models used in TB research should be considered, to ensure the selection of the most suitable animal. Table 2-6 provides a brief summary of the advantages and disadvantages of different animal TB models. When accounting for this, the mouse model provides the best combination regarding practical aspects and similarities to humans. It should be kept in mind however that, i) there are still several differences between mouse models and humans that should be considered, and ii) it is important to choose the mouse strain that is the most relevant to the disease model and outcomes to be assessed.

Table 2-6: Summary of the advantages and disadvantages of different animal tuberculosis models*

Animal model	Advantages	Disadvantages
Mice	• Similar immune response to TB than humans. • <i>Mycobacterium tuberculosis</i> pathogen primarily targets the lungs. • Any aspect of TB pathogenesis in mice can be explored with genetic resources. • Experimental methods available. • Less space required. • Inexpensive. • Responds to TB medication. • Extensive gene knowledge available. • Most sophisticated animal model. • Genetic variant strains. • Reagents available.	• Pathology not very similar to humans. • More resistant to TB infection. • Difficult to perform procedures on due to small size. • Different immunological features than in humans. • Lack of necrosis. • Lack of cell structure. • Lack of true latency. • Some mouse strains may be more susceptible to TB infections than other strains.

Continuation of Table 2-6: Summary of the advantages and disadvantages of different animal tuberculosis models*

Animal model	Advantages	Disadvantages
Guinea pig	• Commercially available inbred strains. • Immunology closest to human. • Pathology closest to humans. • Highly susceptible to TB infection. • Cellular response to aerosol infection is most similar to human response. • Responds to TB medication. • Easy to handle. • Necrosis.	• Expensive. • Large space required. • Limited availability of immunological tools and reagents. • Lack of true latency.
Non-human primates	• Pathology similar to humans. • Immunology similar to humans. • Easy to perform procedures on. • Very susceptible to TB infection. • Responds to TB medication. • Outstanding model to screen new vaccines, diagnostic reagents, and drug treatments for pre-clinical studies.	• Expensive. • The possibility of interspecies cross-contamination. • Large space required. • Difficult to handle. • Dedicated veterinarian staff required. • Ethical concerns.
Minipig	• A pulmonary structure similar to humans. • Lesions similar to humans. • A strong local granulomatous response based on the induction of a fibrotic process, where lesions are encapsulated, and intragranulomatous necrosis and calcification are present.	• Difficult to handle. • Dedicated veterinarian staff required. • Expensive. • Limited availability of immunological tools.
Zebrafish embryo	• Easy to handle. • Excellent to study early steps of granuloma formation.	• Lack of lung structure. • Lack of lymphocytes.

Continuation of Table 2-6: Summary of the advantages and disadvantages of different animal tuberculosis models*

Rabbits	- Cellular response to aerosol infection is similar to humans. - Highly susceptible to TB infection. - Easy to perform experimental procedures on. - Granulomatous lesions similar to that seen in the progressive human disease.	- Non-existing inbred strains. - Large space required. - Limited availability of immunological tools and reagents.
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*Apt & Kramnik, 2009; Flynn, *et al.*, 2003; Guirado & Schlesinger, 2013; Gupta & Katoch, 2005; Karlson & Feldman, 1949; Kaufmann, 2003; Kramnik & Beamer, 2016; Lenaerts *et al.*, 2007; Mustafa *et al.*, 1999; Orme, 2003; Phyu *et al.*, 1998; Rivera & Ganz, 2009; Tsai *et al.*, 2006.

TB: Tuberculosis

2.3.2 Comparing mice to humans

The respiratory system in the body is composed of a tree-like system of branch tubes, which is necessary for air exchange from and to the alveoli (Roth *et al.*, 2017). This process is similar in both mice and humans, but there are important differences between the two models that require consideration in the investigation of TB. Table 2-7 provides some of the differences and similarities that need taking into account.

Table 2-7: Differences and similarities between mouse models and humans*

Differences	Similarities
<i>Mycobacterium tuberculosis</i> infection in humans is a product of long co-evolution, while mice are not.	Similar to the main features of the innate and adaptive immune responses to MTB (the protective role of CD4+ T-cells, IFN- γ , and TNF).
Mice are not naturally infected with MTB.	
Mice do not transmit the bacteria via aerosols.	The lungs are the primary target site.
Some mouse strains are more resistant to TB infection than humans.	
Humans can stay infection-free even in high exposure settings (ranging between 20 and 70 % in different studies).	Both respond to TB medication.
Human infection can result in multiple outcomes, ranging from spontaneous eradication, latent non-symptomatic, mild chronic to rapidly progressing primary or reactivation diseases.	

Continuation of Table 2-7: Differences and similarities between mouse models and humans*

Differences	Similarities
The mouse genome is shorter than that of humans.	<i>Mycobacterium tuberculosis</i> strains of human and mouse models can be manipulated with eg. cytokines or drugs.
The cellular composition and organization of the mouse intrapulmonary airway epithelium resemble only most distal portions of the human conducting airways.	
Mucin-secreting goblet cells are in abundance in human lungs, but rare in mice when they are under laboratory conditions.	

*Apt. & Kramnik, 2009; Kramnik & Beamer, 2016; Rock *et al.*, 2010

CD: cluster of differentiation; T-cells: T-Helper cells; IFN- γ : Interferon- γ ; MTB: *Mycobacterium tuberculosis*; TB: tuberculosis; TNF: tumor necrosis factor

2.3.3 Different mouse models for the study of tuberculosis

When considering the literature on TB mouse models, some previously used models include: i) C3HeB/FeJ, ii) BALB/c, iii) C57BL/6, iv) DBA/2, v) CBA/J, and vi) I/St mice (Kaufmann, 2003; Kramnik & Beamer, 2016). As pointed out earlier, it is important to choose the mouse strain most relevant to the disease model and outcomes to be assessed. For example, mice can be classified as highly resistant or highly susceptible to TB infection. BALB/c mice fall in the highly resistant category and can survive more than 250 days following TB infection, whilst C3HeB/FeJ mice share a common origin with C3H/HeJ, which fall in the highly susceptible TB group (Medina & North, 1998). Table 2-8 provides a comparison between the different mouse strains in terms of TB control, immune mechanisms and necrosis. The table indicates that the two most commonly used strains, C57BL/6 and BALB/c, do not develop necrotising lung lesions, however, necrotising responses to MTB do occur in C3HeB/FeJ, DBA/2, CBA/J, and I/St mice. Overall, if the spectrum of TB and corresponding mouse strains are taken into account, the C3HeB/FeJ mouse model (more commonly referred to as the Kramnik model) were found to be the most sophisticated model, and the immune response is reported to be well known (Kaufmann, 2003).

Table 2-8: Tuberculosis spectrum and corresponding mouse strains*

Strain	MTB control	Clinical forms	Immune mechanisms		Necrosis in TB lesions
			Innate	Adaptive	
BALB/c	- Allow initial MTB replication. - Allow progression towards TB.	Primary progressive TB, disseminated and pulmonary TB.	Immune system not sufficient to eradicate MTB and to control TB progression.	- Immunity cannot control TB progression. - No transparent immune deficiency.	No
CBA/J					Yes
I/St					Yes
DBA/2					Yes
C3HeB/FeJ (well understood immune response)					Yes (lung lesions most similar to humans).
C57BL/6	- Allow initial MTB replication. - Control progression towards TB.	- Latent TB infection. - Reactivation of TB.	Immune system not sufficient to eradicate MTB.	Immunity can control TB progression.	No

*Kramnik & Beamer (2016)

MTB: *Mycobacterium tuberculosis*; TB: Tuberculosis

When comparing the Kramnik model to others, it can be observed that the lung granulomas of the Kramnik strain have more distinguished necrotic areas of degenerating cells and neutrophil infiltrates (this is the expected clinical picture of the human lungs) (Gupta *et al.*, 2013; Irwin *et al.*, 2015). Kramnik *et al.* were the first to describe this highly susceptible mouse model (Kramnik) that develops necrotic lesions solely in the lung, and in no other organ in the body (Kramnik, 2000). Furthermore, it is made known that the Kramnik mice can develop three different types of lung lesions, namely, type i) caseous necrotic lesions, type ii) uncontrolled, neutrophilic lesions, and type iii) cellular lesions (Irwin *et al.*, 2015). The first types of lesions (caseous necrotic lesions) are highly organised with central neutrophilic cores, rims of foamy cells, and distinct fibrotic rims of collagen. These lesions undergo caseous necrosis. Furthermore, mice who display these types of lesions present with high bacterial numbers. The second type of lesions (uncontrolled, neutrophilic lesions) are disorganised and rapidly expand. It is neutrophil dominated, present with few lymphocytes, and no fibrosis. These lesions undergo cellular necrosis/hypoxia. Mice who display these types also present with high bacterial numbers (Irwin *et al.*, 2015). The last type of lesions (cellular lesions) is similar to the lesions developed in BALB/c mice. These lesions are lymphocyte dominated by foamy macrophages, have isolated pockets of neutrophils, and do not undergo any necrosis/ hypoxia. Mice who display these types of lesions only have few bacilli (Irwin *et al.*, 2015).

The Kramnik mice were the chosen type for the current animal experimental study. With this in mind, a variety of studies have suggested that this mouse strain can be utilised to investigate new TB treatments, as these types of lesions are most representative of what is expected in MTB infected humans (Driver *et al.*, 2012; Irwin *et al.*, 2016; Lanoix *et al.*, 2016; Rosenthal *et al.*, 2012; Ordonez *et al.*, 2018; Singh & Gupta, 2018).

2.4 Summary of the literature

As seen in the literature, TB is a problem of considerable magnitude in countries like South Africa. Furthermore, because there are a number of risk factors that contribute to disease progression, this problem is further aggravated. Despite intensive efforts, TB control continues to be highly challenging. The most effective treatment of patients with TB is of utmost importance to help with reduced mortality rates, therapeutic expenses, and to enhance the quality of life of TB survivors. Therefore, more study interventions of supportive treatment may be of great benefit. With this in mind, n-3 PUFAs are modified to produce LM, which play an important role during inflammation and other cellular functions in the body. These fatty acids may benefit host immune response by helping to resolve excessive inflammation and to improve immune cell function during TB infection. Iron status reportedly

plays a very important role during host immune response to infections such as TB (Cherayil, 2011). However, treatment of Fe deficiency can become difficult as Fe supplementation may benefit the pathogen and alter host defense, therefore there is a need for other therapeutic approaches.

Previous research has highlighted the need to examine treatment options to play a supportive role to current standard treatment in order to improve and preserve lung function and reduce long-term inflammatory damage to the lungs (Hawn *et al.*, 2013; Kaufmann & Dorhoi, 2013). Interestingly, studies examining the role of anti-inflammatory medication in the treatment of MTB have shown benefit regarding the size and number of lung lesions, inflammatory response, bacillary load and survival (Marzo *et al.*, 2014; Vilaplana *et al.*, 2013). Furthermore, the administration of other anti-inflammatory medication has been successful as adjunctive therapy in TB patients (Gupta *et al.*, 2013; Maiga *et al.*, 2015). Considering these findings, it may be useful to investigate whether a sufficient n-3 PUFA status may reduce inflammation (and thus AI), thereby improving Fe status, as well as other clinical outcome measures after TB infection.

As it is not possible to investigate the effects of n-3 PUFA status in response to AI and morbidity in TB patients, animal substitutes are used (Kaufmann, 2003). The study of TB has used several different animal models, however, because mouse models provide the best combination regarding practical aspects and similarities to humans in several types of infection, the conclusion is that these models should be sufficient to study AI and morbidity during TB (Rivera and Ganz, 2009). Moreover, in comparison with other mouse models, the C3HeB/FeJ (Kramnik) model was found to be the most sophisticated one, and has been extensively used in other TB studies (Driver *et al.*, 2012; Gupta *et al.*, 2013; Irwin *et al.*, 2015; Lanoix *et al.*, 2015; Marzo *et al.*, 2014; Rosenthal *et al.*, 2012; Vilaplana *et al.*, 2013).

The aim of this animal experimental study was to determine the effect of pre-infection n-3 PUFA status on AI and morbidity in TB infected mice. As no previous study has been performed on this topic, the current study may serve as a basis for further human investigations to contribute in making possible dietary recommendations on n-3 PUFA intake in TB patients.

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CHAPTER 3 RESEARCH METHODOLOGY

In this Chapter, the methodologies and procedures of the animal experimental study are described. Section 3.1 gives information regarding the ethical aspects of the study, whilst Section 3.2 provides detailed information about the study design, methods and materials. Section 3.3 describes the sample collection and all analyses that were performed in the study. Analytical protocols were strictly followed according to the standard operating procedures (SOPs), or the specifications of the manufacturers of the kits used. Lastly, Section 3.4 includes a description of statistical methods that were used to meet the study objectives. The animal experimental study was conducted in two DAFF accredited level P3-safety animal facilities (PCDDP, NWU, Potchefstroom Campus and the Institute of Infectious Disease and Molecular Medicine (IDM), University of Cape Town (UCT), South Africa (SA). Two different infection methods was used in this study and will be explained in greater detail in the Chapter below.

3.1 Ethical aspects

Ethical approval for this sub-study was obtained from the AnimCare Ethics Committee of the Faculty of Health Sciences of the North-West University (NWU), Potchefstroom (Ethics number: NWU-00153-18-S5). As the study was conducted in the Department of Agriculture, a Forestry and Fisheries (DAFF) accredited level P3-safety facility (ANNEXURE B), strict precautions were taken in order to minimise the transfer of the Tuberculosis (TB) bacilli from mice to humans. The safety procedures of this unit are attached in documentation (ANNEXURE B). All sample collections were done under the supervision of a trained animal technologist of the Pre-Clinical Drug Development Platform (PCDDP) from the NWU Vivarium, and strict safety precautions were taken regarding personal protective clothing. All blood and tissue collected were used in analyses to determine the different outcome measures in order to reach the study objectives and were not wasted. The guidelines of the World Health Organization (WHO) TB Laboratory Safety Manual, 2012, were followed with laboratory analysis of samples at the NWU laboratories.

The 3R (Replacement, Reduction, and Refinement) principals were followed to ensure the research was conducted in an ethical and humane way (Singh, 2012). The reason why an *in vivo* (mice) model was used (and not an *in vitro* model) was to be able to consider the complex nature of the effect of n-3 polyunsaturated fatty acid (PUFA) deficiency on the organism. As it is not possible to investigate the influence of n-3 PUFA status on the lungs of humans, a mouse model was used (Section 3.2.1).

The number of animals chosen to be included in this research project was based on previous studies of the same kind, in order not to use excess animals, but also to have enough power to optimise the likelihood to obtain statistically significant results (Section 3.4) (Bouté *et al.*, 2017; Driver *et al.*, 2012; Irwin *et al.*, 2015; Marzo *et al.*, 2014; Vilaplana *et al.*, 2013). The animals were handled with strict precautions and were monitored on a daily basis using a TB-specific monitoring checklist (ANNEXURE C). The entire study was closely monitored by the research team for progress, deviations, incidents, and ethical aspects.

3.2 Study design, materials and methods

The researchers wanted to determine whether there was a difference between TB infected C3HeB/FeJ mice with an n-3 PUFA sufficient or deficient status prior to TB infection, with regard to markers of anaemia of infection (AI), morbidity, and disease progression.

3.2.1 Animals

For this research project, C3HeB/FeJ (Kramnik) mice were used as the animal model. This mouse strain was found to be an excellent TB model, and has been extensively used in previous TB research experiments (Driver *et al.*, 2012; Gupta *et al.*, 2013; Irwin *et al.*, 2015; Irwin *et al.*, 2016; Lanoix *et al.*, 2015; Lanoix *et al.*, 2016; Rosenthal *et al.*, 2012; Ordonez *et al.*, 2018; Singh & Gupta, 2018). As already described in Chapter 2, this model has been deemed the most cost-effective and sophisticated animal model in which the immune response is well understood (Kaufmann, 2003). With the C3HeB/FeJ mouse model, we were able to consider the complex nature of the effect of an n-3 PUFA deficiency on the organism, including the lung, blood, and liver. Previous research has also found the lung histopathology of pulmonary TB in this specific strain of mice is similar to that in humans and can be translatable, thus making it a very useful human substitute model for TB studies (Driver *et al.*, 2012; Marzo *et al.*, 2014; Vilaplana *et al.*, 2013).

3.2.2 Housing

The housing units were equipped with cages, an autoclave, incubators and class II biosafety cabinets. Mice were housed under a 12h/12 hour light/dark cycle (lights on at 06:00) at 22 ± 2 degrees Celsius ($^{\circ}\text{C}$) and $55 \pm 10\%$ relative humidity. The mice were housed in standard type II long mouse individually ventilated cages (IVC) (391 x 199 x 160 millimeter (mm) [WxDxH]) with corncob bedding. Three to four mice were housed per cage. All mice had *ad libitum* access to purified laboratory diets and water, which will be described in the following section.

3.2.3 Diet compositions

Purified experimental diets (AIN-93) were commercially obtained from Dyets Inc. (Bethlehem, USA). All diets were custom prepared in pellet form and stored at -20 °C until it was used. Ingredients of experimental diets based on the AIN-93 diet are listed in Table 3-1. Mice had *ad libitum* access to these diets and to demineralised water (18 MΩ) (Reeves *et al.*, 1993).

Table 3-1: Ingredients of experimental diets based on the AIN-93G diet*

	n-3 Fatty acid sufficient)	n-3 Fatty acid deficient
Cornstarch (g/kg)	397.5	397.5
Casein (g/kg)	200	200
Dextrose – Dextrinised cornstarch (g/kg)	132	132
Sucrose (g/kg)	100	100
Soybean oil (g/kg)	70	-
Hydrogenated coconut oil (g/kg)	30	81
Safflower oil (g/kg)	-	19
Vitamin mix (g/kg)	10	10
Mineral mix (g/kg)	35	35

*Strydom, 2018

g/kg: gram per kilogram; n-3: n-3

As this was a nutritional intervention study, some of the experimental groups received an n-3 PUFA sufficient diet, whilst others received an n-3 PUFA deficient diet. This is necessary in order to mimic what would be expected in a real-life human situation, as n-3 PUFA intake is generally low in the South African population (Richter *et al.*, 2014; Stark *et al.*, 2016). However, all animals received sufficient energy and macronutrients as recommended for mice. Both diets (n-3 PUFA sufficient and n-3 PUFA deficient) were iso-caloric and contained 10% fat with modifications of the fat source (Table 3-1). Gas Chromatography-Mass Spectrometry (GC-MS) analysis, conducted at the manufacturer Dyets Inc. (Bethlehem, USA) confirmed the fatty acid composition of the diets as per product specification. Table 3-2 describes the analysed fatty acid and iron composition of the two diets used in the study.

Table 3-2: Fatty acid and iron composition of the diets

	n-3 Fatty acid sufficient	n-3 Fatty acid deficient
Iron (ppm)	41.3	40.1
Total n-6:n-3 fatty acid ratio	8:1	130:1
Saturated Fatty Acids (g/100g)	3.79	6.77
Total Cis Unsaturated Fatty Acids (g/100g)	5.45	1.55
Monounsaturated Fatty Acids (g/100g)	1.65	0.304
Polyunsaturated Fatty Acids (g/100g)	3.80	1.25
Trans Fatty Acids (g/100g)	0.039	0.033
Omega 3 Fatty Acids (g/100g)	0.436	0.010
Omega 6 Fatty Acids (g/100g)	3.54	1.30
Omega 9 Fatty Acids (g/100g)	1.60	0.302
Total Fatty Acids (g/100g)	9.75	8.85
4:0 Butyric (g/100g)	<0.007	<0.007
6:0 Caproic (g/100g)	0.021	0.048
8:0 Caprylic (g/100g)	0.223	0.541
10:0 Capric (g/100g)	0.178	0.424
12:0 Lauric (g/100g)	1.34	3.25
14:0 Myristic (g/100g)	0.532	1.26
14:1 Myristoleic (g/100g)	<0.007	<0.007
15:0 Pentadecanoic (g/100g)	<0.007	<0.007
15:1 Pentadecenoic (g/100g)	<0.007	<0.007
16:0 Palmitic (g/100g)	1.05	0.831
16:1 Palmitoleic (g/100g)	0.008	<0.007
17:0 Heptadecanoic (g/100g)	0.008	<0.007
17:1 Heptadecenoic (g/100g)	<0.007	<0.007
18:0 Stearic (g/100g)	0.603	0.814
9c 18:1 Oleic (g/100g)	1.58	0.302
18:2 Linolenic (g/100g)	3.54	1.30
18:3 Gamma Linolenic (g/100g)	<0.007	<0.007
18:3 Linolenic (g/100g)	0.436	0.010
18:4 Octadecatetraenoic (g/100g)	<0.007	<0.007
20:0 Arachidic (g/100g)	0.025	0.016
20:1 Eicosenoic (g/100g)	0.023	<0.007
20:2 Eicosadienoic (g/100g)	<0.007	<0.007
22:0 Behenic (g/100g)	0.023	<0.007
21:5 Heneicosapentaenoic (g/100g)	<0.007	<0.007
20:3 Eicosatrienoic (g/100g)	<0.007	<0.007

Continuation of Table 3-2: Fatty acid and iron composition of the diets		
	n-3 Fatty acid sufficient	n-3 Fatty acid deficient
20:4 Eicosatetraenoic (n-3)	<0.007	<0.007
20:4 Arachidonic (n-6) (g/100g)	<0.007	<0.007
20:5 Eicosapentaenoic (g/100g)	<0.007	<0.007
22:1 Erucic (g/100g)	<0.007	<0.007
24:0 Lignoceric (g/100g)	0.010	<0.007
22:2 Docosadienoic (g/100g)	<0.007	<0.007
22:3 Docosatrienoic (g/100g)	<0.007	<0.007
22:4 Docosatetraenoic (g/100g)	<0.007	<0.007
22:5 Docosapentaenoic (n-3) (g/100g)	<0.007	<0.007
22:5 Docosapentaenoic (n-6) (g/100g)	<0.007	<0.007
22:6 Docosahexaenoic (g/100g)	<0.007	<0.007
24:1 Nervonic (g/100g)	<0.007	<0.007
Total 18:1 trans (g/100g)	0.010	0.008
Total 18:1 cis (g/100g)	1.69	0.318
Total 18:2 trans (g/100g)	0.016	0.027
Total 18:3 trans (g/100g)	0.015	<0.007

g: gram; n-3; n-3; ppm: parts per million

As mentioned in Chapter 2, n-3 PUFA consumption in the general adult population should be 1-2% of total daily energy intake (Flock *et al.*, 2013; Harris *et al.*, 2009). Therefore, in order to ensure that the diets we provided to the animals would also translate to deficient and sufficient n-3 PUFA intake in the human situation, we made use of a calculation from a review article which provided a simple guide for dose conversion between animals and humans (Nair & Jacob, 2016). The method for the calculation and instructions are explained in ANNEXURE L.

Great care was taken in monitoring the animals to ensure the prevention of any malnutrition related effects. Food intake was measured on a weekly basis to evaluate the amount of food eaten by the animals (ANNEXURE E). In a previous study conducted by one of our groups in rats, skin problems were seen when n-3 PUFA and iron (Fe) deficiency were combined in one group, but no skin problems were found in the n-3 fatty acid deficient (FAD) group or the control group (Baumgartner *et al.*, 2012). Nevertheless, specific attention was given to the n-3 FAD group to look out for skin problems.

3.2.4 Tuberculosis strain and infection

For the infection, a mouse-passaged H37Rv TB strain was used, which was obtained from the IDM, UCT, and cultivated, harvested and suspended, at the Centre for Human Metabolomics, Biochemistry Department, NWU, Potchefstroom Campus, SA. The inoculum stock solutions were transported in airtight containers following the Red Cross procedure, to the Vivarium NWU P3-safety facility in order to infect animals. The mice were infected intranasally at the NWU (Franzblau *et al.*, 2012) and via the aerosol route at the UCT (Kramnik & Beamer, 2016). Previous research has found C3HeB/FeJ mice to be highly susceptible to TB infection (Lenaerts *et al.*, 2015). It is possible to administer a higher dose of infection with intranasal infection than with aerosol infection. In this study, two different infection doses were administered with the intranasal and aerosol infections to present two different clinical pictures. The infection amounts were based on the effective and tested doses administered in previous C3H/HeJ mice TB research studies (Jordao *et al.*, 2008; Medina & North, 1998; Vilaplana *et al.*, 2013). The infection dose for the intranasal infection was 3.4×10^3 Colony forming units (CFU) (using 50 microliters (μ l) of a 6.8×10^4 CFU/milliliter (ml) stock solution as inoculum) (ANNEXURE H). With this dose, a more acute infection was induced. However, in order to present the clinical picture of what may be expected in a more chronic infection, a lower infection dose was administered by aerosol infection to obtain ~50-100 CFU (ANNEXURE G). By using these two infection doses, the required lung pathology in this mouse strain was attained without severe morbidity. For practical reasons (one being insufficient breeding numbers of female mice for the second stagger), female mice were used for the intranasal infection (first stagger) and males for the aerosol infection (second stagger).

3.2.4.1 Aerosol infection procedure

Prior to the aerosol infection, a mycobacterial 6 ml suspension (24×10^6 CFU) was prepared, and the titer of the starting suspension was confirmed. The animals were then infected by exposure to aerosol (ANNEXURE G).

For this procedure, the acrylic exposure chamber lid of an inhalation exposure system (IES) was firstly unlatched and fully raised. The metal basket support/baffle plate and stainless steel mesh exposure basket were then placed into the chamber. Mice were loaded into the basket and the basket lid was secured. Then the chamber lid was closed. Afterwards, a glass nebuliser-venturi was mounted to the compressed air and main airflow fittings located on the front of the instrument. The nebuliser was then secured into place with clamps, and checked to ensure that all joints appeared to be seated properly and made a sealed fit. The

screw cap was carefully removed above the venturi portion of the nebuliser unit, and the cap was inspected for any wear, cracks, or missing gaskets. With a 10-cc syringe fitted with an 18-G needle, 5 ml mycobacteria suspension was carefully added into the bottom of the venturi. The syringe was then discarded into the sharps container, and the screw cap was replaced to the venturi, ensuring it was threaded properly and fitted tightly.

The main power switch was turned to the on position, and the control keypad displayed the message "Glas-Col Apparatus Co." The UV lamp and programme switches were then turned to the on position and so were lit.

The control keypad displayed several messages and then stopped with the message "Enter When Ready 0." After pressing the enter key on the control keypad, the "Enter Preheat Time 900" message appeared on the keypad display. This indicated that the IES had a preset preheat time of 900 seconds (sec) (15 min) which the incinerator used to decontaminate the exhaust, and air was allowed to raise its core temperature to 790°C before the nebulising cycle began. The 900-sec preheat time setting was accepted, and the nebulising-time message appeared. An "Enter Neb Time 1800" message appeared, indicating a preset nebulising time of 1800 sec. The number 2400 (40 min for mice) was entered to set the nebulising time. The final message to appear before the instrument began operation was "Ent Dec Time 900." This message sets the amount of time in which the UV lamps are turned on and the top surfaces of the basket are exposed to ultraviolet light at 254 nanometers (nm) for surface decontamination. The 900-sec decontamination time setting was selected. Once this time was selected, the IES began to cycle sequentially through preheat, nebulising, cloud decay, and UV decontamination settings for the aerosol challenge for animals within the chamber. The display on the keypad indicated which cycle was in operation.

