Neutrophils and Non-Tuberculous Mycobacteria

By

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A thesis submitted to University College London in fulfilment of the requirement for the degree of Doctor of Philosophy
University College London
November 2023

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Declaration

I, Meyad Alkarni, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

London 2023

Abstract

Recent work suggested that neutrophils may play a role in controlling Non-Tuberculous Mycobacterial (NTM) infection. These cells, however, also contribute to host disease, such as bronchiectasis.

This thesis has investigated neutrophil responses to non-tuberculous mycobacterial infection in three distinct adult human populations: people with antibody deficiency (Common Variable Immune Deficiency, CVID) or X-linked Agammaglobulinemia (XLA), people with NTM pulmonary disease (NTM-PD), and a healthy comparator group. The CVID/XLA cohort was chosen because of their infrequent acquisition of NTM infection despite bronchiectasis.

I evaluated neutrophil activation, surface markers expression, and their phagocytic ability for labelled M. abscessus and M. avium in addition to mycobacterial growth over a 4-day incubation for both species in a whole blood assay. These experiments revealed significant differences in neutrophil activation and immaturity between patient groups and healthy individuals, and greater phagocytosis with a more marked activation response to *M. abscessus* than *M. avium*. Additionally, I assessed opsonization in serum and sputum across groups, finding superior serum opsonic capacity in CVID/XLA patients.

I explored the broader immune response to NTM by assessing cytokines, chemokines, growth factors, neutrophil-derived proteins, and antibodies in various samples, revealing that CVID/XLA patients exhibit notable inflammation, marked by elevated cytokine levels, yet differ in neutrophilic inflammatory markers compared to NTM-PD patients.

In adults with NTM-PD, I examined the relationship between neutrophil markers, humoral immune response indicators, and health status with radiological lung disease severity. Results indicated a significant positive correlation between serum neutrophil markers and bronchiectasis severity CT scan scores. Additionally, transcriptomic analysis identified distinct gene expression patterns associated with bronchiectasis severity and neutrophil-related markers in NTM-PD patients.

My work has potentially important clinical implications including the identification of biomarkers to monitor disease severity and options for host-directed therapy.

Impact Statement

This study examined neutrophil immune responses to non-tuberculous mycobacterial infection in three adult cohorts: common variable immunodeficiency (CVID)/X-linked agammaglobulinemia (XLA), non-tuberculous mycobacterial pulmonary disease (NTM-PD), and a healthy comparator group.

The knowledge, analysis and discoveries presented in this research hold significant potential for beneficial use in both academic and clinical settings. The findings contribute to the growing body of literature on neutrophil response to mycobacterial infection (mainly tuberculosis), elucidating the underlying processes and identifying prospective targets for therapeutic interventions.

The role of neutrophils in non-tuberculous mycobacterial infection is seldom explored, hence this study sought to address that gap. I have also developed robust techniques to study neutrophil-NTM interaction which can be used by others.

Based on my research, neutrophils taken from adults with CVID/XLA or NTM-PD vary significantly from healthy people. Furthermore, there are notable differences in how they react to a mycobacterial infection.

Another novel finding that may have significant implications for clinical practice is that individuals on intravenous immunoglobulin (IVIG) replacement treatment, who typically exhibit low incidences of nontuberculous mycobacterial (NTM) infection, have augmented opsonophagocytosis of *Mycobacterium avium*. The potential use of immunoglobulin therapy in the management or prevention of NTM infection should be explored further, particularly given its immunomodulatory or immunoregulatory properties.

Patients with NTM-PD often present with various co-morbidities, and it is evident that current antimicrobial treatments are frequently poorly tolerated and offer limited effectiveness. The resulting complexity in treatment decisions necessitates a search for new drugs and approaches to address this issue. Therefore, exploring alternative treatments that target neutrophils or their products may hold promise in improving patient outcomes.

I observed a very strong relationship between concentrations of neutrophil products in blood and bronchiectasis severity in NTM-PD: this is a new finding that could have important implications for disease monitoring and treatment decisions.

Understanding correlates of significant lung damage as measured through neutrophil function and markers, antibodies, and transcriptomics could potentially identify candidates for host-directed therapy and help decide when to start treatments.

Overall, my work suggests that an additional understanding of the roles of neutrophils in NTM-PD could guide the development of novel preventive measures and therapies for this important infection.

The future plan to expand on these findings should involve more research exploring the relationship between neutrophil-related products and CT chest scan scores of disease severity, as well as the potential role of immunoglobulin therapy in preventing or treating patients with NTM-PD. Trials could involve testing the effectiveness of treatments that target neutrophils, as well as investigating the mechanisms behind the improved opsonophagocytosis observed in patients on IVIG replacement therapy. Overall, I believe that continued research in this area, involving larger sample sizes and encompassing a broader range of NTM species, has the potential to significantly improve outcomes for people living with this disease.

Acknowledgements

During my Ph.D. journey from February 2019 to 2023, I have been fortunate to receive the support, guidance, and encouragement of numerous individuals who have played a pivotal role in my success. I would like to express my heartfelt gratitude to all of them.

First and foremost, I extend my deepest appreciation to my supervisors, Prof Marc Lipman and Dr David Lowe, they deserve special mention for their unwavering support and guidance throughout my research project. Their patience, dedication, and mentorship have been invaluable to me. I am immensely grateful to them for suggesting this Ph.D. opportunity and for their continuous support in shaping the success of my journey.
My supervisors have played a crucial role in my Ph.D. journey and the successful completion of this thesis. I consider myself fortunate to have had the opportunity to work under their supervision. I will always treasure the valuable lessons and experiences I have gained throughout this journey. Their continuous assistance, especially during the challenging times of the COVID-19 pandemic, has been immensely valuable to me. The pandemic caused major disruptions, including lab closures for six months and difficulty recruiting NTM patients due to limited face-to-face clinics in 2020 and 2021. Despite these formidable obstacles, their support and guidance remained a constant source of motivation and encouragement.

I would like to express special thanks to the members of my thesis committee (Prof Timothy D. McHugh, Dr Paul Pfeffer, Dr Ronan Breen, and Dr Colette Smith) for their valuable opinions and discussions. Their input has greatly influenced the development and structure of my research. In particular, I am indebted to Prof. Tim, the head of The Centre for Clinical Microbiology, for his leadership, support, and guidance. Working under his supervision and with his team has been a valuable experience.

My heartfelt appreciation goes to my colleagues at the Pears building, where I spent three years conducting lab work. Their collaboration, companionship, and support have made my Ph.D. journey more enriching and enjoyable. I want to extend special thanks to Adriana for her invaluable assistance and memorable friendship during my time in London.

I am also grateful to the respiratory clinic team at The Grove Centre, especially Dr James Brown, for their collaboration and support in the clinical part of my research (chapter 6). Their contribution has been crucial to the success of my work.

I extend my sincere appreciation to Prof. Mahdad Noursadeghi, professor of infectious disease, UCL, an Honorary Consultant in Infectious Diseases at UCLH, for his support in bioinformatics, and to Dr Raymond Fernando from the Anthony Nolan Histocompatibility Laboratories for his valuable assistance and expertise.

I would like to express my gratitude to the Royal Embassy of Saudi Arabia Cultural Bureau and King Saud University for their support, which has not only facilitated my academic pursuits but has also paved the way for a promising future in academia. I am honoured and privileged to have been a beneficiary of their support.

I owe my deepest appreciation to my wonderful family— my Mom, whose constant support and love have been my guiding light, and my Dad, whose wisdom and encouragement have shaped my journey. To my best sister ever 'Salwa' and my amazing brothers (Badr, Mohammed, Khalid, Salman, Ahmed, and junior Mohammed)—for their presence, even from afar, during the time I spent studying here in London. Despite the distance that separated us most of the time, their support, constant encouragement, and reassuring contact bridged the gap and kept me strong. Their love and unwavering

belief in my abilities have been the beacon that guided me through the challenges and uncertainties of my Ph.D. journey.

This thesis is not only a testament to my own dedication but also a tribute to their love and belief in me. I wholeheartedly dedicate this achievement to them as a heartfelt expression of my deepest gratitude.

I am incredibly thankful to my brother Khalid, whose immeasurable support and motivation were crucial, especially during the initial days of my journey and throughout. I am also grateful to my beloved nephew, Salman, whose calls and messages always brought joy and positivity to my life. I hope that one day he will have the opportunity to study at one of the prestigious top 10 universities in the world.

I would also like to acknowledge the incredible support and kindness of my cousin, Kholoud, who has been the closest person to me throughout my journey. Your kindness, patience, and support have made a significant difference, and I am grateful for your presence.

Lastly, I want to extend my gratitude to all my friends, including Hala, Reema, Hanin, Arwa, Wasayef, Manal, Ayshah, Nada, and especially Midrar, who has been a source of encouragement, laughter, and support throughout my Ph.D. Your friendship has been a source of strength, and I am grateful for the memories we have shared.

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Abbreviations

ABPA	Allergic Bronchopulmonary Aspergillosis
AFB	Acid-Fast Bacillus
AGPAT1	Acylglycerol-3-Phosphate O-Acyltransferase 1
APCs	Antigen Presenting Cells
ATS	American Thoracic Society
BAFF	B-cell Activating Factor
BMI	Body Mass Index
BTK	Bruton's Tyrosine Kinase
BTS	British Thoracic Society
CF	Cystic Fibrosis
CFU	Colony Forming Unit
CL2	Containment Level 2
COPD	Chronic Obstructive Pulmonary Disease
CSR	Class-Switch Recombination
CT	Computed Tomography
CVID	Common Variable Immune Deficiency
CXR	Chest X-Ray
DC	Dendritic Cells
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
ESID	European Society of Immune Deficiencies
FITC	Fluorescein Isothiocyanate
G-CSF	Granulocyte-Colony Stimulating Factor
GORD	Gastro-Oesophageal Reflux Disease
GPLs	Glycopeptidolipids
HNP	Human Neutrophil Peptides
HRCT	High-Resolution Computerized Tomographic
HSP1L	Heat Shock Protein Family A
IC	Inhaled Corticosteroids
IDSA	Infectious Diseases Society of America
IER3	Immediate Early Response 3
IER3	Immediate Early Response 3
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IIT	Institute of Immunity & Transplantation
IL-8	Interleukin-8
IVIG	Intravenous Immunoglobulin (IVIG)
JRO	Joint Research Office
KANSL1	KAT8 Regulatory NSL Complex Subunit 1
KANSL1	KAT8 Regulatory NSL Complex Subunit 1

LILRB2	Leukocyte Immunoglobulin Like Receptor B2
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MABC	Mycobacterium Abscessus Complex
MAC	Mycobacterium Avium Complex
MAML1	Mastermind Like Transcriptional Coactivator 1
MFI	Median Fluorescence Intensity
MGIT	Mycobacterial Growth Indicator Tube
MTB	Mycobacterium tuberculosis
NCTC	National Collection of Type Cultures
NE	Neutrophil Elastase
NETs	Neutrophil Extracellular Traps
NTM	Non-tuberculous Mycobacteria
NTM-PD	Non-Tuberculous Mycobacterial Pulmonary Disease
OADC	Oleic Acid, Albumin, Dextrose, Catalase
OD	Optical Density
PAGID	Pan American Group for Immune Deficiency
PAP	Pulmonary Alveolar Proteinosis
PCD	Primary Ciliary Dyskinesia
PID	Primary Immune Deficiencies
PLEKHM1	Pleckstrin Homology and RUN Domain Containing M1
RFH	Royal Free Hospital
RGM	Rapid Growing Mycobacteria
ROS	Reactive Oxygen Species
SGM	Slow Growing Mycobacteria
TBC1D3K	TBC1 Domain Family Member 3K
TCR	T Cell Receptor
TTP	Time To Positivity
UKPIN	UK Primary Immunodeficiency Network
XLA	X-Linked Agammaglobulinaemia

Chapter 1: Introduction

1.1 Epidemiology of NTM infection

The term nontuberculous mycobacteria (NTM) refers to the environmental species of mycobacteria other than *Mycobacterium tuberculosis* complex and mycobacteria causing leprosy (1). NTM infections are important causes of pulmonary (nodular and fibro cavitary disease) and extrapulmonary disease (for example cutaneous, lymph node or disseminated) (2). Unlike tuberculosis which typically affects younger people without other co-morbid illness, NTM lung disease commonly occurs in people aged fifty years or above, who may have other on-going conditions, e.g., bronchiectasis, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (3-5).

Non-tuberculous mycobacterial pulmonary disease (NTM-PD) is increasingly prevalent worldwide in healthy and immunocompromised individuals. Affected individuals suffer cough often with sputum production, tiredness and shortness of breath (1, 6-8). The infections cause significant and increasing morbidity, healthcare utilization and death. The reasons for this increase in prevalence are unclear (5, 9).

The criteria for diagnosing NTM-PD use a combination of microbiological, clinical, and radiological evidence. As a result, determining the prevalence of NTM-PD at a national level can be challenging as this requires not only mycobacterial laboratory data but also clinical and radiological information. Equally, the presence of non-tuberculous mycobacteria in respiratory samples cannot be taken as definitive evidence of active disease as NTM can be present without causing symptoms. Finally, the lack of specific symptoms resulting from NTM-PD further complicates the diagnostic process (10).

Challenges in describing the morbidity and mortality of NTM-PD include its varying incidence and prevalence across time (in particular as specific treatment is often deferred for several years or not used at all - making it hard to be clear how to define “incident disease” in people who satisfy the criteria for NTM-PD) and the distribution of infections regionally, nationally, or globally (11).

Few studies report the worldwide prevalence of NTM infection (12, 13) and these might reflect only cases identified in specific cities or clinics (14). Generally NTM-PD is not a notifiable disease, and although some countries count the cases of NTM-PD when de-notified from TB registries, in others they are not captured (11).

The overall global burden of NTM-PD disease is not known (15). However, some studies have demonstrated its considerable economic impact (16, 17) as awareness of the disease has increased (18).

In the UK, the incidence of NTM reported between 1995 and 2006 increased ten-fold by 2012 and the majority of this was due to *Mycobacterium avium complex* (19, 20).

In the US from 2008 to 2015 and Korea from 2003-2016 the incidence and prevalence of NTM-PD increased rapidly among the elderly and women, in particular (21, 22). In a survey reporting the period from 1980 till 2014 the clinically identified incidence of NTM-PD in Japan exceeded the incidence of TB, suggesting a rising prevalence of NTM-PD as a chronic infection (23). European countries vary greatly in their reported burden of NTM-PD, with information limited by a lack of standard reporting methodology (24) and disease outcome measures with or without treatment (24, 25).

1.2 NTM Mycobacteria

Although NTM are a group of environmental bacteria, with more than 150 species recognised as opportunistic pathogens of humans (1), they differ in their pathogenicity and response to treatment (26, 27).

In the United States of America, *Mycobacterium avium* complex (MAC) members are most often isolated in clinical samples, followed by *M. kansasii* and *M. abscessus* (28). However, in the Middle East the majority of isolated species were *M. abscessus*, *M. fortuitum*, and *M. intracellulare* followed by *M. avium* (29). Observations in Singapore and Japan record that MAC accounts for many NTM infections (7).

Other common species causing NTM-PD, in addition to *Mycobacterium avium* complex (most often *M. avium, M. intracellulare* and *M. chimaera* species), include *M. kansasii, M. xenopi, M. abscessus, M. fortuitium* and *M. chelonae* (2, 30). NTM species associated with inflammatory lung damage are classified according to their growth rate into slow growers (SGM, eg *M. avium*) which may need incubation over 1 week for colonies to be visible, and rapid growers (RGM) requiring fewer than 7 days of incubation to grow on agar plates (eg *M. abscessus*). Moreover, the differentiation between RGM and SGM is of clinical importance for diagnosis and treatment planning (1, 31).

1.3 Clinically Significant NTMs

Many cases of NTM-PD are caused by a small number of common species; *Mycobacterium avium* complex (MAC) and *Mycobacterium kansasii* are most common among slow-growing organisms and *M. abscessus* complex (MABC) is the most clinically important of the fast-growing organisms (32-35). However, *M. kansasii* is less frequently encountered in many geographic areas compared to MAC and MABC (36).

MABC and MAC affect patients with underlying airways disease in different ways, and they are associated with independent and different risk factors (37). The prevalence of clinical disease with both species has also increased worldwide even among immunocompetent individuals (38-40).

In people with cystic fibrosis (CF) as the cause of their underlying lung disease, the most prevalent species noted in multicentre studies were MAC and MABC, between them accounting for 95% of NTM-PD (41, 42). Generally, MABC were the cause of more than 80% of pulmonary NTM in CF (43). Additionally, sputum cultures that test positive for Mycobacterium avium complex (MAC) are considered clinically significant in approximately 35% to 42% of cases of NTM pulmonary disease (36, 44).

MABC infection is typically seen in patients with a history of pulmonary disease such as CF or non-CF bronchiectasis, and is associated with the worst outcomes for disease caused by rapidly growing mycobacteria (28, 38, 45, 46). In contrast, MAC often occurs in elderly females and can be much more indolent.

1.4 Diagnostic tests

NTM species isolated from a respiratory sample (sputum, bronchial washings, bronchoalveolar lavage or transbronchial biopsy) are insufficient to confirm NTM-PD; as the diagnosis depends on several criteria including clinical symptoms, bacteriologic culture, and radiographic appearance.

The American Thoracic Society (ATS) and the British Thoracic Society (BTS) have issued guidelines regarding the diagnosis of NTM-PD (Table 1.1). At least two separate sputum samples or one bronchial washing fluid should grow the same NTM species in a patient with compatible symptoms and radiology to confirm NTM-PD (1, 47, 48). CT scanning is therefore a key component when assessing for changes consistent with NTM-PD, such as nodules or cavities in the lung, as well as to rule out other diagnoses and for follow up of NTM-PD over-time.

Table 1.1: Clinical and microbiological criteria for diagnosing non-tuberculous mycobacterial lung disease (28).

Criteria	Clinical	Microbiological
	Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or a high-resolution CT scan that shows multifocal bronchiectasis with multiple small nodules.	Positive culture results from at least two separate expectorated sputum samples; if the results are nondiagnostic, consider repeat sputum Acid-fast bacillus (AFB) smears and cultures.
	OR	**OR**
	Nodular or cavitary opacities on chest radiograph, or a high-resolution CT scan that shows multifocal bronchiectasis with multiple small nodules.	Positive culture results from at least one bronchial wash or lavage.
	AND	**OR**
	Appropriate exclusion of other diagnoses	Transbronchial or other lung biopsy with mycobacterial histopathological features (granulomatous inflammation or AFB) and positive culture for NTM

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The high-resolution computerized tomographic (HRCT) features of NTM-PD are widely reported. Much of the available data relate to MAC (49-52). In Figure 1.1, examples of the two major clinical phenotypes are shown: fibrocavitatory and nodular bronchiectatic. Nodular bronchiectatic disease is the most common type of MAC-associated lung disease and is generally considered a less severe form of NTM infection because of its slowly progressive nature compared to cavitatory disease.

Scoring systems for chest CT scans are used by clinicians to assess the severity of NTM-PD, predict treatment outcomes, and guide therapeutic decisions (53).

These systems take into account factors such as clinical symptoms, microbiology results, and radiological features. However, it's important to consider the limitations of these scoring systems. These include their lack of validation in diverse patient populations, which can limit their generalizability. Also, some scoring systems use subjective assessments, leading to different interpretations and scores among clinicians. Overly complex scoring systems may be difficult to implement in routine clinical practice. Additionally, scoring systems may not always accurately predict outcomes or guide treatment decisions, especially when confounding factors are present.

Bronchiolitis, bronchiectasis, nodules, consolidation, and, less frequently, cavities are seen on CT scans of patients infected with *M. abscessus*: these features can be misdiagnosed as tuberculosis or malignancy (28). Sometimes, the radiographic features of MABC are similar to the nodular bronchiectatic form of NTM-PD caused by MAC (43, 54). In fact, some patients with the nodular bronchiectatic form of NTM lung disease can have mixed infection with MAC and MABC. However, a limited number of studies have been conducted in NTM mixed infection (32).

Generally, it is believed that a test can be useful as a surrogate marker if well-correlated with the severity of disease and can predict response to treatment or disease progression if untreated. CT scanning may meet these criteria in NTM-PD. A retrospective study which included 44 NTM-PD patients, 18 of them (40.9%) experiencing a relapse during follow up after successful treatment revealed that the severity of CT scan findings at the initiation and termination of treatment could predict recurrence of NTM-PD (55).

The use of biomarkers, including neutrophils, C-reactive protein (CRP), and neutrophil/lymphocyte ratios, as predictive tools for prognosis and disease progression in NTM-PD, is an area of ongoing investigation (56). While studies have shown associations between elevated levels of these biomarkers and disease severity in NTM-PD patients with other conditions such as rheumatoid arthritis, their specificity and reliability as prognostic indicators remain the subjects of debate (56, 57). Neutrophil counts, reflecting the inflammatory response, have been linked to disease exacerbations and progression in some studies, but their predictive value in isolation may be limited due to variations in individual immune responses. Similarly, elevated CRP levels, indicative of systemic inflammation, have shown correlations with disease severity in NTM-PD, yet their utility as standalone prognostic markers requires further validation. Neutrophil/lymphocyte ratios, aiming to capture the balance between pro-inflammatory and anti-inflammatory responses, hold potential as prognostic indicators, although their precise predictive value in NTM-PD also remains to be fully elucidated (58). Overall, while these biomarkers offer insights into the inflammatory status of NTM-PD patients, their clinical utility in

predicting disease progression and prognosis and requires further exploration through robust longitudinal studies, and more specific biomarkers are likely to be needed.

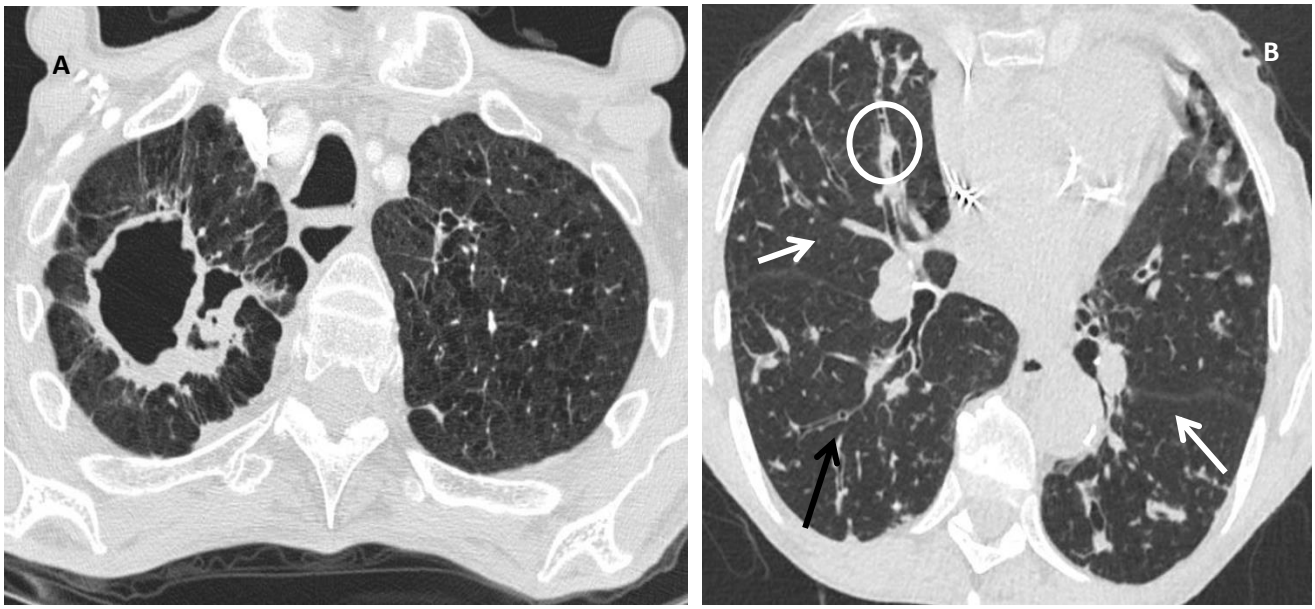


Figure 1. 1: Radiological imaging features of NTM lung infection.

(A) Fibrocavitatory NTM-PD in a patient with *M. kansasii*. Axial CT shows 2 adjacent cavities in the right upper lobe. Additional centrilobular emphysema is also seen in both upper lobes.
(B) Nodular-bronchiectatic NTM-PD in a 73-year-old female patient with MAC. Axial CT shows widespread airways disease. Bronchiectasis with mucus plugging and bubble lucencies (air) in the middle lobe (circle). Bronchiectasis with a non-tapering bronchus in the right lower lobe (black arrow). Additional bronchiectasis and bronchial wall thickening is seen in the middle, lingula and left lower lobes (eg white arrows).

1.5 Challenges in the diagnosis and treatment of NTM-PD

According to Infectious Diseases Society of America (IDSA) and ATS as well as the BTS guidelines, the diagnosis of NTM-PD relies on clinical, laboratory and radiology aspects. Diagnostic challenges in evaluating pulmonary NTM disease occur due to limited clinician awareness and its non-specific clinical presentation, the need for at least two separate sputum cultures or one bronchial lavage culture and the wide spectrum of radiological findings. The organisms can apparently reside causing minimal damage to the lung in some individuals or, in others, can result in complications and poor outcome if not controlled or left untreated for a prolonged period (especially among those with pre-existing conditions or those who are immunocompromised) (30, 35, 59, 60). Conversely, some patients appear to spontaneously clear infection while others are clinically stable without treatment over long periods of time (5).

The decision to treat NTM-PD is not straightforward: antimicrobial therapy is often prolonged, can be poorly tolerated (61, 62) and is not necessarily effective. For instance, NTM-PD caused by *M. abscessus* in many patients is a chronic, untreatable infection and typically has poor treatment outcomes due to high levels of drug resistance and low sputum culture conversion rates even with therapy (63-65). In addition, the treatment failure rate reported for MAC is up to 40% (9).

Clinicians try to treat or manage factors that they feel are contributing to persistence of NTM in the lung or its associated symptoms. Examples of these include colonisation with other pathogens such as *Pseudomonas* spp, symptomatic gastro-esophageal reflux and sinus disease, and stopping the use of local or systemic drugs that impair mucosal immunity such as cortico-steroids (66).

Although adjuvant surgical therapy to resect damaged lung containing a heavy mycobacterial load has been reported as successful, it can be associated with unfavourable outcomes due to underlying pre-existing morbidity and postoperative complications (67).

Treatment for confirmed NTM-PD disease is also challenging because of associated medication toxicity and uncertain outcome. It would be of considerable clinical value, therefore, to identify markers or predictors for disease activity and progression to help guide treatment decisions (9).

1.6 NTM pulmonary disease

The reason for the variable pathogenicity of NTM in humans is unclear. Whilst the host response to *M. avium* is the best understood of the NTM organisms, why the immunological and clinical response to this organism differs between patients remains unknown (68).

Upon entry into the body, NTM usually settle in the lower airways and, if clinical illness develops, it presents as localised inflammation (airways disease, pneumonia, cavitation) due to the release of cytokines and other mediators (69).

1.6.1 Cavitary disease

Cavitary disease is considered a more severe clinical presentation of NTM infection. It is more likely to be associated with sputum AFB smear positivity and greater mycobacterial load (70). If left untreated it can progress to extensive local disease, including in some cases respiratory failure due to fibrosis or progressive cavitation. The lesions are typically in the upper lobes and often arise where there has been previous pulmonary damage including nodular bronchiectasis (71, 72).