Once the instrument started the preheat cycle, the main air flow meter indicated 60 cubic feet per hour (CFH). Furthermore, when the nebulising cycle started, the compressed air flow meter indicated 10 CFH. Afterwards, when the aerosol infection run was completed, the programme was turned off, as well as the UV lamp and power switches on the IES. The instrument signalled the completion of the run with an audible alarm and the message "Process Complete - Remove Specimen" appeared on the keypad. The screw cap on the nebuliser was then carefully removed and the volume of remaining mycobacterial suspension was checked with the aid of a 10-cc syringe fitted with an 18-G needle. The remaining volume was recorded. It is important to keep in mind that there should be no more than 1 ml remaining for infections with mice. The equipment was then disassembled and cleaned.

3.2.4.2 Intranasal infection procedure

Prior to the intranasal infection, work surfaces of the Class II biosafety cabinet inside the BSL3 were cleaned using F10 disinfectant, followed by 70% ethanol. All cleaning materials were then placed in an autoclave bag for disposal. Afterwards, the vial containing the single cell suspension of TB was gently thawed at room temperature. The vial was then vortexed to further disrupt any clumps of bacilli. A stainless steel pipette boat was filled with 5% Lysol to a 1.0- to 2.0 centimeter (cm) depth and placed toward the rear of the work surface. A sharps container was placed to the side to accommodate syringes and needles. Then, the vial was placed in the cabinet, and the top of the butyl rubber septum was disinfected with 70% ethanol. Afterwards, 100 µl of MTB suspension (6.8×10^7 CFU/ml) were removed and carefully injected down the side of a 2 ml tube containing 900 µl saline so that the final volume was 1 ml, and mixed by vortexing; this was a 10x dilution (6.8×10^6 CFU/ml). The process was repeated another two times to reach an inoculum concentration of 6.8×10^4 CFU/ml. Lastly, 80 µl of the 6.8×10^4 CFU/ml TB was transferred into 920 µl to produce 5.4×10^3 CFU/ml inoculum (ANNEXURE H).

After the desired inoculum concentration (6.8×10^3) was reached, mice were weighed and anesthetised for two minutes in an isoflurane chamber with oxygen (to ensure minimal stress in the animals as well as the safety of personnel conducting the infection). The anesthesia was administered by trained laboratory animal technicians who adhered to the study protocol (ANNEXURE F). Afterwards, the mice were randomly infected with a syringe connected to a feeding tube by a trained laboratory animal technologist with a standardised technique (50 µl suspension drop wise into one nostril) (ANNEXURE H).

3.2.5 Medication administration

When working with animal TB models, it is important to keep in mind that not all the results can be successfully extrapolated to human models (Evans *et al.*, 2010; Fuchs *et al.*, 2009). Due to the immunopathology between mice and humans differing, previous studies that investigated anti-inflammatory effects on mouse TB models (specifically the C3HeB/FeJ strain) did not administer regular TB medication (Orme, 2003). Research shows that if mice were to be administrated with medication, TB progression might not be successful, and the typical lung histopathology may not be present (Bonilla *et al.*, 2010b; Jordao *et al.*, 2008; Lanoix *et al.* 2015; Marzo *et al.*, 2014; Vilaplana *et al.*, 2013). Therefore, apart from the non-pharmacological dietary adjustments of the n-3 PUFA content of the diets, the C3HeB/FeJ mice did not receive medication as part of their treatment regime.

3.2.6 Experimental design

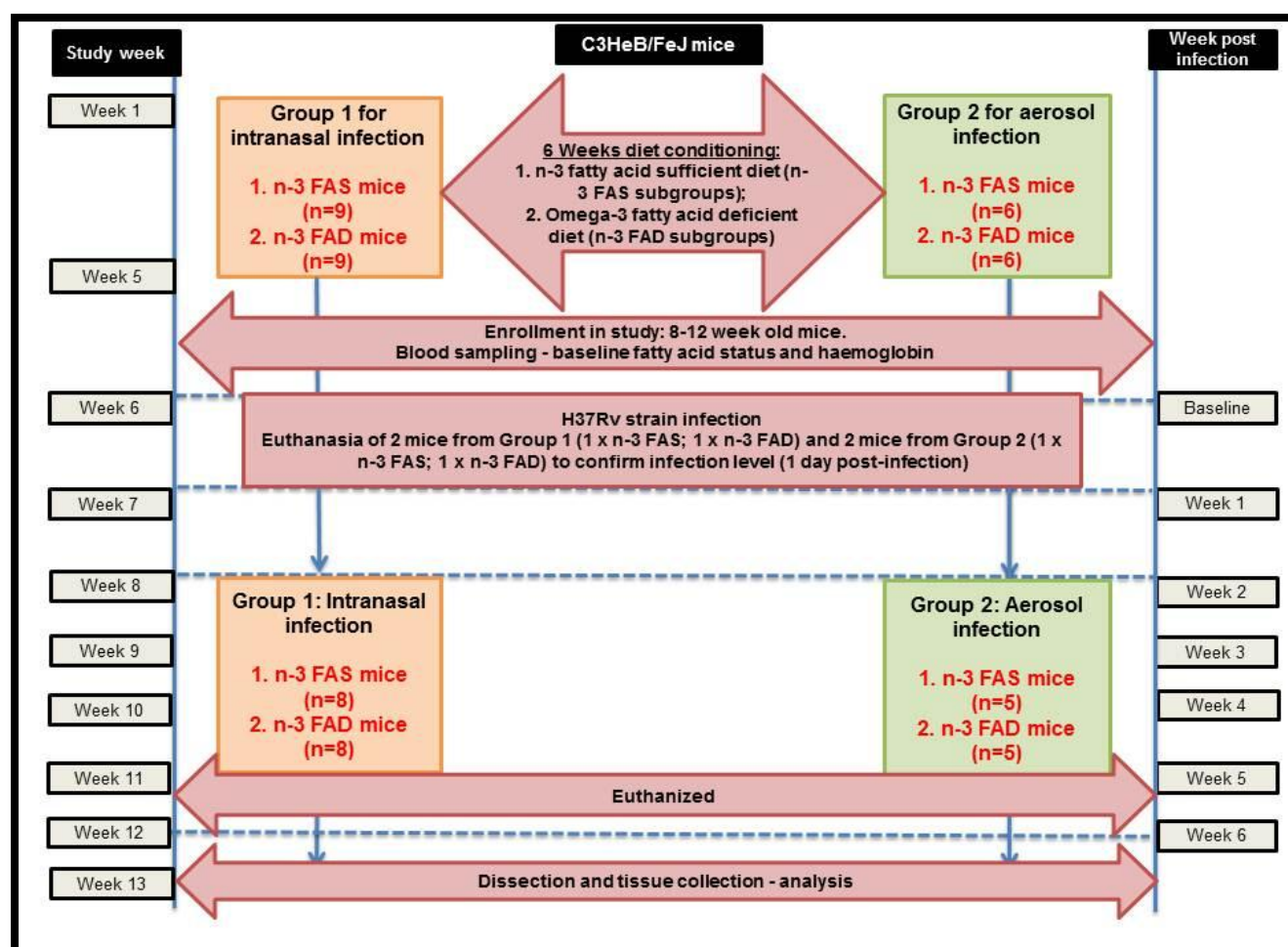


Figure 3-1: Experimental study design

FAD: Fatty acid deficient; FAS: Fatty acid sufficient-3: n-3

Eighteen 8 to 12-week-old mice for intranasal infection, and 12 for aerosol infection were included in the experiment, which was based on previous experiments using this TB mouse model (Driver *et al.*, 2012; Gupta *et al.*, 2013; Irwin *et al.*, 2015; Jordao *et al.*, 2008; Lanoix *et al.*, 2015; Marzo *et al.*, 2014; Rosenthal *et al.*, 2012; Vilaplana *et al.*, 2012). The first group included nine n-3 fatty acid sufficient (FAS) mice, and nine n-3 FAD C3HeB/FeJ mice that were to be intranasally infected. The second group included six n-3 FAS C3HeB/FeJ mice and six n-3 FAD C3HeB/FeJ mice that were to be aerosol infected. The n-3 FAS subgroups were preconditioned on the n-3 FAS diet for six weeks to present with an adequate n-3 PUFA status at the start of study week six (baseline). The mice remained on the FAS diet for the duration of the study. The n-3 FAD subgroups, however, were preconditioned on a n-3

FAD diet for six weeks to present with an n-3 PUFA deficient status at the start of study week six (baseline). These mice remained on the FAD diet for the duration of the study.

After the preconditioning phase (week six), baseline fatty acid status and haemoglobin (Hb), and body weight of the mice were measured. The experimental groups were then infected with the H37Rv TB strain. The first group (n=9/group) were infected intranasally, and the second group (n=6/group) with aerosol infection. One day post TB infection, two mice (1 x n-3 FAS and 1 x n-3 FAD) from group 1, as well as two mice (1 x n-3 FAS and 1 x n-3 FAD) from group 2 were euthanised (ANNEXURE I). The lungs of these mice were harvested, plated, and incubated to determine the bacterial loads and confirm the establishment of infection (ANNEXURE J). More mice (n=8/group) were used for the intranasal infection experiment than the aerosol infection experiment (n=5/group), due to a higher variation of the technique that was observed with test mice in a pilot study prior to the start of this experiment. For example, the intranasal infection method was time-consuming, the animal technologist had to have a very steady hand to successfully inject the droplets into the tiny nostrils of the animals, a higher volume of the TB strain was needed compared to aerosol infection, TB could stay in the mucus of the animals, and some of the TB droplets could have ended up in the stomachs of the mice. Lastly, not all of the administered droplets were successfully inhaled due to the fact that liquid droplets were formed on the noses of the mice when they breathed. All of these examples could result in a variation of TB infection in the lungs, and were observed with test mice in the pilot study. Therefore, it was decided to include more mice for intranasal infection.

Throughout the study period, the mice were monitored daily according to a TB specific morbidity-monitoring sheet, using the Karnofsky score with humane endpoints, where mice had to be euthanised when the endpoints were reached (ANNEXURE C). Table 3-3 provides a brief description of how the different scores were determined.

Table 3-3: Health surveillance of tuberculosis infected mice: key for assignment of Karnofsky score

#	HEALTH INDICATOR	KARNOFSKY SCORE	
		VALUE	EXPLANATION
A.	Appearance	A ₀	Vocalizing; normal appearance of fur and skin at nares and extremities; very active, alert and excitable when observed
		A ₁	Vocalizing; ruffled appearance of fur with normal skin color at nares and extremities; moderate activity and alertness when observed
		A ₂	Very little vocalizing; ruffled appearance of fur with normal skin color at nares and extremities; little activity when observed (lethargic)
		A ₃	No vocalizing, severely ruffled appearance of fur with cyanotic appearance of skin at the nares and the extremities; no visible activity
B.	Breathing	B ₀	<10% increase in mean baseline value with normal breathing pattern
		B ₁	25% increase in mean baseline value with normal breathing pattern
		B ₂	<50% increase in mean baseline value with normal breathing pattern
		B ₃	<100% increase in mean baseline value with heavy/labored breathing pattern
		B ₄	>100% increase in mean baseline value with heavy/labored breathing pattern with visible gasping for breath
C.	Body weight Loss (Based on the comparison of mean body weights of uninfected controls - or- the sudden body weight loss detected in an individual by determining individual body weights twice weekly)	C ₀	Body weight loss of <5%;
		C ₁	Body weight loss of <10%
		C ₂	Body weight loss of <15%;
		C ₃	Body weight loss of >15% but <20%
		C ₄	Body weight loss of >20%
Total Possible Score		26	Consider euthanasia whenever a score of 3 is assigned or get Veterinarian for final assessment. Double frequency of observations for any animal that scored a 2 for any indicator.

The animals were only removed from their cages, in the class II biosafety cabinet, for monitoring and measurements (such as body weight). Researchers have suggested that 28 days post a high dose of infection should be used as the humane endpoint (Marzo *et al.*, 2014; Vilaplana *et al.*, 2013) due to the fact that the Kramnik mouse strain's health may deteriorate significantly faster after 28 days. Due to excessive body weight loss (>20%) and

severe morbidity signs, one mouse from the n-3 FAS subgroup (Group 1 – intranasally infected) was euthanised 3 days before the termination date. However, blood and tissue analysis for this mice were measured at the same time as the other mice. The remaining mice were euthanised at study week 5 (35 days post-infection), all with body weight loss < 20%. Euthanasia took place according to the SOPs for euthanasia in the PCDDP Vivarium P3 safety unit, by decapitation (ANNEXURE I). Following euthanasia, sample collection and laboratory analysis took place as outlined in Section 3.3.

3.3 Sample collection and analysis

As outlined in Section 3.1, strict precautions were taken with all sample collections and analyses in order to minimise the transfer of the TB bacilli from mice to humans. Safety precautions were followed with regard to personal protective clothing, including safety glasses and masks, and the safe handling of samples to ensure the safety of personnel and students. By following the guidelines of the WHO TB Laboratory Safety Manual (2012), sample collection and preparation were done in a class II biosafety cabinet prior to transport for analysis elsewhere. Sample analysis was then performed at the DAFF accredited Laboratory of Infectious Disease Metabolomics at the Centre for Human Metabolomics, the Centre of Excellence for Nutrition (CEN) of the NWU and IDM at UCT.

3.3.1 Sampling

3.3.1.1 Blood sampling

Baseline blood samples were taken from the lateral tail vein using the “tail nick” method. Thereby, the mice were warmed under a warming lamp in order to dilate the blood vessel prior to taking the sample. The mice were then placed in a tube restrainer, and a maximum of 200 µl blood sample was collected after the blood was released as it welled up. The blood was released into 1 ml EDTA coated Minivette® (Minivette® POCT, 1000 µl, Sarsted). The baseline blood samples were used to measure Hb and for fatty acid analysis (ANNEXURE J).

Five weeks post TB infection, mice were euthanised and decapitated (ANNEXURE I). After decapitation, trunk blood was collected and analysed to determine: 1) RBC fatty acid composition, and concentrations of 2) Hb, 3) ferritin 4) transferrin receptor (TfR) in all mice and hepcidin in intranasal infected mice. Trunk blood was collected in 1 ml EDTA-coated vacutainer tubes. Within 1 hour of collection, tubes were centrifuged at 8000 x g for 12 minutes to obtain plasma. Red blood cells (RBCs) were washed twice with 0.15 M NaCl by

centrifugation twice at 8000 x g for 5 minutes. Plasma and RBC aliquots were prepared and stored at -80°C until analysis (ANNEXURE J).

3.3.1.2 Lung sampling

Lungs were collected for the analysis of bacillary load in all mice, as well as lung cytokines in intranasal infected mice. 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 grasped and the lung lobes were cut out. The left lung lobe was placed into 1ml Phosphate-buffered saline (PBS), stored in the fridge at 4°C, and afterwards homogenised for the analysis of the bacillary load (study week 6). The right inferior lung lobe was also placed into 1 ml of PBS stored at -80°C, and defrosted and homogenised when analysed for lung cytokines. Figure 3-2 is a visual representation of the lung lobes that were used for bacillary load and lung cytokine analysis (ANNEXURE J).

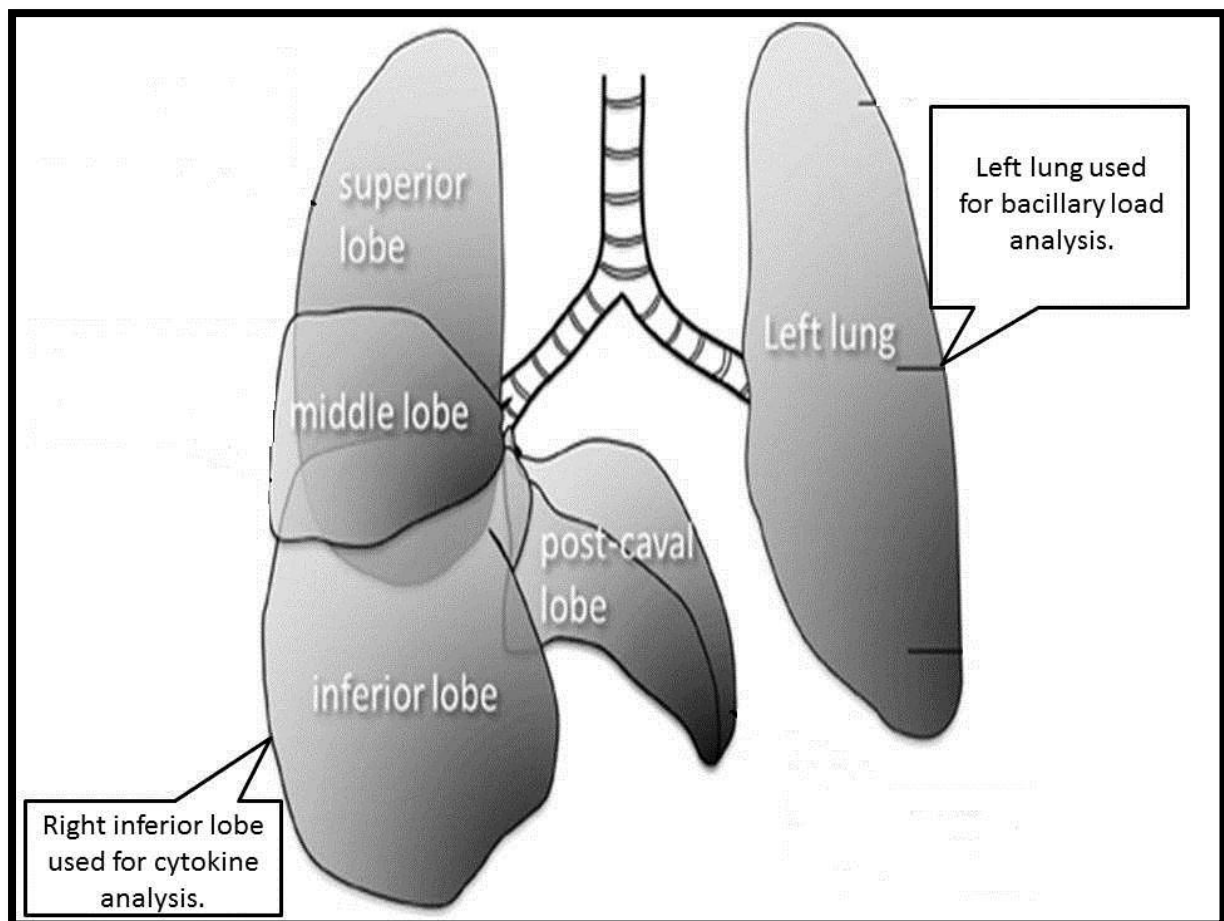


Figure 3-2: Lung lobes used for bacillary load and cytokine analysis

3.3.2 Laboratory analyses

3.3.2.1 Fatty acid analysis

Red blood cell total lipids were extracted from RBC with chloroform: methanol (2:1 vol: vol; containing 0.01% butylated hydroxytoluene) by using a modification of the method of Folch *et al* (1956). Lipid extracts were concentrated, and the neutral lipids were separated from the phospholipids using thin-layer chromatography (silica gel 60 plates, 10x 20 cm; Merck) and eluted with diethyl ether: petroleum ether: acetic acid (30:90:1 vol: vol: vol). The lipid band that contains phospholipids was removed from the thin-layer chromatography plate and transmethylated with methanol: sulphuric acid (95:5 vol: vol) at 70°C for 2h to yield fatty acid methyl esters (FAMES). The resulting FAMES were extracted with water and hexane. The organic layer was collected, evaporated to dryness and the concentrated FAMES re-dissolved in a small volume of hexane.

Fatty acid methyl esters were analysed with a gas chromatograph triple quadrupole mass spectrometer with chemical ionisation on an Agilent Technologies 7890A Gas Chromatograph system, equipped with an Agilent Technologies 7000GC/MS triple quad mass selective detector (Agilent Technologies). The gas chromatography separation of FAMES was carried out on an HP88 capillary column (100 mm x 0.25 mm x 0.20 µm; Agilent) using helium as the carrier gas at a flow rate of 2.2 ml/min. The gas chromatography injector was held at a temperature of 270°C, and the mass spectrometry source at 250°C. The injection volume of the sample solution was 1 µL, using a split ratio of 1:10. The oven temperature was programmed to rise from 50° C to 170 °C at 30 °C/min, then from 170°C to 215°C at 2°C/min, and lastly at 4°C/min to 230°C. After that, the temperature was held isothermally at 230°C for 7 min. The total analysis time was 38.25 min. Mass spectrometry was carried out in the multiple reaction-monitoring mode, with at least two transitions per compound. Quantification of FAMES was performed with Masshunter (B.06.00). FAME peaks were identified and calibrated against a standard reference mixture of 33 FAMES (Nu-Check-Prep) and two single FAME standards (Larodan Fine Chemicals AB). Relative percentages of fatty acids were calculated by taking the concentration of a given fatty acid as a percentage of the total concentration of all fatty acids identified in the sample.

3.3.2.2 Haemoglobin concentration

Haemoglobin concentrations were measured directly after blood collection in whole blood using a portable HemoCue® Hb 201+ photometer (HemoCue AB, Angelholm, Sweden).

3.3.2.3 Serum ferritin, transferrin receptor, and hepcidin concentrations

Mice-specific Enzyme-linked immunosorbent assays (ELISA) kits from ELAB Science (ADD CITY and COUNTRY) were used. The Mouse FE (Ferritin) ELISA Kit was used to analyse 25 µl plasma per assay for ferritin: the plasma was diluted (25 µl sample + 75 µl diluent). For the transferrin receptor, the Mouse TFR1 (Transferrin Receptor) ELISA Kit was used to analyse 75 µl plasma per assay; the plasma was also diluted (75 µl sample + 25 µl diluent). Lastly, the Mouse Hpc (Hepcidin) ELISA Kit was used to analyse Hepcidin by using 20 µl plasma diluted 5 times

For the determination of these three parameters, a standard working solution concentration range was added in duplicate to the first and last plate wells. After this, the diluted samples were added to all the other wells. The plate was then covered with the sealer provided in the kit and incubated for 90 minutes at 37°C. The liquid was subsequently removed from each well. Immediately, 100 µl of Biotinylated Detection Ab working solution was added to each well, and covered with the plate sealer. It was gently mixed and incubated for one hour at 37°C.

After the mix was incubated, the solution from each well was aspirated. Then, 350 µl wash buffer was added to each well. The wells were soaked for 1 to 2 minutes, the solution aspirated from each well. This wash step was repeated three times, thereafter, 100 µl of HRP-conjugated antibody working solution was added to each well. The plate was covered with the plate sealer and incubated for 30 minutes at 37°C. The solution from each well was then aspirated, and the wash process repeated five times. Afterwards, 90 µl of Substrate Reagent was added to each well, covered with a new plate sealer, and incubated for 15 minutes at 37°C. Stop solution (50 µl) was then added to each well, and the optical density (OD) value (of each well) was determined using a microplate reader set to 450 nm.

3.3.2.4 Lung homogenisation process to determine bacillary load and cytokine concentrations

The two lung lobes (left lung lobe and right inferior lobe) were placed in separate pre-weighed 2 ml Eppendorf tubes and their weights recorded. One milliliter of PBS buffer was added to each tube, as well as a stainless steel bead (5 mm). The tubes were then placed in two chilled TissueLyser II Adapter Sets (2 x 24), ensuring the tubes in the two sets were balanced correctly. The TissueLyser II was turned on; the frequency was set at 25 Hertz (Hz) and the time at 12 minutes (min) (required time for lung tissue). The TissueLyser II Adapter Sets (2 x 24) were then attached to the TissueLyser (ANNEXURE J). The entire homogenised sample was collected in one new tube (2 ml) without the beads, and vortexed.

3.3.2.5 Determination of lung bacillary load

For the dermination of bacillary loads, the biosafety cabinet was disinfected with F10 followed by 70% ethanol, and all cleaning materials were placed into autoclave bags for autoclaving. Afterwards, 900 µl of PBS was added into 2 ml tubes. By using sterile filter tips (with a wide bore to prevent blockage) serial dilutions with 100 µl were carefully made. The tips were changed with every tube due to mycobacteria being sticky and remaining in the tips, resulting in carry-over and inaccurate dilutions. The tips were dispensed into the pipette boat. By using a pipette, 100 µl of each homogenate dilution were then dispensed onto Middlebrook 7H11 agar plates and manually dispersed (ANNEXURE K). The procedure was carried out immediately after the dilutions had been prepared (mycobacteria will stick to the plates and tubes and dilutions will not be accurate if left to stand). All materials used in plating (e.g., dilution tubes, homogenisation tubes, pipet boats, test tube racks), were double bagged, removed from the hood, and placed in a large pan for autoclaving. The interior work surfaces of the hood were disinfected with 5% Lysol followed by 70% ethanol. All paper towels in autoclave bags were disposed of. Lastly, the agar plates were incubated for three weeks at 37°C. Afterwards, the bacterial loads were determined by manual counting of CFUs and expressed as CFU/ml (Marzo *et al.*, 2014).

3.3.2.6 Determination of cytokine concentration

After the homogenisation of the right inferior lobe, lung cytokines were measured using the Quansys Biosciences (CITY, COUNTRY) Q-Plex Array (16 plex) for mouse cytokines. Following preparation, all reagents were allowed to equilibrate to room temperature (20-25°C) before they were used. 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 prepared with three different dilutions; i) x3 times diluted (20 µl sample + 40 µl diluent), ii) x6 times diluted (10 µl sample +50 µl diluent), and iii) x10 times diluted (10 µl sample + 90 µl diluent), with enough sample diluent to have 50 µl per well in polypropylene tubes. Afterwards, 50 µl per well of the calibration curve and samples were added by pre-wetting the pipette tips three times, drawing up the liquid into the pipette, and then dispensing back into the original vessel, aspirating 50 µl 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. Afterwards, the plate was washed three times. Then, 50 µl

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 again washed three times, and 50 µl 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. Now the plate was washed six times, where after 50 µl of the previously prepared substrate was added to each well. The plate was placed in the Q-View Imager Pro, Q-View Software was opened, and a project was created. The exposure time was set to 300 sec and the images were captured.

3.3.3 Other parameters considered

Other outcome parameters that may have influenced results were also measured. These included body weight and food intake. Daily morbidity symptoms were also monitored. The body weights of the animals were measured weekly using Sartorius Entris 2202-1S balances, and more frequently as body weight loss increased (ANNEXURE D). However, body weight loss at the infection dose was less than 20% at 5 weeks (35 days) of infection for all except one mouse from the n-3 FAS intranasally infected subgroup. Morbidity signs were recorded daily by using a TB morbidity observational sheet with the Karnofsky score, as described in Section 3.2.6 (ANNEXURE C). Food intake was also measured weekly to ensure sufficient intake, and to evaluate the average food intake per mouse per day. The following equation was used to determine the average food intake per mouse: food intake per cage ÷ 3/4 mice per cage ÷ 7 days per week (ANNEXURE E).

3.4 Statistical methods

The data gathered from this project was analysed using IBM Statistical Programme for Social Sciences (SPSS) Statistics software (version 23) and Excel 2016. The power analysis to determine the appropriate number of animals required was based on previous animal studies that used the C3HeB/FeJ mice strain, with similar research outcomes (Bouté *et al.*, 2017; Driver *et al.*, 2012; Irwin *et al.*, 2015; Marzo *et al.*, 2014; Vilaplana *et al.*, 2013). According to these studies, eight mice per group were decided on as sufficient to investigate research outcomes in intranasal infected mice and five mice per group for the aerosol-infected mice. A biostatistician was consulted to ensure the soundness of the number of animals and statistical methods that were used.

The normality of the data was tested using q-q plots, histograms, and the Shapiro-Wilk test. Homogeneity of variance was examined by the Levene's test. The data was examined for the presence of outliers by using box plots. Results of normally distributed (parametric) data were expressed as means ± standard error of the mean (SEM). Data that was not normally

distributed (non-parametric) were log transformed prior to statistical analysis and reported as geometric means $\pm$ SEM. A *p* value of < 0.05 was considered statistically significant.

The objective was to determine whether there was a statistically significant difference between subgroups with an n-3 PUFA sufficient or deficient status prior to infection, with regard to markers of AI ((iron (Fe) status parameters i.e. serum ferritin, TfR, hepcidin, and Hb), markers of morbidity (monitoring appearance and respiratory rate and measuring body weight change) and markers of disease progression (lung bacillary load, organ indexes, and inflammatory marker profiles). Independent T-tests were used to compare outcomes between the two groups. Dependent paired sample T-tests were used to determine between-subgroup differences from baseline to end. Factors such as baseline body weight and baseline Hb confounded the analysis, therefore analysis of covariance (ANCOVA) was used to analyse the between-subgroup differences, adjusting for these two confounders.

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CHAPTER 4 RESULTS

The following chapter provides the main findings of the research project. The outcomes obtained from the methods followed in Chapter 3 (Research Methodology) are displayed in the subsequent sections. Section 4.1 provides the baseline characteristics of the different mice groups (n-3 fatty acid sufficient (FAS) versus n-3 fatty acid deficient (FAD)), whilst Section 4.2 presents the difference in n-3 polyunsaturated fatty acid (PUFA) status for all groups five weeks post-tuberculosis (TB) infection. Section 4.3 focuses on the effect of n-3 PUFA status on markers of anaemia of infection (AI) in the different groups (Objective 1). Section 4.4 presents the food intake of the mice, determined over the course of five weeks post TB infection. In addition, this Section provides the results obtained indicating the effect of n-3 PUFA status on markers of morbidity (Objective 2). Lastly, Section 4.5 gives information about the disease progression between the different groups (Objective 3).

4.1 Baseline red blood cell total phospholipid polyunsaturated fatty acid status, haemoglobin, and body weight

As previously described in Chapter 3, a total of 18 mice, intranasal infection group (females), and 12 mice, aerosol infection group (males), were included in this animal experimental study. The n-3 FAS subgroups used for intranasal and aerosol infection were preconditioned on the n-3 FAS diet for six weeks to present with an adequate n-3 PUFA status at the start of study week six (baseline level prior to infection). These mice remained on the n-3 FAS diet for the duration of the study (five weeks following infection until euthanasia). The n-3 FAD mice used for intranasal and aerosol infection were preconditioned on the n-3 FAD diet for six weeks to present with an n-3 PUFA deficient status at baseline level prior to infection. The mice remained on the fatty acid deficient diet for the duration of the study (five weeks following infection until euthanasia).

After the preconditioning phase, haemoglobin (Hb), the body weight of all mice, and baseline fatty acid status of a subgroup were measured. Table 4-1 summarises these baseline characteristics of the two groups that were studied. Comparisons were made between n-3 FAS and n-3 FAD mice in the intranasal and aerosol infected subgroups. The baseline red blood cell (RBC) total phospholipid n-6 and n-3 PUFA status did not differ between the intranasal and aerosol groups and was therefore presented together in Table 4-1.

Table 4-1: Baseline red blood cell total phospholipid n-6 and n-3 polyunsaturated fatty acid status (after six weeks of preconditioning prior to tuberculosis infection)

Subgroups of mice included for intranasal and aerosol infection			
Fatty acids (% of total)	n-3 FAS subgroups ^a (males: n=5) (females: n=5)	n-3 FAD subgroups ^b (males: n=5) (females: n=5)	p-value ^c
n-6 PUFA			
Linolenic acid (18:2 n-6) ¹	9.84 ± 0.02	7.24 ± 0.01	<0.001
Arachidonic acid (20:4 n-6) ¹	19.07 ± 0.06	20.73 ± 0.25	0.024
Osbond acid (22:5 n-6) ¹	1.25 ± 0.03	5.05 ± 0.30	0.048
Total n-6 PUFA ¹	33.48 ± 0.01	37.02 ± 0.29	0.006
n-3 PUFA			
Alpha-linolenic acid (18:3 n-3) ¹	0.08 ± 0.01	0.01 ± 0.01	0.001
Eicosapentaenoic acid (20:5 n-3) ¹	0.17 ± 0.01	0.04 ± 0.01	0.022
Docosahexaenoic acid (22:6 n-3) ¹	8.60 ± 0.30	4.27 ± 0.56	0.040
Total n-3 PUFA ¹	9.28 ± 0.36	4.39 ± 0.57	0.028
n-6:n-3 PUFA Ratio			
Total n-6:n-3 ratio ¹	3.61 ± 0.14	8.59 ± 1.18	0.052

^a Characteristics of uninfected groups on diet with normal n-3 PUFA content for six weeks prior to TB infection

^b Characteristics of uninfected groups on diet with low n-3 PUFA content for six weeks prior to TB infection

^c An independent t-test was used to determine between-subgroup differences

¹ Parametric data expressed as mean ± standard error of the mean (SEM) (all such values)

FAD: fatty acid deficient; FAS: fatty acid sufficient; n-3: n-3; n-6: omega-6

The baseline fatty acid status showed differences in all n-6 and n-3 PUFAs between the n-3 FAS and the n-3 FAD subgroups. The n-3 FAD subgroup presented with higher baseline n-6

PUFA (arachidonic acid (AA, $p = 0.024$), osbond acid, $p = 0.048$, and total n-6 PUFA, $p=0.006$) compared to the n-3 FAS subgroup. Conversely, the n-3 FAS subgroup presented with higher baseline n-3 PUFA (alpha-linolenic acid (ALA, $p < 0.001$), eicosapentaenoic acid (EPA, $p = 0.022$), docosahexaenoic acid (DHA, $p = 0.40$), and total n-3 PUFA, $p = 0.028$) compared to the n-3 FAD subgroup. Lastly, a trend towards a lower total n-6:n-3 ratio was present in the n-3 FAS subgroup compared to the n-3 FAD subgroup ($p = 0.052$).

Table 4-2: Baseline haemoglobin and body weight (after six weeks of preconditioning prior to tuberculosis infection)

	Mice included for intranasal infection (females)			Mice included for aerosol infection (males)		
	n-3 FAS subgroup (n=9) ^a	n-3 FAD subgroup (n=9) ^b	p- value ^c	n-3 FAS subgroup (n = 6) ^a	n-3 FAD subgroup (n=6) ^b	p- value ^c
Haemoglobin, g/dL ¹	14.43 ± 1.54 ¹	14.95 ± 0.14	0.371	15.60 ± 0.66	12.86 ± 0.13	0.003
Body weight, g ¹	27.82 ± 1.41	25.28 ± 0.86	0.146	34.90 ± 0.66	31.76 ± 1.11	0.041

^a Characteristics of uninfected groups on diet with normal n-3 PUFA content for six weeks prior to TB infection

^b Characteristics of uninfected groups on diet with low n-3 PUFA content for six weeks prior to TB infection

^c An independent t-test was used to determine between-subgroup differences

¹ Parametric data expressed as mean ± SEM (all such values)

dL: deciliter; FAD: fatty acid deficient; FAS: fatty acid sufficient, g: gram; n-3: n-3

Table 4-2 shows that the n-3 FAD subgroup in the aerosol group presented with lower Hb concentrations ($p = 0.003$), as well as lower baseline body weights ($p = 0.041$) compared to the n-3 FAS subgroup.

4.2 Endpoint red blood cell total phospholipid n-6 and n-3 polyunsaturated fatty acid status

Table 4-3 describes the endpoint n-6 and n-3 PUFA status of the different groups (five weeks post-TB infection). One-day post TB infection, two intranasal infected (one n-3 FAS and one n-3 FAD) mice, as well as two aerosol infected (one n-3 FAS and one n-3 FAD) mice, were euthanised to confirm the infection level, as described in Chapter 3. Thus 16 intranasal infected (n-3 FAS (n=8) and n-3 FAD (n=8)) mice, and 10 aerosol infected (n-3 FAS (n=5) and n-3 FAD (n=5)) mice were included for all statistical analyses five weeks post

TB infection. Due to excessive body weight loss (>20%) and severe morbidity signs, one mouse from the intranasal n-3 FAS subgroup was euthanised three days before the termination date. However, data for this mouse is included in the subgroup the results.

Table 4-3: Endpoint red blood cell total phospholipid n-6 and n-3 polyunsaturated fatty acid status among groups (five weeks post tuberculosis infection)

Fatty acids (% of total)	Intranasal group (females)			Aerosol group (males)		
	n-3 FAS subgroup (n=8) ^a	n-3 FAD subgroup (n=8) ^b	p-value ^c	n-3 FAS subgroup (n=5) ^a	n-3 FAD subgroup (n=5) ^b	p-value ^c
n-6 PUFA						
Linolenic acid (18:2 n-6) ¹	8.37 ± 0.43 ¹	6.01 ± 0.32	<0.001	10.87 ± 0.11	8.74 ± 0.12	<0.001
Arachidonic acid (20:4 n-6) ¹	18.35 ± 0.55	21.79 ± 0.40	<0.001	18.12 ± 0.16	20.41 ± 0.08	<0.001
Osbond acid (22:5 n-6) ¹	1.24 ± 0.05	7.97 ± 0.24	<0.001	1.16 ± 0.04	4.13 ± 0.46	<0.001
Total n-6 PUFA ¹	31.39 ± 0.93	39.74 ± 0.68	<0.001	34.10 ± 0.13	37.87 ± 0.60	<0.001
n-3 PUFA						
Alpha-linolenic acid (18:3 n-3) ¹	0.06 ± 0.01	0.01 ± 0.00	<0.001	0.09 ± 0.00	0.01 ± 0.00	<0.001
Eicosapentaenoic acid (20:5 n-3) ¹	0.17 ± 0.01	0.03 ± 0.01	<0.001	0.24 ± 0.00	0.05 ± 0.01	<0.001
Docosahexaenoic acid (22:6 n-3) ¹	8.40 ± 0.24	2.14 ± 0.25	<0.001	8.36 ± 0.13	3.77 ± 0.55	0.003
Total n-3 PUFA ¹	9.20 ± 0.25	2.30 ± 0.25	<0.001	9.50 ± 0.20	4.01 ± 0.58	<0.001
n-6:n-3 PUFA Ratio						
Total n-6:n-3 ratio ¹	3.41 ± 0.04	18.32 ± 0.71	<0.001	3.60 ± 0.06	10.20 ± 1.76	0.033

^a Characteristics of infected groups on diet with normal n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^b Characteristics of infected groups on diet with low n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^c An independent T-test was used to determine differences between n-3 FAD and FAS subgroups

¹ Parametric data are expressed as mean ± SEM (all such values)

FAD: fatty acid deficient; FAS: fatty acid sufficient; n-3: n-3; n-6: omega-6

At five weeks post TB infection at euthanasia, both the intranasal (females) and aerosol groups (males) showed differences in all n-6 and n-3 PUFAs between the n-3 FAS and n-3 FAD subgroups. The n-3 FAD subgroups in the intranasal and aerosol groups presented with higher n-6 PUFA composition (AA, osbond acid, and total n-6 PUFA, all $p < 0.001$) compared to the n-3 FAS subgroups. Furthermore, the n-3 FAS mice in both subgroups presented with higher n-3 PUFA composition (ALA, EPA, DHA, and total n-3 PUFA, all $p < 0.001$, except DHA in aerosol group $p = 0.003$) compared to the n-3 FAD subgroups. Lastly, a lower total n-6:n-3 ratio was observed in both n-3 FAS subgroups compared to the n-3 FAD subgroups ($p < 0.001$ and $p = 0.033$, respectively) at the end of the study.

4.3 Markers of anaemia of infection

In order to evaluate markers of AI, the different iron (Fe) parameters included in the study objectives were measured five weeks post TB infection. Table 4-4 describes the characteristics of these markers for the different subgroups. Hepcidin data was available for the intranasal group, but not for the aerosol infected group (due to time constraints). At the end of the study, no differences were found in all markers of AI in any of the groups. However, there was a greater decreased amount in Hb in the aerosol infected FAS subgroup from baseline to end ($p = 0.027$), compared to the aerosol infected FAD subgroup (Figure 4-1).

Table 4-4: Markers of anaemia of infection of all groups five weeks post tuberculosis infection at euthanasia

	Intranasal group (Females)			Aerosol group (Males)		
	n-3 FAS subgroup (n=8) ^a	n-3 FAD subgroup (n=8) ^b	p-value	n-3 FAS subgroup (n=5) ^a	n-3 FAD subgroup (n=5) ^b	p-value
Haemoglobin, g/dL ^{c,1}	16.37 ± 0.14	16.08 ± 0.35	0.443	13.20 ± 0.46	12.32 ± 0.30	0.148
Serum ferritin, ng/ml ^{d,1}	91.62 ± 18.77	96.38 ± 18.58	0.860	57.14 ± 1.06	52.65 ± 5.26	0.447
Transferrin receptor, ng/ml ^{d,1}	42.96 ± 9.95	49.58 ± 10.35	0.652	15.91 ± 0.60	16.55 ± 2.36	0.803
Hepcidin, ng/ml ^{d,2}	5452 ± 2926	8179 ± 3754	0.486	-	-	-

^a Characteristics of infected groups on diet with normal n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection ^b Characteristics of infected groups on diet with low n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^c Difference between groups was determined with ANCOVA, correcting for baseline haemoglobin concentrations

^d An independent T-test was used to determine differences between n-3 FAD and FAS subgroups

¹ Parametric data expressed as mean ± SEM (for all such values)

² Non-parametric data expressed as geometric means ± SEM (for all such values)

dL: deciliter; FA: fatty acid; FAD: fatty acid deficient; FAS: fatty acid sufficient, g: gram; n-3: n-3; ng/ml: nanogram per milliliter

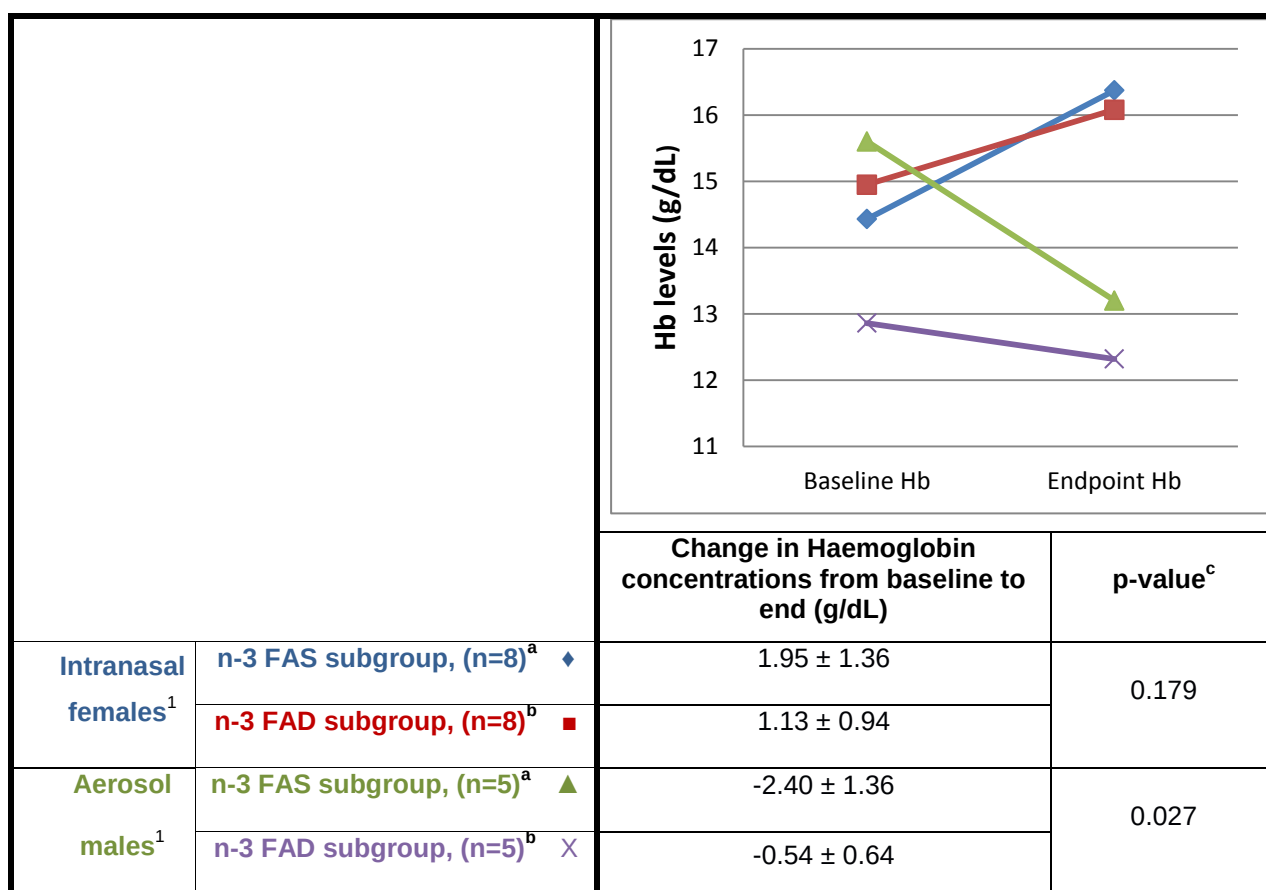


Figure 4-1: Change in haemoglobin from baseline to end

^a Haemoglobin concentrations of infected groups on diet with normal n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^b Haemoglobin concentrations of infected groups on diet with low n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^c An independent T-test was used to determine differences between n-3 FAD and FAS subgroups

¹ Parametric data expressed as mean ± SEM (all such values)

dL: deciliters; FAD: fatty acid deficient; FAS: fatty acid sufficient, g: gram; Hb: Haemoglobin; n-3: n-3

4.4 Food intake and other markers of morbidity

4.4.1 Food intake

Food intake was determined once a week over the course of five weeks post TB infection. Figure 4-2 shows the difference between the n-3 FAS and FAD subgroups in the intranasal and aerosol groups in terms of the food intake per mouse per day.

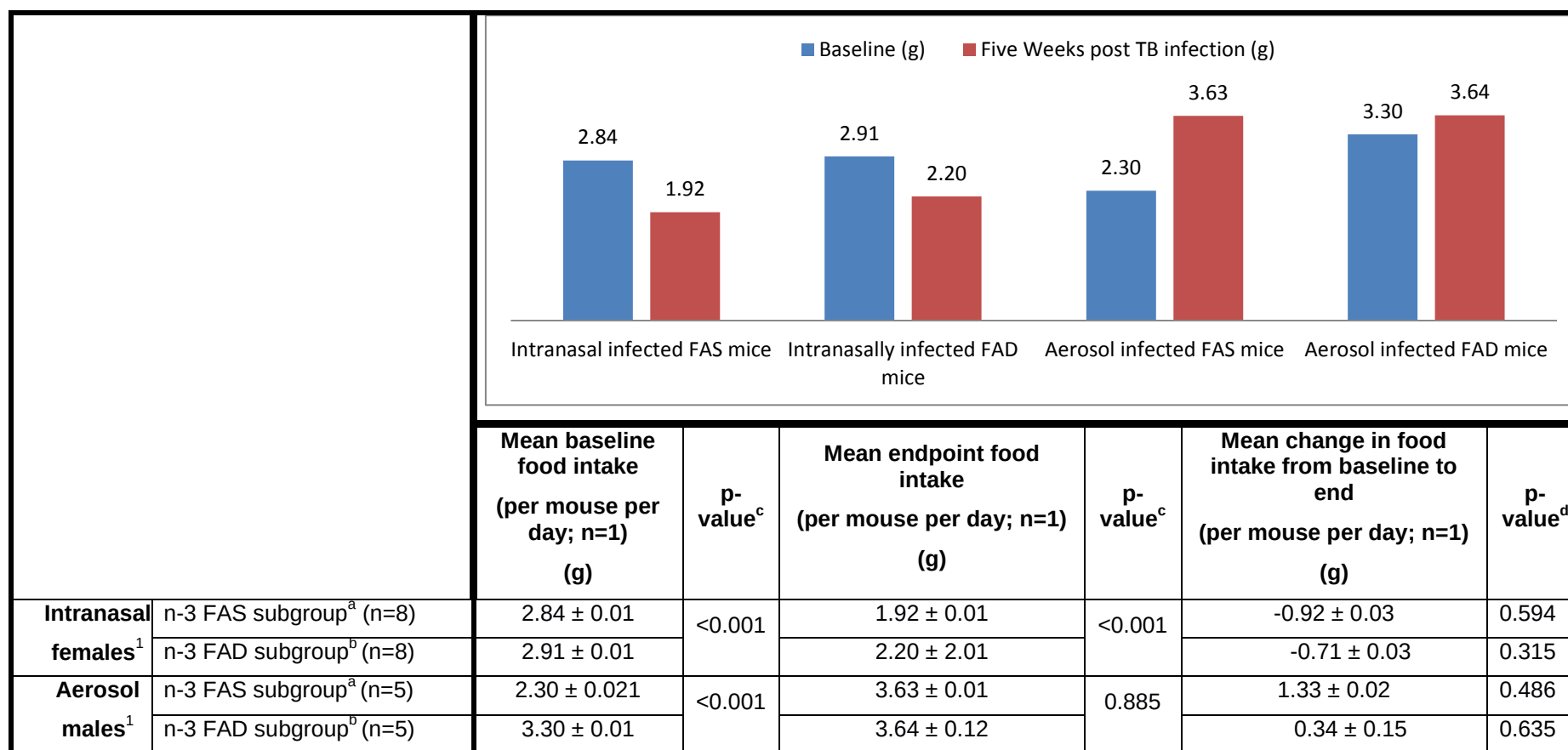


Figure 4-2: Food intake of the different groups at baseline and five weeks post tuberculosis infection

^a Food intake of infected groups on diet with normal n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^b Food intake of infected groups on diet with low n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^c An independent T-test was used to determine between-subgroup differences of n-3 FAD and FAS subgroups

^d A dependent paired sample t-test was used to determine the within-subgroup difference from baseline to endpoint

¹ Parametric data expressed as mean ± SEM (all such values)

FAD: fatty acid deficient; FAS: fatty acid sufficient, g: gram; n-3: n-3

At baseline, the n-3 FAS subgroup presented with lower food intake compared to the n-3 FAD subgroup in the intranasal infected group ($p < 0.001$). A similar result was observed in the aerosol group ($p < 0.001$). Again, at the end of the study, the intranasal n-3 FAS subgroup presented with lower food intake compared to the n-3 FAD subgroup ($p < 0.001$), but not in the aerosol group.

4.4.2 Other markers of morbidity (monitoring appearance and respiratory rate and measuring body weight change)

The appearance and respiratory rate of the mice were monitored daily over the course of the study. The researchers observed no difference in these markers in the n-3 FAS and n-3 FAD subgroups for both intranasal and aerosol infection. Furthermore, body weight change of the aerosol and intranasal infected groups were measured weekly. Figure 4-3 provides a visual representation of the changes in body weight for the different groups five weeks post TB infection. At baseline, as well as at the end of the study, the aerosol infected n-3 FAS subgroup presented with higher body weights (baseline, $p = 0.041$; endpoint, $p = 0.006$) compared to the n-3 FAD subgroup. No differences were observed in body weight changes between the four different subgroups from baseline (pre-infection) to end (five weeks post infection at euthanasia).

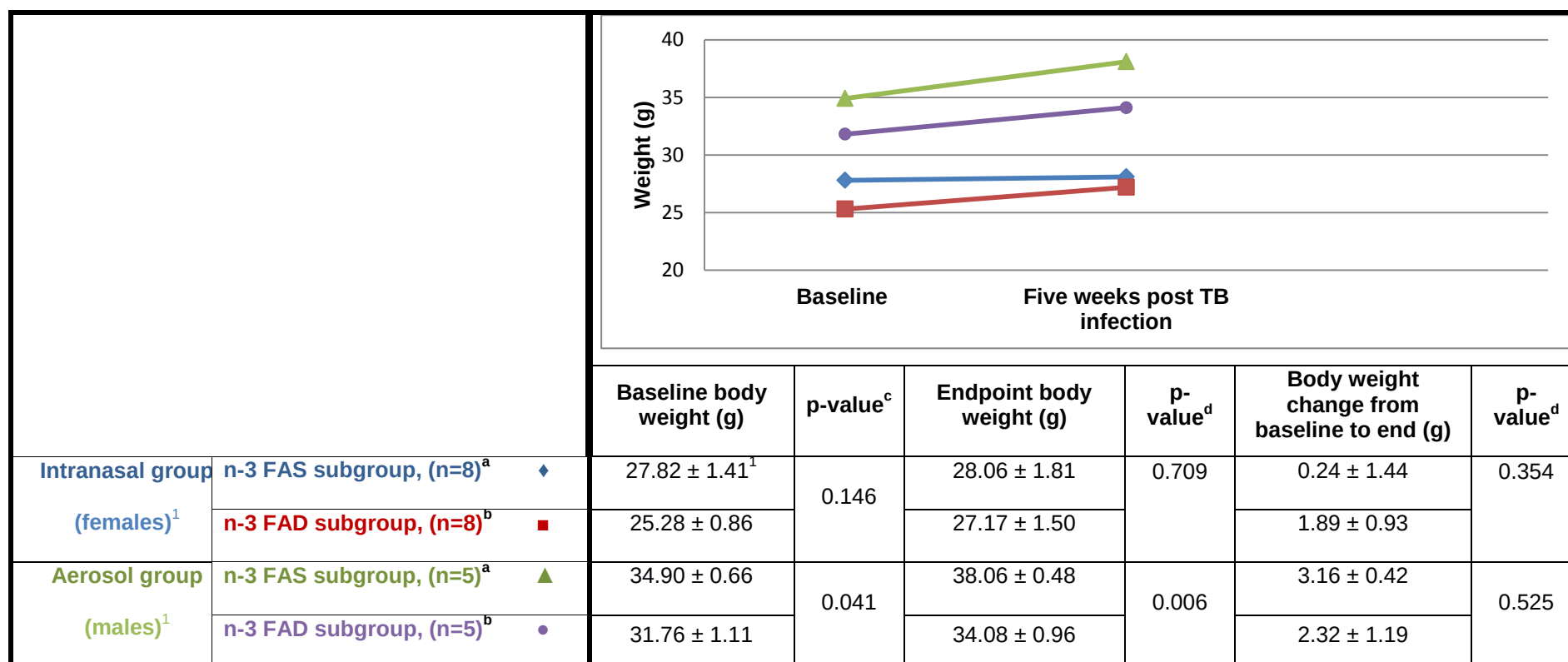


Figure 4-3: The mean body weights of the different groups at baseline and five weeks post tuberculosis infection

^a Characteristics of infected groups on diet with normal PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^b Characteristics of infected groups on diet with low PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^c To determine the difference between groups ANCOVA was used in which baseline body weight was corrected for

^d An independent T-test was used to determine between-subgroup differences of n-3 FAD and FAS subgroups

¹ Parametric data expressed as mean ± SEM (all such values)

FAD: fatty acid deficient; FAS: fatty acid sufficient, g: gram; n-3: n-3

4.5. Disease progression

In order to evaluate disease progression, lung bacillary loads, as well as lung- and spleen-body weight-indexes were measured five weeks post TB infection at euthanasia in all groups. Lung bacillary loads were estimated by the colony forming unit (CFU) assay, as described in Chapter 3. Furthermore, lung-body-weight-index was determined by dividing the lung body weight with the endpoint body weight of each mouse. This same formula was used for the spleen-body-weight-index (spleen body weight/ end body weight). Table 4-5 provides a summary of the lung CFUs, as well as the lung and spleen indexes for the different mice groups.