1.6.2 Bronchiectasis

Bronchiectasis is frequently seen in association with NTM-PD, which may precede or be a consequence of the infection.

In general terms, bronchiectasis can be caused by an underlying condition such as CF or other disorders of ciliary function. It may be associated with immune deficiency (especially antibody deficiency) or can occur secondary to infection (66, 73). In some cases, it is believed to be genuinely idiopathic. The

condition presents with persistent cough with sputum production and is fundamentally characterised by impairment of mucus clearance from the airways. The accumulation of mucus in the damaged airways of the lungs generates a favorable nourishing place for bacteria (including NTM) to grow, leading to further inflammation with consequent damage and dilatation of the airways, especially in the right middle lobe or the lingula segment. This destruction is usually accompanied by clinical manifestations and establishes a 'vicious circle' due to the interaction between persistent or recurrent infection and excess inflammation (Figure 1.2). These changes can be detected by CT scan and, in bronchiectasis associated with NTM disease, serial imaging may help in monitoring the progression of the disease among treated and untreated cases. CT scan images beside the medical history are also useful in the prediction of underlying causes, and the assessment of disease severity (74, 75).

Interestingly, a study conducted by Cowman et al. has shown that the host immune response to NTM-PD can contribute to disease development and severity. Their reported findings suggested that downregulation of 213 genes including IFN-γ mediating T cell responses correlated with progressive radiological changes of bronchiectasis on CT scans (76).

Neutrophils are recognized for their significant role in the inflammatory response present in bronchiectasis. These cells are recruited to the airways, where their activation leads to the release of inflammatory mediators (NE) and the formation of structures called neutrophil extracellular traps (NETs). The chronic and sustained neutrophilic inflammation observed in bronchiectasis contributes to tissue damage and structural changes in the airways (77). While these aspects are well-established, there are still unknowns in the field: researchers are actively investigating the triggers of neutrophil activation, the mechanisms regulating inflammation resolution, the impact of neutrophil diversity, and the association of neutrophils with exacerbations. A more comprehensive understanding of these aspects could pave the way for targeted therapeutic interventions and a deeper comprehension of bronchiectasis pathophysiology.

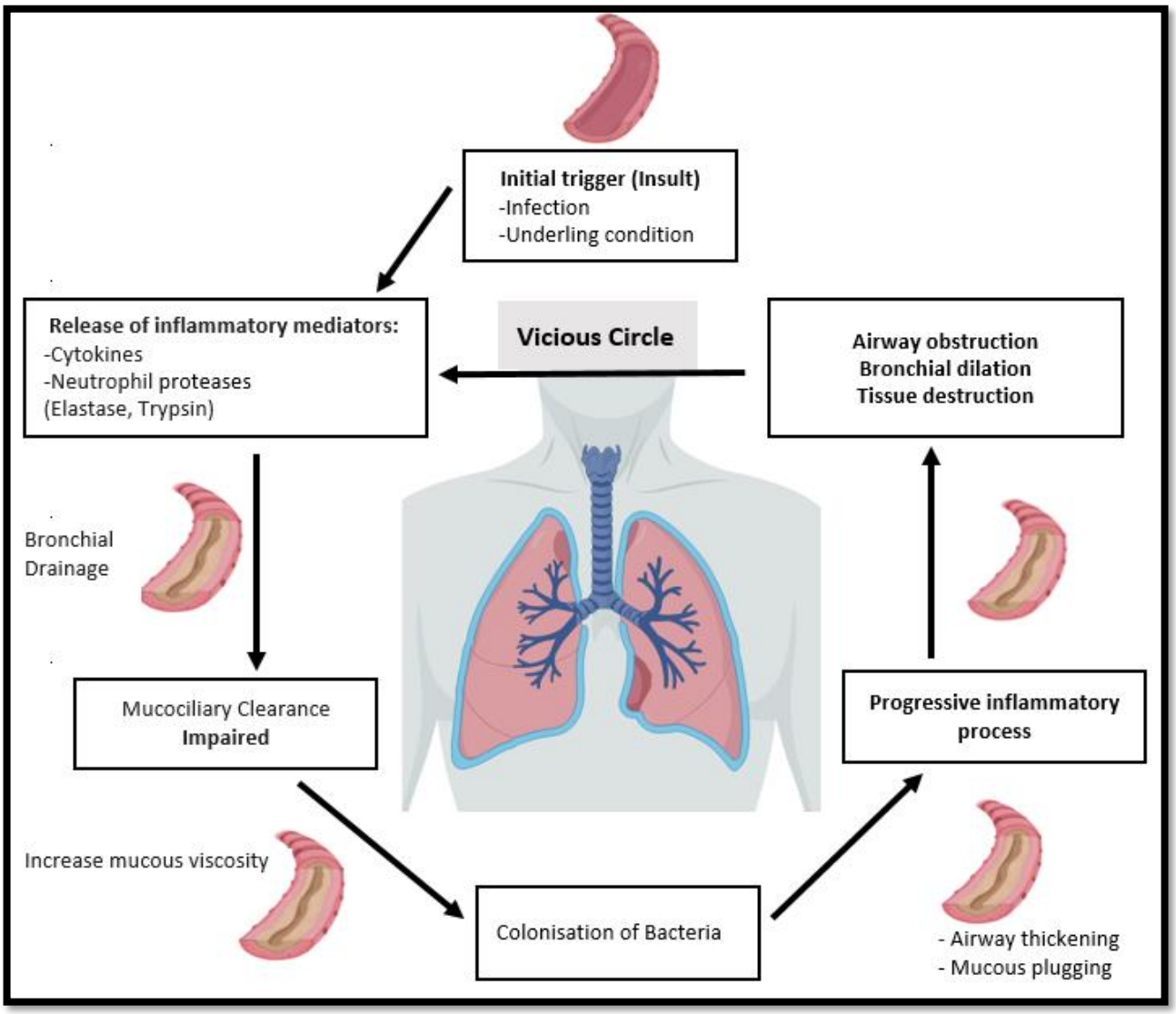


Figure 1. 2: Bronchiectasis: The pathogenesis of bronchiectasis defined as an interaction between distinct phases of infection and inflammation that establish a 'vicious circle' ending with the destruction of the bronchi (damaged airway; widened, scarred and thickened wall with trapped accumulated mucous) and clinical manifestations.

1.7 Neutrophils in the host immune response to NTM

Studies on the immune response to NTM have generally focused on T cells, 'T helper-1' cytokines and mononuclear phagocytes (78). While these are clearly important in protection against NTM (79), their role in either causing or preventing lung damage is less clear.

Generally, professional phagocytes (neutrophils, macrophages, and dendritic cells) are considered as a first line of defence. Neutrophils are a prominent cell type in the bronchial lumen, responsible for tissue damage via the release of granule contents (human neutrophil elastase (NE) in particular) during degranulation. They are strongly implicated in the development of bronchiectasis by releasing mediators (such as inflammatory cytokines, elastases, and matrix metalloproteinases) that damage the bronchial elastin and other lung support structure. Granule-derived molecules could damage host tissues (leading to bronchial dilation) besides their antimicrobial properties which assist in combating the infection (80).

Neutrophils are granulated polymorphonuclear leukocytes produced in the bone marrow and which go through several stages under homeostatic conditions: entry to the circulation, endothelial adherence, migration to tissues, chemotaxis and finally phagocytosis of pathogens. Four groups of granules are found in neutrophils: azurophil (containing enzymes such as NE and antibacterial molecules such as azurocidin and human neutrophil peptides (HNP) 1-3), specific granules, gelatinase granules, and secretory vesicles, each playing specific roles during the response to infection (81). Neutrophils are rapidly recruited to the site of infection when they sense signals from chemoattractant such as Interleukin-8 (IL-8) that are generated by host cells and then migrate through a process called chemotaxis to eventually trap and kill the invading pathogens. Neutrophils' antibacterial functions include phagocytosis (ingestion), degranulation (release of soluble antimicrobials either into the phagosome or extracellularly), and the release of nuclear material in the form of neutrophil extracellular traps (NETs) (82, 83).

Upon a breach of epithelium by bacteria, neutrophils are the first responders to leave the bloodstream and migrate to the site of infection. This recruitment process starts with adherence to activated endothelial cells and rolling, then firm attachment to the endothelium which causes neutrophil arrest before crossing of the endothelial barrier to the infection site. Initiation of neutrophil phagocytosis is significantly enhanced by opsonisation of the bacteria whereby opsonins, for example complement components and immunoglobulins (Igs), coat the bacteria and are recognized by specific surface receptors on neutrophils causing avid binding and triggering ingestion.

Phagocytic cells work to rapidly eliminate NTMs through phagocytosis and intracellular killing; and an impairment in this process can predispose to the development of NTM infection. Despite the fact that the neutrophil response to NTM is poorly studied, previous work has proposed that granulocytes are an important participant in the host defence against mycobacteria (84, 85). These cells can kill several species of mycobacteria (86, 87), yet are also implicated in the pathology of mycobacterial diseases such as tuberculosis (88, 89) – where they are the dominant host cell for infecting organisms (90).

Jones et al., and Majeed et al., reported a high efficacy of mycobacterial phagocytosis by neutrophils through complement-mediated opsonization (91, 92). Conversely, the results of an *in vitro* study by Irina et al., pointed out the low capacity of neutrophils to phagocytise non-opsonized *M. smegmatis* (93). Generally, once phagocytosis has been triggered by engagement of opsonic receptors, internalisation of the pathogen within phagosomes inside the neutrophil occurs in seconds (94).

Subsequently, phagosomal maturation occurs whereby fusion with granules inside the cells takes place. This gives neutrophils unique advantages over other phagocytes as the granules store powerful bactericidal proteins. NADPH oxidase in the wall of secondary granules initiates oxidative burst, leading to the production of antimicrobial reactive oxygen intermediates (superoxide, hydrogen peroxide, hypochlorous acid). However, neutrophil influx may also be associated with pathology through the release of these cytotoxic contents; and if neutrophils are disrupted these processes may lead to damage of neighbouring cells and tissue injury (95, 96).

Several murine models have implicated neutrophils in the host control of NTM infection. TLR-2 deficient mice with *M. avium* infection exhibited defective neutrophil function and a subsequent impairment in controlling the infection in its early stages (97, 98). Conversely, it has been shown that neutrophils might contribute to the pathological dissemination of the infection rather than early clearance among genetically susceptible mice (86), depending on the mycobacterial species (99). Specifically, neutrophils may carry the mycobacteria from the pulmonary surface (90).

Table 1.2 summarises mouse studies suggesting a significant role for neutrophils in the early response to NTM infection.

Table 1.2: Mouse studies suggesting a role for neutrophils in NTM infection and the possible causative mechanism.

Study models/ Reference	Mycobacteria sp	Intervention	Observations
Mouse Intravenously infected 10^6 CFU (100)	*M. avium*	Neutrophils of C57BL/6 mice infused into susceptible beige mice	Decreased the growth rate of *M. avium* compared to control beige mice
		Neutrophil depletion in C57BL/6 mice	Increased growth rate compared to control C57BL/6 mice
Mouse 10^6 CFU or 30mg LPS Intraperitoneally $5x10^4$ CFU or 5mg of LPS Intratracheally (101)	*M. avium*	Gene-disrupted ($CXCR2^{-/-}$) mice infected with *M. avium* or treated with LPS intraperitoneally/ intratracheally	Early and rapid recruitment of neutrophils with *M. avium* infection significantly impaired with CXCR2 chemokine signalling defect compared to controls
Mouse Intraperitoneally infected 10^6CFU (85)	*M. avium*	Inoculation of mycobacteria into CD-l mice.	Neutrophil phagocytosis caused degradation of the bacteria and release of enzymatic granules (lactoferrin) that increase macrophage effectiveness in eliminating mycobacteria and enhancing the further killing process. (Indirect mechanism) .
Mouse Intravenously infected 10^7 CFU (102)	*M. avium*	Administration of G-CSF into C57BL/6 black mice	Neutrophils showed anti-mycobacterial activity. Neutrophil activation inhibited growth compared with control
Mouse Intratracheally inoculated 8×10^7 CFU (103)	*M. abscessus*	Wild type and cystic fibrosis mice inoculated with mycobacteria	High neutrophil number in the bronchoalveolar lavage of mice infected with rough morphotype compared to smooth morphotype

CFU = Colony Forming Unit, **G-CSF =** Granulocyte-Colony Stimulating Factor, **LPS** = lipopolysaccharide

1.7.1 Human neutrophil peptides (HNP) 1-3

HNPs 1, 2 and 3 belong to a family of endogenous cationic antimicrobial and cytotoxic peptides known as 'defensins'. They are localised in the azurophilic granules and considered as one of potential mechanisms by which neutrophils might kill mycobacteria. HNPs also function as immunomodulatory molecules influencing cytokine production as well as inflammatory and immunological responses (104). The ability of HNP-1 to kill *M. tuberculosis* has been studied *in vitro* by Miyakawa *et al*, (105), Sharma et al, (106), Kalita et al, (107), and Martineau et al, (108).

Although the ability of neutrophils to kill mycobacteria remains controversial, and the need for further work is clear, several *in vitro* studies (summarised in Table 1.3) have addressed the capacity of human neutrophils to restrict the growth or kill various NTM species. Little evidence has been reported on the susceptibility to NTM infections among neutropenic patients or those with neutrophil disorders (76), though neutropenia has been associated with disseminated NTM infection (but not pulmonary NTM infection) in patients with haematological malignancy (109).

Table 1.3: *In vitro* studies of neutrophil ability to kill or restrict the growth of NTM.

Organism	Host	Neutrophil purification	Experimental Read out	Observation	Killing (K) / Restriction (R)	Study reference
M. avium	Human (HIV)	Ficoll gradient 98-99% purity confirmed by microscopy	Radiometric assay (Bactec)	Isolated neutrophils from people with advanced HIV responded to exogenously supplied G-CSF by inhibiting the growth of mycobacteria	R 3-10 days	(110)
M. avium	Human	Ficoll sedimentation Purity NR	CFU	Half of the bacteria phagocytosed at 15 min were killed by neutrophils at 45 min, and killing was nearly complete at 120 min.	K 2 hours	(111)
M. avium	Mouse	Ficoll gradient >97% purity confirmed by microscopy	CFU	Neutrophils from mice treated with G-CSF were able to kill *M. avium* ex vivo, compared with controls	K 4 hours	(102)
M. fortuitum	Human	Ficoll gradient Purity NR	CFU	Killing of mycobacteria in the presence of serum, however no killing occurred in the absence of serum	K 2 hours	(112)
M. smegmatis	Human	Percoll, >99% purity confirmed by haematoxylin staining.	CFU	Neutrophils' antibacterial capacities demonstrated with efficient killing of mycobacteria	K Up to 6 hours	(113)
M. abscessus	Human	Percoll, >98% purity confirmed by microscopy	CFU	Mycobacteria activated the neutrophils' bacterial clearance mechanisms, including ROS generation, NET formation, and phagocytosis	K 1 hours	(114)

HIV= Human Immunodeficiency Virus, **K=** Killing (reduction of CFU number), **NR=** Not recorded, **R=** Restriction (slower increase of CFU)

1.8 Neutrophils and adaptive immunity to NTM infection

Neutrophils also have the ability to shape adaptive immunity and make a bridge between the innate and adaptive immune systems (80, 115). Cytokine networks play significant roles in the cell mediated immune response to NTM infection (116). Neutrophils themselves produce cytokines which might impact T cell function, and this might differ in response to the infecting organism. Faldt et al. reported that NTM (*M. avium and M. smegmatis*) induced a significantly higher secretion of TNFα, IL-6, and IL-8 from activated neutrophils than *Mycobacterium tuberculosis* (Mtb) - which suggests some species evoke more effective innate immune reactions that in turn impacts on adaptive immune responses (117).

The T cell response against NTM is regulated by the production of IL-12 following endocytosis of mycobacteria by innate mononuclear phagocytes (eg dendritic cells (DC) and macrophages). Activated $CD4^+$ T cells (T-helper 1) and $CD8^+$ T cells release IFN-γ which is essential for host defence against mycobacteria (118, 119). Therefore, NTM infection which overcomes initial innate mechanisms may be controlled by efficient Th1 responses mediated by IL-12 and IFN-γ. Genetic mutations in the IL-12-IFN-γ pathway increase susceptibility to NTM infection (120, 121). Moreover, IL-17, IL-21, and IL-22 produced by $CD4^+$ T cells (T-helper 17) induce neutrophil influx into sites of inflammation that may arrest the progression of NTM infection (122-124).

However, rather than being protective this neutrophil recruitment may in fact contribute to negative effects – a feature observed in a mouse model of MAC infection where Th1 immunity is impaired (122). Specifically, necrotic neutrophils impair host control of mycobacterial growth in a whole blood model, and increase immunosuppressive IL-10 as well as growth factors and chemokines (125). This may result in further neutrophil accumulation at the site of disease: a pathological cycle that might contribute to the documented association between neutrophilia and poor outcomes (88).

Furthermore, ‘frustrated’ neutrophils that release granule contents extracellularly could drive tissue damage and cause profound effects on T cell differentiation and proliferation (126-128).

The suppressive effect of neutrophils directly or indirectly on T-cell responses are mediated by degranulation of granular constituents or production of chemokines, inactivating T-cell stimulating cytokines, eg IL-2 and IL-6, and speeding up the shedding of IL-2 and IL-6 cytokine receptors on T-cells (80, 129-131). As an example, neutrophil elastase selectively cleaves IL-2 receptor and IL-6 receptor, and leads to a reduction in co-stimulatory molecule expression by DC, thus limiting T cell maturation and affecting the development of the Th1 response (132). Down regulation of T cell receptor (TCR) expression on T-cells can also occur upon release of arginase and the production of reactive oxygen species (ROS) from neutrophils (80, 130). There also appears to be a negative association between baseline neutrophil granule product concentration and downstream Th1 cytokine levels in a human whole blood model of mycobacterial infection, as demonstrated in my supervisor David Lowe's work (Figure 1.3) (unpublished data).

Impaired T cell function of is a risk factor for human NTM infection (133). It has been speculated that attenuation of the immune response, particularly Th1 and innate immune responses among patients without comorbidities, may impact on the pathogenesis of NTM-PD (119).

Conversely, there may also be an action of NETs to reduce T cells' activation threshold (134); while abolition of Th1-specific responses has been reported in a study where neutrophils were depleted during BCG vaccination (135).

In summary, the overall net impact of neutrophils on the T cell response to mycobacterial infection is not known and both positive and negative effects are possible.

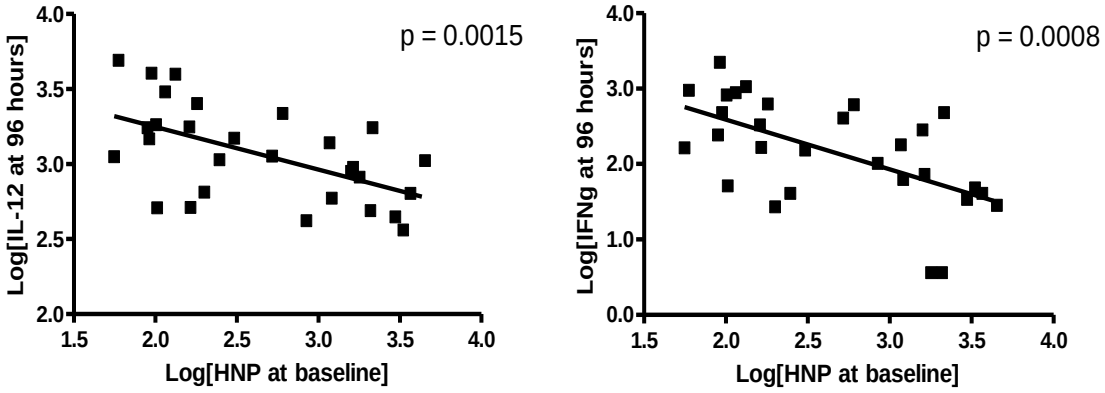


Figure 1. 3: Correlation between baseline Human Neutrophil Peptide 1-3 concentrations and 96-hour Interleukin (IL)-12 and Interferon-γ (IFN-γ).

Whole blood from 10 healthy donors was augmented with autologous necrotic neutrophils, viable neutrophils or medium only and then challenged with *M. bovis*-BCG. HNP 1-3 concentrations were measured in supernatants taken immediately after infection (baseline), and cytokine concentrations were measured in supernatants after 96 hours incubation at 37°C. p values derived from Pearson correlation of log-transformed values. (Unpublished data provided by Dr David Lowe).

1.9 NTM and the humoral immune response

Mycobacteria are intracellular organisms; thus, cell-mediated immunity is considered the major component of the host immunological defence against these bacteria. However, understanding the interaction between antibody-mediated immunity and cellular immunity is useful to determine strategies to combat NTM infection, including treatment and vaccine development. Furthermore, interactions of T cells, B cells and antigen presenting cells (APCs) with neutrophils allows neutrophils to modulate adaptive immunity (128). Neutrophil activation can also play a significant role in B-cell development through the production of B-cell activating factor (BAFF) and granulocyte colony-stimulating factor (G-CSF) (128, 136).

The protective effect of the humoral immune response against mycobacterial antigens has been demonstrated in several models using Mtb. Kunnath *et al*. described the contribution of the humoral immune response to the control of Mtb (137) and Hamasur *et al.* showed the protective effects of mouse monoclonal IgG1 antibody to lipoarabinomannan (SMITB14) against tuberculosis when the mice were infected intravenously (138). Also, Zimmermann *et al.* found that IgA (but not IgG) antibodies specific for different Mtb surface antigens blocked Mtb activity (139). It is unclear whether the same applies to NTM – and this is an area which requires further investigation.

Glycopeptidolipids (GPLs) are a class of glycolipids expressed in the outer layer of several NTM species, including MAC and *M. abscessus.* The GPLs of MAC are highly antigenic and serovar-specific and are associated with MAC virulence (140, 141). A serological diagnostic test measuring the serum IgA antibody against MAC GPLs has been developed and used clinically to diagnose MAC disease. An increase of antibody levels was recorded in patients with NTM-PD caused by MAC and not in patients with Mtb (142).

Subsequent studies suggested that antibody levels might be used to monitor disease activity. One study identified a decrease in antibody level with treatment of MAC-PD, though there was no difference in

patients who did or not respond clinically to drug therapy – suggesting that the measured fall in antibody level was related to a drug-induced perturbation (or that the test had limited clinical value due to its extreme sensitivity) (143).

Overall, many studies have identified the potential role of antibodies against Mtb during the course of infection and have suggested the need to further investigate antibodies in other mycobacterial infections and to explore the mechanisms of their activity (144-147).

1.10 Risk factors for pulmonary NTM disease

Understanding risk factors and identifying those people more likely to develop NTM disease facilitates targeting of clinical interventions to potentially prevent the occurrence of disease.

Direct person to person transmission of NTM has never been convincingly demonstrated, however some cases of inter-individual transmission of *M. abscessus* were identified in CF patients presumably occurring via the clinical environment (9).

Although NTM species can be opportunistic pathogens targeting people with overt immunological abnormalities such as uncontrolled HIV infection, some types of leukemia, and patients taking immunosuppressive medications (148), chronic NTM infection especially of the airways can also be seen in some apparently immunocompetent individuals (149). The underlying reasons that make some people more prone to developing pulmonary NTM infection are poorly understood but there are likely to be defects in local pulmonary or systemic host defence. Table 1.4 summarises the pathogenic mechanisms and other factors that predispose to NTM-PD among immunocompromised and immunocompetent patients.

Table 1.4: Risk factors and features associated with the development of NTM-PD

Mechanism or factor	Dysfunction type and Disease representative / Traits or body and health measurements	Management	References
Pulmonary Defect	Structural & functional pulmonary disease:		
	- Ciliary defect, e.g., CF and PCD (genetic)	Non-modifiable	(150, 151)
	- Mucocilliary dysfunction →Bronchiectasis	Modifiable	(152, 153)
	- Inspissated secretions, e.g., ABPA	Modifiable	
	- PAP	Non-modifiable	(154)
	- Non-CF bronchiectasis & Cavitation	Modifiable	(119)
	- CF (Genetic)	Modifiable	(28, 155)
	- COPD	Modifiable	(156)
	- Pneumoconiosis	Non-modifiable	(157)
	- Congenital → Cartilage & elastin deficiency in the airway	Modifiable	(158) (159, 160)
	- Alpha-1 antitrypsin deficiency (Genetic)	Modifiable	(161)
Immunological factors and genetic disorder	Immune deficiency and dysregulation:		
	- Inherited disorders of IFNγ-IL12 pathway (e.g., IFNγR1 mutations).	Non-modifiable	(162)
	- Cytokine signaling (e.g., STAT mutations).	Non-Modifiable	(163)
	- Mononuclear phagocyte impairment (e.g., GATA2, NRAMP1).	Non-modifiable	(164, 165)
	- Anti-interferon gamma antibodies.	Modifiable	(166)
	Systemic immune deficiency		
	- HIV (low CD4)	Modifiable	(167)
	- Organ transplant	Modifiable	(168, 169)
	- Autoimmune disease, eg rheumatoid arthritis - due to intrinsic T cell dysfunction or using of immunosuppressive treatments	Modifiable	(170-172)
	Iatrogenic Immune suppression:		
	- Immunosuppressive agents e.g., IC and Cytotoxic therapy	Modifiable	(173-175)
	- Targeted therapy e.g., anti TNF-a therapy	Modifiable	(176)
Host association factors in Immunocompetent	Age: Elderly people above 65 years old	Non-modifiable	(177)
	Gender: Slender postmenopausal females - possibly due to abnormal hormonal expression	Non-modifiable	(178)
	BMI: Lower body mass considered as a risk factor; however abdominal fat ratio not related to NTM-PD progression.	Modifiable	(179, 180)
	Vitamin levels: Vitamin D deficiency	Modifiable	(181)
	Stature: Characteristic of greater than average height-thoracic skeletal abnormalities.	Non-modifiable	(182)
	Comorbidities: GORD, Diabetes, Chronic kidney disease and ABPA	Modifiable	(6, 78, 183, 184)

ABPA: Allergic bronchopulmonary aspergillosis, **BMI:** Body Mass Index, **CF:** Cystic fibrosis, **COPD:** Chronic Obstructive Disease, **GORD:** Gastro-oesophageal reflux disease, **IC:** Inhaled Corticosteroids, **PAP:** Pulmonary alveolar proteinosis, **PCD:** Primary ciliary dyskinesia. Modifiable: can be reduced or controlled, non-modifiable: cannot be changed

Increased susceptibility to NTM disease is seen in individuals with immunocompromise due to systemic illness, iatrogenic immunosuppressive agents or primary immune deficiencies (PID) (183). PID, most commonly presenting as antibody deficiency disorders in adults such as common variable immune deficiency (CVID) and X-linked agammaglobulinaemia (XLA), predispose to recurrent and unusual infection. Some PID patients are more vulnerable to NTM infection due to cellular or phagocytic dysfunction (185), but interestingly NTM-PD are rarely seen in patients with CVID and XLA despite a high frequency of co-existing bronchiectasis and other lung diseases (186, 187).