Table 4-5: Lung bacillary loads and organ indexes five weeks post tuberculosis infection

	Intranasal group (females)			Aerosol group (males)		
	n-3 FAS subgroup (n=8) ^a	n-3 FAD subgroup (n=8) ^b	p-value ^c	n-3 FAS subgroup (n=5) ^a	n-3 FAD subgroup (n=5) ^b	p-value ^c
Log ₁₀ lung CFU ¹	6.10 ± 0.36	5.97 ± 0.43	0.512	6.03 ± 0.04	5.44 ± 0.13	0.008
Lung-body- weight-index ¹	0.02 ± 0.00	0.02 ± 0.01	0.823	0.01 ± 0.00	0.01 ± 0.00	0.388
Spleen-body- weight-index ¹	0.01 ± 0.00	0.01 ± 0.00	0.779	0.003 ± 0.000	0.002 ± 0.000	0.006

^a Characteristics of infected groups on diet with normal n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^b Characteristics of infected groups on diet with low n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^c An independent t-test was used to determine between-subgroup differences

¹ Parametric data expressed as mean ± SEM (for all such values)

CFU: Colony Forming Unit; FAD: fatty acid deficient; FAS: fatty acid sufficient, g: gram; n-3: n-3

Five weeks post TB infection, the aerosol n-3 FAS subgroup presented with higher lung CFU counts ($p = 0.008$) and higher spleen-body-weight indexes ($p = 0.006$) compared to the n-3 FAD. The lung-body-weight indexes (a surrogate indicator of inflammation), did not, however, show any differences between the n-3 FAS and n-3 FAD subgroups.

4.5.1 Inflammatory marker profiles

Cytokines were measured in the lungs five weeks post TB infection in the intranasal group (Table 4-6). Interleukin (IL)-12 showed a trend towards a lower concentration in the n-3 FAD subgroup ($p = 0.061$).

Table 4-6: Different cytokine concentrations of the n-3 fatty acid sufficient and n-3 fatty acid deficient subgroups of the intranasal infected mice

	Intranasal infection (females)		
	n-3 FAS subgroup	n-3 FAD subgroup	p-value ^c
Interleukin-1a, pg/ml ¹	2967 ± 376	3188 ± 555	0.747
Interleukin-1b, pg/ml ¹	367 ± 71	354 ± 82	0.907
Interleukin-2, pg/ml ²	32.65 ± 1.54	22.28 ± 1.34	0.361
Interleukin-3, pg/ml ²	10.42 ± 1.24	10.54 ± 1.16	0.758
Interleukin-4, pg/ml ²	10.92 ± 1.19	10.61 ± 1.16	0.889
Interleukin-5, pg/ml ¹	35.79 ± 6.13	25.65 ± 6.03	0.452
Interleukin-6, pg/ml ²	109.95 ± 1.57	164.48 ± 1.60	0.452
Interleukin-12, pg/ml ¹	51.45 ± 7.20	34.58 ± 3.57	0.061
Interleukin-17, pg/ml ¹	43.90 ± 6.87	38.06 ± 3.22	0.454
Monocyte Chemoattractant Protein-1, pg/ml ²	2268.82 ± 1.28	2409.91 ± 1.36	0.714
Interferon-gamma, pg/ml ¹	51.65 ± 17.53	64.25 ± 25.89	0.693
Tumor necrosis factor-alpha, pg/ml ¹	39.20 ± 6.94	37.30 ± 6.83	0.848
Macrophage inflammatory protein-1-alpha, pg/ml ¹	446 ± 70	446 ± 78	0.997
Granulocyte-macrophage colony-stimulating factor, pg/ml ¹	22.01 ± 4.32	22.64 ± 4.33	0.927

^a Cytokines of infected groups on diet with normal n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^b Cytokines of infected groups on diet with low n-3 PUFA content for six weeks prior to TB infection and continued with the same diet five weeks post TB infection

^c An independent t-test was used to determine differences between the n-3 FAD and FAS subgroups

¹ Parametric data expressed as mean ± SEM (for all such values)

² Non-parametric data expressed as geometric means ± SEM (for all such values)

FAD: fatty acid deficient; FAS: fatty acid sufficient; n-3: n-3; pg: picogram

CHAPTER 5 DISCUSSION

In this chapter, the effect of pre-infection n-3 polyunsaturated fatty acid (PUFA) status on all study objectives is discussed. Section 5.1 describes the changes in PUFA composition between the different diet groups (fatty acid sufficient (FAS) versus fatty acid deficient (FAD) after six weeks of diet conditioning (baseline), as well as at the end of the study (after 5 weeks of TB infection). Sections 5.2, 5.3, and 5.4 provide a detailed discussion about the effect of n-3 PUFA status on markers of anaemia of infection (AI) (Objective 1), markers of morbidity (Objective 2), and markers of disease progression (Objective 3), respectively.

The current study is unique in that it is the first report (to our knowledge) to address the role of n-3 PUFA status on AI and morbidity in an animal tuberculosis (TB) model. In order to successfully demonstrate these effects, we chose a TB mouse model (C3HeB/FeJ) that reflected the best combination with regard to practical aspects and pathophysiological similarities to the human lung (Riveira & Ganz, 2009). Our study results showed that a pre-infection n-3 PUFA deficient status resulted in lower haemoglobin (Hb) concentration before infection in lower-dose (aerosol) infected mice. Furthermore, after TB infection, the aerosol infected n-3 FAS subgroup presented with a greater decrease in Hb concentration, compared to the n-3 FAD subgroup. However, n-3 PUFA status did not affect other markers of AI (iron (Fe) status parameters, i.e. serum ferritin, transferrin receptor (TfR), and hepcidin), as well as markers of morbidity (monitoring appearance and respiratory rate and measuring food intake and body weight change). With regards to disease progression (lung bacillary load, organ indexes, and inflammatory marker profiles), differences were observed in lung bacillary loads and spleen-body weight-indexes between the aerosol infected n-3 FAS and FAD subgroups. Nonetheless, in contrast to what we hypothesised, the bacillary loads and spleen-body-weight indexes suggested that an n-3 PUFA deficient profile provided a more protective response, compared to a sufficient profile.

It has been shown that besides their health benefits, the anti-inflammatory and pro-resolving effects of post-infection treatment with n-3 PUFA may be beneficial in response to TB with regards to resistance, disease progression and bacillary load (Jordao *et al.*, 2008; Paul *et al.*, 1997). Due to the fact that TB is known to be a non-resolving inflammatory disease which causes lung damage in the host, we hypothesised that sufficient n-3 PUFA intake and availability would not only be beneficial in reducing inflammation during the course of infection, but also protect the host against the TB pathogen, and restore immune competence (Fullerton *et al.*, 2014; Serhan & Petasis, 2011). In addition, anti-inflammatory medication in the treatment of *Mycobacterium tuberculosis* (MTB) has also shown benefit with regard to the size and number of lung lesions, inflammatory response, bacillary load, and survival (Marzo *et al.*, 2014; Kroesen *et al.*, 2017; Vilaplana *et al.*,

2013). However, contrasting research has found that n-3 PUFA, when supplemented prior to TB infection, may have immunosuppressive effects resulting in an increased susceptibility and reduced ability of the host to control TB infection (Bonilla *et al.* 2010a; Bonilla *et al.* 2010b; McFarland *et al.*, 2008). These studies suggest that even though the anti-inflammatory effects of n-3 PUFA may benefit a number of inflammatory disorders, these same anti-inflammatory properties may be harmful when host immune response is needed to eradicate an invading pathogen such as in TB (Schwerbrock *et al.*, 2009). However, this experiment is the first to investigate the effects of normal, but sufficient n-3 PUFA intake versus a deficient amount, rather than providing supplementation (over and above what is physiologically required), in order to examine the effects of n-3 PUFA status on TB morbidity and AI.

5.1 Red blood cell total phospholipid polyunsaturated fatty acid composition

As described in Chapter 3, 8 to 12-week-old mice were included in this animal study and were conditioned on two different diets (n-3 FAS and n-3 FAD diets) for six weeks prior to TB infection. As would be expected, the n-3 FAD subgroups appeared to be normal and healthy with no additional phenotypic changes other than presenting with lower n-3 PUFA composition (alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and total n-3 PUFA) and higher n-6 PUFA composition (Arachidonic acid (AA), osbond acid, and total n-6 PUFA) compared to the n-3 FAS subgroup prior to TB infection (following six weeks on diet conditioning). The baseline PUFA of the n-3 FAD subgroups were comparable to those in other animal studies where mice were fed a deficient n-3 PUFA diet (Harauma *et al.*, 2010; Moriguchi *et al.*, 2001; Moriguchi & Salem, 2003; Moriguchi *et al.*, 2013). In addition, baseline PUFA in the n-3 FAS subgroups were also comparable to other animal studies where rodents were fed a sufficient n-3 PUFA diet (Baumgartner *et al.*, 2012; Fitsanakis *et al.*, 2009; Grill *et al.*, 2001). The reasoning behind including an n-3 FAD subgroup was to mimic the human situation, where a low n-3 PUFA intake and a resulting low n-3 PUFA status has previously been reported in low-income countries (such as South-Africa), as well as, in Western-type diets of developed countries (Richter *et al.*, 2014; Simopoulos, 2011; Stark *et al.*, 2016). We provided the n-3 FAD subgroups with n-3 PUFA deficient custom-prepared purified experimental diets (AIN-93), which also translated to insufficient n-3 PUFA intake in humans, as described in Chapter 3. In contrast, we provided the n-3 FAS subgroups with the n-3 FAS diet which contained enough n-3 PUFA to translate to what is reported to be sufficient intake in the human population (1-2% of total energy intake) (Flock *et al.*, 2013; Harris *et al.*, 2009). The same n-3 FAS and FAD diets used in this animal study have previously been used in other animal experimental studies to investigate sufficient intake, as well as n-3 PUFA deficiency in rodents (Baumgartner *et al.*, 2012; Fitsanakis *et al.*, 2009; Grill *et al.*, 2001; Harauma *et al.*, 2010; Moriguchi *et al.*, 2001; Moriguchi & Salem, 2003; Moriguchi *et al.*, 2013).

At the end of the study, the aerosol and intranasal infected n-3 FAS subgroups still presented with these higher amounts of ALA, EPA, DHA, and total n-3 PUFA compared to the n-3 FAD subgroups, as expected. Furthermore, all n-6 PUFAs (LA, AA, osbond, total n-6 PUFA, and total n-6:n-3 ratio) were affected, with the n-3 FAD subgroups presenting with higher amounts in all, except LA, compared to the n-3 FAS subgroups. With this in mind, it was evident in this experiment that the n-3 PUFA status was influenced by dietary intake, and the C3HeB/FeJ (Kramnik) model shows these changes in PUFA composition in red blood cell (RBC) membranes as a marker of dietary type-of-fat intake. The current study reported PUFA status in RBC, as the determination of RBC fatty acid status is a steady, long-term marker, considering that fatty acids are stable in the membranes of RBCs for up to three months (Hooper *et al.*, 2017). These findings agree with previous studies reporting effects if diets were fed from two to five or six weeks prior to the start of the experiment (Husson *et al.*, 2016; Jones & Roper, 2017). Furthermore, humans' manipulation of n-3 PUFA in the diet is also reported to influence RBC PUFA composition (Hooper *et al.*, 2017; Iizuka-Hishikawa *et al.*, 2017; Lauritzen *et al.*, 2016).

It is reported that different amounts of n-3 PUFA consumption and availability have different levels of action on immune function, as n-3 PUFA intake affects the immune cell membrane composition, thereby influencing inflammatory and immune outcomes (Fritsche, 2006; Malan *et al.*, 2014). Polyunsaturated fatty acid composition (n-3 versus n-6) has an effect on the structural and functional aspects of the cell membrane, as the released PUFA influences lipid mediator (LM) production to either be pro-inflammatory or anti-inflammatory and pro-resolving during infection (Calder, 2010). Therefore, by altering the PUFA composition of cell membranes of the mice, the goal was to investigate a non-pharmaceutical approach where the sufficient n-3 PUFA status in the n-3 FAS subgroups might have positively influenced anti-inflammatory and pro-resolving pathways compared to a deficient n-3 PUFA status, but with fewer side effects than anti-inflammatory medication.

With regard to inflammatory response, the n-3 FAD subgroups presented with higher baseline total n-6 PUFA content in their cell membranes and a higher n-6:n-3 ratio, together with the resulting higher AA content, which is the principal precursor for the production of pro-inflammatory LM (Calder, 2015; Serhan & Petasis, 2011). Therefore, it could be postulated that in TB infection, the n-3 FAD subgroup would have presented with a more pro-inflammatory LM profile (Hidaka *et al.*, 2015), whilst it is hypothesised that the n-3 FAS subgroup would have had a higher production of anti-inflammatory and pro-resolving LM (derived from n-3 PUFA) in order to protect the host against exaggerated inflammation caused by the TB pathogen (Croasdell, 2016; Kaufmann & Dorhoi, 2013). However, the effect of LM generation during TB infection was not addressed in this work and remains to be determined.

5.2 The effect of n-3 polyunsaturated fatty acid status on anaemia of infection

Anaemia of infection is reported to be the most common type of anaemias presented in TB patients (Lee *et al.*, 2006; Rivera & Ganz, 2009). Previous research showed that AI is associated with significant morbidity, as well as increased mortality rates during infectious diseases such as TB (Minchella *et al.*, 2014; Oliveira *et al.*, 2014), even though AI is a protective strategy for the host. As discussed in Chapter 2, MTB takes advantage of the inflammatory process, while thriving on Fe and using it as an essential nutrient to grow (Boelaert *et al.*, 2007). Excessive inflammation (induced by pro-inflammatory cytokines) ultimately leads to decreased oxygen-carrying capacity, as well as AI (Cherayil, 2010; Jonker & Van Hensbroek, 2014). The current study investigated whether a sufficient n-3 PUFA status would affect markers of AI (Objective 1), thereby contributing to reduced Fe depletion, and mitigated anaemia (and its resulting negative effects). With this in mind, markers of AI during TB infection include: i) normal to increased serum ferritin, ii) normal TfR, iii) increased hepcidin, and iv) decreased Hb (Oliveira *et al.*, 2014; Weiss & Goodnough, 2005).

According to Raabe *et al.* (2011), Hb levels below 13.6 g/dL can be considered anaemic for male mice, and values below 13.9 g/dL for female mice. The same researchers described the Hb parameter as being “the most direct and sensitive measure for detecting anemia” (Raabe *et al.*, 2011). Following diet conditioning, prior to TB infection, the n-3 FAD subgroup included for aerosol infection presented with a mean Hb of 12.9 g/dL. This subgroup could thus be considered as being anaemic, whilst this was not the case in the FAS subgroup, even though the two different diets that the mice were conditioned on for six weeks prior to TB infection had the same Fe content (differing only in fatty acid content) (Chapter 3). With this in mind, some studies have proved that higher n-3 PUFA status is associated with a lower risk of Fe depletion, that the availability of each nutrient may influence the other’s mechanistic performance, and that a deficiency of a combination of these nutrients might have additive effects on immune function (Clauss *et al.*, 2008; Jamieson *et al.*, 2013; Mutanen *et al.*, 2016; Ochoa *et al.*, 2016). It is suggested that n-3 PUFA consumption supports Fe status through the down-regulation of inflammatory processes in the body, and in this manner contributes to improved dietary Fe absorption (Jamieson *et al.*, 2013). The lack of n-3 PUFA (after six weeks of diet conditioning) in the n-3 FAD subgroup included for aerosol infection may have been the result of inflammation in the body, which caused lower erythropoietin and Hb synthesis (Jamieson *et al.*, 2013). However, this contradicts results from previous studies that indicated that n-3 PUFA caused reduced Fe status, decreased serum ferritin, as well as the development of Fe deficiency (Lukaski *et al.*, 2001; Miret *et al.*, 2003; Rao & Larkin, 1984; Van Dokkum *et al.*, 1983). It should be taken into account that these studies did not provide any evidence regarding the effect of n-3 PUFA on blood Hb. Moreover, the researchers focused on the effect of n-3 PUFA enriched diets/supplementation on Fe status, which could have had a U-shaped

result, as too little, but also too much n-3 PUFA may contribute to Fe deficiency (as demonstrated in Chapter 2). In addition, no differences in Hb were observed in the n-3 FAS and FAD subgroup included for intranasal infection.

The fact that the Hb prior to infection were only affected in the aerosol group may relate to them being males, as opposed to the intranasal group being females. Indeed, it is reported that factors such as gender can influence Hb (Murphy, 2014). Raabe *et al.* (2011) reported significantly higher Hb concentration in healthy female mice compared to male mice during weekly blood collection. In addition, male mice did not adequately recover from these weekly blood collections compared to females. Recovery was based on their change in Hb (a return to at least 13.6 g/dL for male mice and 13.9 g/dL for female mice), and was compared to the human definition of anemia, defined by the World Health Organization (WHO). Furthermore, one animal study was found where the researchers investigated the change in Hb throughout the lifespan of mice (Goulden & Warren, 1944); the researchers observed that female mice presented with higher Hb concentrations at all ages, compared to male mice, and the concentrations of the female blood pigments were higher at all stages.

Contributing to AI during TB infection is the overproduction of the pro-inflammatory cytokine interleukin (IL)-6, which causes increased production of hepcidin (the principle mediator during AI), and ultimately the internalisation and degradation of ferroportin (Rivera & Ganz, 2009). Therefore, with adequate n-3 PUFA status, an n-3 PUFA x Fe interaction during TB infection is accomplished, because the suppression of IL-6 (via n-3 PUFA derived LM) causes decreased production of hepcidin and consequently lessens AI. With this in mind, we hypothesised that the n-3 FAS subgroup compared to the FAD subgroup would have presented with a more favourable Fe profile (i.e. lower serum ferritin [as marker of inflammation – not of Fe storage], and lower TfR and hepcidin, and higher Hb) at the end of the study, due to the fact that n-3 PUFA, with its anti-inflammatory effects, would have caused a reduction in inflammation (IL-6) and lowered the up-regulation of hepcidin, thereby decreasing AI. However, hepcidin was not measured due to time constraints but would have been of value in this experiment.

A greater decrease was observed in Hb concentration from baseline to end in the aerosol infected n-3 FAS subgroup, compared to the n-3 FAD subgroup. These mice presented with a mean Hb of 15.6 g/dL at baseline. However, at the end of the study, the Hb of these mice decreased to below 13.2 g/dL, and the mice were therefore considered as being anaemic. With this in mind, the pro-inflammatory response and diminished capacity to resolve inflammation in the n-3 FAS subgroup may have contributed to decreased Hb after aerosol infection. No animal or human studies were found that investigated the effect of n-3 PUFA on Hb concentration after TB infection to support this. However, contrasting effects have been observed in other inflammatory diseases when

patients consumed n-3 PUFA. For example, Perunicic-Pekovic *et al.* (2007) studied the effect of n-3 PUFA on haemodialysis patients' nutrient intake and nutritional status at university hospitals in Serbia and Montenegro. The researchers reported that n-3 PUFA supplementation resulted in increased Hb concentrations in haemodialysis patients (mean age 55 ± 8 years) with premature atherosclerosis and presenting with AI. Conversely, Gharekhani *et al.* (2014) studied the effect of n-3 PUFAs on markers of AI in haemodialysis patients (mean age 56 ± 13 years) in Iran, and did not observe any difference in Hb between the n-3 PUFA supplemented group and the control group. It is important to take note of the fact that the n-3 FAD subgroup presented with lower bacillary loads and spleen-body-weight indexes after TB infection (Section 5.4), therefore the TB pathogen did not affect these mice as severely as the n-3 FAS subgroup who presented with higher loads and indexes. This could also be the reason why the n-3 FAS mice presented with a greater decrease (change) in Hb concentration from baseline to end.

Our data further suggested that pre-infection, n-3 PUFA status that was maintained throughout TB infection did not affect serum ferritin, TfR or hepcidin (only measured in the intranasal group), as no differences were observed in the n-3 FAS subgroups compared to the n-3 FAD subgroups in both aerosol and intranasal infected groups. The reason for findings not supporting our hypothesis may be that a threshold amount for EPA and DHA was perhaps not reached in the n-3 FAS mice in order to induce changes in the investigated markers as the diets were not supplemented with DHA and EPA, but only had normal or deficient amounts. This is related to the fact that EPA is a competitive precursor of AA for LM synthesis (Schmitz & Ecker, 2008; Serhan, 2017), and that DHA is reported to have a relatively low turnover (56-fold to 556-fold slower than all other PUFA turnover) (Metherel *et al.*, 2018). In contrast to our study, other studies investigated n-3 PUFA supplementation and not sufficiency, in response to TB infection in rodents and therefore provided a much higher amount, compared to our study (Bazinet *et al.*, 2004; Bonilla *et al.*, 2010a; Bonilla *et al.*, 2010b; Jordao *et al.*, 2008; McFarland *et al.*, 2008; Paul *et al.*, 1997). This may suggest that increased incorporation of EPA and DHA into cell membranes during infection (for the production of pro-resolving LM) may only occur when a higher, therapeutic amount of n-3 PUFA are given post-infection. Thus, n-3 PUFA supplementation after TB infection may cause increased incorporation of EPA and DHA into cell membranes, thereby inducing higher levels of anti-inflammatory and pro-resolving LM in response to TB, contributing towards decreased AI and its resulting negative effects (Jordao *et al.*, 2008; Paul *et al.*, 1997).

5.3 The effect of n-3 polyunsaturated fatty acid status on food intake and morbidity

Malnutrition during TB infection is a risk factor that is associated with early mortality (Sattler *et al.*, 2018). When infected with TB, the body begins to utilise increased energy in order to fight the pathogen, and in addition, TB infection leads to a decrease in appetite, macro- and micronutrient

malabsorption, and altered metabolism (Gupta *et al.*, 2009). This ultimately results in body weight loss, muscle wasting, and therefore, a poor nutritional status. (Sattler *et al.*, 2018). In the current study, food intake was measured on a weekly basis to evaluate the amount of food eaten by the animals (as described in Chapter 3). During the course of the infection, the different subgroups followed similar patterns regarding the change in food consumption from baseline to end. Therefore, no difference in food intake was observed between n-3 PUFA status subgroups, in neither the higher dose intranasal infected nor the lower dose aerosol infected mice, therefore, did not alter appetite in these animals.

Body weight loss can be seen as a crucial indicator of the extremity of TB infection in experimental animals, as well as humans (Lyadova *et al.*, 2010). For this reason, we monitored body weight loss on a weekly basis. Other markers of morbidity that were measured included appearance and respiratory rates. During the course of the study, we suspected that the intranasal infected mice would have shown body weight loss from baseline to end, due to the fact that these mice were infected with a higher dosage to present a more acute infection. Also, other studies found a decrease in body weights when mice were intranasal infected with high dosages of TB infection (Bouté *et al.*, 2017; Guler *et al.*, 2015). Due to excessive body weight loss (>20%) and severe morbidity signs, one mouse from the intranasal infected n-3 FAS subgroup was euthanised three days before the termination date. The remaining mice were euthanised at study week 5 (35 days post-infection), all with little change in appearance and respiratory rates, and body weight loss of less than 20%. Accordingly, it can be proposed that a sufficient pre-infection n-3 PUFA status did not impair nor improve markers of morbidity after TB infection (Objective 2) in this study, as morbidity and survival were similar in all groups. This study was, however, not designed to measure mortality, which may have been different between groups.

The TB morbidity sheet (used to monitor morbidity) was a modified monitoring sheet, using the Karnofsky score (Chapter 3) with humane endpoints where mice had to be euthanised when these were reached. The Karnofsky score was introduced by David A. Karnofsky and Joseph H. Burchenal in 1949, and was originally used to determine the functional status of hospital patients (Péus *et al.*, 2013). Ian Orme (Colorado State University) modified this score in order to assess animal morbidity (ANNEXURE C & D), however, the reliability and validity of this score for animals are yet to be determined. In our animal study, using the C3Heb/FeJ TB sensitive mice, the modified Karnofsky score seemed to be a limitation, as it was not sensitive enough to successfully monitor morbidity symptoms and the score did not reflect disease progression in the mice as required. The mice only started showing signs of morbidity right at the end of the study. For example, when the scoring sheet picked up the signs of morbidity in the early-euthanised mouse, it had to be euthanised within hours. Further research to improve the use of this score in animal experimental studies, and specifically for the C3Heb/FeJ mice, is thus needed.

5.4 The effect of n-3 polyunsaturated fatty acid status on disease progression

5.4.1 Lung bacillary load and organ indexes

As described in the literature and Section 5.1 above, it is evident that n-6 PUFAs lead to the production of pro-inflammatory LM, while anti-inflammatory and pro-resolving LM are derived from n-3 PUFAs (Hidaka *et al.*, 2015). In this experiment the goal was to manipulate these pathways in order to influence host inflammatory response against TB infection, thereby contributing to altered markers of disease progression (Objective 3). At the end of the study, lower bacterial loads ($\log_{10}$ colony forming units (CFUs)) were observed in the lungs of the low dose aerosol infected FAD subgroup, compared to the FAS subgroup. The results obtained were unexpected, as we hypothesised that n-3 PUFAs, with their anti-inflammatory effects, would have contributed to the resolution of inflammation, thereby addressing inflammatory lung tissue damage, and restoring immune competence (Divangahi *et al.*, 2013; Donoghue *et al.*, 2017; Fullerton *et al.*, 2014; Serhan, 2017; Serhan *et al.*, 2007). This would then result in lower bacterial loads in the FAS subgroup, which was not the case in our experiment. In addition to decreased bacterial loads, the aerosol infected n-3 FAD subgroup presented with lower spleen-body-weight-indexes compared to the n-3 FAS subgroup. These results suggest that splenomegaly (a sign of TB infection in the spleen) was less severe in the n-3 FAD subgroup (Lee & Hahn, 2017; Pottakkat *et al.*, 2010). An enlarged spleen may be the result of splenic lesions due to excessive inflammation caused by the TB infection (Gupta *et al.*, 2008). Therefore, an n-3 FAD diet with increased pro-inflammatory LM may contribute to better host immune response and control of the TB pathogen. Unfortunately, data for the bacterial loads in the spleens of the aerosol infected mice were not available, as one could speculate that higher MTB loads in the spleens of the n-3 FAS subgroup could, in fact, be a key event leading to higher spleen-body-weight indexes. Supporting this speculation, splenomegaly was more severe in another animal dietary (n-3 PUFA) TB study, when the spleens of the mice presented with higher bacterial loads (Paul *et al.*, 1997). We anticipated that splenomegaly would be more severe in the aerosol infected mice, as these mice were infected with a lower TB dosage to present a more chronic infection. The aerosol group was, therefore, more representative of TB progression inside the host with well-defined changes in the immune response. Differences between the n-3 FAD and FAS intranasal infected mice were not necessarily expected due to the fact that these mice were infected with a higher TB dosage, therefore the infection was more acute and subtle differences were masked by an acute host-response.