1.11 NTM infection in CVID and XLA

CVID is a heterogeneous disorder presenting with impaired antibody production that increases susceptibility to bacterial infection, resulting in recurrent and often severe disease primarily affecting the respiratory tract. CVID presents in both children and adults, however the diagnosis is usually made between the ages of 20-40 and cannot be confirmed in children under 4 years old. The broad spectrum of clinical manifestations and unknown molecular defects in the majority of patients make the identification of CVID cases challenging. There is an increased risk of autoimmune disorders and cancer due to underlying dysregulation of the immune system.

A compatible clinical picture with supportive laboratory evidence forms the new proposed diagnostic criteria of the European Society of Immune Deficiencies (ESID) and the Pan American Group for Immune Deficiency (PAGID). A reduced serum IgG level with poor serological responses to polysaccharide vaccines plus a history of recurrent infections or autoimmune / inflammatory disease are the key features (188). CVID should be suspected in all patients with recurrent bacterial infections especially those localized in the respiratory tract. Other primary or secondary immunodeficiencies which present with clinical findings similar to CVID should be ruled out. CVID is managed with regular follow up and immunoglobulin replacement therapy is administered to prevent complications (189-192).

Many patients also receive prophylactic antibiotics, especially in the context of underlying lung disease.

Similar to CVID, XLA is a genetic disorder that also results in patients exhibiting antibody deficiency, in this instance (usually) complete agammaglobulinemia. XLA affects the body's ability to fight infection due to a defect in the Bruton's Tyrosine Kinase (BTK) gene, which prevents B cells from developing normally (187).

Studies suggest that due to impaired antibody production in CVID, there are alterations in neutrophil chemotaxis and phagocytosis. These defects may contribute to the increased susceptibility of individuals to recurrent bacterial infections (193, 194).

The compromised immune system in CVID and the affected individual's inability to control pathogens, results in persistent bacterial respiratory tract infections. Common pathogens include *Haemophilus influenzae* and *Streptococcus pneumoniae*. Over time, these repeated infections can result in bronchiectasis and other structural lung changes. The chronic lung disease associated with CVID significantly impacts the quality of life for affected individuals, emphasizing the importance of clinical interventions to manage both the immune deficiency and the associated pulmonary complications (195).

XLA and CVID are severe primary immunodeficiencies, and many patients develop progressive bronchiectasis due to their antibody deficiency (196, 197). However, despite severe immune system impairment, XLA and CVID patients in our clinic at the Royal Free Hospital do not frequently have NTM-PD and in the UK Primary Immunodeficiency Network (UKPIN) registry, there are no recorded diagnoses of NTM infection among these patients (198).

There was also not a single reported case of NTM-PD case among 252 CVID patients in a cohort study investigating the types of infection in CVID cases (186). In a similar manner, another study summarized infectious complications within a United States Registry of 201 XLA Patients, and only one patient with pulmonary disease caused by *M. avium* was identified (187).

1.12 Conclusions

In this chapter I have sought to outline the epidemiology, clinical presentation and immunology of NTM, and in particular the roles of neutrophils and antibodies. To summarise:

- Neutrophils may have the ability to eliminate NTM through phagocytosis and intracellular killing (including ROS generation), or via NET formation with the aid of granular proteins, and any defect in this process might lead to the progression of NTM infection.
- Neutrophils influence the development and shaping of adaptive immunity by acting as a bridge between the innate and adaptive immune systems.
- Neutrophils are implicated in the pathology of bronchiectasis by releasing mediators (including cytokines, elastases and proteinases) which destroy the bronchial elastin, leading to permanent dilatation of the bronchi.
- Antibodies may be important in the host response to NTM infection and will help to mediate opsonophagocytosis by neutrophils.
- Patients with antibody deficiency syndromes (CVID and XLA) rarely suffer NTM infection despite frequent bronchiectasis.

Chapter 2: Aims and hypotheses

Recent work has suggested that neutrophils may play an important role in controlling NTM infection. However, neutrophils can also contribute to pathology in NTM-PD, for example in the development of bronchiectasis**.** Although experiments to understand the ability of neutrophils to internalise and kill mycobacteria exist, the results are still controversial and inconsistent and there is debate in the literature about the role of neutrophils in mycobacterial infection.

Aim:

In response to the identified research gap, the aim of my thesis was to investigate neutrophil response to mycobacterial infection in three clinically-different populations: (1) healthy adults who did not develop NTM-PD, (2) adults with CVID/XLA who are at risk of developing lung disease but do not develop NTM-PD, and (3) adults with NTM-PD.

Hypotheses:

- Adults with CVID/XLA exhibit differences in their innate immune response to NTM compared to adults with NTM-PD and healthy comparators. Specifically, their phagocytic activation and restricting mechanisms are more effective in eliminating NTM.

- The opsonisation capacity of heat-inactivated and normal (non-heat-inactivated) serum and sputum differs between the study populations and is highest in CVID/XLA patients.

- The levels of neutrophilic inflammation markers (in serum, sputum and whole blood supernatant) and/or humoral immune response indicators (in serum and sputum) differ between the groups (NTM-PD patients, CVID/XLA patients and healthy donors).

- The expression of inflammatory cytokines is greater in whole blood samples from NTM-PD patients compared to CVID/XLA patients and healthy comparators before and after exogenous mycobacterial infection.

- Neutrophil markers in sputum, serum and whole blood supernatant, and humoral immune response indicators in serum and sputum obtained from patients with NTM-PD are associated with clinical symptom and radiological (CT scan) severity scores in NTM-PD.

- Among NTM-PD patients, those with severe disease, indicated by high CT scan scores and high concentrations of host neutrophil markers in serum, will have a distinct gene expression as evidenced by a whole blood host RNA transcriptomic signature compared to those with less severe lung disease.

Objectives:

- Investigate the activation of neutrophils and their ability to phagocytose and restrict NTM in the three study populations (Chapter 4)
- Determine the opsonisation capacity of participants' serum and sputum (Chapter 4).
- Measure relevant antibodies (anti-GPL-core IgA in serum, and total IgG in both serum and sputum), neutrophil-derived products (MMP-8/-9, S100A8/A9, Neutrophils Elastase, and HNP1-3 in serum, sputum, and whole blood supernatant), and cytokines and chemokines present in whole blood supernatant (Chapter 5).
- Investigate adults with NTM-PD patients and untreated NTM infection to assess how the severity of lung disease, defined by a radiological score, relates to: (i) measures of neutrophil activity and function in blood and sputum, and to (ii) health status assessed by standard respiratory and general health questionnaires (Chapter 6).

- Explore whether a transcriptomic signature in blood can be identified that might differentiate severe and less severe radiological disease in people with NTM-PD (Chapter 6).

Chapter 3: Materials and Methods

3.1 Health and safety consideration

NTM are categorised as Hazard Group 2 pathogens. For this reason, all experiments involving NTM have been carried out following risk assessments in a Containment level 2 Laboratory (CL2).

3.2 Human Participants

All samples were collected from healthy adult donors, adults with CVID/XLA at the Institute of Immunity & Transplantation (IIT) and adults with NTM-PD at the Grove Centre Mycobacterial Clinic in the Royal Free Hospital. All samples were taken under the guidelines of the Human Tissue Authority Act 2004 and only from patients who had given prior consent following NHS Research Ethics Committee approvals (for immunodeficient patients (REC 04/Q0501/119), for healthy donors (08/H0720/46) and for NTM-PD patient (20/PR/0118)).

Venous blood samples were collected from participants directly into sodium heparin tubes or EDTA tubes to be used for the mycobacterial restriction assay assessment, neutrophil activation, phagocytosis assay and neutrophil isolation. All serum tubes were centrifuged at 800g for 5 minutes, and the serum stored at -80°C for neutrophil-related products and immunoglobulin level measurement via enzyme-linked immunosorbent assay (ELISA). The cytokines in the whole blood supernatant were analysed using Luminex.

Whole blood samples for RNA extraction were collected from NTM-PD patients into Tempus tubes (3 ml blood per tube) according to the manufacturer's instructions (Applied Biosystems™, 4342792) and stored at -20°C.

Sputum samples were collected from CVID/XLA patients and NTM-PD patients and centrifuged at 18,000xg for 60 minutes at 4^{o}C, the supernatant aspirated and stored for immunological assessments. The sputum pellets were stored in 500µl RNA Later for further research.

3.2.1 Participants characteristics and Exclusions

Summary of the participant characteristics in Table 3.

a. Adults with CVID/XLA:

A clinical proforma was completed by patients (and the treating clinician) including how frequently they receive immunoglobulin infusions, current medication and antibiotics, (Appendix 1).

Inclusion Criteria

- Clinically diagnosed with CVID or XLA
- Able to produce sputum spontaneously
- Able to provide written informed consent
- Age ≥ 18 years

Exclusion Criteria

- Known HIV infection, chemotherapy, corticosteroid treatment equivalent to ≥20mg prednisolone.
- Known or suspected active malignancy, with the exception of non-metastatic non-melanoma skin tumors (eg basal cell carcinoma)
- Stage IV chronic kidney disease or dialysis
- Decompensated liver failure
- Patients who cannot provide written informed consent

b. Adults with NTM-PD:

Patients with untreated NTM-PD were recruited over a period of 12 months. They were identified via the isolation in sputum of a slow-growing NTM species and approached by investigators who are part of their regular care team during their visit to clinic (or occasionally on an inpatient ward).

Following the approved protocol for the NTM study, eligible patients were invited to participate in the study and provided with a patient information sheet. The study was verbally explained to the patients, and those willing to participate signed a consent form, (Appendix 2, 3, and 4).

Inclusion Criteria

- Two sputum cultures with consistent isolation of the same slow-growing NTM species on different days or a single positive culture from bronchoalveolar lavage (BAL)
- Able to expectorate and spontaneously produce sputum samples, or willing to undergo sputum induction procedure
- Able to provide written informed consent
- Age ≥ 18 years

Exclusion Criteria

- Known or suspected significant primary immunodeficiency (including if diagnosed on tests in the research study), HIV infection, chemotherapy, biologic therapy, corticosteroid treatment equivalent to ≥20mg prednisolone for at least 4 weeks, other immunosuppressive medication at direction of study Steering Committee
- Contraindication to sputum induction procedure if required to obtain samples
- Known or suspected active malignancy, with the exception of non-metastatic non-melanoma skin tumors (eg basal cell carcinoma)

- Contra-indication to diagnostic X-ray procedures, including previous adverse reaction
- Stage IV chronic kidney disease or dialysis
- Decompensated liver failure
- Patients involved in any ongoing drug intervention study
- Patients who cannot provide written informed consent

c. Healthy comparators

A group of healthy adults served as a comparator to the two other groups. They provided a valuable reference for evaluating and understanding the results obtained in the other study populations. Importantly, all individuals in the healthy comparator group had no underlying immune compromise and were not taking any medications at the time of the study.

Table 3. 1: Participant demographics.

	CVID/XLA (n=18)	NTM-PD (n=15)	Healthy Comparators (n=16)
Age (years) -Range -Median [IQR]	 25-81 61(37-74)	 63-94 77(68-80)	 25-63 35 (26-58)
Gender	M (9) – F (9)	F (8) – M (7)	F (7) – M (9)
-Using systemic corticosteroids -Dosage	(n=4) 5mg/day (n=2) 10mg/day (n=2)	-	-
-Macrolide prophylaxis (Azithromycin/Clarithromycin) -Dosage	7 Azithromycin 250mg 3 times a week (n=4) Azithromycin 500mg 3 times a week (n=2) Clarithromycin 250mg twice a day (n=2)		
Other antibiotic prophylaxis	6		
-Ig preparation	Gamunex 10% (n=6) CUVITRU (n=1) Gammaplex (n=1) Flebogamma (n=1) Intratect 10% (n=4) Privigen (n=2)		
Neutrophils count (x10^9/l)*	4.1 (2-6.2)	5.3 (4.4-6.6)	-
Lung disease/bronchiectasis -Bronchiectasis -COPD -Fibrosis -Cavitation -Nodules -GLILD -Emphysema -Bronchial wall thickening	 n(13) - - - - n(2) n(1) n(1)	 n(14) n(1) n(2) n(3) n(2) - - -	-
Respiratory infection -Pseudomonas aeruginosa (PA) -Haemophilus Influenzae -Staphylococcus Aureus -Stenotrophomonas maltophilia -Streptococcus pneumoniae **Other infection** -Aspergillus -UTI	 - n(4) - - n(1) - -	 n(4) n(2) n(2) n(1) n(1) n(1) n(1)	-
Other immune suppressive drugs -Hydroxychloroquine -Steroid inhalers -Infliximab -MMF -Mesalazine	 n(1) n(3) n(1) n(1) n(1)	 n(1) n(1) - - -	-
Significant Inflammatory conditions -Low Ig state following NHL treatment -Colon cancer -Renal cell carcinoma -GLILD and AIHA -SLE	 - - - n(2) n(1)	 n(1) n(1) n(1) - n(1)	-
Anti-NTM treatment	-	-	-

* The normal range of neutrophils count is (1.5 – 8.0) X 10^9/l , Participants' data presented as Median values and interquartile range.
NHL=Non Hodgkins Lymphoma, MMF= Mycophenolate Mofetil, GLILD= Granulomatous Lymphocytic Interstitial Lung Disease, AIHA= Autoimmune Hemolytic Anemia

3.3 Bacterial strains

Mycobacterium avium ATCC 25291 (NCTC13034) and *Mycobacterium abscessus* ATCC 19977 (NCTC 13031) were obtained from the National Collection of Type Cultures (NCTC) operated by Public Health England. Mycobacterial strains were both transferred to growth media (Middlebrook 7H11 agar (7H11), Middlebrook 7H9 broth (7H9) and Lowenstein-Jensen (LJ) media) upon receipt and incubated at 37°C for 2-7 days for sub-culturing.

3.3.1 Mycobacterial culture preparation and storage

M. avium (slow growing NTM species) and *M. abscessus* (fast growing) require specific environments. 7H9 liquid media was prepared by dissolving 2.3g of dried powder Middlebrook 7H9 broth base (Sigma-Aldrich M0178) in 450ml dH_2O together with 5ml of 50% glycerol (Sigma-Aldrich G7757) and 2.5ml 20% Tween 80 (Sigma-Aldrich P5188) then autoclaved at 121°C and 15psi for 1 hour. After cooling, the broth was stored in the fridge for up to a month and before using the media, Middlebrook OADC (Oleic acid, Albumin, Dextrose, Catalase) growth supplement (Sigma-Aldrich M0678) was added at a ratio of 1ml per 9ml 7H9 media. 7H11 solid media was prepared by dissolving 10.35g of dried powder Middlebrook 7H11 agar base powder (Sigma-Aldrich M0428) in 450ml dH_2O together with 4ml of 50% glycerol (Sigma-Aldrich G7757). Autoclaving was performed at 121°C for the same pressure and time as for 7H9. After cooling, the media was enriched with 100ml of Middlebrook OADC growth supplement, then the content was mixed thoroughly before being dispensed into agar plates and allowed to solidify under sterile conditions. Plates were stored in the fridge until use.

3.3.2 Colony Forming Unit

Colony Forming Units (CFU) were established by pipetting 20µl of bacterial suspension onto 7H11 agar media and streaking with a sterile loop spreader. 10-fold serial dilutions (using 7H9) were plated, taking 3 aliquots from each concentration. The plates were kept inside plastic bags in the incubator at 37°C until the colonies became visible to be counted; for *M. avium* this usually occurred four to six days from inoculation and for *M. abscessus* two to three days from inoculation. The colonies were counted with the help of a magnifying glass (Figure 3.1).

3.3.3 Optical Density

Optical Density (OD) measurements were performed using a 7200 Visible 72 Series Diode Array Scanning Spectrophotometer (Jenway, WZ-83056-01) at 600 nanometers (nm) as a standard method for estimating the mycobacterial concentration in a broth medium. This instrument measures the light intensity passed through the sample at different time points. Absorbance readings were adjusted to zero using un-inoculated medium (7H9) in a 1 ml sterile cuvette. The digital absorbance readings for bacterial suspension were taken in triplicate and the average was recorded for growth curve estimation. The growth curves for both *M. avium* and *M. abscessus* were plotted using both CFU and OD/600 (Figure 3.2).

3.3.4 Mycobacterial Growth Indicator Tube

The Mycobacterial Growth Indicator Tube (MGIT; Becton Dickinson Microbiology Systems), used for the rapid detection of mycobacterial growth, was employed for detecting time to positivity (TTP) for *M. avium* and *M. abscessus* over 25 days (Figure 3.3). The MGIT contains 7ml of modified Middlebrook 7H9 broth base; 800µl of MGIT 960 Growth Supplement was added to make the medium complete before adding the bacterial suspension. MGIT tubes were entered into a MGIT 960 instrument where they were incubated and monitored for increasing fluorescence as an indicator that mycobacteria were reaching a threshold of bacterial growth. The instrument declared a tube positive when there were

approximately 10^5 CFU per ml of medium. The growth of mycobacteria results in light visual turbidity (while contaminating bacteria generally produce heavy turbidity).

3.3.5 Preparation of mycobacterial stocks

Working stocks of both mycobacterial strains were made by inoculating 100µl of each strain into 9ml of 7H9 broth with 1ml OADC and incubating at 37°C until they reached mid-log phase; for *M. avium* this occurred at 7 days and for *M. abscessus* at 5 days. The cultures were then transferred into screw-cap tubes to be stored at -80°C until use

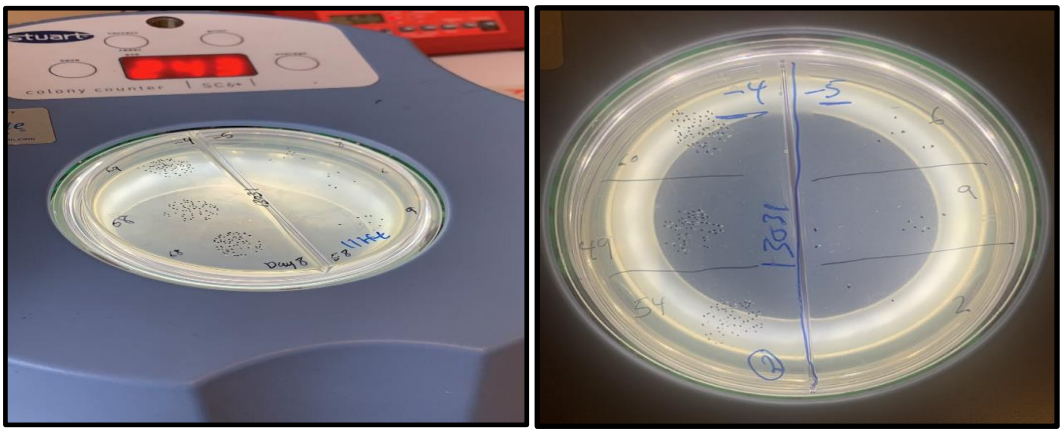


Figure 3. 1: Culture of *M. abscessus* (13034) and *M. avium* (13031) on 7H11.
The plates were incubated at 37°C, then the visible colonies were counted with the help of a magnifying glass; Colony forming unit per ml (CFU/ml) calculation = Average number of colonies for a dilution x Dilution factor x 50.

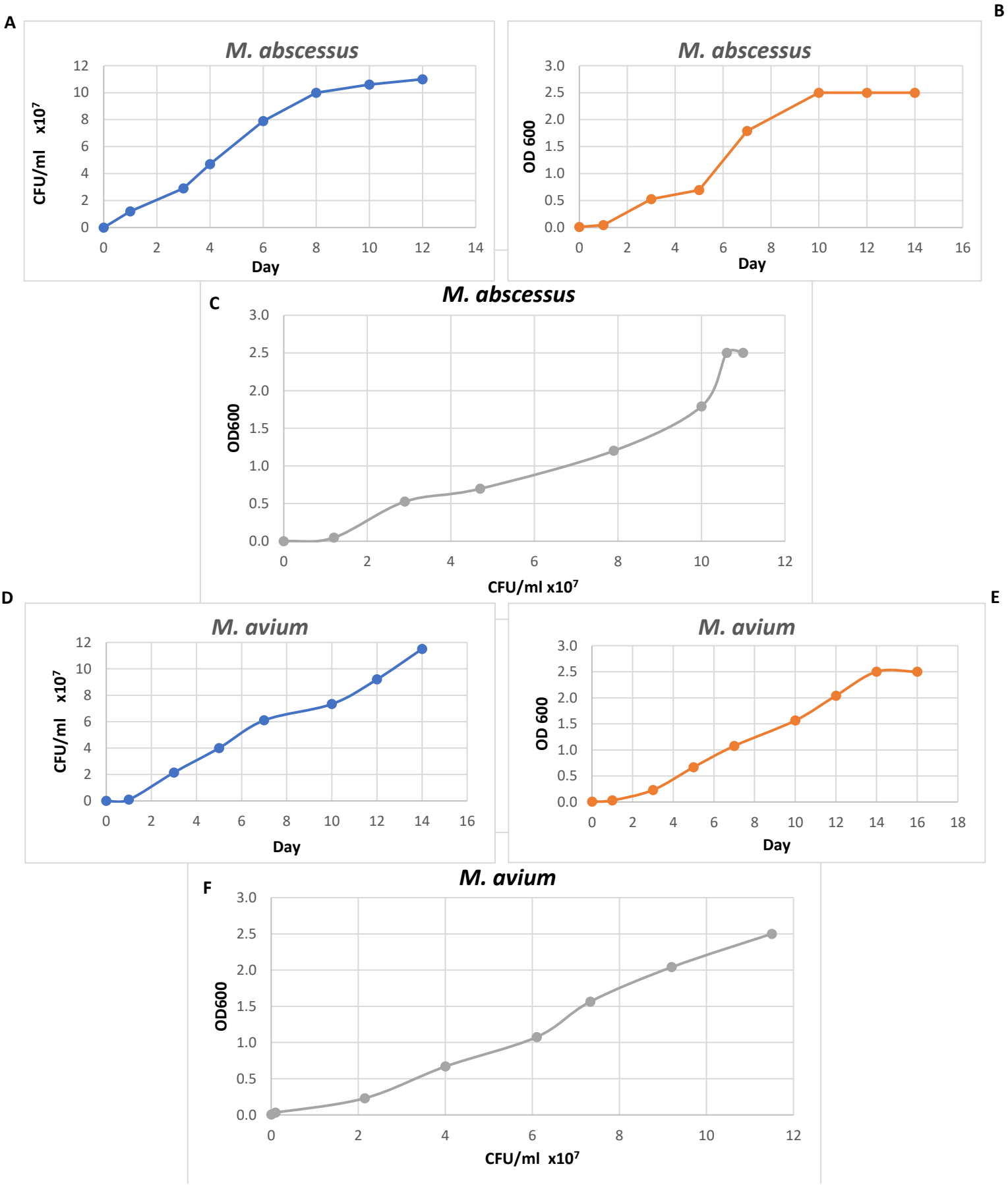


Figure 3. 2: *M. abscessus* and *M. avium* curves.
(A and D) *M. abscessus* and *M. avium* growth curves at 37°C over 12-14 days. The colonies become visible and countable for *M. abscessus* between day 2 and day 3 when they reach around 4.25x10^7 CFU/mL (as expected for an RGM). For *M. avium* (an SGM) this occurred between day 3 and day 4 with 2.72x10^7 CFU/m. The growth becomes limited starting from day 10 for RGM and day 12 for SGM (the stationary phase). **(B and E)** Optical Density measurement at 600nm for both species. An OD of 2.5 indicates that the mycobacterial growth has reached a plateau. **(C and F)** CFU/mL (x10^7) plotted against optical density at 600nm. These graphs are from one representative experiment out of two separate experiments

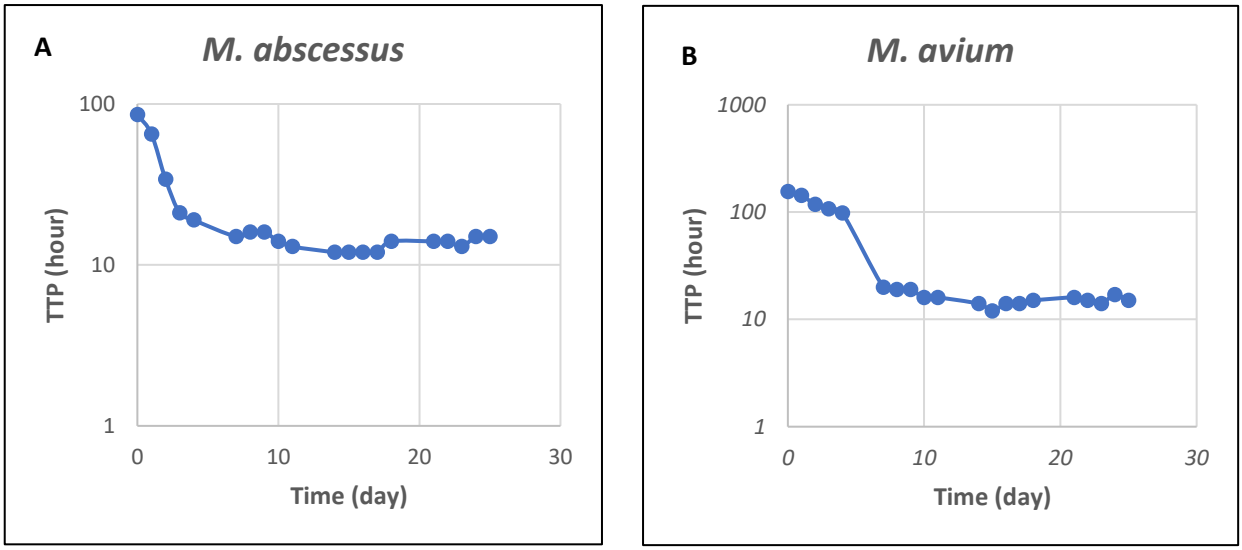


Figure 3. 3: Time To Positivity (TTP) standard curve for *M. abscessus* (A) and *M. avium* (B) by MGIT incubated at 37°C at different times up to 25 days on logarithmic scale. These plots are from one representative experiment out of three separate experiments.

3.4 Antibodies and fluorochromes

All human antibodies were monoclonal and of mouse origin (Table 3.1). eFluor450 Fixable Viability Dye was used for gating of dead cells. Trypan blue, which stains both dead and fixed cells (signal seen maximally in APC channel), was used in flow cytometry with phagocytosis assays to 'quench' extracellular fluorescence indicating surface-bound organisms (199).

Table 3. 2: Fluorochromes and dyes used; CD14-PE, CD15-PE Texas Red, CD16-FITC, CD62L-BUV737, CD64-APC, CD66b-PE-Cy7, FVD

Antibody	Fluorophore	Clone	Working concentration/ Volume per sample	Manufacture/ Catalogue
Human CD14	PE	M5E2	20µl / sample	BD 555398
Human CD15	PE-Texas Red	CF594 W6D3	1µl / sample	BD 562372
Human CD16	FITC	-	30µl / sample	BD 335035
Human CD62L	BUV737	DREG-56	5µl / sample	BD 741843
Human CD64	APC	10.1 RUO	5µl / sample	BD 561189
Human CD66b	PE-Cy7	G10F5	5µl / sample	eBioscience 25-0666-41
eFluor450 FVD	Pacific Blue	-	1µl / sample	eBioscience 65-0863-14
Trypan Blue	APC	-	12.5µl / sample	Sigma-Aldrich T8154-100ML

CD = Cluster of Differentiation; **PE** = Phycoerythrin; **PE-Texas Red** = Phycoerythrin Texas Red; **FITC** = Fluorescein isothiocyanate; **APC** = Allophycocyanin; **BUV737**= BD Horizon Brilliant™ Ultraviolet**; PE-Cy7**=Phycoerythrin with cyanine dye; **µl**=microliters; **FVD**=Fixable Viability Dye eFluor™ 450

3.4.1 Optimisation of assays and titration of fluorochrome concentrations

All antibody volumes were individually determined by titration and spectral overlap was avoided as far as possible. The concentrations tested showed separation between the positive and negative populations. The best resolution in the context of the full panel used was defined as shown in Figure 3.4 and Figure 3.5.

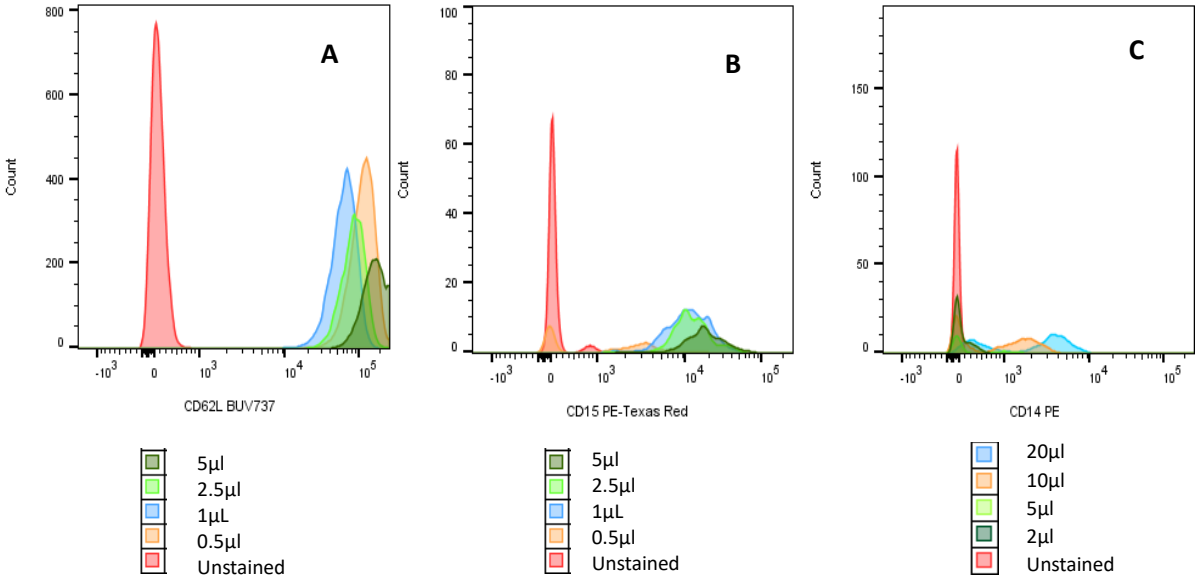
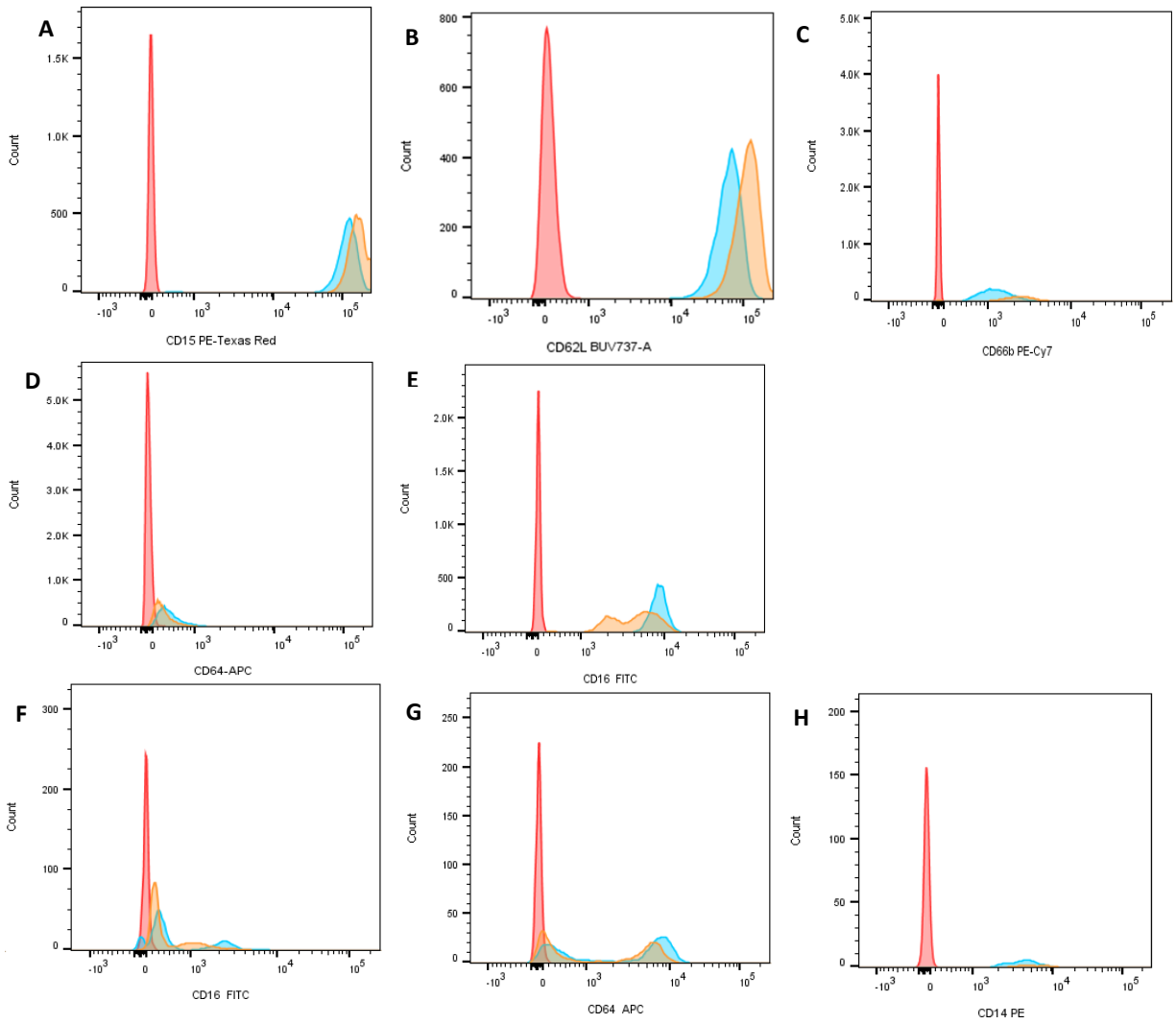


Figure 3. 4: Preliminary comparison of histograms using four different concentrations of fluorochrome-conjugated antibody; (blue) histogram demonstrated the maximum or best separation compared with other concentrations and at a distance from the unstained (red) histogram but without over-staining. **(A)** CD62L for neutrophils, **(B)** CD15 for neutrophils, and **(C)** CD14 for monocytes; optimal concentrations of 1µl, 1µl, and 20µl, respectively. These histograms are from one representative experiment out of three separate experiments.



Red = Control Unstained - Blue = Optimal concentrations - Orange = Other tested concentration

Figure 3. 5: Determining optimum antibody concentrations.
A – E. Histograms are presented indicating fluorescence intensity for the described surface markers/fluorochromes on neutrophils, using a single fluorochrome-conjugated antibody at varying concentrations (blue, orange) or unstained (red). **F-H**. As above but for monocytes. In all cases, the concentrations used in the histograms marked in blue were more reliable than those marked in orange and were chosen for future experiments. These histograms are from one representative experiment out of three separate experiments.

3.5 Fluorescein isothiocyanate (FITC) labelling of mycobacteria

3.5.1 Preparation of stock solutions:

a. Carbonate bicarbonate buffer, pH 9.6

One capsule of carbonate bicarbonate (Sigma-Aldrich, C3041) was dissolved in 100ml deionized water and pH adjusted to 9.6. The buffer was sterilized using a 0.22µm syringe filter and stored at room temperature until use for no longer than 24 hours.

b. FITC

FITC stock solution was freshly prepared for each assay by mixing 10mg of FITC (Sigma-Aldrich, 46950) with 1 ml Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D8418) and vortexing vigorously until dissolved.

3.5.2 Preparation of FITC-labelled mycobacteria bacteria

For FITC labelling of mycobacteria, 10 ml of mid log-phase organisms in 7H9 were harvested by centrifugation at 10,000g and 4°C for 5 minutes. Bacterial cells were re-suspended in 1ml of carbonate bicarbonate buffer containing 0.05% Tween 80 (Fisher Scientific Ltd, 11493601). 5µl of FITC stock solution previously made to 10mg/ml was added to the cell suspension and immediately vortexed, the cells were then incubated in the shaking incubator for 30 min at 37°C. The labelled mycobacteria were spun in a micro-centrifuge at 4000g for 2 minutes, the supernatant was discarded, and the pellet was re-suspended in 1ml PBS pH 7.2 (Thermo Fisher Scientific, 20012019) containing 0.05% Tween 80. This washing step was repeated twice to remove unbound dye and finally the mycobacteria were re-suspended in 7H9. Optical density was measured at 600nm to confirm the concentration of suspension reached $2x10^7$ CFU/ml; otherwise, it was appropriately diluted with 7H9.

3.6 Phagocytosis assay

The assay was performed similarly to previous descriptions (199). 250µl of whole heparinised blood and 250µl RPMI-1640 were aliquoted into sterile 5ml flow cytometry tubes. 100µl of FITC-labelled *M. avium* or *M. abscessus* were added to the blood samples. Samples were mixed well on a vortex before being incubated on the shaking incubator (150rpm) at 37°C for 1 hour. After incubation, 200µl of sample was transferred into new tubes. 20µl CD14-PE (BD 555398), 1µl CD15-PE Texas Red (BD 562372), 1µl eFluor450 Fixable Viability Dye (eBioscience, 65-0863-14) and 12.5µl of 0.2 micron syringe filtered Trypan blue were added to the tubes and kept on ice for 20 minutes. Single stained controls for each antibody were also prepared, as well as an unstained tube. 3ml of cold FACS Lysing Solution (BD Biosciences, 349202) was added to the tubes (having been prepared previously by combining 10ml of 10x solution and 90ml distilled water). Samples were placed on ice and incubated in the dark for 10 minutes. Whilst samples were incubating, the centrifuge was cooled to 0°C. Samples were then spun at 400g for 5 minutes. The supernatant was aspirated, and the pellets resuspended in 1ml of cold Stain Buffer (BD Biosciences 554656). After a further centrifuge spin (400g for 5 minutes), the pellets were resuspended in 500µl 20% paraformaldehyde. All samples were acquired immediately on a Becton Dickinson LSR Fortessa flow cytometer running on FACS Diva 8 software.

3.7 Activation assay

400µl of whole heparinised blood and 400µl RPMI-1640 were aliquotted into sterile 5ml flow cytometry tubes. 100µl of (unlabelled) *M*. *avium* or *M*. *abscessus* were added to the blood samples. All samples, including those chalenged with *M. avium and M. abscessus*, were mixed and incubated for 2 hours in the shaking incubator at 37C before staining.

After incubation, 200µl of sample was transferred into a new tube. 1µl CD15-PE Texas Red (BD 562372), 1µl l eFluor450 Fixable Viability Dye (eBioscience, 65-0863-14), 30µl CD16-FITC (BD 335035), 1µl CD62L-BUV737 (BD 741843), 5µl CD64-APC (BD 561189) and 5µl CD66b-PE-Cy7 (eBioscience 25-0666-41) were added.

The samples were lysed, washed, and fixed similarly to the phagocytosis assay. However, in this instance the whole assay was carried out at room temperature. Fresh, unchallenged samples from healthy donors and patients were also processed without 2 hours incubation for comparison and to assess baseline levels of neutrophil activation. All samples were also acquired on a Becton Dickinson LSR Fortessa flow cytometer, using FACS Diva 8 software.

3.8 Gating Strategy

Fluorochrome compensation was performed using unstained and single-stained samples acquired contemporaneously with each experimental sample. The gating strategy used for all samples was to first define singlets using forward scatter area (FSC-A) versus forward scatter Height (FSC-H), followed by the exclusion of dead cells (Viability dye positive events).

For the phagocytosis assay, neutrophils were defined as positive for PE-Texas Red conjugated CD15 with high side scatter. Neutrophils were divided into quadrants on the basis of CD15 (PE- Texas Red) and FITC signal.

When a sample was challenged with FITC-labelled mycobacteria, events positive for FITC among the PE Texas Red (CD15)-positive events were counted to assess neutrophil phagocytosis (mycobacterial uptake). This percentage was compared between patients and healthy donors for the two different mycobacterial strains (Figure 3.6). Phagocytosis by monocytes was determined as the percentage FITC-positive among events positive for PE-conjugated CD14 (Figure 3.7).

For activation, neutrophils were defined among live cells. Neutrophil activation was determined by the expression of CD66b, CD15, CD16 and CD62L among healthy donors, CVID or XLA patients, and NTM-PD patients (Figure 3.8). The expression of each surface marker was calculated by the Median Fluorescence Intensity (MFI) and/or the proportion positive or negative, depending on the marker.

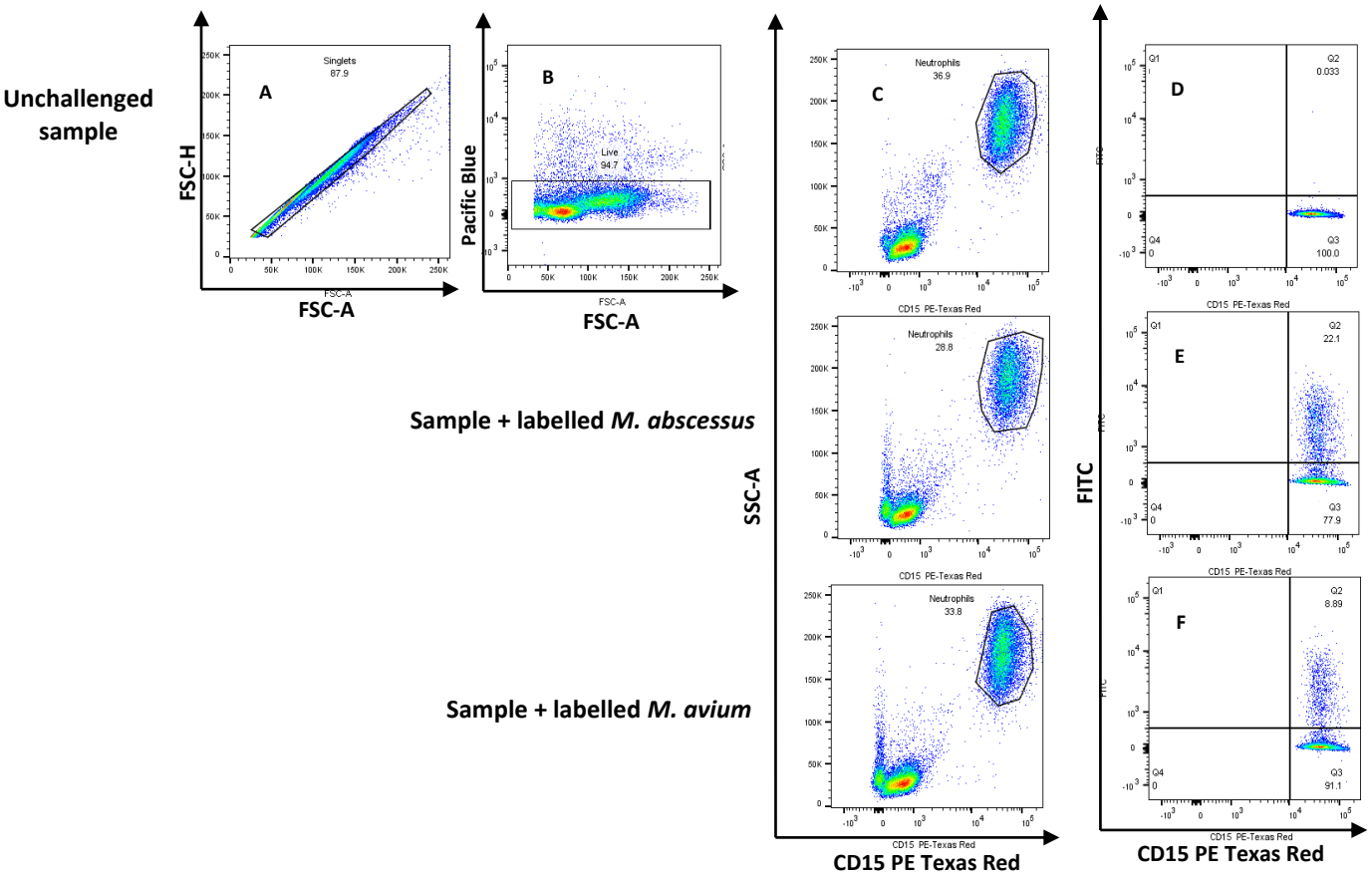


Figure 3. 6: Phagocytosis gating strategy.
(A) First; a gate was set on singlet events using Forward Scatter Area versus Forward Scatter Height (FSC-A vs FSC-H). **(B)** All live cells were selected from a FSC-A vs Pacific Blue plot, and dead cells were excluded on the basis of positivity for eFluor450 Fixable viability dye. **(C)** Neutrophils were gated as positive for PE-Texas Red conjugated CD15 with high side scatter **(D)** Unchallenged sample; neutrophils are defined as positive for PE Texas Red conjugated CD15 (Q3) with no FITC signal as no labelled organisms are present. **(E and F)** samples challenged with *M. abscessus* and *M. avium* respectively; Q2 (FITC positive, PE Texas Red positive) mycobacterial uptake by neutrophils (phagocytosis), Q3 (FITC negative, PE Texas Red positive) neutrophils which have not interacted with labelled mycobacteria. These graphs are from one representative experiment out of two separate experiments.

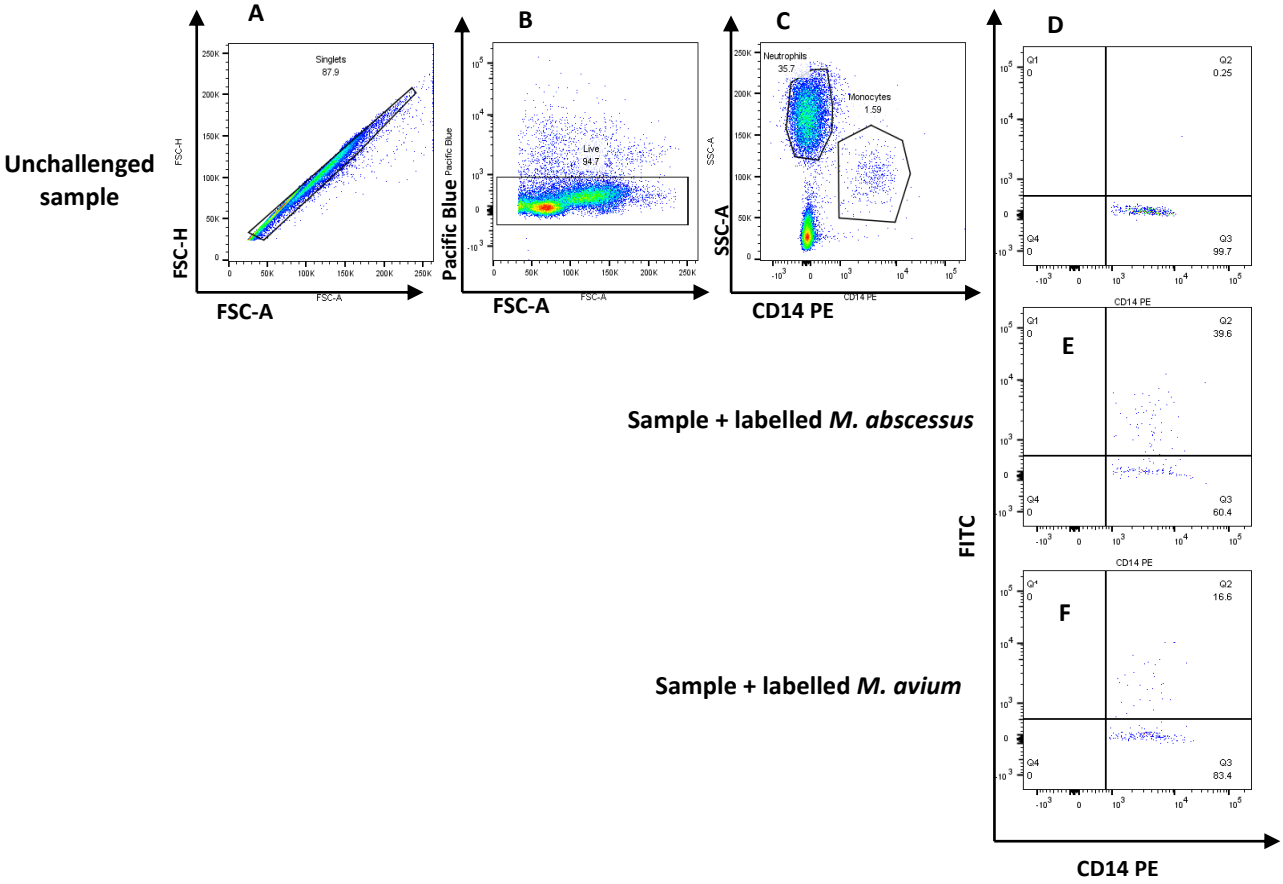


Figure 3. 7: Flow cytometry gating strategy for phagocytosis by monocytes.
(A) Singlet events were defined; **(B)** Dead cells were excluded on the basis of positivity for eFluor450 Fixable viability dye, **(C)** Monocytes were gated as positive for PE conjugated CD14 **(D)** Monocytes with no FITC signal (unchallenged samples), **(E and F)** Sample challenged with *M. abscessus* and *M. avium* respectively, Q2 phagocytosis; mycobacterial uptake by monocytes (FITC positive and PE positive). This is one representative experiment out of two separate experiments.

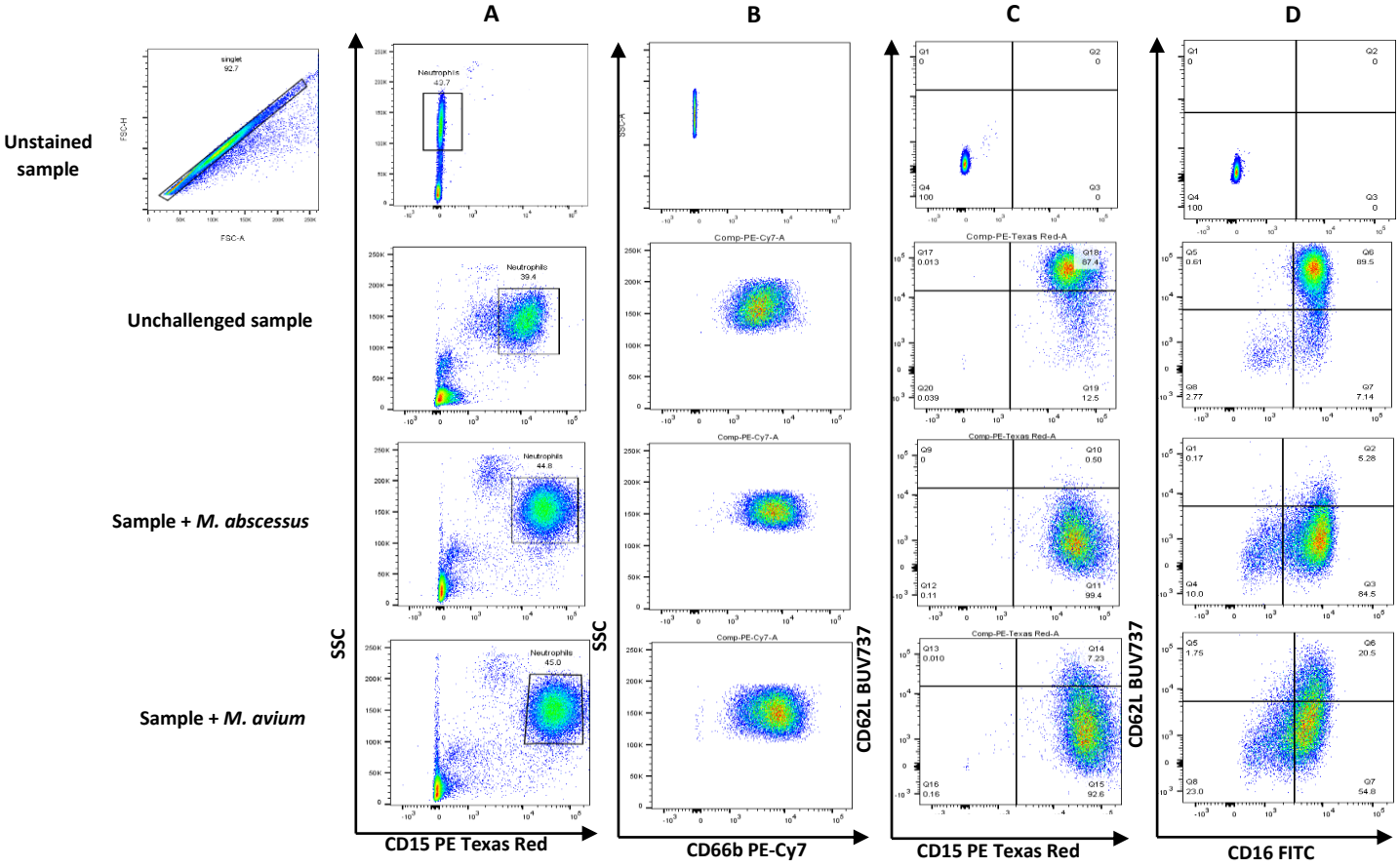


Figure 3. 8: Activation gating strategy.

The plots demonstrate the activation assay using CD66b, CD15, CD16 and CD62L to observe the variation in the expression of surface markers for unchallenged samples and challenged samples within heathy donors and patients.

All gates were first set on singlet events (FSC-A vs FSC-H), followed by gating on target cell population (Neutrophils in **A** defined by PE-Texas Red conjugated C15 vs SSC and **B** CD66b expression on neutrophils as defined by CD15 positivity. Neutrophils' expression of CD62L conjugated BUV737 vs CD15 indicated in **C**. **D** representative FACS plot of CD16 vs. CD62L expression for the total neutrophil population). In all cases, the upper panels represent an unstained control. This is one representative experiment out of two separate experiments.

3.9 Confocal Microscopy

Experiments were performed by infecting isolated neutrophils from healthy comparator group and patients using the EasySep Direct Human Neutrophil Isolation Kit (StemCell Tech, #19666) with FITC-labelled *M. avium* and *M. abscessus* following the staining protocol previously described for the phagocytosis assay. In the last step of staining, challenged cells were fixed and resuspended in 20µl of PBS.

5µl of cell suspension was placed on top of a microscope slide and one drop of ProLong Gold Antifade Mountant (ThermoFisher, P36930) on a Fibronectin-coated *c*overslip 1.5 thickness 13mm round (ScientificLabs, MIC3336D2). Slides were stored to dry for 3-5 days in the dark at room temperature. The edge of the coverslip was sealed then stored in the fridge.