With regard to lower bacterial loads as well as lower spleen-body-weight indexes in the FAD subgroup, our results showed that n-3 PUFA deficiency may reduce disease progression, especially in a chronic TB model. The ability of the n-3 FAD subgroup to induce better bacterial killing (as evidenced by lower lung bacterial loads) during TB infection could be ascribed to the

higher n-6:n-3 ratio and higher n-6 PUFA content of cell membranes in these mice, leading to the production of pro-inflammatory LM. Supporting these results, Bonilla *et al.* (2010b) and McFarland *et al.* (2008) reported that n-3 PUFA enrichments led to increased bacterial loads in the lungs, as well as in spleens. These researchers suggested that n-3 PUFA causes the suppression of immune activation and decreased resistance to MTB through diminished activation and antimycobacterial responses in cells (Bonilla *et al.* 2010b; McFarland *et al.*, 2008). Furthermore, Jordao *et al.* (2008) suggested that EPA should be avoided during latent TB, due to the fact that an increased risk for disease reactivation might be observed with EPA consumption. The same researchers hypothesised that pro-inflammatory LM might have therapeutic effects against TB infection. It should be kept in mind that n-3 PUFA were supplemented in these studies prior to TB infection, whereas the current study focused on the effect of pre-infection n-3 PUFA status that was maintained during the course of TB infection. Nevertheless, the findings of this study also support these earlier studies, indicating that a lower n-3 PUFA status prior to and during infection may contribute to an enhanced immune response and better control of the bacteria under conditions of low-dose chronic infection.

5.4.2 Lung inflammatory marker profiles

Cytokines (inflammatory markers) are essential low molecular weight regulatory proteins that initiate effective macrophage killing activities and develop protective responses against TB infection (Bonilla *et al.*, 2010a). The immune response against TB is dependent on T-Helper cell activity (consisting of IL-12, interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α)) (Romero-Adrian *et al.*, 2015). As described in Chapter 2, TB infection takes advantage of the inflammatory process and causes the body to continually synthesise pro-inflammatory LM (in the latent and active states) (Dorhoi *et al.*, 2011). This leads to altered immune function, cell damage, disrupted T-Helper cell balance, and altered memory T-cell and antibody production (Kaufmann & Dorhoi, 2013; Lowe *et al.*, 2012; Walzl *et al.*, 2011). Alterations of these cells during TB infection cause insufficient clearance of the pathogen (Wan & Flavell, 2009).

We speculated that during the course of TB infection, IL-1-alpha (α), IL-1 β , IL-6, and TNF- α would have been affected in the intranasal infected mice, as these cytokines are highly involved in acute inflammation (Dinarello, 1991; George *et al.*, 2015; Ordway *et al.*, 2007). It was also postulated that an n-3 FAS diet during the course of TB infection would contribute to lower pro-inflammatory TNF- α , IL-1, IL-6, IL-17, whilst causing increased anti-inflammatory transforming growth factor beta (TGF- β) and IL-10. However, no differences were observed in any cytokines between the n-3 FAS and the n-3 FAD subgroups, but there was a trend towards lower pro-inflammatory cytokine IL-12 in the n-3 FAD subgroup compared to the n-3 FAS subgroup. Due to the fact that IL-12 is reported to be one of the factors in MTB-induced granuloma formation, a lack of this cytokine production may contribute to increased bacterial loads during TB infection (Sasindran & Torrelles, 2011). This

is supported by two studies who reported that IL-12 deficient mice showed increased bacterial loads, as well as increased mortality during TB infection (Cooper *et al.*, 1997; Flynn *et al.*, 1995). It can, therefore, be suggested that n-3 FAS may contribute to increased IL-12 production, contributing to better immune response (innate and adaptive) and survival following TB infection, as Interleukin-12 is responsible to mediate early cluster of differentiation 4 (CD4+) T-cell activation, polarisation and survival (Domingo-Gonzalez *et al.*, 2016; McNab *et al.*, 2014; Sasindran & Torrelles, 2011). However, the higher bacterial load in the FAS subgroup does not support this reasoning, and higher n-3 PUFA (brought upon by supplementation) that result in higher IL-12 production may be needed to support this statement.

Other studies that supplemented with n-3 PUFA found significant effects on cytokine production (Bhattacharya *et al.*, 2006; Jordoa *et al.*, 2008; Kang, 2007; Kesavalu *et al.*, 2007; Robinson *et al.*, 1996; Simopoulos, 2002). Interestingly, previous animal experimental studies observed reduced pro-inflammatory IL-6 production in *fat-1* transgenic mice. These mice are capable of producing n-3 PUFA from n-6 PUFA, causing higher n-3:n-6 PUFA ratio in their organs and tissues, without any dietary n-3 PUFA consumption (Bhattacharya *et al.*, 2006; Kang, 2007; Simopoulos, 2002). Previous n-3 PUFA dietary studies also showed decreased IL-6 during TB infection (Jordao *et al.*, 2008; Kesavalu *et al.*, 2007; Robinson *et al.*, 1996). As described in Section 5.3 above, the suppression of IL-6 can contribute to decreased production of hepcidin, ultimately causing reduced inflammation, as well as AI. Further research is needed to investigate whether n-3 PUFA supplementation (i.e. providing n-3 PUFA at higher dosages) show promising results by successfully reducing pro-inflammatory IL-6 as part of the inflammation resolving process during TB infection, thereby contributing towards decreased AI and its resulting negative effects.

Immunosuppressive effects have also been observed when n-3 PUFAs were supplemented prior to TB infection, resulting in decreased cytokine production, as well as reductions in T-cell activation, signalling and colony proliferation (Bonilla *et al.*, 2010a; Bonilla *et al.*, 2010b; Fan *et al.*, 2003; Jordao *et al.*, 2008; McFarland *et al.*, 2008; Meydani, 1996). It is suggested that because n-3 PUFAs are precursors for anti-inflammatory LM (lipoxins (LX), protectins (PD), and resolvins (Rvs)), these n-3 PUFAs could play a down-regulating role during macrophage response to MTB. In addition, anti-inflammatory cytokines induced by DHA are suggested to suppress macrophage functions (Bonilla *et al.*, 2010a; Bonilla *et al.*, 2010b). Lastly, the absence of AA-derived LM, for example prostaglandins (PGs) and leukotrienes (LTs) (due to the antagonism between n-3 and n-6 PUFA), in n-3 PUFA-treated cells may contribute to the observed phenotype, as these pro-inflammatory LM seem to play a role in control of TB infection (Mayatepek *et al.*, 1994).

Whether n-3 FAS supplementation may successfully impact cytokines (especially IL-12) as part of the inflammation resolving process during TB infection, remains to be confirmed by more research

including higher n-3 PUFA dosages and perhaps more animals. Moreover, it can be recommended to investigate the effect of n-3 PUFA status on inflammatory cytokine production during the course of aerosol infection (chronic infection), as these data were not available in the current study. It can be hypothesized that IL-12, TNF-beta (β), and IFN- γ would be impacted in the aerosol infected subgroups, as these cytokines play a significant role during chronic inflammation (George *et al.*, 2015; Manca *et al.*, 2001; Ordway *et al.*, 2007).

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CHAPTER 6 CONCLUDING REMARKS

The current chapter completes this mini-dissertation, based on the key objectives that have been identified. To provide a reference as to what was addressed in the research project, the research aim and objectives are repeated in Section 6.1. Section 6.2 describes the main findings emanating from this research project. Lastly, the limitations of this study, practical recommendations, as well as future research are discussed in Sections 6.3, 6.4, and 6.5, respectively.

6.1 Aim and objectives

6.1.1 Research aim

The aim of this experimental animal study was to determine the effect of pre-infection n-3 polyunsaturated fatty acid (PUFA) status on anaemia of infection (AI) and morbidity in tuberculosis (TB) infected mice.

6.1.2 Research objectives

The objectives of this study were to determine whether there was a difference between subgroups with a pre-infection n-3 PUFA sufficient or deficient status after TB infection, with regard to markers of:

1. AI (Fe status parameters i.e. serum ferritin, transferrin receptor (TfR), hepcidin, and haemoglobin (Hb));
2. morbidity (monitoring appearance and respiratory rate and measuring food intake and body weight change);
3. disease progression (lung bacillary load, organ indexes, and inflammatory marker profiles)

6.2 Main findings emanating from this research project

As specified in Chapter 2, host resistance to TB depends on the development of an effective host immune response. Additionally, nutrition is by all accounts one of the critical determinants of the quality of the innate as well as the adaptive immune response against TB infection (Escobar, 2009). Polyunsaturated fatty acids have been found to serve as structural membrane components, a source of energy, as well as regulators of the host immune response (Calder, 2015; Croasdell, 2016; Dennis & Norris, 2015). N-3 PUFA has

specifically been found to influence multiple aspects of the immune system and cause the resolution of inflammation (Calder, 2015; Serhan & Petasis, 2011). It is for this reason that we investigated whether this class of dietary fat has an effect on markers of AI, markers of morbidity, and markers of disease progression of TB.

6.2.1 Pre-infection n-3 polyunsaturated fatty acid status

In this study it was evident that fatty acid status was influenced by dietary intake, and that a sufficient n-3 PUFA diet results in higher alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and total n-3 PUFA content in RBC which reflects other cell membrane fatty acid composition, compared to an n-3 PUFA deficient diet, which causes higher arachidonic acid (AA), osbond acid, and total n-6 PUFA content prior to, as well as after (including higher linolenic acid (LA)) TB infection. These results mimicked the human situation, where low-income countries and people consuming Western-type diets ingest higher amounts of n-6 PUFA and lower amounts of n-3 PUFA (Richter *et al.*, 2014; Scaioli *et al.*, 2017; Simopoulos, 2011; Stark *et al.*, 2016).

6.2.2 The effect of n-3 PUFA status on anaemia of infection

It is well known that AI and Fe deficiency is highly prevalent and affects clinical outcomes of TB infected individuals (Minchella *et al.*, 2014; Oliveira *et al.*, 2014). It is especially challenging to improve Fe status and reduce anaemia as TB infection is known to be influenced by Fe availability, resulting in cautious supplementation in order to prevent increased morbidity and mortality rates (Jonker *et al.*, 2012; Jonker & van Hensbroek, 2014; Roth *et al.*, 2010). However, due to the fact that Fe status and anaemia affect clinical outcomes and the improvement thereof is problematic in settings where TB is prevalent (Roth *et al.*, 2010; Soofi *et al.*, 2013), it is worthwhile to investigate the possibility of a safer approach. In this experiment, the Hb status was lower in healthy mice that were fed an n-3 FAD diet for six weeks prior to infection. . In contrast, mice that were fed the n-3 sufficient diet for five weeks post aerosol route TB infection presented with a greater decrease in Hb status from baseline. Due to these contrasting results, and the fact that different effects on Hb concentrations (decreased and increased) have been observed in other inflammatory diseases when patients consumed n-3 PUFA, there is a need for further research on the effect of n-3 PUFA status on Hb concentrations after TB infection. Furthermore, it could not be demonstrated that a sufficient or deficient n-3 PUFA status provided a protective or a detrimental effect on the other markers of AI (serum ferritin, TfR, and hepcidin) (Objective 1).

6.2.3 The effect of n-3 PUFA status on morbidity

When investigating markers of morbidity, it was found that a sufficient pre-infection n-3 PUFA status that was maintained throughout TB infection did not improve markers of morbidity (monitoring appearance and respiratory rate and measuring body weight change) when compared to mice with a n-3 FAD diet in this experiment (Objective 2). The TB morbidity monitoring sheet used was a modified Karnofsky score. However, this score seemed to be a limitation as it was not sensitive enough to successfully monitor morbidity symptoms in the mouse model used, as signs of morbidity were only evident at the end of the study. The scoring sheet picked up the signs of morbidity only hours before mice had to be euthanised.

6.2.4 The effect of n-3 PUFA status on disease progression

It can be concluded that an n-3 PUFA deficient diet led to decreased disease progression in TB infection (Objective 3). This was demonstrated by lower lung bacterial loads (Log_{10} colony forming units (CFUs)), as well as lower spleen-body-weight indexes in the FAD subgroup of aerosol infected mice (the clinical picture of a chronic infection response to TB) when compared to mice fed a n-3 PUFA sufficient diet. Supporting these findings, other studies strongly indicate the impairment of host defense against TB infection when supplemented with dietary n-3 PUFA prior to TB infection (Bonilla *et al.* 2010a; Bonilla *et al.* 2010b; McFarland *et al.*, 2008). It is known that n-3 PUFA reduces the generation of pro-inflammatory LM (prostaglandins (PGs), leukotrienes (LTs) and lipoxins (LXs)) and increases anti-inflammatory and pro-resolving LM (protectins (PD), resolvins (Rvs) and maresins (MaR)) (Fullerton *et al.*, 2014; Hidaka *et al.*, 2015; Serhan & Petasis, 2011). With this in mind, research shows that pro-inflammatory PGs and LTs play crucial roles in controlling TB infection, which explains these findings (Levy *et al.*, 2005; Peres-Buzalaf *et al.*, 2011; Weiss & Schaible, 2015).

It is reported that n-3 PUFAs exert immunosuppressive effects, which result in decreased cytokine production in response to TB infection (Bonilla *et al.*, 2010a; Bonilla *et al.*, 2010b; Fan *et al.*, 2003; Jordao *et al.*, 2008; McFarland *et al.*, 2008; Meydani, 1996). However, the inflammatory profiles of the current study showed a trend towards a higher pro-inflammatory cytokine Interleukin (IL)-12 concentration in the n-3 FAS subgroup compared to the n-3 FAD subgroup (intranasal infection (acute infection)), suggesting that a higher pre-infection n-3 PUFA status may be associated with a improved inflammatory response to TB. Nevertheless, no other cytokines were affected by n-3 PUFA status in this experiment (Objective 3). Additionally, data was not available for the aerosol infected mice. More

research may, therefore, be needed to investigate the effect of n-3 PUFA on cytokine production in a more chronic TB infection. We speculate that after a low dosage of aerosol infection (chronic infection), the model would elicit less aberrant changes in the immune response, and therefore lead to clearer distinguishable results between groups.

6.3 Limitations of the research project

Based on our animal TB model and some dietary factors, this research project had some limitations. These limitations include the following:

6.3.1 The animal tuberculosis model (C3HeB/FeJ mice) used in this experiment:

- i. We used a TB morbidity monitoring sheet containing a modified Karnofsky score in order to monitor appearance, respiratory rate, and body weight. However, this score seemed to be a limitation as it was not sensitive enough to successfully monitor morbidity symptoms in this specific mouse model.
- ii. The C3HeB/FeJ mice have been extensively used as a TB model. However, this model has been reported to develop different types of lesions with regards to cellular composition, immunopathology, and control of bacterial replication, therefore resulting in heterogeneous bacterial loads, immune response, and mortality rates as a result of uncontrolled granulocytic response in the lungs (Irwin *et al.*, 2015). Even though the current mouse model is a remarkable TB-sensitive model, it may be suggested to use animal models that better reflect the homogeneity of lesion types after TB infection and thereby a possibly better or more sensitive modelling of the AI parameters.
- iii. Fatty acid status in red blood cells (RBCs) were reported, and not the lung PUFA status itself. Nevertheless, other studies observed significant changes in lung PUFA status when n-3 PUFA diets were fed for five to six weeks prior to chronic lung infections (Pierre *et al.*, 2007; Portal *et al.*, 2018). This methodology also relates closer to a human study. Red blood cells are commonly used as a proxy for other membrane fatty acid status in the human body due to the fact that the other tissues (e.g. lung and liver) cannot be measured as is the case in animals.
- iv. We used males in the aerosol infection group, and females in the intranasal infection group due to practical reasons. This can be seen as a limitation due to the fact that potential confounding factors of gender-specific hormones have been reported in previous studies of TB (Bini *et al.*, 2014; Neyrolles & Quintana, 2009). However, the

fact that different results may have been obtained if the aerosol group consisted of females cannot be excluded.

- v. The number of animals that we used could have been a limitation. It is reported that higher numbers can lead to statistical significance due to the fact that the C3HeB/FeJ mice react heterogeneously (Lenaerts *et al.*, 2015). However, previous research has observed significant results when using the same number of animals from this strain (Bouté *et al.*, 2017; Driver *et al.*, 2012; Irwin *et al.*, 2015; Marzo *et al.*, 2014; Vilaplana *et al.*, 2013).
- vi. We did not measure baseline serum ferritin, TfR, and hepcidin in all groups due to time constraints. Therefore, we could not conclude the effect of pre-infection n-3 PUFA status on the change of these single markers after TB infection (from baseline to end).

6.4 Recommendations

- i. In order to gain a better holistic picture of the effect of n-3 PUFA on disease progression, it can be recommended to include histopathology in the investigation, especially in the TB model that we used where lung lesions are similar to those found in humans (Rivera & Ganz, 2009).
- ii. Another recommendation can be to perform Reporter Enzyme Fluorescence on the infected animals. Reporter enzyme fluorescence allows a quick evaluation of lung bacillary loads from the infected mice for imaging or detection by fluorescence or bioluminescence (Sharan *et al.*, 2018). The same group of mice can be imaged multiple times, without having to euthanise the animals. This method can reduce costs, rapidly quantify bacteria, and permit novel observations during interactions between the host and the pathogen.
- iii. Lastly, the researchers observed a higher variability in the measured parameters when mice were infected intranasally compared to aerosol infection (as discussed in Chapter 3). Therefore, the aerosol infection route is recommended as a better technique to use as it has less variability and more closely resembles human exposure to the TB infection.

6.5 Future research

Additional research beyond the scope of this animal experimental study may be required to elucidate the following gaps in the available literature:

- i. Both male and female C3HeB/FeJ mice can be included for intranasal or aerosol infection in future research to evaluate gender-specific influences on the effect of n-3 PUFA after TB infection.
- ii. Further research for the use of the Karnofsky score (for morbidity monitoring) in our specific mouse model (C3HeB/FeJ) is needed, as the reliability and validity of this score for animals are yet to be determined.
- iii. Furthermore, the safety of n-3 PUFA supplementation together with TB medication can be studied (to establish any possible drug-nutrient interactions). These studies can be followed up with human intervention studies to suggest a nutrient-based supportive treatment in order to help reduce lung damage and improve outcomes in TB patients.
- iv. Reportedly we have the capacity to manipulate LM pathways (Divangahi *et al.*, 2013; Fullerton *et al.*, 2014). If future researchers can gain a better understanding of exactly how TB alters the regulation of these pathways, it may lead to a novel approach to intervening therapeutically. Also, the effect of LM generation on markers of disease progression (including histology) was not addressed in this work and remains to be determined.
- v. Lastly, due to the fact that there are many gaps in the available literature, future investigations to harness our knowledge about the exact mechanisms that underlie the relationship between Fe and TB are clearly needed. Further research on AI management and the treatment of Fe deficiency during TB may also be of great benefit.

The overall aim of this research project was to add to current literature to ultimately make a contribution to the better care and clinical outcomes of TB patients. In conclusion, the aim and objectives of this animal experimental study were reached. The findings of this research will contribute to current literature regarding the effect of pre-infection n-3 PUFA status on disease progression, AI and morbidity after TB infection. Our findings show that a sufficient pre-infection n-3 PUFA status, although exerting anti-inflammatory and pro-resolving actions, did not improve markers of AI and morbidity after TB infection in this experiment. However, with regard to disease progression, our results proved that an n-3 PUFA deficient status may, in fact, provide a protective role in the host immune response against TB infection. Future research emanating from this study would be to investigate the safety of n-3 PUFA supplementation together with TB medication, rather than normal sufficient status, on

disease progression, markers of AI and morbidity after TB infection. Also, to translate findings to the human situation, by investigating whether n-3 PUFA supplementation added to the standard treatment regimen would benefit TB patients.

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ANNEXURES

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



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Research Ethics Regulatory Committee

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ETHICAL CLEARANCE LETTER OF STUDY

Based on approval by AnimCare Animal Research Ethics Committee (AREC-130913-015) on 11/07/2018, the North-West University Research Ethics Regulatory Committee (NWU-RERC) hereby **approves** your study as indicated below. This implies that the NWU-RERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: The effect of pre-infection n-3 fatty acid status on anaemia of infection and morbidity in tuberculosis infected mice.

Study Leader/Supervisor: Dr L Malan

Student: M Britz

Ethics number:

N	W	U	-	0	0	1	5	3	-	1	8	-	S	5
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Institution Study Number Year Status

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

Application Type: Single study

Category: 4

Commencement date: 11-07-2018

Expiry date: 31-07-2018

Approval of the study is initially provided for a year, after which continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.

Special conditions of the approval (if applicable):

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 study leader (principle investigator) must report in the prescribed format to the AnimCare:
 - annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study, whereby a letter of continuation will be provided, 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.
- Annually a number of studies may be randomly selected for an external audit.
- The approval applies strictly to the proposal as stipulated in the application form. Should any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the AnimCare committee, 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.
- The date of approval indicates the first date that the study may be started.
- In the interest of ethical responsibility the NWU-RERC and AnimCare 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 AnimCare or that information has been false or misrepresented; and/or
 - the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,
 - new institutional rules, national legislation or international conventions deem it necessary.
- AnimCare can be contacted for further information or any report templates via Ethics-AnimCare@nwu.ac.za or 018 299 2197.

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

Yours sincerely

Prof. Tiaan Brink

Chair NWU AnimCare Research Ethics Committee

ANNEXURE B: Biological Safety Manual for the level P3 safety unit

		Biosafety Manual	
Title	Biosafety Manual for BSL 3 Unit		
Effective date	9 October 2017	Page no	Page 150 of 230

COMPILATION and AUTHORISATION

Action	Name	Designation	Signature	Date
Compiled by	Kobus Venter	Senior Laboratory Technician		
Reviewed by	Cor Bester	Head: Vivarium		
Authorised by	Prof AF Grobler	Director: PCDDP		

1. ABBREVIATIONS and/or DEFINITIONS

Abbreviation/ Definition	Description
Anteroom	A small room that separates TB isolator area from passage or outside
BSL	Biological Safety Level
BSC	Biological Safety Cabinet
HEPA	<i>High Efficiency Particulate Air</i>
IVC	Individually Ventilated Cages
MTB	<i>Mycobacterium tuberculosis</i> Bacillus
N/A	Not Applicable
PPE	Personal Protection Equipment
SOP	Standard Operating Procedure
SPF	Specific Pathogen Free
UPS	Uninterruptable Power Supply

2. PURPOSE

The purpose of this document is to explain the biohazards associated risks that are present at the BSL 3 Unit of the PCDDP and what facilities, equipment and procedures are in place to minimize the risks to an acceptable level.

3. SCOPE

The Biosafety Manual applies to all staff members and visitors that need to work within the BSL3 Unit of the PCDDP.

4. RESPONSIBILITIES

4.1 Vivarium Head / Designee

The Vivarium designee has the responsibility, authority and support for assessing risks, establishing policies and procedures, training personnel and maintaining the facility and equipment.

The Vivarium designee ensures appropriate assessment and reassessment of risks for research projects as needed.

The Vivarium designee also approves research personnel to work in the laboratory and assures that personnel are competent to conduct the work.

4.2 Research Personnel

All research h personnel engaged in BSL3 research must:

- complete the requirements detailed in the BSL3 personnel training for approval to work independently in the laboratory;
- ensure that all work is conducted in compliance with all applicable guidelines;
- learn the operating procedures for the laboratory, the potential hazards of the infectious agents in use and emergency procedures;
- help maintain the facility in good working condition;
- report to the Vivarium designee any medical restrictions, reportable illnesses, and any event that may be an exposure or result in the creation of a potential hazard;
- report any irregular laboratory conditions or accidents must be reported immediately to the Vivarium designee.

5. MATERIALS and EQUIPMENT

- Autoclave;
- Biological Safety Cabinet Class II;
- F10 (Active ingredient is Quaternary ammonium and biguanide compound) Veterinary Disinfectant products;
- Personal Protective Equipment (nitrile hand gloves, overshoes, hairnets, heat insulated gloves, safety goggles, 3M 1860/ N95 respirator and silverguard antimicrobial laboratory coats)

6. RISK ASSESSMENT FOR BSL3 UNIT

6.1 Hazards, associated risks and measures to reduce risks at BSL3 Unit

The following table lists hazards, describes risks associated with these hazards and indicates measures that are in place to prevent/ reduce risks.

HAZARD	RISK	SAFETY MEASURES
Biological Hazards		
MTB H37Rv (laboratory strain of MTB; considerable less virulent than clinical strains; fully susceptible to first line anti-TB drugs and F10 disinfectant)	Development of active disease (tuberculosis). Risk of infection is low if safety measures are followed. Can be effectively treated with first-line anti-TB drugs should infection occurs.	BSL 3 Facility for containment of MTB; Work with MTB in BSC II, PPE, BSL 2 & BSL 3 practices are followed; TB infected animals within IVC; F10 disinfectant; Autoclave; Incineration of MTB infected waste by accredited Bio-hazardous Waste Removal Company
<i>Clostridium tetani</i>	Development of tetanus if bitten by mice/ cuts by surgical instruments during dissection/ needle pricks during anaesthesia of mice. Risk of infection is low if vaccinated against tetanus and SPF animals are used.	All personnel working with animals are vaccinated against tetanus. SPF mice are used in experiments.
Hepatitis B virus	Development of Hepatitis B if in contact with human blood. Risk is low if vaccinated.	All personnel working in BSL3 unit are vaccinated against Hepatitis B

HAZARD	RISK	SAFETY MEASURE
Chemical Hazards		
Quaternary ammonium and biguanide compounds (active compounds of F10 Veterinary Disinfectant)	Can damage skin and eyes if used undiluted. No risk if use at recommended dilutions.	Use F10 products at recommended dilutions of manufacturer; eye wash station is available

HAZARD	RISK	SAFETY MEASURE
Ergonomic Hazards		
Poor posture (work in BSC II)	Lower back and neck pain; musculoskeletal disorders. Risk is medium to high.	Personnel receive training by supplier in correct posture; an adjustable chair is used when working with BSC
Lifting and moving of heavy objects (animal cages, bedding, water bottles, animal food)	Back injuries. Risk is medium to high.	Inform responsible personnel about risks involved if too heavy loads are handled.
Physical Hazards		
Heat exposure (autoclave)	(i.) Burns caused by handling loads immediately after sterilization, touching of hot surface of autoclave, steam that is released by exhaust or upon opening lid of autoclave after completion of sterilization/drying cycle; boiling liquid in bottles under pressure, or release of steam due to failure of doors/ lids; (ii.) cuts by broken glass due to explosion of bottles/ flasks (if caps are closed tight). Risks are low if required PPE is worn and correct procedures are followed.	Personnel are trained in correct procedures by following instructions in operations manual and SOP and are obliged to wear eye protection, heat insulated gloves and laboratory coat when operating autoclave.
Safety Hazards		
Confined space of anteroom	Bruises when moving objects in and out of facility. Risk is medium to high if one person is performing tasks.	Personnel are informed about risks when performing these tasks without assistance.
Electrical equipment	All electrical equipment in use at BSL 3 Unit poses risk of electrocution if not handled in accordance with operational manual. Risk is low if instructions are followed and training provided.	Personnel are obliged to follow instruction manuals; SOP's and receiving training in correct operation of electric equipment.