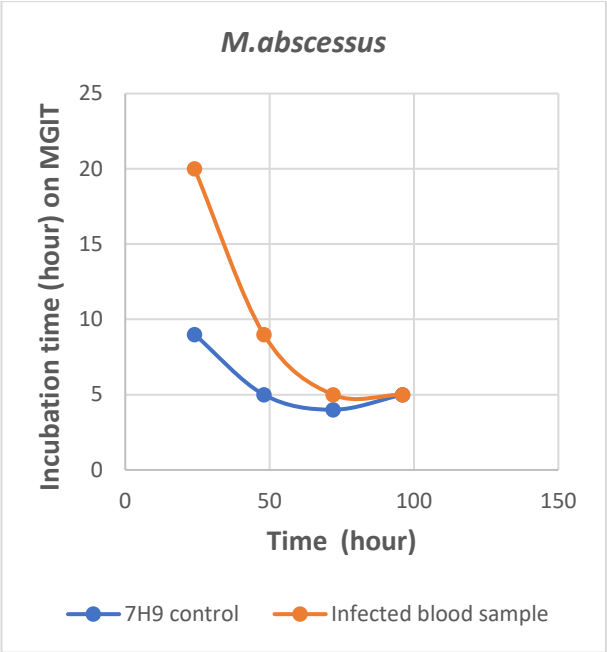
Images were obtained on live cells using a Zeiss LSM 710 inverted confocal microscope and demonstrated internalization of *M. avium* and *M. abscessus* by CD15+ neutrophils. I was supervised in all confocal microscopy by Dr Dale Moulding.

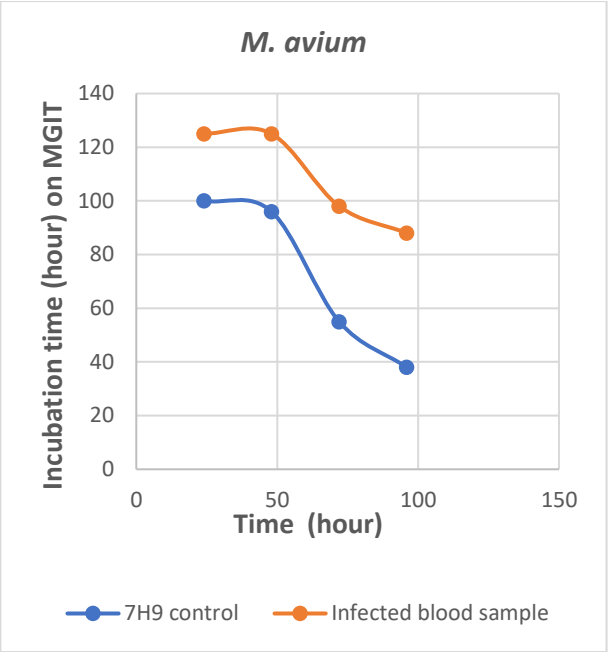
3.10 Mycobacterial restriction assay

Venous blood samples were collected from healthy donors and consenting patients (CVID/XLA and NTM-PD) directly into sodium heparin tubes to be used for the *M. avium* and *M. abscessus* mycobacterial restriction assay. The assay, similar to those described elsewhere (200) where NTM proliferation in whole blood can be detected by measuring the TTP in MGIT, was performed in duplicate in Sarstedt Micro Tubes (Sarstedt, 72.694.006) containing 300µl fresh whole blood and 300µl RPMI, inoculated with 50µl (10^7cells/ml) of each individual mycobacterial species. For each experiment, there was a positive control tube containing 300µl of 7H9 broth instead of blood and 300µl RPMI-1640 to compare the direct growth with the blood samples.

Tubes were incubated in a shaking incubator at 140 rpm for 96 hours at 37°C. After 4 days of incubation the cells in each tube were lysed with 1ml sterile water and centrifuged at 15,300 g for 10 minutes. The supernatant was aspirated and stored in a new tube and the pellet resuspended in 500µl 7H9 broth. The entire volume was inoculated into BACTEC MGIT (BECTON DICKINSON, 245122). TTP was calculated for all samples and compared with the positive control (Figure 3.9).

For NTM infection-related cytokine analysis and neutrophils associated products, 300µl of whole heparinised blood in 300µl RPMI-1640 (samples challenged with 50µl NTM or unchallenged) were incubated for 96hrs in the shaking incubator. After incubation, samples were centrifuged at 2000g for 10 minutes at 37°C, the supernatants aspirated and stored at -80°C and the pellet re-suspended in 500µl RNA Later (Thermo Fisher Scientific, AM7021), again stored at -80°C.





Time	7H9 control	challenged blood sample
24	9	20
48	5	9
72	4	5
96	5	5

Time	7H9 control	Challenged blood sample
24	100	125
48	96	125
72	55	98
96	38	88

Figure 3. 9: *M. abscessus* and *M. avium* mycobacterial restriction assay over the course of 4 days (96hrs). *M. abscessus* and *M. avium* mycobacterial restriction assay over the course of 4 days (96hrs). Every 24 hrs the samples (challenged blood and 7H9 control) were assessed by directly inoculating MGIT tubes and were incubated at 37°C until 'positivity' was detected, indicating a certain threshold of mycobacterial growth (proliferation). The Time to Positivity (TTP) data in the representative graphs and tables below indicate the times from the beginning of incubation to the detection of mycobacterial growth in hours. These graphs are from one representative experiment out of three separate experiments.

3.11 Opsonophagocytosis assay (OPA)

This assay was done to investigate the impact of opsonisation of *M. avium* with serum or sputum from healthy comparators and patients (CVID/XLA and NTM-PD) on phagocytosis by neutrophils that had been isolated from a healthy donor.

3.11.1 Culture and FITC labelling of bacteria

M. avium were cultured in 7H9 and labelled with FITC (as previously described). OD600 was measured to confirm the concentration of suspension reached $2x10^6$ CFU/ml.

3.11.2 Serum generation, Heat Inactivation, and pre-opsonisation

Stored serum from healthy comparator group, CVID/XLA and NTM-PD patients were serially diluted in six 2-fold dilutions. Heat inactivation of serum was performed in a water bath at 56^{o}C for 30 minutes while normal (non-heat-inactivated) serum was used directly from the stock without heating.

For pre-opsonisation, 50µl *M. avium* suspension in 7H9 at a concentration of $2x10^6$CFU/ml was incubated at a 1:1 volume ratio with 50µl serum (or PBS in serum-free experiments) for 20 minutes at 37^{o}C on an orbital shaking incubator.

3.11.3 Neutrophils isolation

Neutrophils were isolated using the EasySep Direct Human Neutrophil Isolation Kit according to the vendor's protocol (StemCell Tech, #19666). Neutrophils were resuspended in RPMI 1640. The mean number of neutrophils isolated from 20ml of blood collected in EDTA tube was 2 x 10^6 cells/ml as confirmed visually by light microscopy.

To perform the assay, 100µl CD15+ granulocytes in RPMI-1640 at a concentration of 2x 10^6/ml were aliquotted into sterile 5ml flow cytometry tubes containing pre-opsonised 50µl serum with 50µl *M. avium* suspension, MOI was approximately 1:2 (0.5).

Samples were capped and mixed before being incubated on a rocking plate (150 rpm) at 37°C for 1 hour. After 1-hour, samples were placed immediately on ice and 0.5µl CD15-PE Texas Red and 1µl eFluor450 Fixable Viability Dye was added to the tubes and kept on ice for 20 minutes. Samples were then centrifuged at 400g for 5 minutes. The supernatant was discarded, and the pellets were resuspended in 2ml FACS buffer and 500µl 20% paraformaldehyde for 15min, samples were Spun at 400xg for 5 minutes in the pre-chilled centrifuge after each incubation. Pellets in the last step were resuspended in 200µl FACS buffer and acquired immediately on a Becton Dickinson LSR Fortessa flow cytometer running on FACS Diva 8 software.

3.12 Enzyme Linked Immunosorbent Assays (ELISA)

Enzyme Linked Immunosorbent Assays (ELISA) is a simple, low-cost and sensitive test that can be used to detect and quantify substances including antibodies, antigens, proteins, glycoproteins and hormones. In this thesis, all adult groups were included in the sample analysis using commercially available ELISA kits and following the manufacturer's protocols. Serum samples and supernatant of whole blood samples incubated for 96hrs from all cohorts, and the sputum samples from CVID/XLA individuals and people with NTM-PD.

The ELISA assay is based on the sandwich principle or indirect ELISA principle; the absorbance at 405nm was measured with a spectrophotometer. A standard curve was obtained by plotting the absorbance (linear) versus the corresponding concentrations of the given standards. The concentration of samples was determined from the standard curve using GraphPad Prism. All assays were optimised for the best dilution in preliminary experiments.

- The human HNP1-3 (Neutrophil Defensins) ELISA kit assay (HycultBiotech, HK317-01) was used for quantitative determination of human HNP1-3 in all samples.

- The Human Matrix Metallo Proteinase 8 (MMP8) level was measured by Hu MMP8 kit (Thermofisher, EHMMP8)
- Human Calprotectin ELISA kit (S100A8/S100A9) was used for the quantitative determination of Calprotectin (ABCAM PLC, ab267628)
- ProteaseTag® Active Neutrophil Elastase Immunoassay utilises ProteaseTag® technology to specifically detect and quantify active NE (ProAxsis Ltd, PA001).
- Capilia™ MAC Ab Elisa (GENTAUR, CAMC8170) was used to detect serum IgA antibodies to Glycopeptidolipids (GPL) core derived from *Mycobacterium avium complex* (MAC).
- Human Total Immunoglobulin G ELISA kit was used to determine total IgG quantities in human samples (Invitrogen, BMS2091).

3.13 Luminex®

a. Samples

Supernatants from whole blood samples that were unchallenged or challenged with *M. abscessus* or *M. avium* and incubated for 96hrs at 37C were used for the Luminex assay.

b. Luminex assay

A customised Human Magnetic Premixed Multi-Analyte (10-Plex) kit from R&D SYSTEMS (Bioteche, LXSAHM-10) was used to evaluate the measurements of multiple cytokines and chemokines in a single sample among all patient cohorts.

The Human Cytokine/Chemokine Magnetic Bead Panel included: G-CSF, GM-CSF, IFN-γ, IL-1, IL-8, IL-10, IL-12, IL-17A, PD-L1 and TNF-alpha.

Sample were tested in preliminary experiments to find the best dilution to perform the assay. Plates were read on a Luminex 200 Instrument with xPONENT 4.2 software. I was assisted in reading the plate by Dr Raymond Fernando.

3.14 Questionnaires

The London - Chelsea Research Ethics Committee, Health Research Authority (HRA) and Health and Care Research Wales (HCRW) formally reviewed and approved the questionnaires and research work plan, including the collection of samples from NTM-PD patients (20/PR/0118), (Appendix 5 and 6).

Patients' medical and medication history, demographic details and health status measures were obtained from the participants during recruitment, recorded in a proforma and entered into a database under an allocated study number. These, together with the QOL-B +NTM questionnaire, MRC breathlessness scale, EQ-5D-5L, COPD Assessment Test (CAT) and the GAD-7/PHQ-9 questionnaire were collected from patients with NTM-PD to assess the burden of respiratory symptoms (Appendix 7, 8, 9, 10, 11, 12, and 13).

3.15 Transcriptomic analysis

In this work, RNA sequencing was performed in collaboration with UCL Genomics, and the transcriptomic analysis was done by Professor Mahdad Noursadeghi (UCL Honorary Consultant in Infectious Diseases, UCLH) and his bioinformatics team.

3.15.1 RNA extraction

a. Samples

Blood samples from 15 NTM-PD patients were collected in Tempus™ Blood RNA Tubes (Applied Biosystems, 4342792) and stored at -20°C.

b. RNA Extraction method

RNA was extracted and purified using the Tempus™ Spin RNA Isolation Kit (Invitrogen, 4380204). Stabilized blood was transferred to a 50-mL tube and diluted with 1X PBS. The tube was then vigorously vortexed for at least 30 seconds to ensure proper mixing, with the lysate traveling to the top of the tube. Subsequently, the tube was centrifuged at 4°C at 3000 × g, and the supernatant was carefully poured off. The RNA pellet was resuspended, and the RNA was isolated using a purification filter. The resuspended RNA was then purified and eluted using a microcentrifuge. The purified RNA was stored in Nucleic Acid Purification Elution Solution and kept at -80°C for further analysis. RNA quantity and quality were assessed using a NanoDrop ONE (ThermoScientific) and stored at -80°C.

3.15.2 RNA-seq method

This work was performed by UCL Genomics following the protocol of KAPA RNA HyperPrep Kit with RiboErase (HMR) Globin (Appendix 3.14)

3.15.2.1 Sequencing Methods

The quantification and integrity of total RNA were assessed using Agilent's 4200 Tapestation (Standard Total RNA assay) prior to library preparation. The RNA Integrity Numbers (RINs) were within the range, around 9.0, indicating variable integrity RNA suitable for library preparation after ribosomal RNA and globin depletion.

a. Library preparation

The library preparation involved processing 200ng of total RNA for each sample, following the manufacturer's instructions for the KAPA RNA HyperPrep Kit with RiboErase (HMR) Globin for Illumina® Platforms (Roche p/n KR1520).

Briefly, the protocol involved depleting rRNA and globin mRNA transcripts from the total RNA by hybridizing them with complementary DNA oligonucleotides. RNase H and DNase treatments were then applied to remove the duplexed rRNA and mRNA from the DNA oligonucleotides. The depleted RNA was subsequently fragmented using chemical hydrolysis (heat and divalent metal cation) and primed with random hexamers. Reverse transcriptase was used to generate strand-specific first strand cDNA in the presence of Actinomycin D, allowing RNA-dependent synthesis while preventing spurious DNA-dependent synthesis. The second cDNA strand was synthesized using dUTP instead of dTTP to mark it. The resulting cDNA was "A-tailed" at the 3' end to prevent self-ligation and adapter dimerization. Full-length xGen adaptors (IDT) containing sample-specific indexes (two unique 8bp indexes), a unique molecular identifier (N8), and a T overhang were ligated to the A-tailed cDNA. The successfully ligated cDNA molecules were then enriched using limited cycle PCR (13 PCR cycles). The PCR employed a high-

fidelity polymerase that cannot extend through uracil, resulting in amplification of only the first strand cDNA for sequencing, thereby ensuring the library's strand specificity (first-strand).

b. Sequencing

To confirm high-yield libraries without adaptor dimers, the libraries were analyzed using the Agilent TapeStation 4200 with the High Sensitivity Agilent DNA 1000 assay. Quantification of the samples was performed using the Qubit High Sensitivity DNA assay, and the concentrations were normalized to 10nM. Equal volumes of each library were pooled and re-quantified using Qubit. The pooled library was then sequenced on the NovaSeq instrument (Illumina, San Diego, US) using the SP 100-cycle kit at a concentration of 275pM. A 56bp single-read run was performed, accompanied by 8bp dual sample index reads and 8bp unique molecular index reads.

3.15.3 An overview of the data processing steps involved in analyzing RNA-seq data.

The study used R, a language and environment for statistical computing, to process and analyse data (https://www.R-project.org). Paired-end reads were mapped to the Ensembl human transcriptome reference sequence (homo sapiens GRCh38), (https://feb2023.archive.ensembl.org/Homo_sapiens/Info/Index). Kallisto was used for mapping and generation of read counts per transcript based on pseudoalignment (201). The mapped counts data was imported and summarized into gene-level data using the R/Bioconductor package Tximport (202). DESeq2 and SARTools packages were used for differential gene expression analysis (203). The study used a false discovery rate (FDR) <0.05 to analyse the data, which was normalized with a negative binomial generalised linear model. Flow chart for the main steps of analysis process outlined in Figure 3.10.

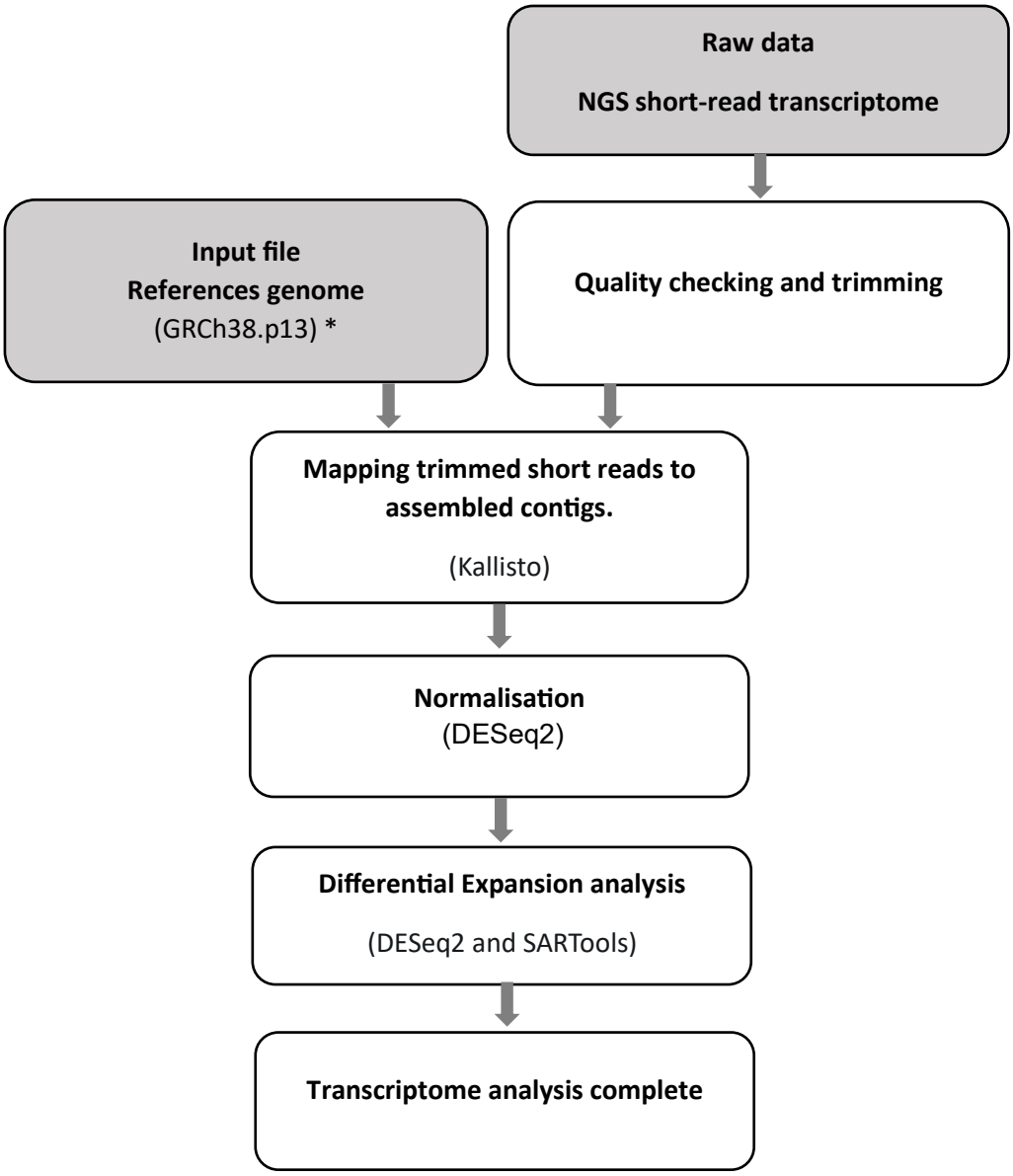


Figure 3. 10: Flowchart outlining the steps of RNA-seq data analysis (transcriptome analysis).

This includes the following steps: trimming, alignment, counting and normalization of the sequenced reads, and differential expression (DE) analysis across conditions.

*RNA sequencing data were mapped to human reference genome (GRCh38.p13) release 109. https://feb2023.archive.ensembl.org/Homo_sapiens/Info/Index

3.16 HRCT Image Analysis

Eligibility for NTM-PD patients included a recent CT scan (within the last 6 months). Two specialist respiratory radiologists from the Radiology Department, Royal Free Hospital scored the patients' images to systematically characterise disease severity according to accepted protocols. Severity was assessed using an NTM-specific scoring system previously described (53, 204). The scoring of patients' images were part of the study. For bronchiectasis extent, bronchiectasis severity, tree-in-bud opacification, nodules, and consolidation—were used to classify the features as low, medium, or high. Aspergilloma scores, severe cavitation, and cavitating nodules were categorised as either present or absent. The total scores and the scores specifically related to bronchiectasis, or those related to other signs of active infection, were calculated (Appendix 15).

3.17 Data analysis

Flow cytometry data were analysed using FlowJo software (v10). Graphs were generated using Graphpad PRISM GraphPad software v9. Parametric unpaired statistical analysis (t-test) or non-parametric (Mann Whitney test) were used to determine differences between two groups and (One way ANOVA) or the Kruskal–Wallis test (non-parametric) to assess the difference between multiple groups followed by Dunn's tests for post-hoc comparisons of changes within- and between-groups. A two-tailed p-value of less than 0.05 was considered significant. The relationships between the different tools and measures were determined using Pearson's correlation coefficient.

Chapter 4: The activation of neutrophils and their ability to phagocytose and kill NTM

4.1 Introduction

Neutrophils are prominent innate cells involved in defence against infection (through for example phagocytosis and killing of invading microbes). However, as discussed in Chapter 1, they can also contribute to pathological effects and progression of lung disease when they become activated and die (205).

Unlike other immune cells, neutrophils are activated easily, and cryopreservation unavoidably damages the cells. Consequently, ex vivo studies are limited in this area. Although experiments to understand the ability of neutrophils to internalise and kill mycobacteria exist, the results are still controversial and inconsistent.

While it is recognized that a compromised immune system and the use of immunosuppressive drugs can contribute to a weakened immune response against mycobacterial infections and an increased risk of NTM infections, it is noteworthy that CVID/XLA patients appear to exhibit a low frequency of NTM infection. The reason for this phenomenon remains unclear (183, 185-187).

Assessing the host response to NTM infection using surrogate markers would be of considerable clinical value if it facilitated the diagnosis of NTM-PD and enabled disease monitoring by predicting who requires treatment and how individuals are progressing with their treatment. The response of neutrophils to mycobacteria by measuring the surface expression of cellular markers is useful to verify whether phagocytes become activated during the infection (113).

Phagocytosis of mycobacteria at the site of infection by neutrophils can occur directly via the recognition of microbial antigens or through opsonization. The flow cytometry-based neutrophil phagocytosis assay can identify the presence and location of fluorescently-stained mycobacteria. Trypan blue is used to

distinguish between internalised mycobacteria and mycobacteria attached to the cell surface by quenching the external fluorescence. However, it is important to confirm the internalisation of mycobacteria by neutrophils, and confocal microscopy can differentiate between surface adherent organisms and internalised organisms (206).

Previous studies on Mtb have reported the potentially protective role of humoral immune responses in human mycobacterial defence against this mycobacterium. However, it is unclear whether the same applies to NTM. Flow cytometry can determine the contribution of antibodies and complement to neutrophil opsonophagocytosis by comparing serially diluted normal serum to heat-inactivated serum (30 min at 56°C). Here, healthy donor neutrophils are co-cultured with serum challenged by *M. avium* (pre-opsonised) to assess the ability of serum samples to opsonise mycobacteria at different concentrations.

Whether neutrophils are able to restrict or kill the mycobacteria they have internalised is still controversial. Previous approaches conducted to investigate neutrophils' ability to kill or restrict the growth of various NTM species (summarised in Chapter 1, Table 1.3), based on CFU or radiometry assay, require prolonged culture of organisms and are subject to contamination (207). Also, many other cells and molecules contribute to the control of mycobacteria. MGIT is an automated rapid technique for detecting mycobacteria in a liquid media, e.g. whole blood samples, which conforms more to *in vivo* reality.

In this Chapter, I investigated differences in neutrophil behaviour following *in vitro* exposure to NTM, serum opsonisation capacity and whole blood growth restriction in all three study populations.

4.2 Aim

The aim of this chapter was fourfold.

1) The activation state of neutrophils and the expression of surface markers were evaluated in unchallenged whole blood samples and in response to the challenge with *M. abscessus* and *M. avium* among all study groups.
2) Neutrophil phagocytosis of labelled *M. abscessus* and *M. avium* was assessed by flow cytometry in all groups using a whole blood assay to evaluate the capability of neutrophils to internalize mycobacteria.
3) Using the whole blood mycobacterial restriction assay, I sought to measure the time-to-detection of growth with both mycobacterial species (*M. avium* and *M. abscessus*) over 4 days of incubation.

Such findings would also help to investigate any changes in TTP between the CVID/XLA patients receiving macrolide prophylaxis and those who did not. It is unclear whether azithromycin (AZM) prophylaxis protects patients against mycobacterial infection, while the receipt of IVIG might also have a protective role. These therapies, commonly administered to adults with CVID/XLA, might help to explain lower infection rates and might lead to differences in the mycobacterial growth detected by MGIT between the patient groups (CVD/XLA group and NTM-PD group).

4) I wanted to gain insight into the impact of serum and sputum soluble components in the phagocytosis of mycobacteria by neutrophils by measuring the opsonisation capacity of heat-inactivated and normal (non-heat-inactivated) serum and sputum among the study groups.

I hypothesized that adults with CVID/XLA will show distinct innate immune responses including enhanced phagocytosis, neutrophil activation and restricting mechanisms in response to NTM, while the opsonization capacity of serum and sputum components would offer insights into differences between my study populations.

4.3 Materials and Methods

Blood samples from consenting CVID/XLA and NTM-PD patients plus healthy comparator group were used to assess *in vitro* neutrophil activation and neutrophil phagocytosis of mycobacteria by flow cytometry. The investigation of whole blood mycobacterial restriction assays, as well as the development of the opsonophagocytosis assay are described in Chapter 3 and briefly summarised again here in the Figure legends.

The results are presented as mean ± SD. I employed parametric unpaired statistical analysis (One-way ANOVA) to assess differences among multiple groups, followed by Bonferroni's tests for post-hoc comparisons within and between groups. Additionally, a two-tailed t-test was used to compare two groups.

4.3.1 Study participants and clinical data

Eighteen adults with CVID/XLA, 8 with NTM-PD and 16 healthy comparators were studied.

As fresh blood samples were required, only 8 patients with NTM-PD were recruited for the work described in this chapter. This was in part due to logistical issues during the COVID-19 pandemic as clinics and patients were encouraged to limit face to face contact unless absolutely necessary. Also, the experiments took several hours to perform; and therefore patients could only be recruited to the study if they were seen at a planned clinic appointment occurring at certain specific times of the day.

7 CVID/XLA patients were using prophylactic macrolide antibiotics and a further 6 were on other antibiotic prophylaxis. Several Ig preparations were assessed in our study, including Gamunex 10% (6 cases), CUVITRU (1 case), Gammaplex (1 case), Flebogamma (1 case), Intratect 10% (4 cases), and Privigen (2 cases). These preparations represent a diverse range of Ig options with different concentrations and formulations. The clinical features are summarized in Table 3.1 (Chapter 3). *M. avium* and *M. abscessus* were used to challenge the blood samples.

4.4 Results

4.4.1 *M. abscessus and M. avium* promote neutrophil activation, as indicated by the shedding of cell surface CD62L and CD16 when they are challenged and this is especially pronounced with *M. abscessus*

Flow cytometric assessment for the expression of surface markers was performed to estimate the activation of neutrophils in blood challenged *in vitro* with mycobacteria.

Distinct surface shedding of CD62L during challenged was observed in both healthy comparators and patient groups.

The difference in L-selectin (CD62L) expression was most marked between unchallenged samples, and those challenged with *M. abscessus* and *M. avium*; (mean ± SD MFI 36107±30764, 1970±1674, and 13461±29138 respectively, p=0.001 for healthy comparator group), (33138±29040, 1755±1421, and 17918±24636, p=0.0004 for CVID/XLA patients), and (23310±26888, 2610±1958, and 7880±10600, p=0.05 for NTM-PD) (Figure 4.1A).

The expression of CD16 and its shedding with activation was analysed. There were significant differences in CD16 expression between unchallenged samples and samples challenged with *M. abscessus;* (6352±1643 vs 3458±2886, p=0.001 in CVID/XLA and 6210±1646 vs 2245±1412, p=0.0004 in NTM-PD group).

In addition, increased neutrophil CD16 expression was observed in patient groups compared to healthy comparators in the unchallenged samples calculated by one-way ANOVA (p=0.03); (6352±1643 in CVID/XLA vs 4584±2337 in healthy comparators, p = 0.02 and 6210±1646 in NTM-PD vs 4584±2337 in healthy comparators, p=0.1) (Figure 4.1B).

I also compared the expression (i.e. positive/negative) of CD16 and CD62L between healthy subjects and the two patient groups (CVID/XLA group and NTM-PD group) (Figure 4.2 shows representative flow cytometry plots).

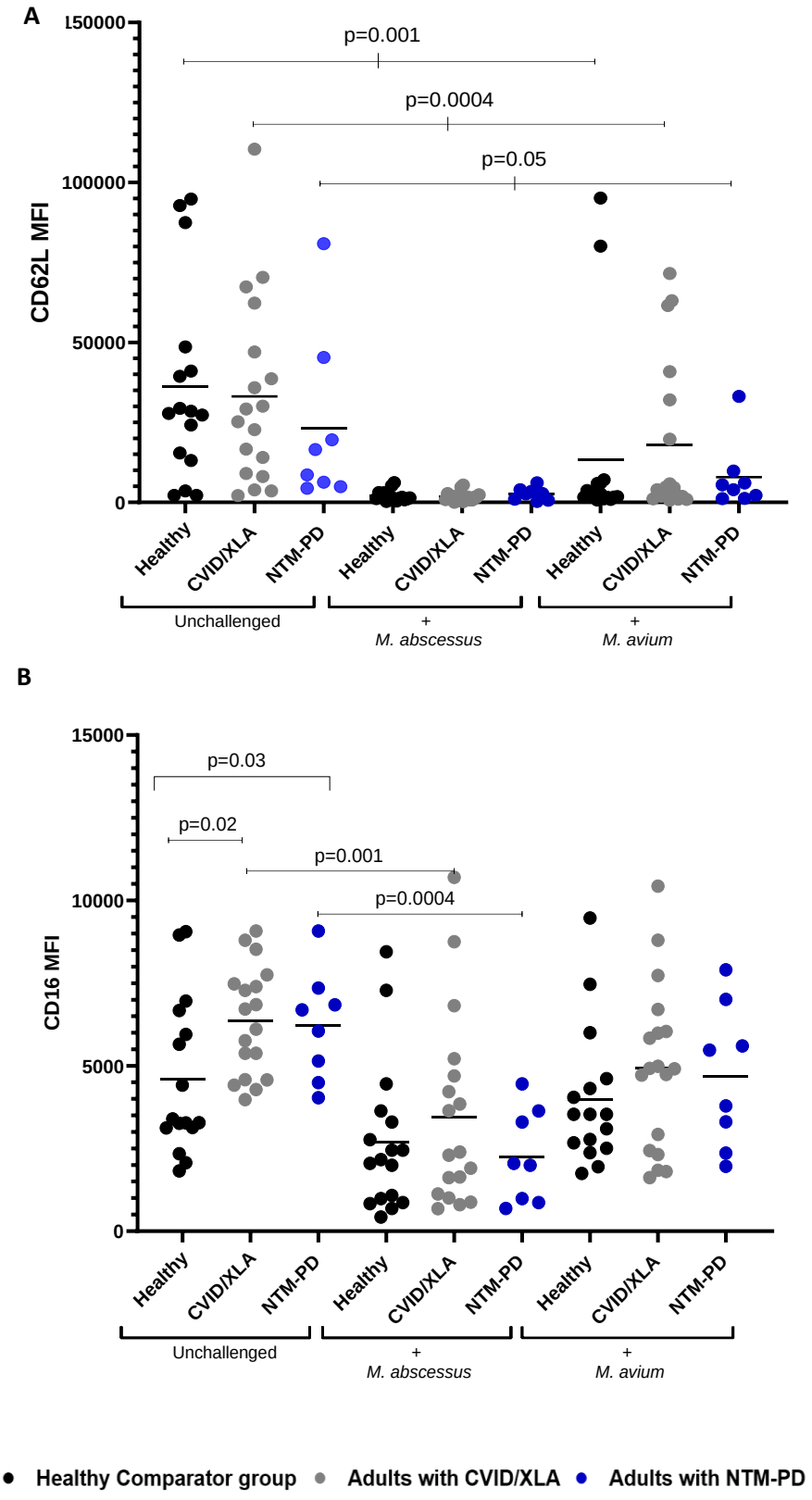


Figure 4. 1: The expression of CD62L and CD16.

The expression of CD62L (A) and CD16 (B) on neutrophils in whole blood in unchallenged samples, samples challenged with *M. abscessus,* and samples challenged with *M. avium* after 2hrs of incubation at 37◦C was analysed by flow cytometry. In all groups CD62L expression altered markedly following infection. A significant difference was noted for CD16 between unchallenged samples in patient groups and the healthy comparator group. These graphs are from one representative experiment out of 18 separate experiments.

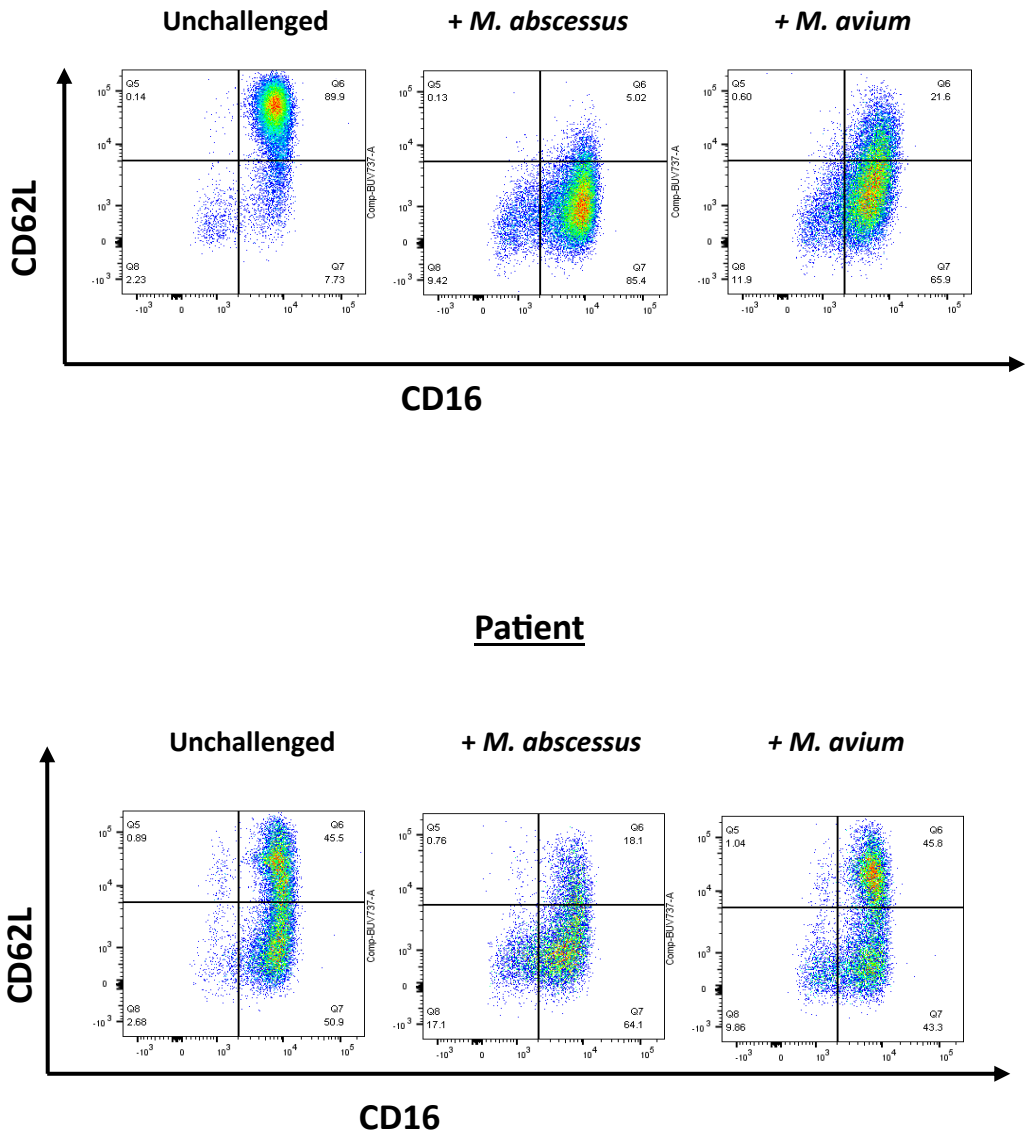


Figure 4. 2: Representative FACS plots of CD16 vs CD62L expression on neutrophils in whole blood from one healthy comparator and one patient using unchallenged whole blood samples, samples challenged with *M. abscessus,* and samples challenged with *M. avium*.

Figure 4.3 presents the percentages of neutrophils negative for CD62L (reduction of CD62L) and neutrophils negative for CD16 (reduction of CD16). This shows that during challenge with *M. abscessus* there is a reduction in CD16 and CD62L (24±26% and 95±4.8% for CD16 neg and CD62L neg respectively) and *M. avium* (12±15% for CD16 neg and 71±30% for CD62L neg) when compared to unchallenged samples (3.7±4.6% CD16 negative and 31±19% CD62L negative, in all groups, $p < 0.0001$).

The proportion of CD62L negative neutrophils (or CD62L reduction) was higher with *M. abscessus* challenge compared with *M. avium;* (96±3.3, 94±6.2, and 96±3.8 vs 78±28, 58±33, and 85±14, $p=0.02$, $p=0.0002$, and $p=0.07$ among healthy subjects, CVID/XLA patients and NTM-PD patients respectively) (Figure 4.3).

For CD16 expression, there were no significant differences between groups although the percentage of cells negative for the markers often tended to be higher in NTM-PD patients. CVID/XLA patients exhibited considerable heterogeneity, especially following the challenge.

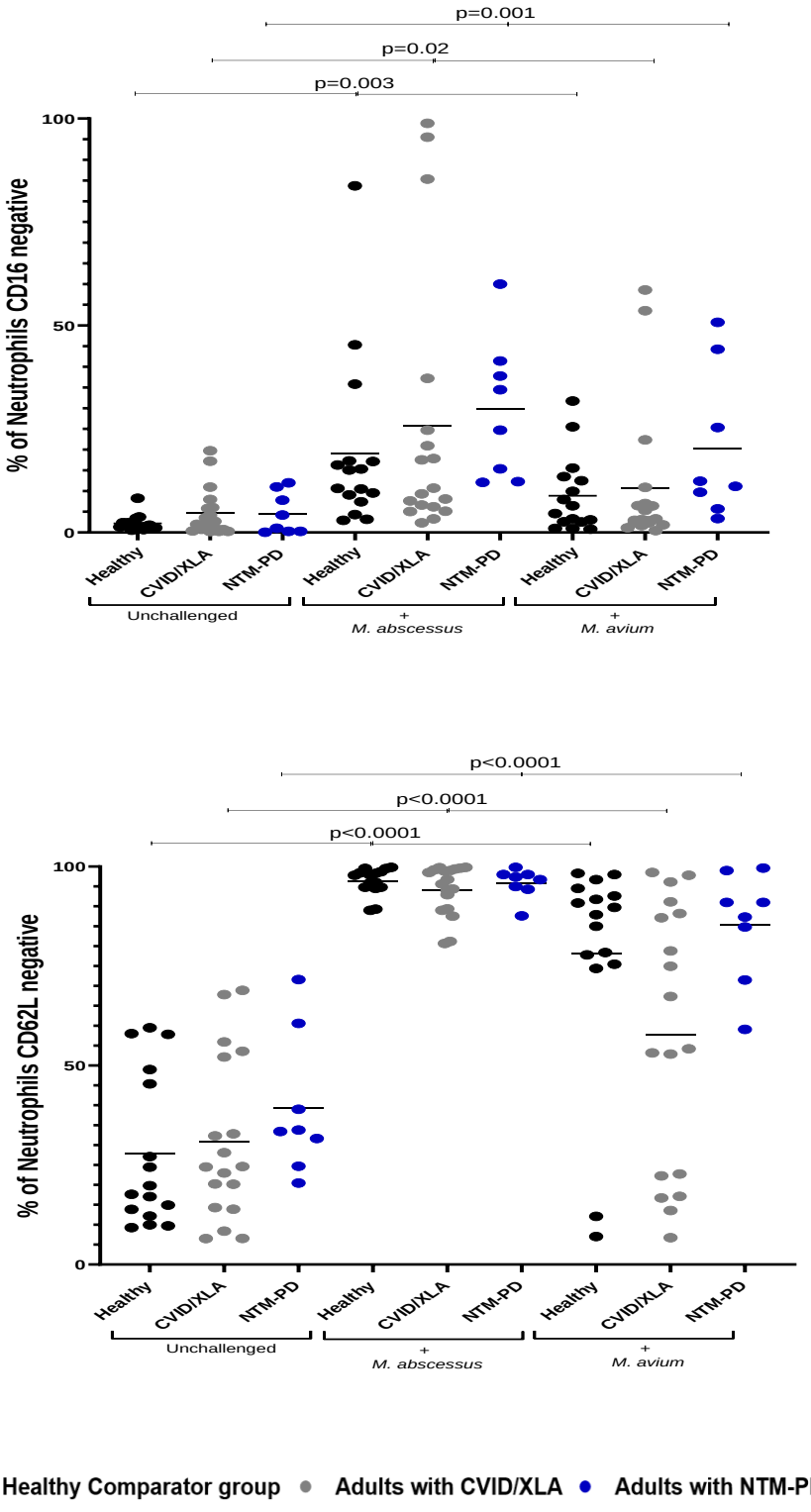


Figure 4. 3: The percentages of neutrophils in fresh whole blood from 16 healthy donors, 18 CVID/XLA patients, and 8 NTM-PD patients that were CD16 negative and CD62L negative on flow cytometry for unchallenged samples (blood and 7H9 media), samples challenged with *M. abscessus*, and samples challenged *with M. avium;* (blood and 10^6 CFU organisms cultured in 7H9 media), after 2 hours of incubation at 37◦C. These graphs are from one representative experiment out of 18 separate experiments.

4.4.2 Neutrophil activation and immaturity seen among the patient groups as indicated by increased expression of CD66b at baseline and decreased expression of CD15 with and without challenge.

I compared surface expression of CD64, CD66b and CD15 on neutrophils using median fluorescence intensity. Both patient groups' neutrophils had lower expression of CD15 compared to healthy comparators with or without mycobacterial challenge. One-way ANOVA) was used to assess the difference between multiple groups followed by Bonferroni's tests for post-hoc comparisons of changes both within and between groups.

- Unchallenged samples (p=0.01); (28675±11875 in CVID/XLA vs 48755±26559 in HC, p=0.01), and (28439±19453 in NTM-PD vs 48755±26559 in HC, p=0.045).
- Samples challenged with *M. abscessus* (p=0.02); (39171±16623 in CVID/XLA vs 67002±42038 in HC, p=0.03), and (38972±26751 in NTM-PD vs 67002±42038 in HC, p=0.07).
- Samples challenged with *M. avium* (p=0.02); (35464±13133 in CVID/XLA vs 60589±34511 in HC, p=0.009), and (40199±18750 in NTM-PD vs 60589±34511 in HC, p=0.1) (Figure 4.4).

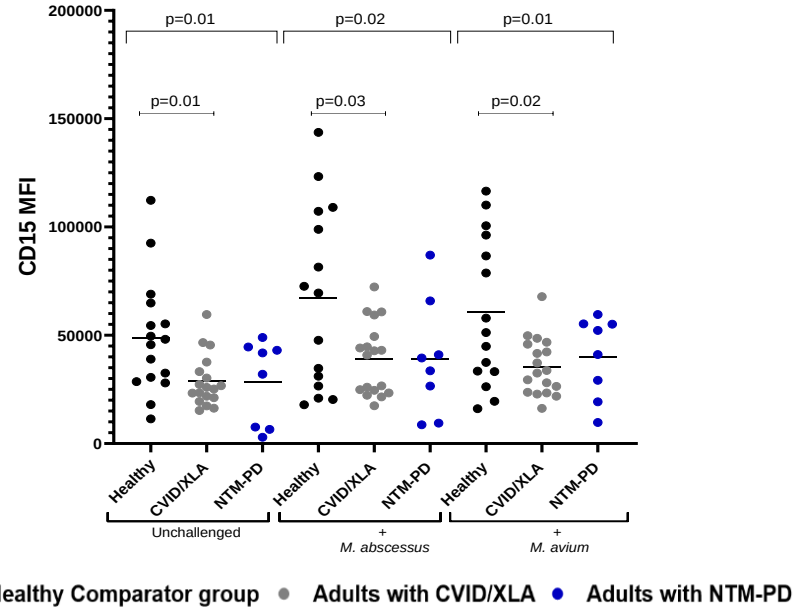


Figure 4. 4: Mean Fluorescence Intensity (MFI) of CD15 expression on neutrophils in whole blood in unchallenged samples, samples challenged with *M. abscessus*, and samples challenged with *M. avium*. All samples were incubated for 2hours at 37◦C. The variation between the groups and within groups was assessed using one-way ANOVA. (The P values at the top of the graph indicate the statistical significance between the groups in the first comparison (ANOVA). At the top of the bars, the lower set of P values shown in the figure are for the second comparison (Post Hoc test), indicating the significance of the differences between the groups. This graph represents one out of 18 experiments.

An increased expression of neutrophil CD66b was observed in patient groups compared to healthy comparators in the unchallenged samples (p=0.004); (4976±3241 in CVID/XLA vs 2831±2391 in comparator group, p=0.09), and (7604±4122 in NTM-PD vs 2831±2391 in comparator group, p=0.002). Moreover, in all groups CD66b was markedly different between the samples with and without challenge (unchallenged samples vs samples challenged with *M. abscessus* vs samples challenged with *M. avium*); healthy group (p<0.0001), CVID/XLA patients (p=0.003), and NTM-PD patients (p=0.01) (Figure 4.5).

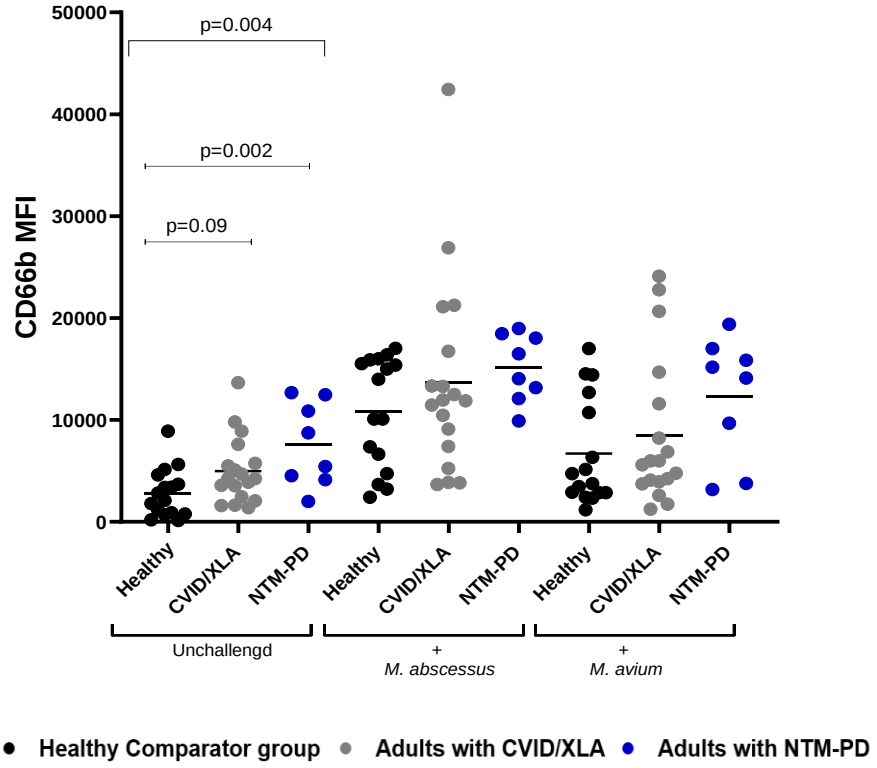


Figure 4. 5: The expression of CD66b on neutrophils in whole blood samples in unchallenged samples, samples challenged with *M. abscessus,* and samples challenged with *M. avium* after 2hrs. of incubation at 37◦C was analysed by flow cytometry. Unchallenged samples in patient groups were significantly different to healthy comparators.
The heterogeneity of the results is because amalgamation of a variety of experiments performed on different days, this graph presents summary data from 18 separate experiments.

The expression of CD64 tended to be higher among the CVID/XLA patients, in comparison with healthy donors. However, a significant difference between the groups was only seen during challenge with *M. avium* (p=0.03); (462±272 in CVID/XLA vs 267±125 in healthy comparators, p=0.01) (Figure 4.6).

Together, these data imply greater baseline activation of neutrophils in both patient groups and also suggest that, regardless of the immunology of the host, there is a difference in how neutrophils respond to individual NTM species.

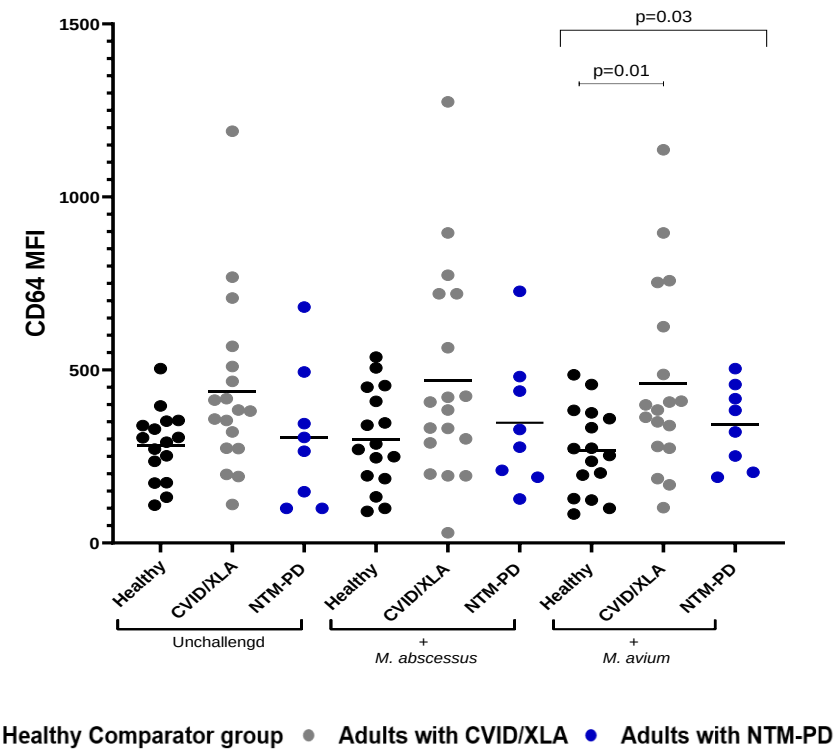


Figure 4. 6: CD64 expression with and without challenge. CD64 expression on neutrophils in whole blood samples challenged with *M. avium* among all groups was significant (p=0.03).
The heterogeneity of the results is because amalgamation of a variety of experiments performed on different days, this graph presents summary data from 18 separate experiments.

4.4.3 Greater neutrophil phagocytosis for *M. abscessus* compared to *M. avium,* particularly among patient groups

The phagocytosis assay demonstrated clear populations of neutrophils positive for FITC, indicating the presence of mycobacteria (Figure 4.7).

There was no significant difference in neutrophil or monocyte phagocytosis of *M. abscessus* and *M. avium* between healthy subjects, CVID/XLA patients, and NTM-PD patients (Figure 4.8). However, despite using the same MOI, there was greater phagocytosis by neutrophils of *M. abscessus* compared to *M. avium* among patient groups and the healthy comparator group (52±25% for *M. abscessus* versus 26±22% for *M. avium* among healthy subjects, p=0.005, 54±25% for *M. abscessus* versus 24±27% for *M. avium* among CVID/XLA patients, p=0.001, and 40±12% for *M. abscessus* versus 21±16% for *M. avium* among NTM-PD patients, p=0.02). The difference in monocyte phagocytosis between *M. abscessus* and *M. avium* was also significant in healthy samples (p=0.003), CVID/XLA adult samples (p=0.01), and NTM-PD adult samples (p=0.02).