7.) MEDICAL RESTRICTIONS:

7.1) IMMUNOSUPPRESSIVE CONDITIONS:

People with immunosuppressive conditions, such as pregnancy, HIV/AIDS or who receive immunosuppressive therapy are at increased risk to become infected upon exposure to MTB and need to consider risks in consultation with healthcare professional before engaging in research involving exposure to MTB.

8 FEATURES OF PCDDP BSL3 FACILITY

- 8.1 The BSL3 Container Unit located at in the courtyard of the PCDDP Vivarium was designed, manufactured, erected and commissioned (in March 2017) by LIS (Laboratory Infrastructure Solutions). LIS is also responsible for scheduled maintenance to BSL3 Unit.
- 8.2 Only personnel authorized in writing by the director of the PCDDP have access to BSL3 laboratory. A lock and key system is fitted to main access door and authorized personnel must sign key out and in on key control log sheet. All users and visitors to the BSL 3 unit need to sign in and out on entering log sheet. Cargo doors (for movement of large furniture in and out of container) are locked and sealed.
- 8.3 Laboratory room is maintained under negative pressure by extracting air with extractor fan unit through a HEPA filter (that captures all potential air borne pathogens) without recirculation of air back to room.
- 8.4 A supply air fan unit condition air at specific relative humidity ($55 \pm 10\%$) and temperature ($22 \pm 2^{\circ}\text{C}$) levels before pumping air in succession through primary, secondary (pocket) and HEPA filters into room of Isolator unit.
- 8.5 Air supply and air extraction units as well as air conditioning system controls are supported by an UPS to prevent loss of pressure in the event of power failure until emergency power generator starts to provide power.
- 8.6 Air pressure within laboratory room is maintained at negative pressure of $-34 \pm 5\text{Pa}$ while air in anteroom is maintained at positive pressure of $10 \pm 5\text{Pa}$ to ensure unidirectional airflow from the outside to inside of laboratory.
- 8.7 An electronic interlock prevent simultaneous opening of outside and inside doors of the anteroom thereby preventing loss of negative pressure in containment area. A battery backup ensures that interlock is functional during power disruptions.

- 8.8 An audible and visible alarm is activated when there is pressure loss to the isolation room and users of isolator room are trained to cease activities and ensure rectification of condition before resuming activities in isolation room.
- 8.9 Interior wall and ceiling panels of container consist respectively of 38 mm polystyrene 79 mm polystyrene both enveloped in 0.58mm galvanised electrostatically coated metal sheeting; intersections of panels are sealed with non-shrinking sealant and panels can be cleaned by means of disinfectant and cloth. The isolator unit contains no windows.
- 8.10 Floors consist of monolithic polyurathane coved in corners and can be cleaned routinely by germicidal detergent.
- 8.11 A hand washing basin is located near exit of isolator unit that is operated by elbow.
- 8.12 The BSL3 isolator unit is furnished with a 85L vertical Hirayama autoclave for sterilization of all MTB contained material; an ESCO Class 2 BSC for working with MTB infected mice; a TECHNIPLAST IVC System consisting of a frame for 63 mouse IVC cages connected to Air Supply Unit (for housing of experimental mice), a 37°C incubator, a refrigerator (for keeping special mouse diet), a dunk tank and dry rack of stainless steel for disinfection of mouse cages, eye wash station, first aid kit and fire extinguisher.
- 8.13 All required mandatory, prohibitory, and informative signs are displayed on the outer and inner doors of the BSL 3 Isolator Unit.

9 PROCEDURES

9.1 Laboratory Access

a) Entry procedures

Entry into the facility is restricted to authorized individuals. Only individuals required for program or support needs are authorized to enter the facility while research is in progress. All authorized personnel are informed about hazards and correct procedures before entry is allowed.

- Obtain and sign for key to BSL3 Unit at custodian of key.
- Before entering the laboratory, check the reading of the magnehelic pressure gauge. If the BSL3 laboratory is positive to the anteroom do not enter the laboratory and notify the Vivarium designee.

- Upon entering the anteroom, sign the BSL3 Entry/Exit Log. Before walking into the laboratory, put on a, don gloves (2 pairs), shoe covers, hair net and 3M 1860/ N95 respirator.

b) Exit procedures

- Before leaving the laboratory, spray outer gloves with F10 Skin Prep before removal of all gloves, dispose gloves in biohazardous waste box for incineration.
- Wash hands with F10 Hand Scrub
- Enter the anteroom and make sure door is closed.
- Remove respirator and place within labelled ziplock bag, place silverguard antimicrobial laboratory coat on hook, dispose hair nets and overshoes in autoclavable bag.
- Sign the BSL3 Entry/Exit Log, exit anteroom, check all doors are closed and locked.
- Return key, sign key in at custodian of key.

9.2 PERSONAL PROTECTIVE EQUIPMENT

- Wear compulsory protective clothing/gear such as nitrile gloves N95 respirator, overshoes and hairnets and laboratory coat at all times while inside isolator unit.
- Wear safety goggles and heat insulated when handling hot autoclave or removing hot objects from autoclave;
- Keep long hair tied back or covered.

9.3 GENERAL PROCEDURES

- Mice are removed from, handled and returned to home cages within BSC II.
- Transfer mice to clean cages within BSC II.
- Transfer dirty bedding to autoclavable bags with heat sensitive tape, autoclave the latter for 20 minutes at 121°C and 1 atm and place autoclaved bags containing dirty bedding within biohazardous waste box for incineration.
- After removal of dirty bedding, spray mouse cages with F10SXD Veterinary Disinfectant/ Cleaner at dilution of 1:100 in water before submerging cages in dunk tank containing F10SC Veterinary Disinfectant at dilution of 1:100 in water for 15 minutes. Allow cages to dry, pack cages in plastic bags and transfer cages to washing room.

- When working with infectious sample material or animals, arrange with another lab partner to accompany you. This identified lab partner should be fully informed as well and should know what to do in case of a spillage or accidental exposure to the infectious substance.
- Disinfect and clean BSC II with F10 Skin Prep.
- After cleaning dispose of the blue nitrile laboratory gloves in the appropriate Bio hazardous waste container supplied in the Class II Biosafety Cabinet.
- The protection provided by work practice controls is based upon employee behaviour and attitude.
- Organize and plan work procedures with safety in mind and keep an uncluttered work space.
- Always make sure all necessary safety materials and exposure control equipment are available and in good working order.
- Prepare two response spill kits; one for placement outside the containment laboratory and one inside the laboratory. Each spill kit consists of spray bottle with F10SC Veterinary Disinfectant diluted 1:100 with water, Respirators (1 Box), Gloves (1 Box), Goggles (2 pairs), 4-6 disposable gowns, Dustbin and brush, Liquid Soap/ Detergent, 1 Paper Towel Roll and Bio-hazardous Bags and Sharps Container.
- Always remove personal protective equipment (such as gowns, gloves, boot covers) and wash hands when leaving the facility.
- Eating, drinking, smoking, applying cosmetics and lip balm, and handling contact lenses and using cell phones are prohibited in the laboratory.
- Post the biohazard door sign on the access door to the facility. Ensure that any specific entry requirements (PPE and vaccination), the name of the agent, the Biosafety Level, and the name of an emergency contact person is posted on the sign.
- Label all equipment (e.g., incubators, freezers) used to store or ship infectious materials with biohazard warning labels and agent(s) name.
- Avoid the use of needles and other sharps whenever possible. Needles shall never be recapped, removed, sheared, bent or broken. Needles and syringes must be discarded promptly in a needle sharps container after use. For BSL3 experiments, this requires placement of the sharps containers within the biosafety cabinet.
- Avoid the use of glass items and use plastic alternative items.
- For transport to the autoclave, package contaminated materials in closed, durable, leak-proof containers or autoclavable bags.

9.4. MATERIALS TRANSPORTED TO AND FROM THE UNIT

- Transport infectious materials to and from the BSL3 facility in sealed leak-proof containment carriers inside labelled secondary containers, provided the transport is to another BSL3 facility.
- Open containers of infectious materials and handle infectious materials within BSC II.
- Decontaminate/ sterilize all items before leaving the BSL3 facility.
- Decontaminate all contaminated laboratory wastes and equipment before disposal or washing. Store contaminated materials that can be autoclaved in closed, leak-proof containers containing suitable disinfectant solution. Allow an appropriate length of contact time before removal from the laboratory.

9.5 WORK AREAS

- Decontaminate work surface of BSC II after any spills, contamination and at the end of every work session with F10SC Veterinary Disinfectant diluted 1:100 in water.

9.6 WASTE HANDLING

- All disposable waste that was not in contact with MTB are sprayed with F10 SC Veterinary disinfectant diluted 1:100 in water and placed in Biohazardous waste box for incineration by biohazardous waste removal company.
- All dirty animal bedding are first transfer to autoclavable bags labelled with heat sensitive tape within BSC before autoclaving for 20 minutes 121°C and 1 atm before placing in biohazardous waste box for incineration by biohazardous waste removal company.
- Liquid solutions containing MTB are transferred to Schott bottles labelled with heat sensitive tape and autoclaved for 1 hour at 121°C and 1 atm before transferring to liquid waste containers for incineration by biohazardous waste removal company.
- Carcasses, animal tissue, blood etc. are sprayed with F10SC Veterinary Disinfectant and transferred to anatomical waste containers that are stored within freezer containing biohazard signal until collection for incineration by biohazardous waste removal company.
- For treatment of waste other than biological waste refer to Waste Management SOP (SOP_BSL3_Waste_1).

9.7 EMERGENCY PROCEDURES FOR EXPOSURE INCIDENTS:

An "exposure incident" is specific contact (eye, mouth, other mucous membrane, non-intact skin, percutaneous or aerosol exposure) to potentially infectious materials that results from the performance of an employee/researchers duties.

An employee/researcher who sustains a known or potential "exposure incident" must wash the area immediately with soap and water or immediately leave the laboratory in the event of an aerosol exposure. This incident must be immediately reported to the BSL 3 safety officer.

9.7.1 Percutaneous Injury Response

Wash well with F10 Skin Prep disinfectant or F10 Hand Scrub and water for 15 minutes.

9.7.2 Face Splash Response

Flush affected area in eye wash for 15 minutes. If an eyewash is not immediately available use another source of clean potable water and follow up with a full 15 minute eyewash as soon as feasible.

NOTE: If the accident generates an aerosol (i.e. spill outside the biosafety cabinet) leave the area promptly and follow the response procedures at a safer location. Identify a back up location prior to initiation of work.

9.7.3 Aerosol Exposure

9.7.3.1 Prevention

Homogenization

- The homogenization of tissue must take place in a closed system, namely the TissueLyser II, Qiagen, within the Class II Biosafety cabinet. This system allows for the homogenization to be done within a closed Eppendorf tube in a closed container.
- Care should be taken to only open the Eppendorf tubes in the Class II Biosafety cabinet following homogenization.
- The machine and all equipment used for homogenization must be decontaminated after use (See SOP_BSL3_Decontamination_1).

Pipetting

- Mouth pipetting is not permitted; use mechanical or electronic pipettes.
- All pipetting should be conducted within the Class II Biosafety cabinet.

- Filtered tips should be used when pipetting.
- Pipetting should be conducted with autoclavable pipets.
- Pipets should be autoclaved after pipetting and tips discarded in the correct waste container (See SOP_BSL3_Waste_1).
- Eppendorf tubes should be closed whilst still inside the Class II Biosafety cabinet and decontaminated prior to removal and storage (See SOP_BSL3_Decontamination_1).

Centrifuging

- All centrifuging should be conducted within the Class II Biosafety cabinet.
- All centrifuging should be done in a sealed safety cup, which should only be opened inside the Class II Biosafety cabinet.
- The caps of the bucket and Eppendorf tubes must be closed properly prior to centrifuging.
- A wide nonporous seal should be used to ensure that the lid is closed tightly and the lid must not be opened until the rotor has stopped completely.
- Decontaminate centrifuge buckets by soaking them in a suitable disinfectant after use.
- Centrifuges should be placed towards the rear end of the Class II Biosafety cabinet.

9.7.3.2 Response

- Hold breath and immediately leave the room. Quickly remove outer gloves before leaving and drop gloves on floor before entering the ante room.
- Remove PPE carefully: turn exposed areas inward and place in a biohazard bag.
- Wash hands well with soap and water. Also wash any exposed skin with F10 Skin Prep disinfectant soap and water. Remove contaminated personal clothing and place in a separate biohazard bag.
- Post biohazard spill sign and record time of spill on sign. Lab should be evacuated for at least 30 minutes.

10. ADDENDUMS

ADDENDUM A: WHO Tuberculosis laboratory biosafety manual, 2012

ADDENDUM B: WHO Laboratory biosafety manual, 2004

11. DOCUMENT HISTORY

Date	Issue no	Reason for revision
	Draft 1	First draft of safety manual based on initial process
	1	First safety manual issued

ANNEXURE C: Daily monitoring sheet: appearance and respiratory rate

TB INFECTED MICE LOG SHEET FOR DAILY ASSIGNMENT OF KARNOFSKY HEALTH CHECK SCORES

DATE _____

Select score for each health indicator by circling appropriate score using the KARNOFSKY Score Key for assignment of Health Check Scores

ID	Ear Map	(A.) Appearance				(B.) Breathing					Total Score	Time	Notes
Intranasal													
1.1		0	1	2	3	0	1	2	3	4			
1.2		0	1	2	3	0	1	2	3	4			
1.3		0	1	2	3	0	1	2	3	4			
1.4		0	1	2	3	0	1	2	3	4			
1.5		0	1	2	3	0	1	2	3	4			
1.6		0	1	2	3	0	1	2	3	4			
1.7		0	1	2	3	0	1	2	3	4			
1.8		0	1	2	3	0	1	2	3	4			
2.1		0	1	2	3	0	1	2	3	4			
2.2		0	1	2	3	0	1	2	3	4			
2.3		0	1	2	3	0	1	2	3	4			
2.4		0	1	2	3	0	1	2	3	4			
2.5		0	1	2	3	0	1	2	3	4			
2.6		0	1	2	3	0	1	2	3	4			
2.7		0	1	2	3	0	1	2	3	4			
2.8		0	1	2	3	0	1	2	3	4			
Aerosol													
3.1		0	1	2	3	0	1	2	3	4			
3.2		0	1	2	3	0	1	2	3	4			
3.3		0	1	2	3	0	1	2	3	4			
3.4		0	1	2	3	0	1	2	3	4			
3.5		0	1	2	3	0	1	2	3	4			
4.1		0	1	2	3	0	1	2	3	4			
4.2		0	1	2	3	0	1	2	3	4			
4.3		0	1	2	3	0	1	2	3	4			
4.4		0	1	2	3	0	1	2	3	4			
4.5		0	1	2	3	0	1	2	3	4			
HEALTH SURVEILLANCE PERFORMED BY:													
Name:							Signature:					Date:	

ANNEXURE D: Monitoring sheet: body weight

TB INFECTED MICE LOG SHEET FOR DAILY ASSIGNMENT OF KARNOFSKY HEALTH CHECK SCORES

DATE _____

Select score for each health indicator by circling appropriate score using the KARNOFSKY Score Key for assignment of Health Check Scores

ID	Ear Map	Body Mass	(A.) Appearance				(B.) Breathing					(C.) Loss in Body Mass					Total Score	Time	Notes
Intranasal																			
1.1			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
1.2			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
1.3			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
1.4			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
1.5			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
1.6			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
1.7			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
1.8			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
2.1			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
2.2			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
2.3			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
2.4			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
2.5			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
2.6			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
2.7			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
2.8			0	1	2	3	0	1	2	3	4	0	1	2	3	4			
Aerosol																			
3.1																			
3.2																			
3.3																			
3.4																			

ID	Ear Map	Body Mass	(A.) Appearance				(B.) Breathing					(C.) Loss in Body Mass					Total Score	Time	Notes
3.5																			
4.1																			
4.2																			
4.3																			
4.4																			
4.5																			

HEALTH SURVEILLANCE PERFORMED BY:		
Name:	Signature:	Date:

ANNEXURE E: Weekly monitoring sheet: food intake

Date of measurement 1 _____

Date of measurement 2 _____

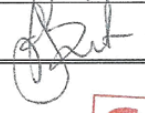
Cage number	Measurement 1	Measurement 2	Difference	Amount per mice per day (Food amount/ 3 or 4 mice/ 7 days)

ANNEXURE F: Standard Operating Procedure: Isoflurane anaesthesia of mice

 PCDDP DSTANWU Preclinical Drug Development Platform		Standard Operating Procedure	
Title Isoflurane anaesthesia of mice			
SOP no	SOP_Viv_Anim 4	Issue no	3
Issue date	23-06-2014	Revision date	24-06-2016
Effective date	25-06-2014	Page no	Page 1 of 4

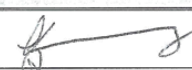
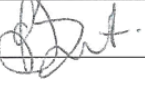
1. COMPILATION AND AUTHORISATION

COPY

Action	Designated Person	Signature	Date
Compiled by:	Mrs A Fick		20/06/2014
Authorised by:	Mr CJJ Bester		20-06-2014

Controlled Document

2. DISTRIBUTION

Department	Name	Signature	Date
PCDDP QA Manager	L Scholtz		24-06-2014
Head: Vivarium	CJJ Bester		24-06-2014

3. DOCUMENT HISTORY

Date	Issue no	Reason for revision
31-08-2012	Draft 1	First draft of SOP based on initial process
03-04-2013	Issue 1	First issue of SOP
14-02-2014	Issue 2	Addition of test principle
23-06-2014	Issue 3	Training Removed

PCDDP DST/NWU Preclinical Drug Development Platform		Standard Operating Procedure	
Title	Isoflurane anaesthesia of mice		
SOP no	SOP_Viv_Anim 4	Issue no	3
Issue date	23-06-2014	Revision date	24-06-2016
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4. POLICY and OBJECTIVE

Short term anaesthesia of small rodents is best achieved through the use of inhalation anaesthetics as the action of the anaesthetic agent is quickly reversible. This allows rapid recovery times as well as rapid induction times. This SOP establishes the techniques required to anaesthetise mice with Isoflurane.

5. SCOPE

This SOP establishes the techniques required to anaesthetise mice with Isoflurane for short surgical procedures (up to 90 seconds)

6. REFERENCE DOCUMENTS

- Mouse management – A practical guide to the care of laboratory mice, L M Scott editor, Office for Research, The University of Melbourne, 1991
- Use of isoflurane in a drop system. AE Goldenthal, Lab Animal, May 1992, Vol 21(5), 47-48
- SANS 10386:2008 The Care and Use of Animals for Scientific Purposes
- SOP_All_Wast 1 - Disposing of Hazardous and Biohazardous Waste

7. ABBREVIATIONS and/or DEFINITIONS

Abbreviation	Description
GLP	Good laboratory practice
N/A	Not applicable
SOP	Standard operating procedure
SANS	South African National Standard
MSD	Material Safety Data Sheet

8. RESPONSIBILITIES

This SOP must be followed by all people who have been assessed by the head of the Vivarium or designate, as fully competent to apply the procedure below following a period of training and experience. This SOP applies mainly to staff employed and researchers using the GLP facility. Staff in the process of gaining competence may undertake the procedure if supervised by a member of the GLP area who is fully competent in the procedure.

PCDDP DST/NWU Preclinical Drug Development Platform		Standard Operating Procedure	
Title	Isoflurane anaesthesia of mice		
SOP no	SOP_Viv_Anim 4	Issue no	3
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9. TEST PRINCIPLE

N/A

10. MATERIALS and EQUIPMENT

- Plastic container with lid
- Cotton wool
- 5ml syringe
- Isoflurane
- Timer

11. SAFETY

11.1. Required Qualifications

- a) All personnel performing anaesthesia must be appropriately trained and the training recorded.

11.2. Required Safety Equipment

- a) Isoflurane should only be used in a properly ventilated room, preferably in a fume cupboard.

11.3. Exclusions

- a) Isoflurane anaesthesia must not be performed by or in the presence of a pregnant woman.

11.4. Reagents and Chemicals

- a) All chemicals, etc. must be handled appropriately and not in contradiction of any MSD sheet notification.

11.5. Hazards

- a) All material in contact or consisting of biological materials must be disposed of in an appropriately labelled biohazards container. (SOP_All_Wast 1)

12. PROCEDURES

- 12.1. The animal's identity is verified by examining its tattoo, dyed marking, ear notching or other identification.
- 12.2. Gather all necessary equipment, and wash hands and put on gloves prior to the onset of the procedure.
- 12.3. Approximately 5 ml of isoflurane is poured onto a cotton wool pad and placed inside the container beneath a wire platform. The animal is placed inside the container

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and the lid closed. The mouse will stay in the container until it loses its righting reflex and remains immobile (approx. 60 to 90 seconds; the use of a timer is recommended). The animal can now be removed from the jar and the procedure carried out. Anaesthesia will persist for 30 to 90 seconds; full recovery of consciousness will take a few minutes.

12.4. The most important requirement for successful anaesthesia using volatile anaesthetics is careful observation of the mouse during induction. It is therefore mandatory to have two people performing the roles of anaesthetist and surgeon. The anaesthetist is designated to monitoring the depth of anaesthesia.

12.5. A record should be maintained of volumes withdrawn from the bottle. This record may be in the form of an adhesive label attached to it.

13. ADDENDUM

N/A

ANNEXURE G: Aerosol infection of mice with *Mycobacterium tuberculosis*

Because the natural route of tuberculosis infection in humans is the lung, the most precise animal models try to mimic this route. Intranasal or intratracheal inoculation of mice can give rise to pulmonary infection, but the most reproducible technique is to generate an aerosol that is inhaled by the animal.

Animals are exposed to *M. tuberculosis* using bacterial stocks with established cfu titers and specific lot numbers (see Support Protocol). Familiarity with the use of the inhalation exposure system (IES) used to infect mice is important to the success of this procedure. The procedure described below provides detailed instructions for using the inhalation system to infect mice with *Mycobacterium*.

CAUTION: All airborne infections of animals using *Mycobacterium tuberculosis* must be performed in an appropriate ABL-3 laboratory.

Materials

Class II Biosafety cabinet

Mice or guinea pigs to be infected

1.5-ml frozen stock suspensions of *M. tuberculosis* (5.0×10^7 to 5.0×10^8 cfu/ml)

5% (v/v) Iysol (VWR)

70% (v/v) ethanol

Sterile H₂O

7H10 or 7H11 agar quadrant petri plates (Fisher)

Laboratory glassware detergent (VWR)

Pan sonicator (Branson)

Absorbent hood blotters with plastic backing (VWR)

Paper towels

Disposable surgical gown (VWR)

Stainless steel pipet boat with cover (VWR)

1-cc disposable tuberculin syringes fitted with 26½-G needles (VWR)

Sharps container (VWR)

50-ml conical polypropylene centrifuge tube (Fisher)

12 × 125-mm culture tubes with caps

1.0-ml disposable serological pipets (Falcon)

Resealable polypropylene bags

Middlebrook inhalation exposure system (IES; Glas-Col model 099C A4212)

RACAL AC3 or PAPR or Breathe Easy respirator unit (Lab Safety Supply)

Glass nebulizer-venturi (Glas-Col), sterilized and wrapped

10-cc disposable syringes fitted with 18-G needles (VWR)

Stainless steel pan and cover, of sufficient size to accommodate nebulizer-venturi (VWR)

Infection baskets (Glas-Col), sterilized

Prepare mycobacterial aerosol suspension

1. Remove a vial containing the mycobacterial species of interest, and gently thaw at room temperature by placing in water in a pan sonicator. Thaw completely.
2. Turn on the sonicator for 10 to 15 sec to disperse any clumps of mycobacteria that may have formed during freezing. Vortex momentarily to further disrupt any clumps of bacilli.
3. Disinfect the work surfaces of a Class II biosafety cabinet using 5% Lysol followed by 70% ethanol. Place all cleaning materials into an autoclave bag for proper disposal. Place absorbent hood blotters with plastic backing on the work surface of the cabinet and soak with 5% Lysol. This is done to minimize/neutralize any spills or splatters that occur during manipulations with mycobacteria.
4. Fill a stainless steel pipet boat with 5% Lysol to a 1.0- to 2.0-cm depth and place toward the rear of the work surface. Place a sharps container to the side to accommodate syringes and needles.
5. Place the vial in the cabinet, disinfect the top of the butyl rubber septum with 70% ethanol.
6. Carefully remove the mycobacterial suspension from the vial with a 1-cc tuberculin syringe fitted with a 26½-G needle and expel back into the vial. Repeat back and forth into the vial ten to twenty times without removing the needle to mix the suspension and break up any small clumps of bacilli.

NOTE: *Tuberculin syringes that have a pre-attached needle are highly recommended.*

7. Remove the desired volume of mycobacterial suspension with the syringe and carefully inject down the side of a 50-ml conical polypropylene centrifuge tube containing sterile water so that the final volume is 5 ml and the final dilution results in the desired working titer

(cfu/ml) for use in the IES. Place the syringe in the sharps container for disposal. The volume of mycobacterial suspension depends on the numbers of animals to be exposed. The exposure chamber basket can accommodate ~125 mice or 10 small (500-gram) guinea pigs. The basket has five equal compartments for preparation of experimental groups of animals (25 mice or 2 guinea pigs per compartment). The number of animals (animals per group and number of time points following aerogenic challenge) depends on experimental design. In most experiments involving the infection of mice with *M. tuberculosis*, the standard challenge dose is 50 to 100 cfu deposited in the lungs. Guinea pigs, due to their inherent susceptibility to tuberculosis, routinely receive a challenge dose of 10 to 50 cfu (sometimes even lower). To result in an uptake of 100 cfu in mice, the mycobacterial suspension should be at 2.0×10^6 cfu/ml in a total volume of 5.0 ml sterile water (1.0×10^7 cfu total, or five logs higher than the anticipated cfu delivery). About 80% to 100% (4 to 5 ml) of this suspension should be nebulized over the 40-min cycle. The preparation of mycobacterial suspensions for guinea pigs also follows the five-log algorithm, but the duration of the aerosol exposure is shortened 5 min and only 0.5 to 1.0 ml of suspension is delivered through the nebulizer at completion of the aerosol exposure. As guinea pigs are more susceptible and have a larger lung volume than mice, they inhale a larger volume of air per unit time, and thus require a shorter exposure time.

Confirm titer of starting suspension

8. Assemble five sterile 12 × 125-mm culture tubes each containing 4.5 ml sterile saline. Label three 7H10 or 7H11 agar quadrant petri plates to give duplicate quadrants of six dilutions as follows: 100, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} . Label the tubes as 10^{-1} to 10^{-5} . Although steps 8 to 13 can be omitted, they are strongly recommended as a means of confirming the challenge dose loaded into the nebulizer.