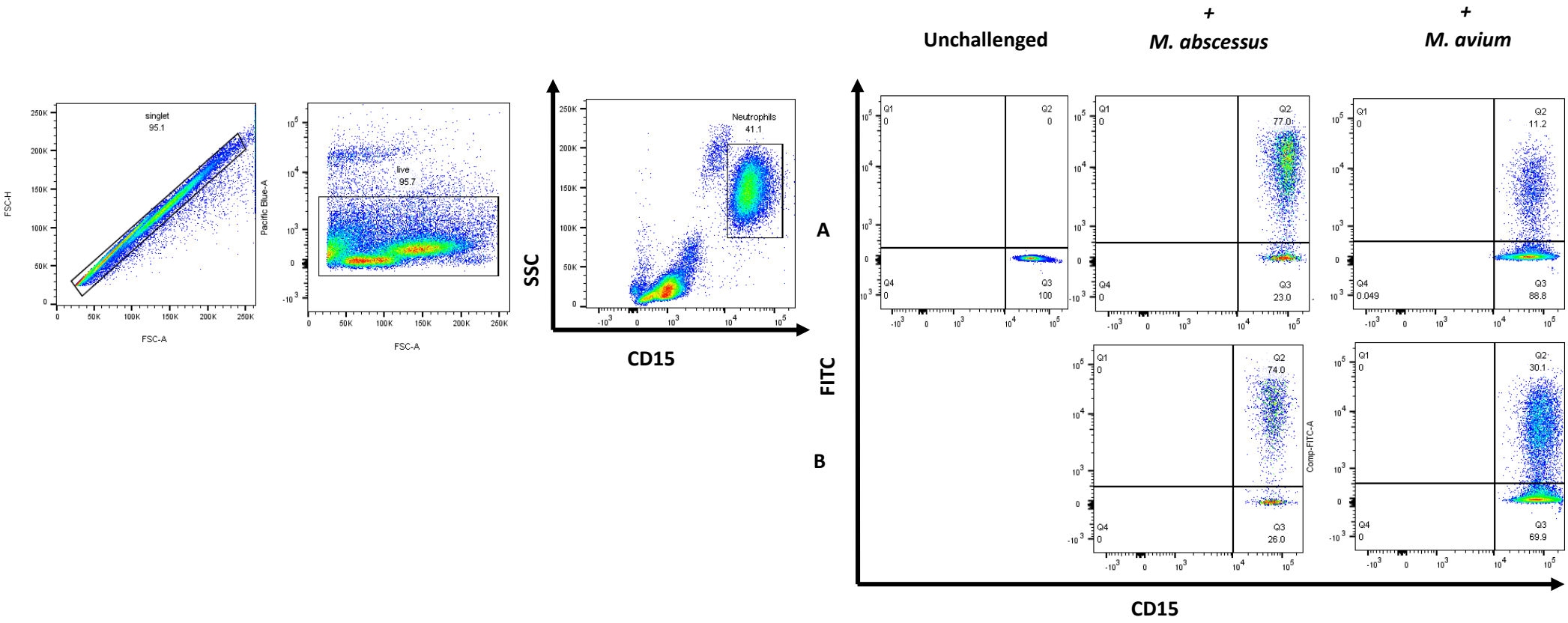


Figure 4. 7: *M. abscessus* and *M. avium* phagocytosis. Representative results of *M. abscessus* and *M. avium* phagocytosis by neutrophils among healthy samples (A) and patients' samples (B) are shown. The flow plots are divided into quadrants on the basis of CD15 signal (PE TexasRed) and FITC signal; Q2 (FITC positive, CD15 positive) represents the proportion of neutrophils which have taken up FITC labelled mycobacteria

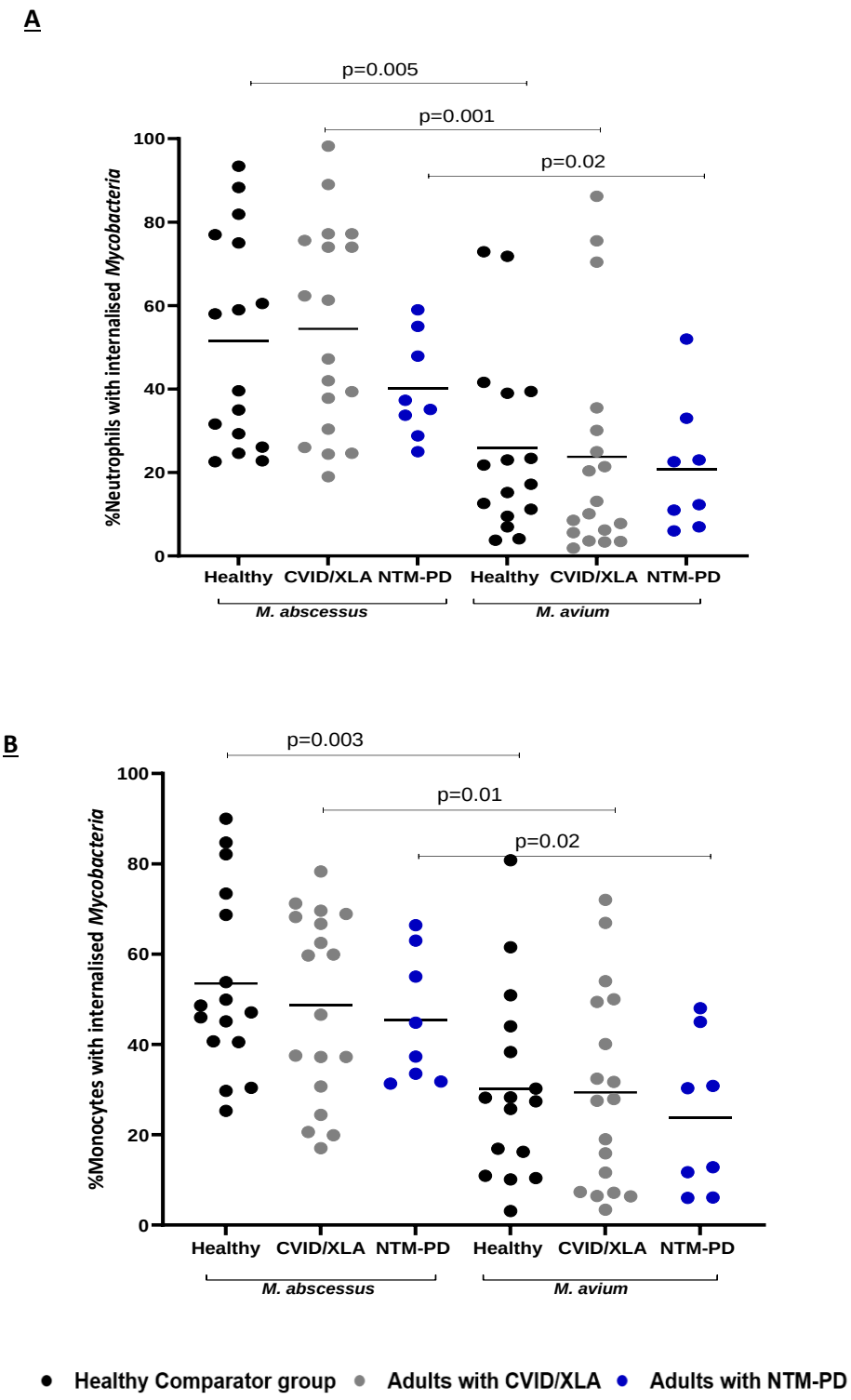


Figure 4. 8: Comparison of phagocytosis by neutrophils and monocytes between healthy comparator group and patients. Data are presented as percentage of neutrophils **(A),** and monocytes **(B)** with internalised organisms for both *M. abscessus* and *M. avium.*
The heterogeneity of the results is because amalgamation of a variety of experiments performed on different days, these graphs present summary data from 18 separate experiments.

4.4.4 Visualisation of neutrophil phagocytosis for *M. abscessus* and *M. avium* via confocal microscopy

I confirmed the results of phagocytosis observed in the whole blood flow cytometry assay by capturing the image of organisms inside isolated CD15+ neutrophils (internalisation of FITC labelled *M. abscessus* and *M. avium*).

Confocal microscopy images confirm in three dimensions that organisms are internalised by neutrophils using an MOI of 10. FITC labelled *M. abscessus* were taken up by neutrophils more than *M. avium* as seen in Figure 4.9.

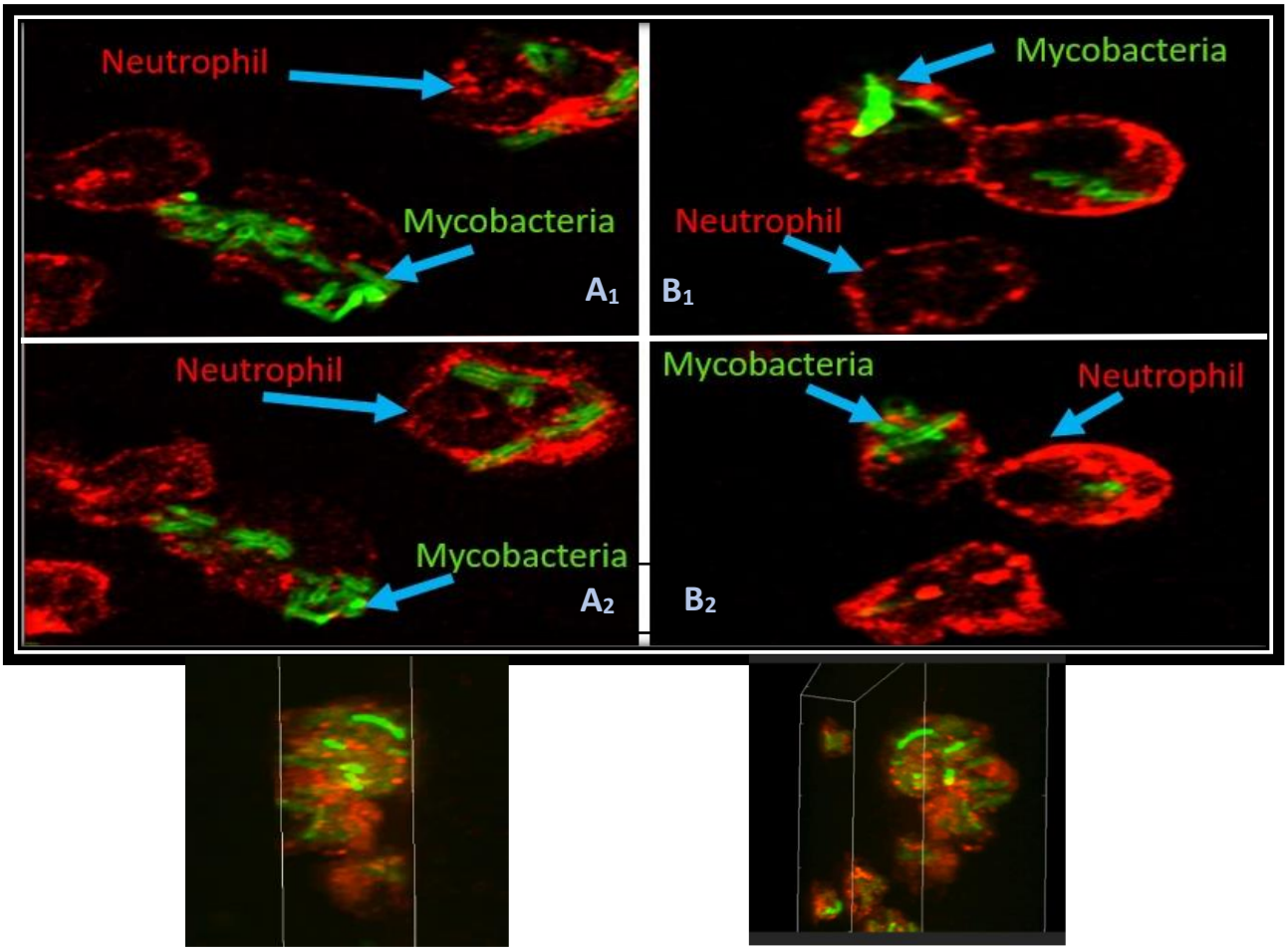


Figure 4. 9: Confocal microscopy image demonstrates the presence of organisms inside neutrophils (green fluorescence represents individual mycobacteria, FITC-labelled) and red fluorescence neutrophils. Neutrophils phagocytose numerous *M. abscessus* mycobacteria (A_1 and A_2), however for *M. avium* (B_1 and B_2) the neutrophils contain few bacilli. *Mycobacteria at an MOI of 10 (10^6 CFU mycobacteria and 10^5 neutrophils). *These results are a composite of 2 experiments.

4.4.5 The whole blood mycobacterial restriction assay demonstrates similar time to positivity between patient and healthy comparators samples for either *M. abscessus* or *M. avium*

TTP in liquid culture media for both mycobacterial species was determined for *in vitro* challenged blood samples from patients and healthy donors in parallel with control 7H9 culture.

Results presented in Figure 4.10 indicate that the shortest TTP was observed for rapidly growing NTM *M. abscessus* (median 5 hours for all), followed by slow growing NTM *M. avium* (median 77 hours for NTM-PD patients, 72 hours for CVID/XLA patients, and 78 for healthy comparators).

There was no significant difference in TTP between the groups, for both species. However, *M. abscessus* and *M. avium* differ significantly in the healthy and patients' samples (p-value < 0.0001). There was no significant difference in TTP between the patients using macrolide prophylaxis and those who did not.

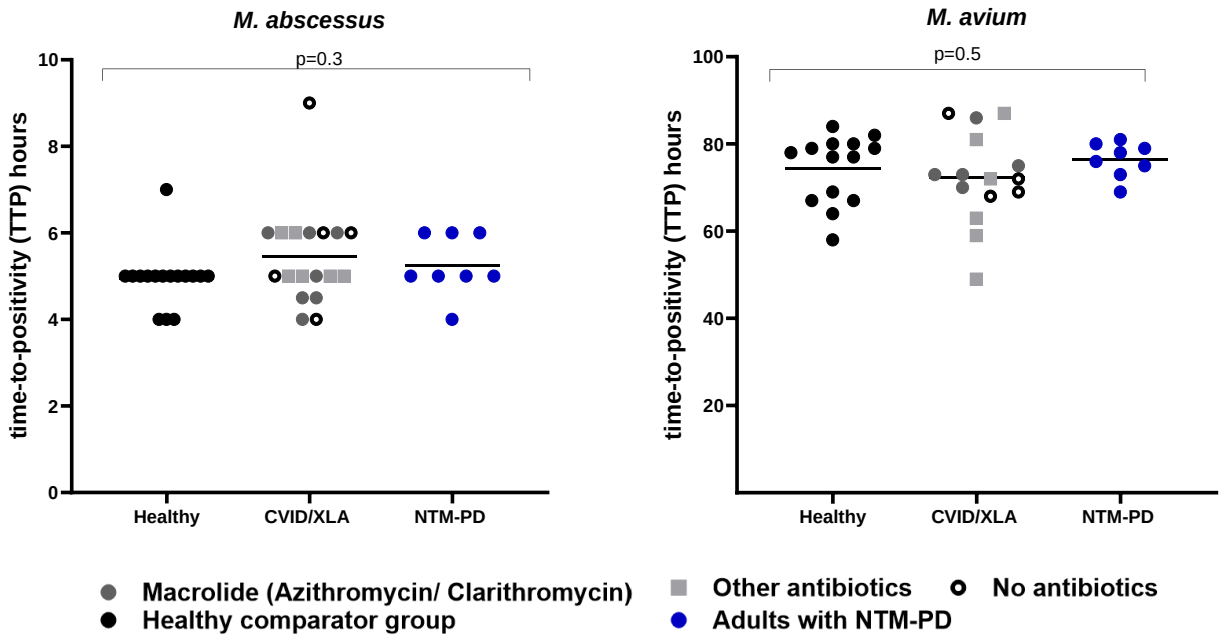


Figure 4. 10: Comparison of time-to-positivity (TTP) for mycobacterial culture *(M. abscessus* and *M. avium)* after 96 hours incubation at 37°C in blood from healthy donors, CVID/XLA and adults with NTM-PD.
Note that for CVID/XLA patients: grey solid circles represent patients using macrolides, grey squares patients taking other antibiotics and black empty circles patients on no preventive antibiotics. These graphs present summary data from three separate experiments.

In two experiments, the results for *M. avium* TTP were extremely low, and I decided to exclude these values as not biologically plausible. However, including these results I observed that the TTP remained similar between patients and healthy comparators for *M. avium* (Median 77 hours for NTM-PD patients, 71 hours for CVID/XLA patients, and 77 for healthy comparators).

Figure 4.11 includes all experimental results for 18 CVID/XLA patients, 8 NTM-PD patients and 16 healthy donors.

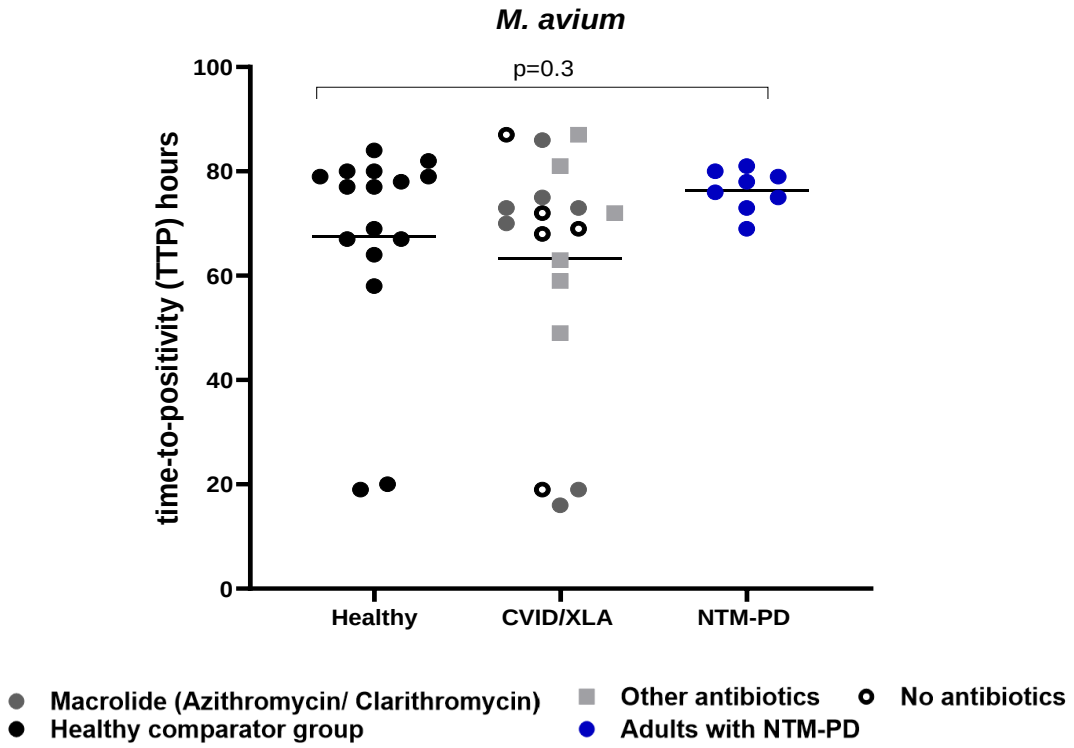


Figure 4. 11: Time-to-positivity (TTP) for *M. avium* after 96 hours of incubation at 37C° in blood from 16 healthy donors, 8 people with NTM-PD, and 18 people with CVID/XLA. This graph present summary data from 18 separate experiments

4.4.6 Opsonophagocytosis of mycobacteria by isolated neutrophils with serum or sputum, with and without pre-opsonisation.

I analysed neutrophil phagocytosis using cells isolated from freshly drawn blood from healthy donors and incubated with FITC-labelled *M. avium* in either heat-inactivated serum or non-heat inactivated serum (normal serum). Pre-opsonisation tended to yield non-significantly higher and more consistent results and was therefore chosen for further experiments (Figure 4.12).

Similar preliminary experiments using serially diluted normal sputum supernatant samples and heat-inactivated sputum supernatant samples for opsonisation showed no difference in both patient groups at varying concentrations (Figure 4.13). Therefore, the opsonophagocytosis assay was performed using pre-opsonisation with sputum samples with heat inactivation at 1:8 and 1:16 dilutions only and without heat inactivation at 1:32 and 1:128 dilutions only.

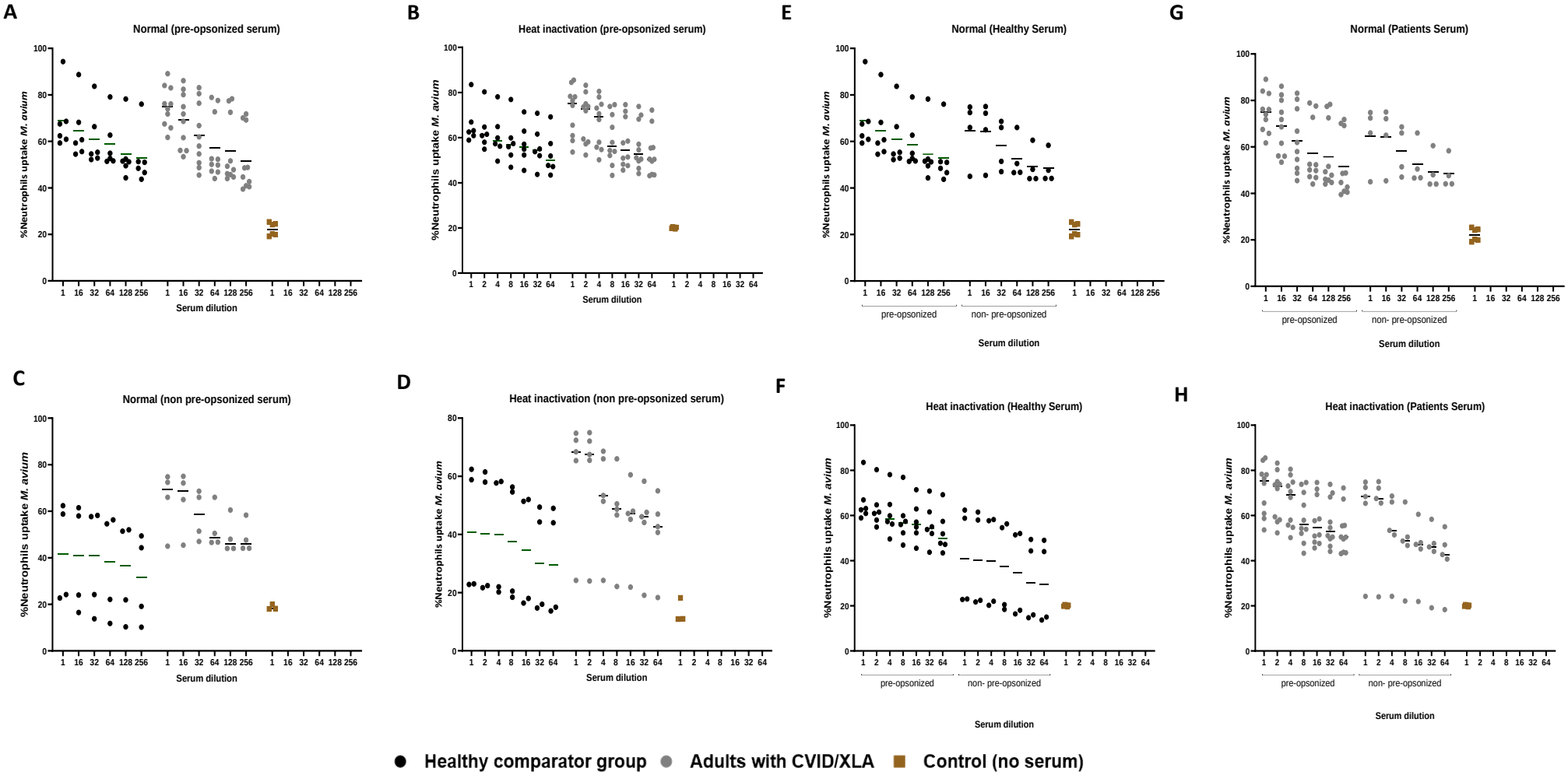


Figure 4. 12: Flow cytometry analysis of *in vitro* opsonophagocytosis assay to evaluate the opsonisation capacity of serially diluted serum. 50µl of FITC-labelled *M. avium* suspension in 7H9 at a concentration of 2 million CFU/ml was incubated at a 1:1 volume ratio with serum for 20 minutes at 37° C. EasySep-direct isolated neutrophils of $2x10^6$ cells/ml were added to the suspension. For 'Pre-opsonised' – organisms were incubated with serum for 20 minutes at 37◦C before challenge of cells. 'Non-pre-opsonised' – organisms and serum were added at the same time. 'Control no serum' – organisms were added without serum. **(A)** and **(B)**; (Healthy n=6, CVID/XLA n=10, Control n=6), **(C)** (Healthy n=4, CVID/XLA n=4, Control n=3) and **(D);** (Healthy n=4, CVID/XLA n=5, Control n=3). The y axis represents the proportion of neutrophils with internalised organisms after 60 minutes incubation. Each dot represents one subject; lines represent means. **(E)** Healthy normal Serum and **(F)** Healthy heat inactivated Serum, pre-opsonized and non pre-opsonized. **(G)** Patients normal Serum and **(H)** Patients heat inactivated Serum, pre-opsonized and non pre-opsonized. These graphs are from one representative experiment out of four separate experiments

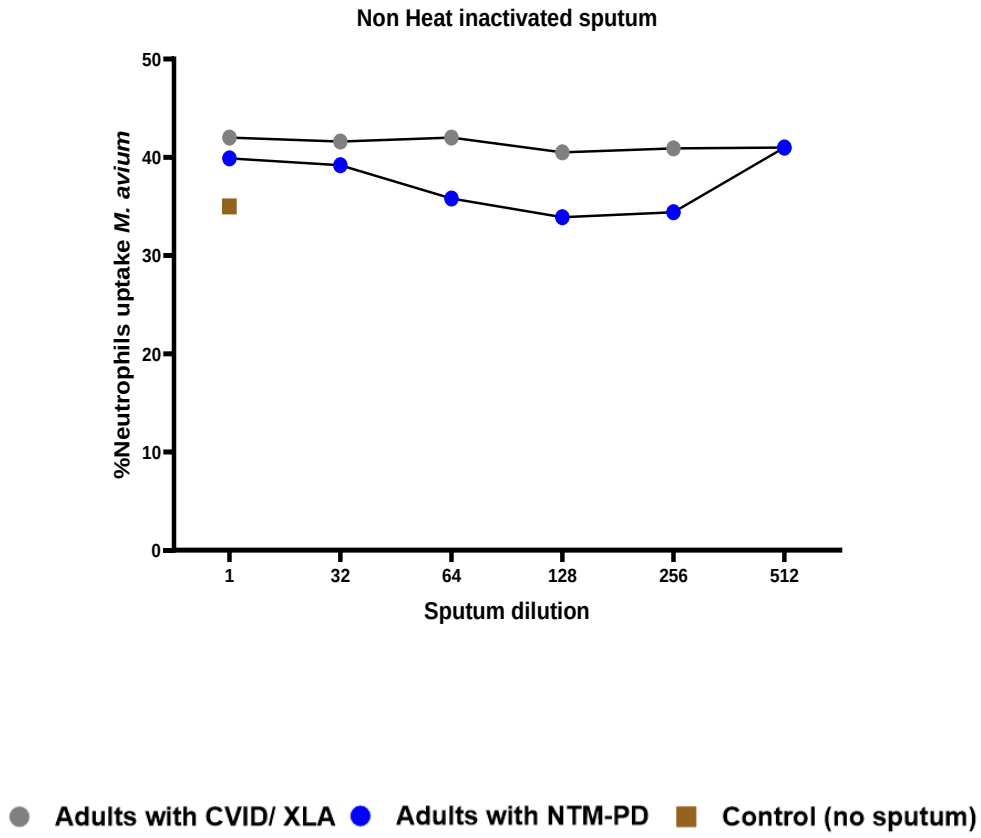


Figure 4. 13: Optimisation of two-fold serial dilutions of sputum (1/32 to 1/512) for normal sputum from patient groups; (CVID/XLA n=1, NTM-PD n=1). The y axis represents the proportion of neutrophils with internalised organisms after 60 minutes incubation.

4.4.6.1 Opsonisation capacity of serum is higher in CVID/XLA patients compared to other groups although there are no differences in opsonisation capacity of sputum supernatant.

The opsonisation capacity of neat serum with and without heat inactivation differed between the groups. CVID/XLA patients had a higher opsonization capacity than NTM-PD patients and healthy comparators though this was only significant between CVID/XLA and NTM-PD patients. The proportion of neutrophils with internalised organisms 70±6% for CVID/XLA vs 64±4.9% for healthy comparators, p=0.1, and 70±6% for CVID/XLA vs 59±6.1% for NTM-PD patients, p=0.002 for normal neat serum; 69±5.6% for CVID/XLA vs 63±7.1% for healthy comparators, p=0.17, and 69±5.6% for CVID/XLA vs 59±8.2% for NTM-PD patients, p=0.009 for heat-inactivated neat serum (Figure 4.14).

No appreciable difference was seen between the patient groups with serially diluted sputum in both NTM-PD and CVID/XLA (using neat heat inactivated sputum supernatant, 41±12% of neutrophils internalising organisms for NTM-PD and 36±11% for CVID/XLA patients; using neat normal sputum supernatant, 39±15% of neutrophils internalising organisms for NTM-PD and 35±11% in CVID/XLA patients (Figure 4.15)).