9. Using a 1.0-ml sterile serological pipet, remove 0.7 ml working mycobacterial suspension (from step 1), add 0.5 ml to the first culture tube (10^{-1} dilution), and plate the remaining 0.2 ml onto the duplicate quadrants labeled 100. Discard the pipet into the stainless steel pipet boat containing 5% Lysol. Pipetting operations during the serial dilution process should be done with extreme care in order to prevent aerosol generation. Any small spills or drops during the pipetting process should be immediately disinfected with 5% Lysol.

10. Gently swirl the contents of the 10^{-1} dilution tube. Use a new 1.0-ml serological pipet to remove 0.7 ml, transfer 0.5 ml to the next culture tube (10^{-2}), and plate the remaining 0.2 ml onto the duplicate quadrants labeled 10^{-1} .

11. Repeat this process until the final 10^{-5} dilution has been plated.

12. Place plates upright in a resealable polypropylene bag and incubate in the dark at 37°C for ~3 weeks. After incubation mycobacterial colonies should become visible and countable (see step 37).

13. Disinfect the biological containment cabinet after use. Double bag culture tubes, stock vials, and the pipet boat in autoclave bags and tape with heat-sensitive autoclave tape.

Infect animals by exposure to aerosol

14. Unlatch and fully raise the acrylic exposure chamber lid of an IES and place the metal basket support/baffle plate and stainless steel mesh exposure basket into the chamber.

CAUTION: *A Racal AC3 or Breathe Easy respirator should be in use when operating the IES.*

15. Load mice or guinea pigs into the basket and secure the basket lid and cover assembly. Close the chamber lid and press firmly next to each latch until each one snaps with an audible click.

16. Mount a glass nebulizer-venturi to the compressed air and main air-flow fittings located on the front of the instrument. Secure the nebulizer into place with clamps provided, and check to make sure that all joints appear to be seated properly and make a sealed fit.

17. Carefully remove the screw cap above the venturi portion of the nebulizer unit. Inspect the cap for any wear, cracks, or missing gaskets.

18. With a 10-cc syringe fitted with an 18-G needle, carefully add 5 ml mycobacteria suspension into the bottom of the venturi. Discard the syringe into the sharps container. Replace the screw cap to the venturi, making sure it is threaded properly and fits tightly.

19. Turn the main power switch to the on position so it is lit, and wait for the control keypad to display the message "Glas-Col Apparatus Co."

20. Turn the UV lamp and program switches to the on position so they are also lit. The control keypad will display several messages and will then stop with the message "Enter When Ready 0".

21. Press the enter key on the control keypad. The "Enter Preheat Time 900" message will appear on the keypad display. This indicates that the IES has a preset preheat time of 900 sec (15 min) in which the incinerator used to decontaminate the exhaust air is allowed to raise its core temperature to 790°C before the nebulizing cycle begins.

21. Press enter on the keypad to accept the 900-sec preheat time setting. The next message is for the nebulizing time. The display will appear with the message "Enter Neb Time 1800," indicating a preset nebulizing time of 1800 sec.

22. Enter 2400 (40 min; for mice) or 600 (10 min; for guinea pigs) and press enter to set the nebulizing time. If an error is made, press the Esc key and reenter the correct

number. The display for the cloud decay cycle will appear next as “Enter C. D. Time 1800.”

23. Set 2400 sec (for mice and guinea pigs) or 3600 sec (for multi-drug resistant, or MDR, strains of *M. tuberculosis*) for the cloud decay cycle and press enter to continue. The final message to appear before the instrument begins operation is “Ent Dec Time 900.” This message sets the amount of time in which the UV lamps are turned on and the top surfaces of the basket are exposed to ultraviolet light at 254 nm for surface decontamination.

24. Press enter to accept the 900-sec decontamination time setting. Once the decontamination time is selected, the IES will begin to cycle sequentially through preheat, nebulizing, cloud decay, and UV decontamination settings for the aerosol challenge of animals within the chamber. The display on the keypad will indicate which cycle is in operation.

25. Once the instrument has started the preheat cycle, check to see that the main air flow meter indicates 60 cubic feet per hour (CFH). If it is not, adjust vacuum control valve until the flow reaches 60 CFH.

27. When the nebulizing cycle starts, check that the compressed air flow meter indicates 10 CFH. If not, adjust the compressed air control valve until the float in the flow meter indicates 10 CFH.

28. When the aerosol infection run is complete, turn off the program, UV lamp, and power switches on the IES. The instrument will signal the completion of the run with an audible alarm and the message “Process Complete - Remove Specimen” on the keypad.

29. Carefully remove the screw cap on the nebulizer and check the volume of remaining mycobacterial suspension with the aid of a 10-cc syringe fitted with an 18-G needle. Record the volume remaining. There should be no more than 1.0 ml remaining for infections with mice. Infections with guinea pigs should have 4.0 to 4.5 ml suspension remaining.

Disassemble and clean equipment

30. Discard the syringe in the sharps container. Remove the nebulizer-venturi carefully from the IES, wrap in paper towel, and place in a stainless steel pan containing 5% Lysol to a depth of ≥ 5 cm. Place the stainless steel pan in an autoclave bag and autoclave at 121°C and 15 psi for 40 min on slow exhaust.

31. Clean the nebulizer thoroughly with laboratory glassware detergent, rinse well, dry, wrap, and autoclave again to sterilize for future use. Disinfect the fittings where the nebulizer attaches to the IES by wiping with 5% Lysol followed by 70% ethanol. Place all

cleaning materials in autoclave bags and autoclave. Carefully open the lid, remove the animals from the chamber basket, and return them to their cages.

32. Remove the basket and its support/deflector from the IES chamber, double bag in very large autoclave bags, autoclave, and wash thoroughly with soap and water.

33. Disinfect the interior surfaces of the IES chamber by wiping them twice with 5% Lysol and twice with 70% ethanol. Bag and autoclave all cleaning materials (paper towels).

34. One day after aerogenic challenge, harvest a statistically significant number of aerogenically exposed animals and verify the uptake of mycobacteria into the lungs.

35. Count colonies on the quadrant plates ~20 days after incubation at 37°C (step 12) and calculate the initial titer of the suspension as follows: where MCCQ is the mean colony count of replicate countable plate quadrants, VP is the volume plated per quadrant, and DFQ is the dilution factor of the sample in the quadrant.

36. Calculate the mycobacterial load delivered by the IES as $\text{cfu} = \text{cfu/ml} \times \text{DFA} \times \text{VD}$, where cfu/ml is the titer (step 36), DFA is the dilution factor used to prepare the aerosol suspension (step 7), and VD is the volume delivered (5 ml minus the volume remaining in step 29).

BIBLIOGRAPHY

Ordway, D.J. & Orme, I.M. 2011. Animal models of mycobacteria infection. *Current protocols in immunology*, 94(1):19.15.

ANNEXURE H: Intranasal infection of mice with *Mycobacterium tuberculosis*

PREPARED BY:	DATE IMPLEMENTED:	SUPERSEDES SOP#
Linda Malan	May 2017	

REVIEW DATE:	REVISION DATE:	SIGNATURE:

APPROVAL OF STANDARD OPERATING PROCEDURE:	
Requires the signature of the following persons:	
Department Manager	Signature:..... Date:.....
Author	Signature:..... Date:.....

CONTENTS:

- 1) Amendment record
- 2) Confirmation of starting suspension concentration
 - a. Materials
 - b. Procedures
- 3) Intranasal infection
 - a. Materials
 - b. Procedures

1) AMENDMENT RECORD

DATE:	REASON FOR AMENDMENT:	SECTION AMENDED:	REPLACES EDITION:	SIGNATURE:

2) Confirmation of starting suspension concentration

a. Material

- Class II biosafety cabinet in BSL3 laboratory
- 1ml pipette plus filter tips
- 10 ml glass pipette, sterile (if making dilutions in 10 ml volumes)
- 1 ml or 10 ml sterile PBS per dilution (depend if 1 ml or 10 ml dilutions are done), x 7 dilutions
- 7H11 Agar plates; 7 x 3 (triplicates) per inoculum
- paper towels
- container for dirty tools
- sharps container
- 2ml eppies/24-well plates/10 ml sterile tubes for dilution
- eppendorf centrifuge 10 000 RPM
- vortex (+ sonicator/ 26/27 gauge needles if TB not already in single cell suspension)

b. Procedures

1. Assemble five sterile eppendorf tubes each containing 900 μ l sterile saline. Label 7H10 or 7H11 agar petri plates (12) or quadrant petri plates (3) to give duplicates of six dilutions as follows: 10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} .

Label the tubes as 10^{-1} to 10^{-5} . Although steps 1 to 5 can be omitted, they are strongly recommended as a means of confirming the challenge dose loaded into the nebulizer.

2. Using a 1.0-ml sterile serological pipet, remove 0.3 ml working mycobacterial suspension (from step 1), add 0.1 ml to the first culture tube (10^{-1} dilution), and plate the remaining 0.2 ml onto the plates labeled 10^0 . Discard the pipet into the stainless steel pipet boat containing 5% Lysol. Pipetting operations during the serial dilution process should be done with extreme care in order to prevent aerosol generation. Any small spills or drops during the pipetting process should be immediately disinfected with 5% Lysol.

3. Gently swirl the contents of the 10^{-1} dilution tube. Use a new 1.0-ml serological pipet to remove 0.3 ml, transfer 0.1 ml to the next culture tube (10^{-2}), and plate the remaining 0.2 ml onto the duplicate quadrants labeled 10^{-1} .

4. Repeat this process until the final 10^{-5} dilution has been plated.

5. Place plates upright in a resealable polypropylene bag and incubate in the dark at 37°C for ~3 weeks. After incubation mycobacterial colonies should become visible and countable.
6. Disinfect the biological containment cabinet after use. Double bag culture tubes, stock vials, and the pipet boat in autoclave bags and tape with heat-sensitive autoclave tape.
7. Count colonies on the quadrant plates ~20 days after incubation at 37°C and calculate the initial titer of the suspension as follows: where MCCQ is the mean colony count of replicate countable plate quadrants, VP is the volume plated per quadrant, and DFQ is the dilution factor of the sample in the quadrant.

$$\text{cfu/ml} = \frac{\text{MCC}_Q}{V_P \times \text{DF}_Q}$$

3) Intranasal infection

a. Material

- Class II biosafety cabinet in BSL3 laboratory
- 1 ml frozen stock suspension of M tuberculosis (6.8×10^7 cfu/ml) to be diluted to inoculation concentration of 5.4×10^3 / 6.8×10^4 cfu/ml
- F10 disinfectant
- 70% (v/v) ethanol
- 200 µl pipette plus filter tips / 1 ml syringes with feeding tube
- 1ml pipette plus filter tips
- 250 ml sterile PBS
- paper towels
- container with F10 for dirty tools
- sharps container
- 2ml sterile eppies
- eppendorf centrifuge 10 000 RPM
- vortex (sonicator and not necessary, because TB already in single cell suspension)

- syringes
- isofluorane
- oxygen
- anaesthetizing apparatus

b. Procedures

1. Remove a vial containing the mycobacterial species of interest, and gently thaw at room temperature. Thaw completely.
2. Vortex momentarily to further disrupt any clumps of bacilli.
3. Disinfect the work surfaces of a Class II biosafety cabinet using F10 followed by 70% ethanol. Place all cleaning materials into an autoclave bag for proper disposal.
4. Fill a stainless steel pipet boat with 5% Lysol (F10) to a 1.0- to 2.0-cm depth and place toward the rear of the work surface. Place a sharps container to the side to accommodate syringes and needles.
5. Place the vial in the cabinet, disinfect the top of the butyl rubber septum with 70% ethanol.
6. (if TB not already in single cell suspension) Carefully remove the mycobacterial suspension from the vial with a 1-cc tuberculin syringe fitted with a 26½-G needle and expel back into the vial. Repeat back and forth into the vial ten to twenty times without removing the needle to mix the suspension and break up any small clumps of bacilli.

NOTE: Tuberculin syringes that have a pre-attached needle are highly recommended.

7. Remove the 100 µl of mycobacterial suspension (6.8×10^7 cfu/ml) and carefully inject down the side of a 2 ml tube containing 900 µl sterile water or saline so that the final volume is 1 ml. This is a 10 x dilution (6.8×10^6 cfu/ml). Repeat another 2 times to reach the desired inoculum concentration (6.8×10^4) for infection plan 1 or transfer 80ul of 6.8×10^4 TB in 920ul of saline in FACS tube with cap to produce 5.4×10^3 inoculum.

Place the syringe in the sharps container for disposal.

Preparation of inoculum

- Vortex the cryovial for 15 sec.
- Transfer 1 ml of TB culture in Eppendorf tube
- Spin at 10000 rpm for 10 min.
- Discard supernatant and resuspend in 1 ml PBS

This stock should be 6.8×10^7 cfu/ml

Prepare 10-fold dilutions up to 6.8×10^4 cfu/ml

To make 5.4×10^3 cfu/ml,

- transfer 80ul of 6.8×10^4 TB in 920ul of saline in FACS tube with cap
- Plate -1 dilution of this inoculum to determine actual CFU before infection
- Use this inoculum to infect 14 mice 1st mouse of each cage then infect 2nd mouse from G7 to G1
- Then plate -1 dilution of remaining inoculum to determine cfu after infection (i.e. 14th mice)

Then prepare new inoculum as above for next 14 mice from same vial (6.8×10^4 cfu/ml). Basically preparing 2 inoculum from last diluted stock.

For all subsequent inoculums, dilute again from 10^7 to 10^4 and prepare as above to plate before and after

Infection plan 1

	G1	G2	G3	G4	G5	G6	G7
Inoculum 1							
							
Inoculum 2							
							
Inoculum 3							
							
Inoculum 4							
							

Preparation of inoculum

- Defrost TB on ice and vortex the cryovial for 15 sec

This stock (1) should be 6.8×10^7 cfu/ml

Prepare 10-fold dilutions up to 6.8×10^3 cfu/ml

(9ml PBS + 1ml) x 4

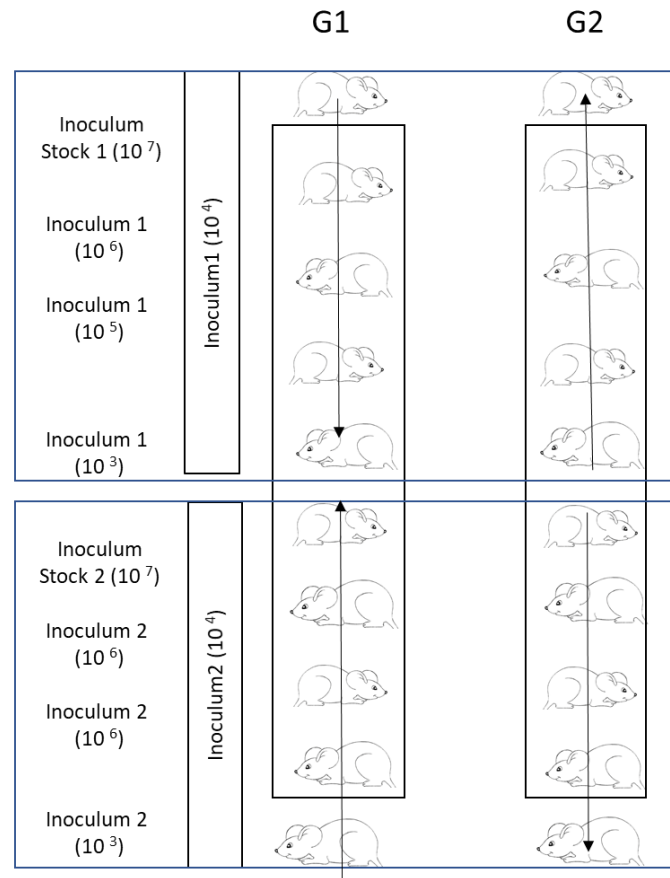
Plate serial dilutions of 2 stocks before and after infection in triplicate

- Use 6.8×10^4 inoculum to infect 10 mice, 1st test mouse, 4 from Group1cage1, 4 from Group2cage1, 2nd test mouse

Then prepare new inoculum (2) as above for next 10 mice as above from new stock 6.8×10^7 cfu/ml dilution.

- Infect 10 mice with 6.8×10^4

Infection plan 2



8. Put cage into biosafety cabinet. Anaesthetize mice one by one (1 from each group) in isofluorane chamber with oxygen for 2 minutes.
9. With a 100µl pipette plus filter tip or 1 ml syringes with feeding tube, infect mice drop-wise with 50 µl into the same nostril. Note down the number of each mouse infected next to its position of infection (1st, 2nd etc). Hold mouse until it wakes up and gently release it back into the cage.
10. Repeat until all mice are infected.

ANNEXURE I: Standard Operating Procedure: Euthanasia method

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Title	Euthanasia of Rodents		
SOP no	SOP_Viv_Anim 1	Issue no	4
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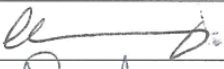
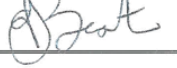
Controlled Document

1. COMPILATION AND AUTHORISATION

Action	Designated Person	Signature	Date
Compiled by	Mrs A Fick		17-06-2015
Checked by	Mr H Buntting		17-06-2015
Authorised by	Mr CJJ Bester		17-06-2015

AnimCare ethics approval number	NWU-0076-15-A5
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2. DISTRIBUTION

Department	Name	Signature	Date
PCDDP QA Manager	L Scholtz		19-06-2015
Head: Vivarium	CJJ Bester		19-06-2015

3. DOCUMENT HISTORY

Date	Issue no	Reason for revision
20-09-2012	Draft 1	First draft of SOP based on initial process
13-05-2013	Issue 1	First issue of SOP
17-02-2014	Issue 2	Addition of Test Principles
23-06-2014	Issue 3	Training Removed
18-06-2015	Issue 4	Changes as recommended by ethics committee

PCDDP DST/NWU Preclinical Drug Development Platform		Standard Operating Procedure	
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4. POLICY and OBJECTIVE

The purpose of this Standard Operating Procedure (SOP) is to ensure that the humane killing of rats and mice is performed with the minimum possible discomfort, pain or distress to the animals; that death is confirmed by an appropriate method; and that the procedure does not endanger human health or safety.

5. SCOPE

This SOP concerns the humane euthanasia of mice, rats, and guinea pigs, including new-born pups and foetuses.

6. REFERENCE DOCUMENTS

- SANS 10386:2008 The Care and Use of Animals for Scientific Purposes
- SOP_Viv_Anim 2: Anaesthesia of rats for Surgical Procedures.
- SOP_Viv_Anim 4: Isoflurane Anaesthesia in Mice.
- SOP_All_Waste 1: Waste Management.
- SOP_Viv_Eqp_Opr 2: Weighing of Animals and Materials.

7. ABBREVIATIONS and/or DEFINITIONS

Abbreviation	Description
CO ₂	carbon dioxide
DST	Department of Science and Technology
GLP	Good laboratory practice
MSDS	Material Safety Data Sheet
N/A	Not applicable
NWU	North West University
PCDDP	Pre-Clinical drug development platform
QA	Quality assurance
SANS	South African National Standard
SOP	Standard operating procedure
SPF	Specific pathogen free

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Definition	Description
rodent	Mouse, rat & guinea pig.

8. RESPONSIBILITIES

Only competent, experienced and authorised persons may perform this procedure. This SOP must be followed by all personnel and researchers who are authorised by the SAVC to perform the procedure following a period of training and experience. Persons in the process of gaining competence may undertake the procedure if supervised by a member of the Vivarium who is fully competent and authorised in the procedure.

9. TEST PRINCIPLES

- See section 12.5 – Confirming Death

10. MATERIALS and EQUIPMENT

- Anaesthetic such as Isoflourane, Ketamine and Sodium Pentobarbital
- Compressed medical carbon dioxide (CO₂) gas in cylinder with valve
- Large container with lid, e.g. a garbage can
- Connecting hose from the cylinder to the can
- Scissors
- Portable guillotine with V-shaped blades
- Level, flat and stable operating surface
- Biohazard disposal container
- Sharps disposal container
- 1ml syringes
- 26G needles
- Protective clothing including eye protection

11. SAFETY

11.1. Required Qualifications

- a) All persons performing euthanasia must be appropriately trained and the training recorded. All persons must be authorised by the SAVC to perform the procedure.

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11.2. Required Safety Equipment

- a) Isoflurane and CO₂ should only be used in a properly ventilated room, isoflurane preferably in a fume cupboard.
- b) Technicians performing the euthanasia must wear appropriate personal protective equipment.

11.3. Exclusions

- a) Isoflurane anaesthesia must not be performed by or in the presence of a pregnant woman.

11.4. Reagents and Chemicals

- a) All chemicals, etc. must be handled appropriately and not in contradiction of any MSDS sheet notification.

11.5. Hazards

- a) All needles and syringes must be disposed of in an appropriately labelled sharps container.
- b) All material in contact or consisting of biological materials must be disposed of in an appropriately labelled biohazard container.
- c) Animals must be restrained adequately before an intraperitoneal injection is administered.
- d) Euthanised animals are put in plastic bio-hazard containers and frozen away until removed by an appointed contractor.

12. PROCEDURES

12.1. General

- a) The animal's identity is verified by examining its tattoo, dyed marking, ear notching or other identification.
- b) Gather all necessary equipment, and wash hands and put on gloves prior to the onset of the procedure. All equipment used must be functional and appropriate to for the right use.
- c) The animal is weighed according to SOP_Viv_Eqp_Opr 2: Weighing of Animals and Materials, if necessary to calculate a dose.
- d) Depending on the species, age and size of the animal and the specific requirements of the study, different euthanasia procedures can be used.
 - i. CO₂ overdose (12.2)
 - ii. Anaesthetic overdose (12.3)
 - iii. Decapitation (~~11.4~~)

*12.4 did
wrong number*

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- e) In selecting a method, it is important to take into account the purpose for which the animal is being euthanised. Whichever method of euthanasia is to be used, a prime requisite is that it must be carried out humanely, causing only the absolute minimum amount of stress or pain to the animal.
- f) Depending on the requirements of the study, dead animals will either be retained for further analysis or be disposed of in an appropriately labelled biohazards container. See SOP_All_Waste 1: Waste management for detailed procedures.

12.2. CO₂ overdose;

- a) Ensure that the euthanasia chamber is clean, and that there is sufficient gas in the CO₂ cylinder to complete the procedure (confirm by opening the cylinder's regulator valve).
- b) Place the animals inside the euthanasia chamber and close the lid firmly.
- c) Introduce CO₂ gas into the euthanasia chamber. Open the CO₂ flow-meter to deliver **20% of the euthanasia chamber's volume per minute**, and start the timer. Higher flow-rates must not be used, since this causes distress to the animals. The correct CO₂ flow-rate to use depends on the euthanasia chamber's size (volume), as in the table below:

Euthanasia chamber volume	CO ₂ flow-rate to use
2 litres	400 ml/minute
5 litres	1 litre/minute
7.5 litres	1.5 litres/minute
10 litres	2 litres/minute

- d) Allow approximately **5 minutes** for the euthanasia chamber to fill, and then close off the flow-meter.
- e) Leave the animals inside the euthanasia chamber for another **3 minutes**.
- f) **Neonates** for rats or mice younger than 21 days: must be left in the chamber for at least **30 minutes**. Neonates of guinea pigs must be treated as adults.
- g) **Confirm the death** of each animal (see section 12.5 - *Confirming Death*).
- h) Dispose of the animals in the prescribed manner for the facility.

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- on side*
- i) Turn the euthanasia chamber upside down for 3 minutes to allow CO₂ to flow out of the chamber (CO₂ is heavier than air), and then clean the chamber before another batch of animals is euthanised.
 - j) Once all animals have been euthanised, close off the CO₂ cylinder's regulator valve.
 - k) Once all animals have been euthanised, clean the euthanasia chamber, turn it upside down and store the chamber in this position.

12.3. Anaesthetic overdose

- a) Anaesthetic overdose is recommended in situations where the animals are already under anaesthesia, such as for terminal blood sampling procedures.
- b) An overdose of any of the anaesthetic compounds used in (SOP_Viv_Anim 2) and (SOP_Viv_Anim 4) will euthanize the animals after they first pass into a gentle sleep.
- c) In the case of inhaled agents, the rodent will remain in the anaesthetic chamber or the nose cone will remain on the rodent's face until palpation confirms that the heart has stopped beating.
- d) In the case of injectable anaesthetics such as sodium pentobarbital, double the dose recommended for anaesthesia will be sufficient to euthanize the animal.

12.4. Decapitation

- a) Decapitation done on new-born pups (up to 7 days old) and foetuses are done by means of the operator holding the foetus or pup with one hand and using a pair of sharp scissors of appropriate size to decapitate the animal. It is important that the procedure is undertaken skilfully and rapidly.
- b) Decapitation of adult rats ~~and mice~~ (older than 21 days) is done with a guillotine.
 - i. The operator must wear protective clothing including eye protection.
 - ii. Place the guillotine on a level, flat and stable surface as well as at a comfortable height, preferably at the height of the operator's hips.
 - iii. Lift the handle of the guillotine to free the blades and open the space between the blades.
 - iv. Grasp the animal firmly over and behind the shoulder blades with one hand so that the front legs crisscross under the animal's chin.
 - v. Secure the animal's head within the bottom of the V-shaped blades so that the blades are positioned over the back of the animal's neck.

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- vi. Press the handle on the top V-shaped blade down quickly and with enough force to decapitate the animal in one smooth motion.
- vii. With the hand holding the animal, press the skin of the neck firmly over the wound to prevent spraying of blood.
- viii. If needed, restrain the animal by firmly pinning down the hind legs on the operating surface with your free hand until convulsions cease.
- ix. All dead animals or tissue are put in bio-hazard containers and frozen away until the appointed contractor removes them.

12.5. Confirming Death

- a) Following euthanasia, the death of every animal must be confirmed by one of the following methods:
 - i. Decapitation,
 - ii. Dislocation of the neck,
 - iii. Exsanguination (i.e. bleeding out, e.g. by cardiac puncture),
 - iv. Permanent cessation of the circulation (e.g. remove heart or transect aorta),
 - v. Destruction of the brain (e.g. crush cranium with artery forceps in neonates),
 - vi. Confirmation of rigor mortis (i.e. general muscle stiffness with blue membranes).

13. ADDENDUM

N/A

ANNEXURE J: Standard Operating Procedure: Blood and tissue harvest

PREPARED BY:	DATE IMPLEMENTED:	SUPERSEDES SOP#
Linda Malan	May 2017	

REVIEW DATE:	REVISION DATE:	SIGNATURE:

APPROVAL OF STANDARD OPERATING PROCEDURE:	
Requires the signature of the following persons:	
Department Manager	Signature:..... Date:.....
Author	Signature:..... Date:.....

CONTENTS:

- 1) Amendment record
- 2) Baseline
 - a. Apparatus and reagents
 - b. Procedure
- 3) Day after infection
 - a. Apparatus and reagents
 - b. Procedure
- 4) Endpoint
 - a. Apparatus and reagents
 - b. Procedure

1) AMENDMENT RECORD

DATE:	REASON FOR AMENDMENT:	SECTION AMENDED:	REPLACES EDITION:	SIGNATURE:

2) Baseline blood in sub-sample

Tail vein blood:

Hb (10ul)

Centrifuge in EDTA tube

RBC fatty acids (50ul)

Plasma (50 µl), iron parameter method development

a. Materials

- Small EDTA tubes, labelled
- permanent marker
- Hemocue Hb, controls, cuvettes
- labelled eppendorfs (2/mouse = RBC and plasma)
- list with animal numbers

b. Procedures

1. Collect blood samples from the lateral tail vein using the “tail nick” method. To do this, put the mice under a warming lamp in order to dilate the blood vessel prior to taking the sample.
2. Place mice in a tube restrainer.
3. After blood is released, collect a maximum of 200 µl blood sample in 1 ml EDTA coated Minivette® (Minivette® POCT, 1000 µl, Sarsted) tubes.

3) Infection test mice on day 1 (or 2) after infection

1 Lung: homogenise - bacillary load (Plating of whole lung + 10x dilution)

Material

- Ice
- Tissue lyser,
- Tissue lyser beads – autoclaved (steel)
- 2ml round bottom eppies (9/sample) - autoclaved
- 100 ml PBS and PBS/Tween 80 (sterile)

- analytical scale
- agar plates: $n = \text{mice} \times 4$ (250ul $\times 4 = \text{total}$)
- Incubator (Vivarium BSL3 unit takes 153 standard 20 ml plates, BCH takes 600)
- extra petri dishes to cut tissue on

Removal of lungs:

-Make an incision through the thoracic cavity with surgical scissors and reflect the thoracic wall, so that the lungs are readily visible on either side of the heart. With small toothed forceps, grasp the bronchial tree close to the heart and cut the lung lobes away.

Homogenization of the mouse tissue samples (GUT, lung, liver, brain) with Qiagen TissueLyser:

- 1) Fill a polystyrene container with crushed ice.
- 2) Cut the tissue with a scalpel on a clean watch glass in little pieces (on ice).
- 3) Place lungs pieces in of approximately in pre-weighted 2 ml Eppendorf tubes.
- 4) Weigh tubes and combine to total lung weight, note weight
- 5) Add 1 ml PBS
- 6) Add a bead (5 mm) to each tube.
- 7) Make sure all tubes are tightly closed.
- 8) Place the tubes in the two chilled TissueLyser II Adapter Sets (2 x 24) (balanced!).
- 9) Turn on the TissueLyser II (power switch on the back).
 - Frequency: 25 Hz; time: e.g. 12 min for lung tissue.
- 10) Attach the TissueLyser II Adapter Sets (2 x 24) to the TissueLyser as follows:
 - Pull the locking pin upward out of its slot, and rotate it by 90°.
 - Turn the hand wheel counterclockwise until the maximum clamping range is reached.
 - Rotate back the locking pin by 90° until it engages with its slot.
 - Insert the adapter, and press it lightly into the indentation in the clamp.
 - Turn the hand wheel clockwise with two fingers until the adapter is seated in the clamp and level, and does not move freely. Continue to rotate the hand wheel clockwise until 6-8 audible clicks are heard. Locking pin should be in the slot.
 - To remove the adapter lift the locking pin and turn the hand wheel counterclockwise.
- 11) Close the hood and press START.

12) The tissue homogenates are ready for serial dilution and plating on 7H11 agar plates.

Culture of tissue homogenates

1. Disinfect the biosafety cabinet with F10 followed by 70% ethanol and place all cleaning materials into autoclave bags for autoclaving.
2. Dispense 900ul of sterile saline (PBS/Tween) into 2 ml eppies.
3. Using a sterile filter tips (with a wide bore to prevent blockage) carefully make serial 100ul dilutions. Change tips with every tube as mycobacteria are very sticky and will remain in the tips resulting in carry-over and inaccurate dilutions. Dispense tips into the pipette boat.
4. Using a pipette, dispense 100ul of each homogenate dilution onto the agar plates. This procedure should be carried out immediately after the dilutions have been prepared as over time mycobacteria will stick to the plates and dilutions will not be accurate.
5. Double bag all materials used in plating (e.g., dilution tubes, homogenization tubes, pipet boats, test tube racks), remove from the hood, and place in a large pan for autoclaving out of the ABL-3 facility.
6. Disinfect the interior work surfaces of the hood with 5% Lysol followed by 70% ethanol. Dispose of all paper towels in autoclave bags.
7. Incubate the plates at 37°C in the dark for ~3 weeks.
9. Determine the bacterial load within the organ using the following formula:

$$\text{cfu/organ} = \frac{CC_Q \times V_H}{V_P \times DF_Q}$$

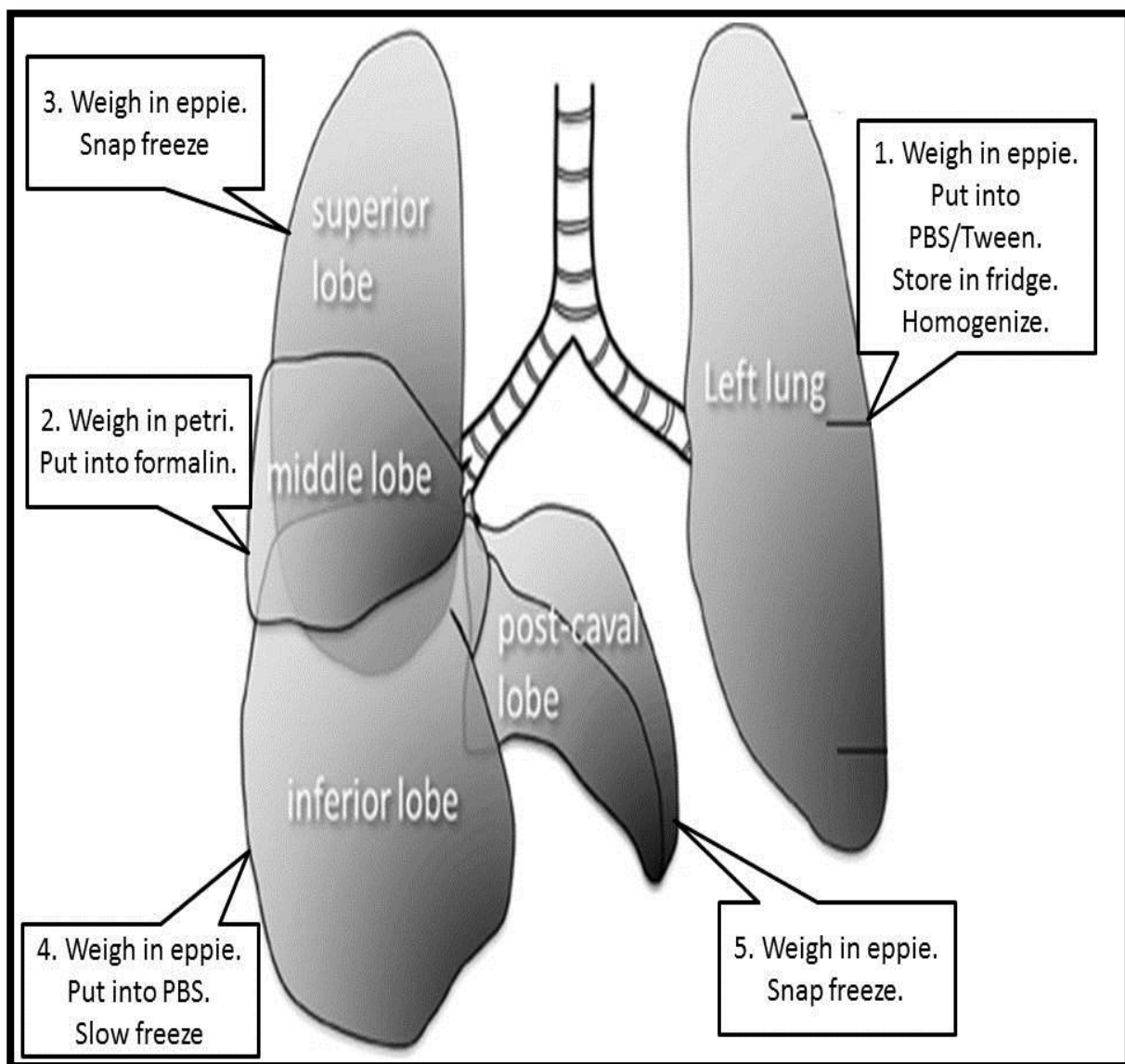
where CCQ is the colony count in one quadrant, VH is the total volume of the homogenate, VP is the volume plated onto the quadrant, and DFQ is the dilution factor of sample in the quadrant.

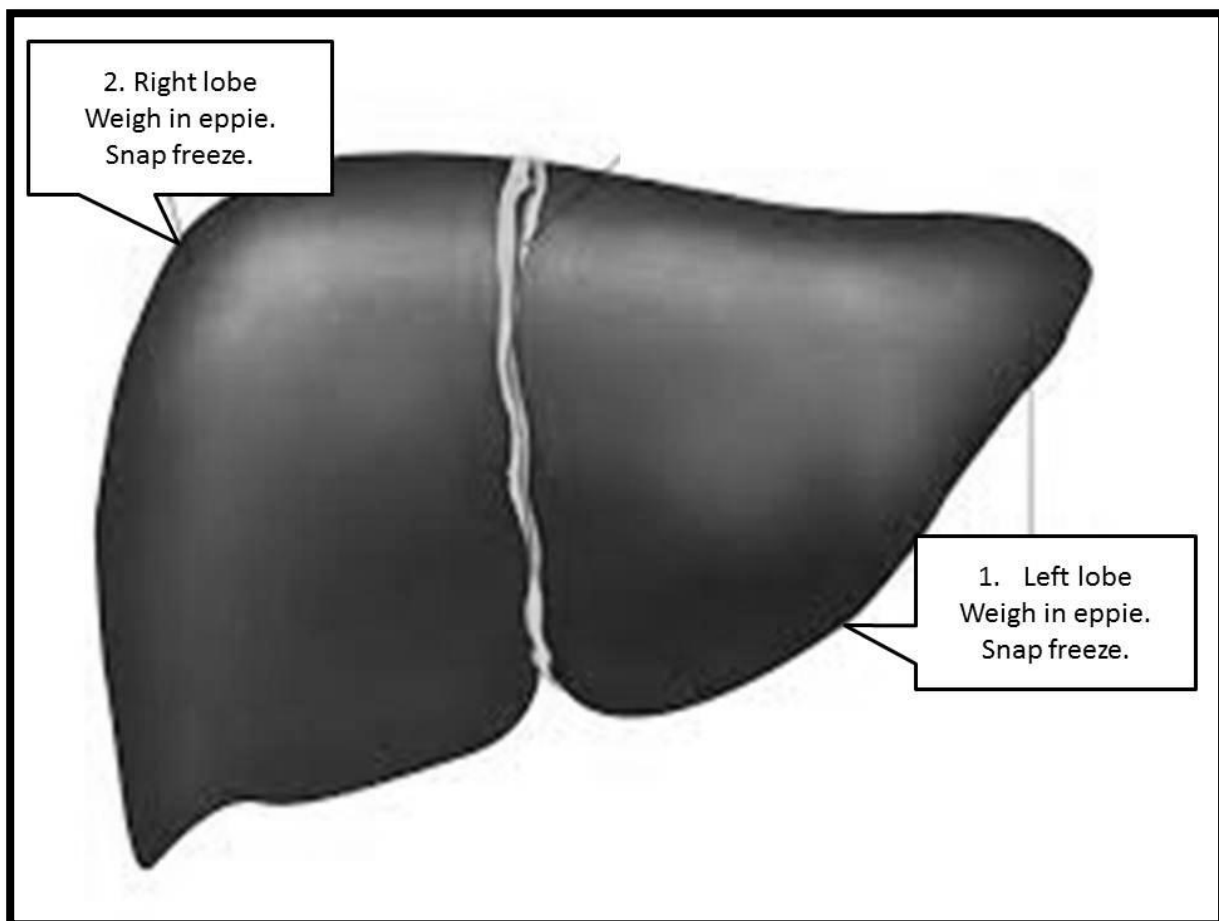
As an example, suppose that a quadrant contains 26 colonies (CCQ) of a sample that was plated in a 0.1-ml volume (VP) and diluted by 1.0×10^{-3} (DFQ) from an original homogenate volume of 10.0 ml (VH). Using the above formula the bacterial load in cfu/organ for this sample would be $(26 \times 10.0)/(0.1 \times 1.0 \times 10^{-3}) = 2.6 \times 10^6$.

left lung	lobe	homogenise immediately - acillary load, centrifuge leftover – freeze for cytokines
right superior ½ Lung lobe		snap freeze, homogenise later, lipid mediators (~50mg)
right middle lobe		in formalin for histology and immunohistochemistry
right inferior lobe		Freeze in 1ml PBS and save for backup bacillary load, -80°C OR prepare for FACS analysis
post-caval lobe (5 th lobe)		snap freeze, homogenize later, metabolomics

= 5 eppies + 2 x liver, + 1 spleen

4) Endpoint tissue collection





Liver (right and left lobe) snap freeze, homogenise later, hepcidin levels and expression
Cytokines (20µl)

Trunk blood: Centrifuge in EDTA tubes

Use plasma for:

Hb (50ul)

Ferritin (50ul)

TfR (50ul)

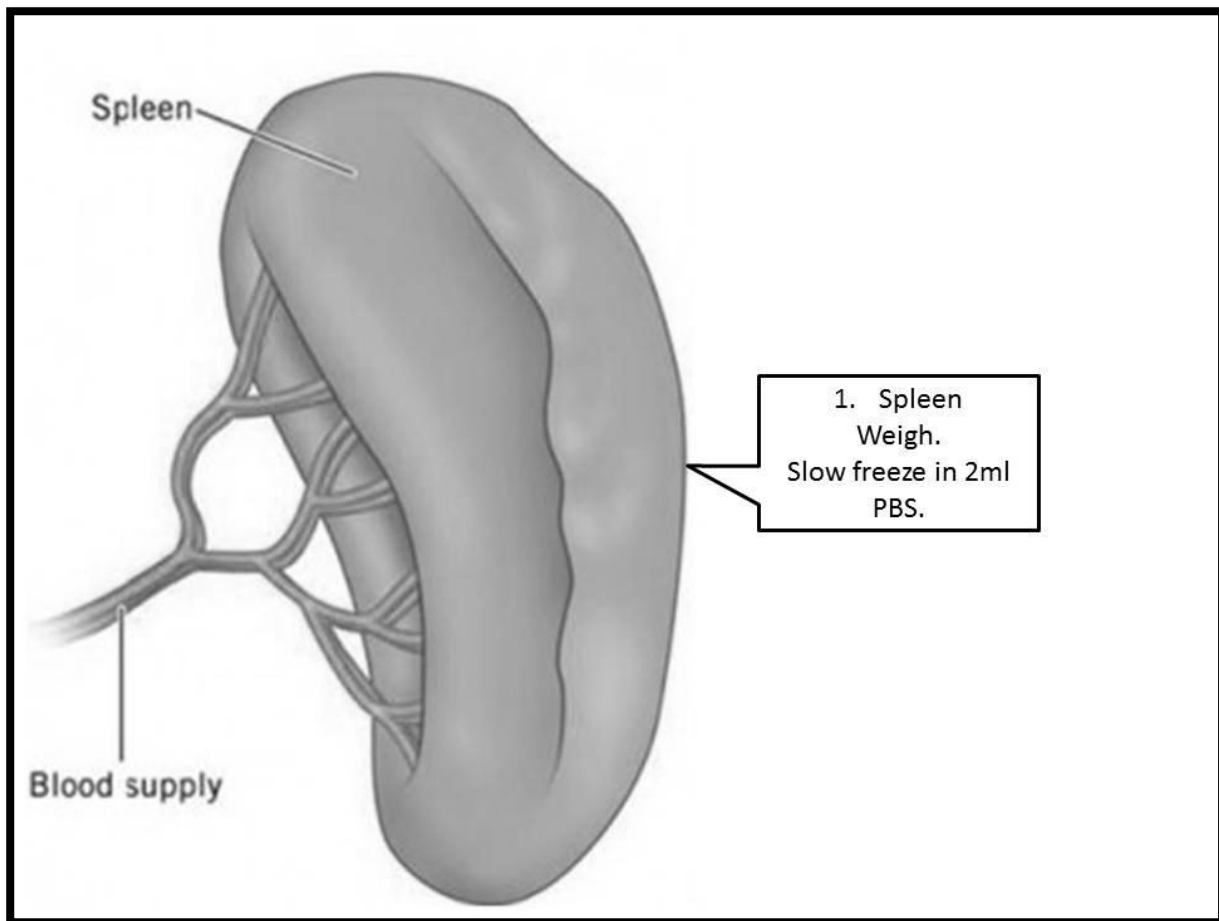
Hepcidin (50ul)

RBCs:

Fatty acids (50ul)

Buffy coat:

Fatty acids



Spleen

Freeze in 1ml PBS and save for backup bacilliary load, -80°C

a. Material

General

- 70% ethanol in spray bottle
- 2 ml safelock Eppendorf eppies, labelled
- mark pen and tape
- large beaker filled with F10 for waste
- extra petri dishes to cut tissue on
- Ice
- plastic bags to move samples in
- cryoboxes
- Hemocue + Hb cuvettes

Blood samples

- EDTA collection tubes, small funnels, EDTA in spray bottle
- eppendorf centrifuge
- saline
- 1ml and 100/200ul pipettes plus tips

Snap freeze samples

- liquid nitrogen + holder

Histology samples:

- histology cups with 10 % formalin, 1 per group

Tissue to save for plating backups

- 1ml PBS in eppie

Plating and Homogenisation of lung tissue and check inoculum cfu:

- Tissue lyser,
- Tissue lyser beads – autoclaved (steel)
- 2ml round bottom eppies (9/sample) - autoclaved
- 10 ml PBS and PBS/Tween 80 per sample - autoclaved
- analytical scale
- plates:

Lung plates: n = mice x 3 dilutions x 3 triplicate

- Incubator (Vivarium BSL3 unit takes 153 standard 20 ml plates, BCH takes 600)

For removal of lungs:

Make an incision through the thoracic cavity with surgical scissors and reflect the thoracic wall, so that the lungs are readily visible on either side of the heart. With small toothed forceps, grasp the bronchial tree close to the heart and cut the lung lobes away.

b. Procedure:

- 1) Fill a polystyrene container with crushed ice
- 2) Autoclave, weight and label eppies

- 3) Pre-pack eppies per mouse number in racks – and histology cup
- 4) Select mice randomly as infected from each group
- 5) Weigh mouse, note weight
- 6) Anaesthetize mouse, dislocate neck, cut head off and collect blood into EDTA tube through funnel sprayed with EDTA. Put in rack.

6.2 Measure Hb.

- 7) Spray mouse abdomen and chest with 70% Ethanol.
- 8) Remove spleen, place in eppie, weigh, add 1 ml PBS, place on ice
- 9) Remove liver, dissect into left and right, place each in eppie, weigh, snap freeze. (Cut the tissue with a scalpel on a clean watch glass
- 10) Remove lung (dry on tissue paper), weigh total weight on clean petri dish (zero the scale before each weight).
- 11) Dissect lung:
 - Left lung lobe: place in eppie, weigh (+ note), add PBS+ 0.005% Tween. Put in fridge/ on ice. Homogenise according to SOP
 - right sup lung and post caval lobe: place in eppie, weigh, snap freeze,
 - right inferior lobe: place in eppie, weigh, add 1 ml PBS, place on ice
 - right middle lobe: Weigh on petri dish, place in formalin
- 12) Serial dilute (3 x dilutions) and plate homogenates on 7H11 agar plates.
- 13) Centrifuge leftover, aliquot 100ul supernatant into eppie and snapfreeze (lung cytokines)
- 14) Process blood.
- 15) Snap frozen samples – Store at -80 degrees.

Blood preparation

1. Hb
2. Centrifuge 8000 rpm - plasma A
Buffy coat
Wash RBC x 2 times

Culture tissue homogenates – as above.

ANNEXURE K: Preparation of agar plates for culturing of TB colony forming units

PREPARED BY:	DATE IMPLEMENTED:	SUPERSEDES SOP#
Linda Malan	May 2017	

REVIEW DATE:	REVISION DATE:	SIGNATURE:

APPROVAL OF STANDARD OPERATING PROCEDURE:	
Requires the signature of the following persons:	
Department Manager	Signature:..... Date:.....
Author	Signature:..... Date:.....

CONTENTS:

- 1) Amendment record
- 2) Apparatus and reagents
- 3) Procedure

1) AMENDMENT RECORD

DATE:	REASON FOR AMENDMENT:	SECTION AMENDED:	REPLACES EDITION:	SIGNATURE:

2) Apparatus and reagents

(1L ~ 50 plates – 20ml/petri dish)

-Autoclave, Incubator, fridge

-Pipetboy + tips

-Magnetic stirrer + magnets

-1 L Schott bottles with rim

-1 L measuring cylinder

-860 ml dd H₂O per 1L agar

-5.0 ml glycerol per 1 L agar

- 21.0 g Middlebrook 7H11 agar powder (7H11 agar contains a pancreatic digest in the base, whereas 7H10 does not; this additive seems to enhance the growth of many strains of mycobacteria and hence is the preferred medium)

-100ml OADC/ 1L agar

-2 antimicrobial tablets/1L agar

-Plastic bags (20 x 30 cm) if moving plates to store elsewhere

-Freezer gloves

3) Procedure

(A) PREPARATION OF 7H11 AGAR MEDIUM:

1.) Pre-set water bath at 50°C.

2.) Place magnet in clean 1L SCHOTT bottle (check that neck of bottle is fitted with a seal).

3.) Stick heat sensitive tape to side of bottle and label: "7H11 Agar" plus date.

3.) Fill bottle with 860 ml of deionised H₂O.

4.) Add 5 ml of glycerol (note brand - Sigma 49767, batch number and expiry date) swirl to get smooth suspension.

- 5.) Weigh out 21 g of 7H11 Agar Base (Sigma M0428; record mass, note expire date and lot number) and suspend in bottle with deionised H₂O/ glycerol.
- 6.) Cap bottle without tightening, cover cap and bottle neck with tin foil and autoclave for 15 minutes at 120°C and 1 atm (first check level of water in autoclave).
- 7.) While bottle with agar medium is in autoclave, prepare selective antimicrobials (see B).

(B) PREPARATION OF SELECTIVE ANTIMICROBIALS (KIRCHNER MEDIUM):

Either tablets or vials can be used.

Use two vials of Mycobacteria Selectavial (SV24) per 1L of 7H11 agar. Add 5 ml of sterile water to each of two vials with Kirchner media using 10ml syringe and 21G needle. Dissolve medium by inverting vials several times (avoid shaking). Record number of vials used, batch number and expiry date of Kirchner Medium in data sheet for preparation of 7H11 agar plates.

OR

Use two tablets of Mycobacteria Selectatab (MS24) per 1L of 7H11 agar. Record number of tablets used, batch number & expiry date in data sheet. Add two tablets by means of sterile forceps to 1 L of 7H11 agar after it is cooled in 50°C water bath for 10 minutes.

(C.) PREPARATION OF 7H11 AGAR PLATES:

- 1.) Upon completion of autoclaving, remove bottle with 7H11 agar medium, tighten cap and cool to 50°C in pre-set water bath (10 minutes). You should be able to handle bottle with gloved hand.
- 2.) Place on magnetic stirrer until well mixed.
- 3.) Wipe bottle with 70% ethanol and transfer to Biosafety Cabinet.
- 4.) Uncap 100ml bottle with Middlebrook OADC (oleic acid albumin dextrose catalase), wipe bottle with 70% Ethanol and add to bottle with 7H11 agar (all done within biosafety cabinet).
- 5.) Place bottle on magnetic stirrer, wipe with 70% ethanol and return to biosafety cabinet for addition of two vials of dissolved Kirchner media.
- 6.) Mix again on stirrer, wipe with ethanol and pour (within biosafety cabinet) 20ml per petri dish (Any brand, 90mm diameter) using 20 ml Pipette and sterile 20ml pipette tip.
- 7.) Place petri dishes with agar on clean bench top that was wiped with 70% Ethanol and allow setting for at least two hours.
- 8.) Place 6 agar plates within plastic bags (20 x 30 cm), place in box (labelled "7H11 agar plates" plus date) and incubate overnight (24h) in 37°C incubator for sterility check.
- 9.) After overnight incubation allow to cool to room temperature before storing at 4°C.
- 10.) Use within a month.

ANNEXURE L: Dose conversion between animals and human

The dose by factor method is an empirical approach and use the no observed adverse effect levels (NOAEL) of drug from preclinical toxicological studies to estimate human equivalent dose (HED).

Key Points in Scaling of Dose

- Larger animals have lower metabolic rates
- Physiological process of larger animals is slower
- Larger animals required smaller drug dose on weight basis
- Allometry accounts the difference in physiological time among species
- Do not apply allometric scaling to convert adult doses to kids.

Dose Calculations and Examples

The dose by factor method applies an exponent for body surface area (0.67), which account for difference in metabolic rate, to convert doses between animals and humans. Thus, HED is determined by the equation:

$$\text{HED (mg/kg)} = \text{Animal NOAEL (mg/ kg)} \times \text{weight}_{\text{animal}} \text{ (kg)} / \text{Weigth}_{\text{human}} \text{ (kg)}^{(0.33)}$$

Example:

The NOAELvalue in rat weighing approximately 150 g is 18 mg/kg:

To calculate the starting dose for human studies, use the eqation:

$$\text{HED (mg/kg)} = 18 \times (0.15\text{kg}/60\text{kg})^{(0.33)} = 2.5 \text{ mg/kg}$$

Thus, for a 60 kg human, the dose is 150 mg. This HED value is further divided by a factor value of 10; thus, the initial dose in entry into man studies is 15 mg.

Dose is equally related to body weight although it is not the lone factor which influences the scaling for dose calculation. The correction factor (K_m) is estimated by dividing the average body weight (kg) of species to its body surface area (m_2).

Example:

The average human body weight is 60 kg, and the body surface area is 1.62 m². Therefore, the K_m factor for human is calculated by dividing 60 by 1.62, which is 37. The K_m factor value of various animal species is used to estimate the HED as:

$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \times \text{Animal K}_m / \text{Human K}_m$$

As the K_m factor for each species is constant, the K_m ratio is used to simplify calculations. Hence, Equation 2 is modified as:

$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \times \text{K}_m \text{ ratio}$$

K_m ratio values is easily obtained by dividing human K_m factor by animal K_m factor or vice versa. For instance, the K_m ratio values for rat is 6.2 and 0.162, obtained by dividing 37 (human K_m factor) by 6 (animal K_m factor) and vice versa, respectively. Thus, usually to obtain the HED values (mg/kg), one can either divide or multiply the animal dose (mg/kg) by the K_m ratio.

Example:

The NOAEL in rats is 50 mg/kg. HED is calculated either by multiplying or dividing the animal dose with the K_m ratio values. Accordingly, divide the rat dose (50 mg/kg) by 6.2 or multiply by 0.162, the HED is 8.1 mg/kg.

Bibliography:

Nair, A.B. & Jacob, S. 2016. A simple practice guide for dose conversion between animals and human. *Journal of basic and clinical pharmacy*, 7(2):27.

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Work Certificate

To	Melinda Britz
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Date	13/11/2018
Subject	Dissertation: The effect of pre-infection n-3 fatty acid status on anaemia of infection and morbidity in tuberculosis-infected mice
Ref	GS/MB/01

I, Gill Smithies, certify that I have proofed the following dissertation,
Chapters 1 to 6: The effect of pre-infection n-3 fatty acid status on anaemia
of infection in tuberculosis-infected mice,
to the standard as required by NWU, Potchefstroom Campus.

Gill Smithies

13/11/2018