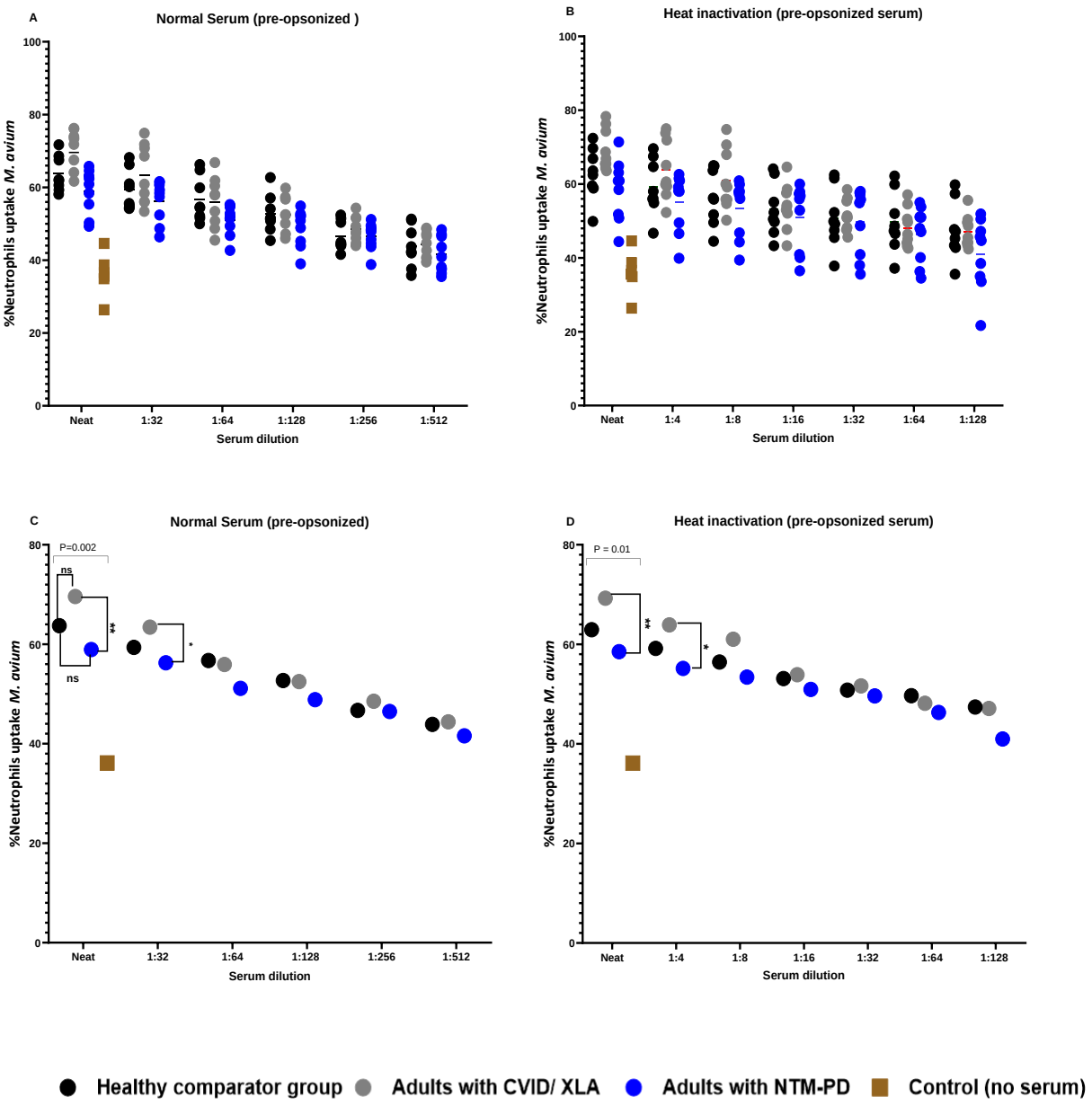


Figure 4. 14: Opsonophagocytosis using a flow cytometry assay. Serum samples at different concentrations from healthy donors (n=8), people with CVID/XLA (n=9), and people with NTM-PD (n=9) were incubated with FITC-labelled *M. avium* and added to EasySep-direct isolated neutrophils at 2x10^6cells/ml (MOI 0.5); serial two-fold dilutions from 1:32 to 1:512 for normal serum and 1:4 to 1:256 for heat inactivated serum including neat in both conditions. The y axis represents the proportion of neutrophils with internalised organisms after 60 minutes incubation. A and B present all data, C and D present mean values only. One-way ANOVA demonstrated significant differences among the 3 groups in neat serum for normal serum and heat-inactivated serum (p=0.002 and p=0.01 respectively) and two-tailed t test between two groups; *p<0.05, **p<0.01

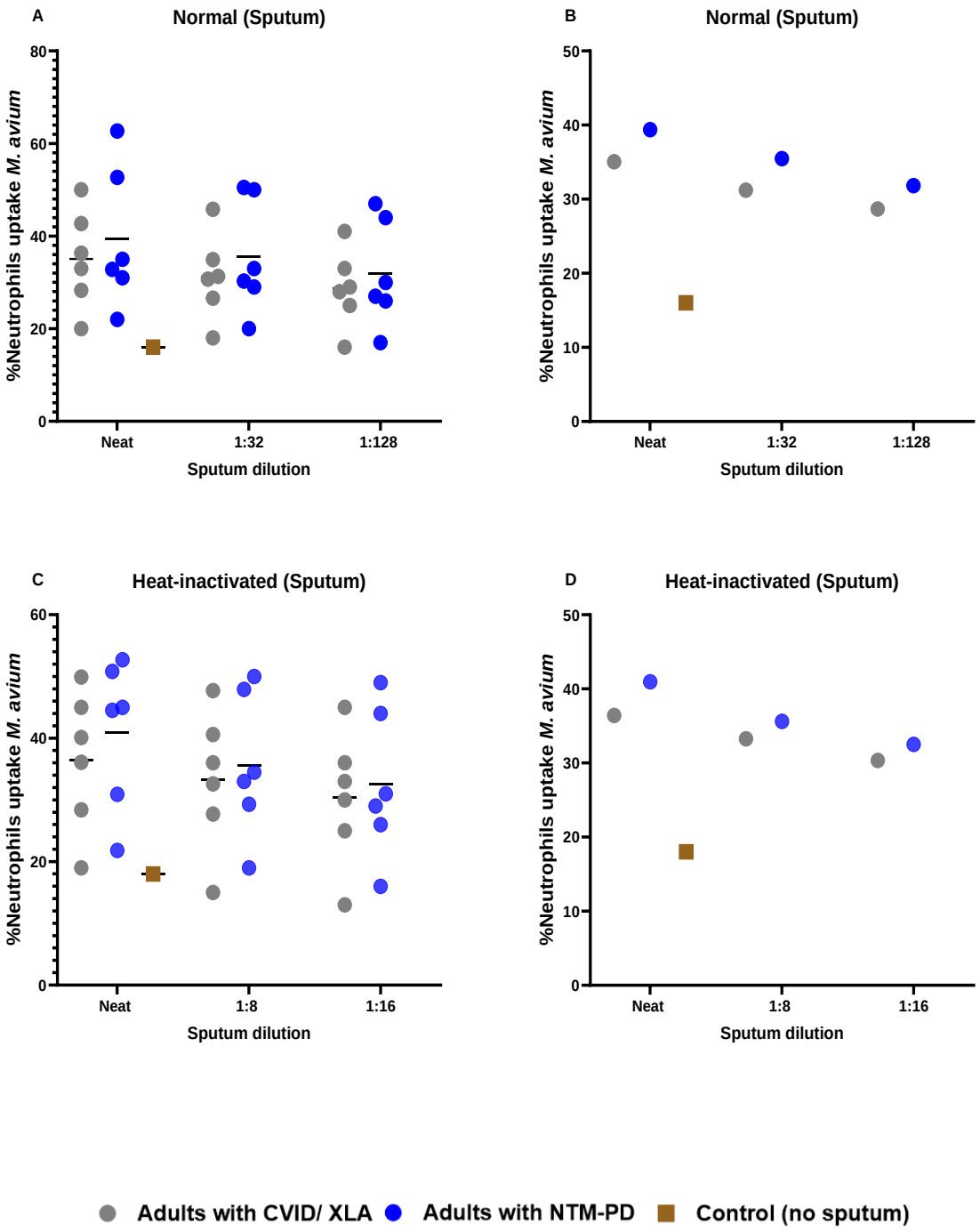


Figure 4. 15: Opsonisation capacity of patients' sputum samples. (A and C); 50µl Sputum supernatants at different concentrations from people with CVID/XLA (n=6) and, people with NTM-PD (n=6) were incubated with 50µl FITC-labelled *M. avium* and added to 100µl of EasySep-direct isolated neutrophils (2x10^{6}Cells/ml); dilution 1:32-1:128 and neat for normal sputum, 1:8-1:16 and neat for heat inactivated sputum. The y axis represents the proportion of neutrophils with internalised organisms after 60 minutes incubation. A and C present all data, B and D present mean values only. No significant difference was found between the patient groups.

4.5 Discussion & Conclusion

In this chapter, experiments were performed to assess differences, if any, in neutrophil behaviour and host defence against NTM in CVID/XLA and NTM-PD patients (both groups that often have bronchiectasis), and a healthy comparator population. I designed experiments to explore the role of neutrophils in NTM-PD, investigating why NTM disease is less common in CVID/XLA and if NTM-PD patients have neutrophil defects contributing to their infection and disease. It is noteworthy that two large independent studies involving CVID/XLA have either reported no instances of this disease or merely a single case (186, 187), suggesting a relative rarity within the studied cohorts. Nevertheless, it is important to acknowledge the potential existence of undetectable cases.

I first assessed the activation of neutrophils and their ability to phagocytose mycobacteria using fresh blood samples. The internalisation of mycobacteria by neutrophils was confirmed by confocal microscopy. Then, I investigated the ability of blood samples from CVID/XLA patients, patients with NTM-PD and healthy comparators to kill or restrict growth of NTM. Finally, I evaluated the opsonisation capacity of serum and sputum samples to facilitate neutrophil phagocytosis.

The activation state of neutrophils has been studied in several chronic diseases. Upregulation of CD66b and CD15 or downregulation of CD62L (L-selectin) have been evaluated in COPD and asthma (208). Given the inflammatory airway disease seen with bronchiectasis, I selected these surface markers to assess neutrophil activation states and responsiveness within a whole blood assay. CD15 and CD66b are specific neutrophil activation markers (209), while CD62L is involved in neutrophil attachment to endothelium at sites of inflammation, but is shed with cellular activation.

As expected, the adhesion molecule CD62L showed significantly lower expression on neutrophils' surface during challenge with *M. abscessus* and *M. avium* compared with unchallenged samples. This was particularly marked for *M. abscessus*. Shedding of CD62L prevents the accumulation of leucocytes at sites of inflammation. Limiting L-selectin levels on activated neutrophils that might contribute to the pathology

of the infection e.g., through worsening lung damage and ultimately the development of bronchiectasis, could have a positive consequence for the host (205, 210, 211).

CD16 and CD64 are Fcγ receptors. Nesterova et al. have reported that they are triggering molecules which induce phagocytosis and killing processes (212). A decreased expression of CD16 was seen among patients with neutropenia and certain other medical conditions associated with inflammation (213, 214). Moreover, It has been suggested that CD64 expressed on the surface of neutrophils can be a valuable indicator of infectious diseases, particularly bacterial infections (215, 216), and monitoring the activity and progression of NTM-PD (217).

Watanabe et al. reported a strong correlation between neutrophil CD64 levels in sputum and the severity of MAC disease as evaluated by pulmonologists through examining spontaneous sputum smears, cultures, and chest X-rays (218). Additionally, there was a positive correlation between CD64 levels and MAC disease severity assessed by radiography among patients with NTM-PD (218). In my experiments, CD64 tended to be more highly expressed in CVID/XLA patients but there was no obvious impact of *in vitro* challenge with mycobacteria. A higher expression level of FcγRIII (CD16) which is normally seen on resting neutrophils was found in patients' samples at baseline compared to healthy comparators. It is intriguing that CD16 expression was increased in patients, consistent with a resting state, while other markers such as CD66b and CD15 (see below) point towards greater activation or immaturity.

On other hand, as CD16 is an FcγR, there may be an as yet uncharacterised relationship with immunoglobulin replacement therapy – which also helps to explain higher CD64 expression in CVID/XLA patients (219). A previous study recorded lower expression of CD16b in CVID patients compared to controls, but the samples were taken at least 21 days from the last immunoglobulin infusion i.e. at a point where one might expect to see less IVIG effect (193). Despite the differences in CD16 expression detected by MFI, there were no differences in the percentage of cells expressing the marker and this result should not be over-interpreted.

In humans, CD15 is a glycan component expressed on mature neutrophils, and may mediate interactions with dendritic cells (220, 221). In my experiments, both sets of patients' neutrophils had a consistently lower expression of CD15 compared to healthy comparator group, with or without challenge.

A previous study by Casulli et al. postulated altered granulopoiesis in CVID patients could result in neutrophil abnormalities including lower expression of CD15 (193, 194), while Ndlovu et al also implicated lower neutrophil maturity as the explanation for lower CD15 expression in a cohort of patients with tuberculosis (222).

CD66b is a marker of activation for neutrophils, mediating the adhesion to endothelial cells and involved in degranulation (223, 224). Higher expression of CD66b was seen in some mild bacterial infections as reported by Manz and Boettcher (225)

In CVID/XLA and NTM-PD patients' unchallenged samples, baseline expression of CD66b was significantly higher than in healthy comparators which implies greater activation of neutrophils. Similarly, XLA patients had an increased expression of neutrophils CD66b compared to healthy donors in work by Cavaliere (226).

In the phagocytosis assay, I found no significant difference between the healthy comparators' and patients' samples in terms of the proportions of neutrophils or monocytes which internalised mycobacteria. In contrast, there was a marked difference between the mycobacterial species, with greater phagocytosis seen for *M. abscessus*. It is possible that this may have been confounded by the inoculum size – which can vary for a variety of reasons including changes in the metabolic activity of mycobacteria during storage at -80C° or the incubation time. However, I attempted to standardise the concentration as far as possible and made new stocks every three months. It is possible that phagocytes are more efficient at internalising *M. abscessus* - and this was seen on confocal microscopy where the clear discrimination of internalisation between the two species *M. avium* and *M. abscessus* by neutrophils was observed in three dimensions. However, this intriguing finding requires further investigation and formal analysis by determining the number of bacteria present inside the cells at specific MOIs.

In the mycobacterial restriction assay, there was a much shorter time to detect *M. abscessus* (hours) as compared to *M. avium* (days) in blood samples from all groups. This most likely reflects the organisms' respective growth rates. However, the results revealed that TTP for both mycobacterial species were similar in each group.

Whilst many factors in whole blood can affect mycobacterial growth such as neutrophil count (108), antibody-mediated responses (227, 228), complement (229), T-cells and mononuclear phagocytes (230), other elements may also be relevant. The regular use of prophylactic therapies such as macrolide antibiotics beyond their antibiotic properties, possess anti-inflammatory and immune-modulating effects that might impact the ability of blood to control the infection. In my experiments, no appreciable difference was noticed in TTP between CVID/XLA patients using and not using macrolide prophylaxis. There were no instances of prolonged TTP among those using macrolides. This is consistent with several scenarios: macrolides given at prophylactic doses may not significantly affect the *in vitro* control of mycobacterial growth or their impact may vary widely among individuals. Additionally, since it was a whole blood assay, variations in macrolide concentration can occur. Several studies have shown that azithromycin did not affect neutrophil phagocytosis or cytokine production in response to *M. abscessus*. Moreover, restriction of *M. abscessus in vitro* was similar for neutrophils treated with azithromycin and untreated neutrophils in both healthy and patient groups (231-234). My results indicate that even in whole blood, the use of macrolide antibiotics does not appear to affect the control of NTM. However, it remains possible that we would observe a difference if using a lower infecting inoculum (or a larger study population).

The results for *M. avium* in two mycobacterial restriction assay experiments on CVID/XLA patients were not biologically plausible as the TTP was too short. Possible explanations for this include contamination, incorrect inoculation or the instrument temperatures.

To ensure that my lab technique was not contributing to this finding, I carefully compared all other results from experiments performed on the same donors, but these appeared valid and within the range of other donors.

As in other experiments, there was a difference in phagocytosis between *M. avium* and *M. abscessus* among the healthy comparators and CVID/XLA patients, implying that the correct organisms were added. Overall, this issue did not affect the statistical results and there were still no differences between healthy comparator group and patients, so I am confident that the rest of my results are robust.

I also investigated the opsonisation capacity of serum and sputum from the various groups in mediating neutrophil phagocytosis of mycobacteria. Although a period of pre-opsonisation did not show any statistical difference in the proportion of neutrophils internalising organisms compared to no pre-opsonisation, overall pre-opsonisation yielded more reliable results. The opsonisation capacity of serum was different in terms of internalisation between the groups, with and without heat inactivation: CVID/XLA patients demonstrated a greater capacity for opsonization when compared to both NTM-PD patients and healthy comparators (see Figure 0.14). However, the difference was only significant between NTM-PD and CVID/XLA patients.

Recently, Lenhart-Pendergrass et al reported that complement-mediated opsonophagocytosis promotes efficient killing of *M. avium* by neutrophils (235). In addition, previous work confirmed the critical role of complement as a mediator of neutrophil-Mtb opsonophagocytosis (92, 199). However, I found that the percentage of neutrophils internalising *M. avium* was quite similar with or without heat inactivation.

Intriguingly, opsonic capacity of serum at the highest concentrations in CVID/XLA patients was enhanced compared to other groups (healthy donors and NTM-PD patients), perhaps suggesting an important role for antibodies among this group. This might be explained by the regular use of therapeutic immunoglobulin preparations in CVID/XLA patients, who are therefore consistently being given antibody replacement that could enhance mycobacterial opsonisation. No appreciable differences were seen in the

opsonisation capacity of sputum supernatant (heat-inactivated or not) between the patient groups. However, sputum is a less consistent biological fluid to work with and there may be more artefactual or experimental differences. Even so, sputum supernatant improved mycobacterial uptake suggesting that opsonins were present.

A more comprehensive comparison may be achieved by including sputum samples from healthy subjects. However, healthy donors do not spontaneously produce sputum and other methods to obtain lung fluids or sputum (such as bronchoscopy or induced sputum induction) are inevitably more challenging.

There are limitations to the current work. These include the difficulty in ensuring a standard inoculum of mycobacteria used in each experiment. I have sought to control this as much as possible by working with similar stock and standardising the inoculation size by measuring OD. Another limitation relates to the FITC labelling technique, and the potential variability in the intensity of fluorescence emitted by FITC-labelled mycobacteria. However, I have mitigated this potential issue by including a healthy comparator for each experiment. In addition, it should be noted that the abundance of phagocytic cells could influence the total number of cells internalising mycobacteria, as many of my experiments were performed using whole blood rather than isolated cells. This would generate a different effective MOI; however, this is unlikely to create a systematic bias unless there was a consistent difference between healthy donors' and patients' neutrophils counts. it's important to acknowledge that for practical and logistical reasons I used data from multiple experiments conducted on different days. This approach introduces some experimental variability, which is a limitation of the study, and means one needs to be careful when looking to extrapolate these results to other populations.

Conclusion

- Neutrophils phagocytose NTM and become activated, especially with *M. abscessus*, in all groups.
- Significant shedding of CD62L expression occurs with mycobacterial challenge (*M. abscessus* in particular) among all groups.
- Neutrophils in patient groups tend towards greater activation and immaturity with higher expression of CD66b and consistent reduction in expression of CD15.
- There is a clear difference in the way that neutrophils respond to NTM species in terms of phagocytosis, regardless of the underlying host immunology within our study populations.
- No differences were observed between the groups within the whole blood mycobacterila restriction assay.
- *M. abscessus* has a significantly shorter TTP than *M. avium*, as anticipated
- The use of macrolide antibiotics does not appear to impact upon the control of NTM in whole blood.
- Adults with CVID/XLA have the best serum opsonic capacity compared with NTM-PD patients and healthy donors. This may reflect the use of therapeutic immunoglobulin in the CVID/XLA population. However, it's important to note that this comparison was significant only between CVID/XLA and NTM-PD patients and did not show a similar difference with healthy donors.

Chapter 5: Antibodies, neutrophil-derived proteins, and cytokines

5.1 Introduction

In Chapter 4, I identified differences in neutrophil behaviour (namely serum and sputum opsonisation capacity and whole blood growth restriction) on exposure to NTM in the laboratory in adults with CVID/XLA compared to people with NTM-PD and bronchiectasis, and a healthy comparator group. I also assessed neutrophil activation. In this chapter I explore the immunological response to NTM infection by measuring specific IgA (anti-MAC antibodies) in serum, markers of neutrophil activation, abundance and degranulation, e.g. NE, MMP-8 and MMP-9, S100A and HNP1-3 (in sputum, serum or supernatants of challenged blood), as well as cytokines, chemokines and growth factors associated with: (i) neutrophil activation (eg IL-8, G-CSF, GM-CSF), (ii) protection against mycobacteria (eg TNF, IFN-γ, IL-12) and (iii) immunosuppression (IL-10).

An association has been reported between neutrophil elastase (NE) activity and both clinical and radiological extent of disease, as well as lung function in bronchiectasis. Also patients with high neutrophil elastase activity are at a greater risk of exacerbations and hospitalization (236). I aimed to measure NE in both serum and sputum; and used, Protease Tag@® as a reliable quantitative method for detecting NE activity based on NE-Tag, with subsequent antibody steps providing additional signal amplification and increased sensitivity (236, 237).

HNP1-3 are antimicrobial peptides (AMPs) with activity against mycobacteria (including *M. Avium*) (238). Neutrophils are the only source of HNP1-3, and the activation of neutrophils leads to rapid release of HNP1-3 that can be measured in plasma and other body fluids during infection and inflammation (239).

S100A8 and S100A9 are mediators of various inflammatory conditions, secreted as a heterodimeric complex (S100A8/A9) from neutrophils (240). It has been reported that S100A8/A9 expression correlates with active disease and lung inflammation in humans with Mtb (241).

Cytokine networks are central to the cell mediated immune response to NTM infection. The T cell response against NTM is regulated by the production of IL-12 following endocytosis of mycobacteria by innate mononuclear phagocytes (eg dendritic cells (DC) and macrophages). In turn, activated $CD4^+$ T cells (T-helper 1) and $CD8^+$ T cells release IFN-γ which enhances killing by mononuclear phagocytes and is essential for host defence against mycobacteria (118, 242). Therefore, NTM infection which overcomes initial innate mechanisms may be controlled by efficient Th1 responses mediated by IL-12 and IFNγ (243). It has been reported that defects in IL-12 and IFN-γ pathways can predispose to pulmonary (and disseminated) NTM infections (149). Notably, interleukin-12-induced IFN-γ production from T cells also activates neutrophils to phagocytose and/or kill NTM (244). Moreover, TNF-a also has a crucial protective role in the immune response to mycobacterial infections (245).

IL-8 is an important neutrophil chemokine. MAC induces the release of IL-8 (and MMP-8 and -9) from neutrophils themselves, causing further neutrophil accumulation and further inflammation, which may promote progression of pulmonary infection (246). The expression of MMPs is stimulated by pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-12, and IL-17. Increased MMP-activity during TB has also been associated with extensive tissue damage (247).

Studies on IL-10 quantification in patients with NTM-PD have been inconsistent (248). IL-10 could be a beneficial regulator of inflammation in mycobacterial disease; however excessive expression may have adverse effects on innate immunity, development of acquired immunity and control of mycobacterial infection (249).

There may be differences in cytokine response between mycobacterial species. For example, it was found that MABS-stimulated PBMC produced greater amounts of TNF-α, IFN-γ, and IL-1 than MAC stimulated PBMC (250).

Neutrophil phagocytosis is enhanced by opsonisation and previous results demonstrated differences in the opsonophagocytosis capacity of samples from the different participant groups. I therefore also investigated anti-mycobacterial antibody concentrations. Nithichanon et al evaluated anti-GPL-core IgA in

the diagnosis of disseminated NTM infection patients with anti-IFN-γ autoantibodies in a Thai population and found higher levels among those with NTM infection and anti-IFN-γ autoantibodies compared to other groups, namely patients with tuberculosis, melioidosis, and control subjects, (251). Moreover, a recent study reported that a positive serum anti-GPL-core IgA antibody result in individuals with radiologically-suspected NTM-PD who already had one positive sputum culture test could serve as a valuable laboratory diagnostic marker for faster detection of MAC-PD or MAB-PD (252).

5.2 Aim

In this chapter I investigated neutrophil-derived proteins, antibody concentrations and cytokine/chemokine/growth factors in serum, sputum, and whole blood supernatants (unchallenged, challenged with *M. abscessus* and challenged with *M. avium*) from adults with CVID/XLA, NTM-PD, and healthy comparators. My aim was to identify any differences between the bronchiectasis patients with and without underlying immune compromise and healthy comparators.

I hypothesized that there is higher inflammatory cytokines expression in whole blood samples from both CVID/XLA and NTM-PD patients compared to healthy comparators before and after exogenous mycobacterial infection. Additionally, I hypothesized that neutrophil-related products differ between the study groups, and that this could help explain their differential response to mycobacterial infection.

5.3 Materials and Methods

The samples used in this section were serum or supernatants harvested from whole blood samples from all study groups and challenged with *M. avium* or *M. abscessus* (or without challenge) that were incubated over 96 hours at 37^{o}C and stored. Sputum samples were collected only from patient groups (CVID/XLA and NTM). The levels of total IgG, specific IgA for MAC, neutrophil elastase, antimicrobial peptides (HNP1-3 and S100A8/S100A9), and the proteolytic enzyme MMP-8, in addition to human cytokines/chemokines/growth factors, were measured using commercially available ELISA kits and Luminex (10-Plex) kit according to the manufacturer's instructions. An ELISA-reader and Luminex Instrument were used for quantitative determination as discussed in Chapter 3 (Sections 3.12 and 3.13).

The results are presented as median and IQR. One-Way ANOVA or the Kruskal-Wallis test were used to compare between the three groups (Healthy comparators, adults with CVID/XLA, and adults with NTM-PD). Dunn's tests for post-hoc comparisons were used to compare within- and between-group changes. Mann-Whitney test was used to compare two patient groups, specifically for sputum analysis. All statistical tests assumed a two-sided alternative with a 5% level of significance.

5.4 Results

5.4.1 Anti-GPL core IgA is increased in adults with NTM-PD

Anti-GPL-core IgA antibodies titres were measured in serum samples from all groups; with results exceeding the cut off value of 0.7 U/mL were considered 'positive' following the Capilia MAC Ab ELISA kit instructions. All adults with NTM-PD had detectable anti-GPL core IgA antibodies with values >2 U/mL, (mean=3.3 U/mL). However, healthy comparator group and CVID/XLA group had antibody concentrations ≤ 0.7 U/mL (Figure 5.1A).

Healthy subjects had significantly lower levels of total IgG compared to CVID/XLA, and NTM-PD patients ($p=0.006$ and $p=0.01$ respectively) (Figure 5.1B).

Sputum IgG concentrations did not exhibit a significant difference between adults with NTM-PD and those with CVID/XLA (Figure 5.1C).

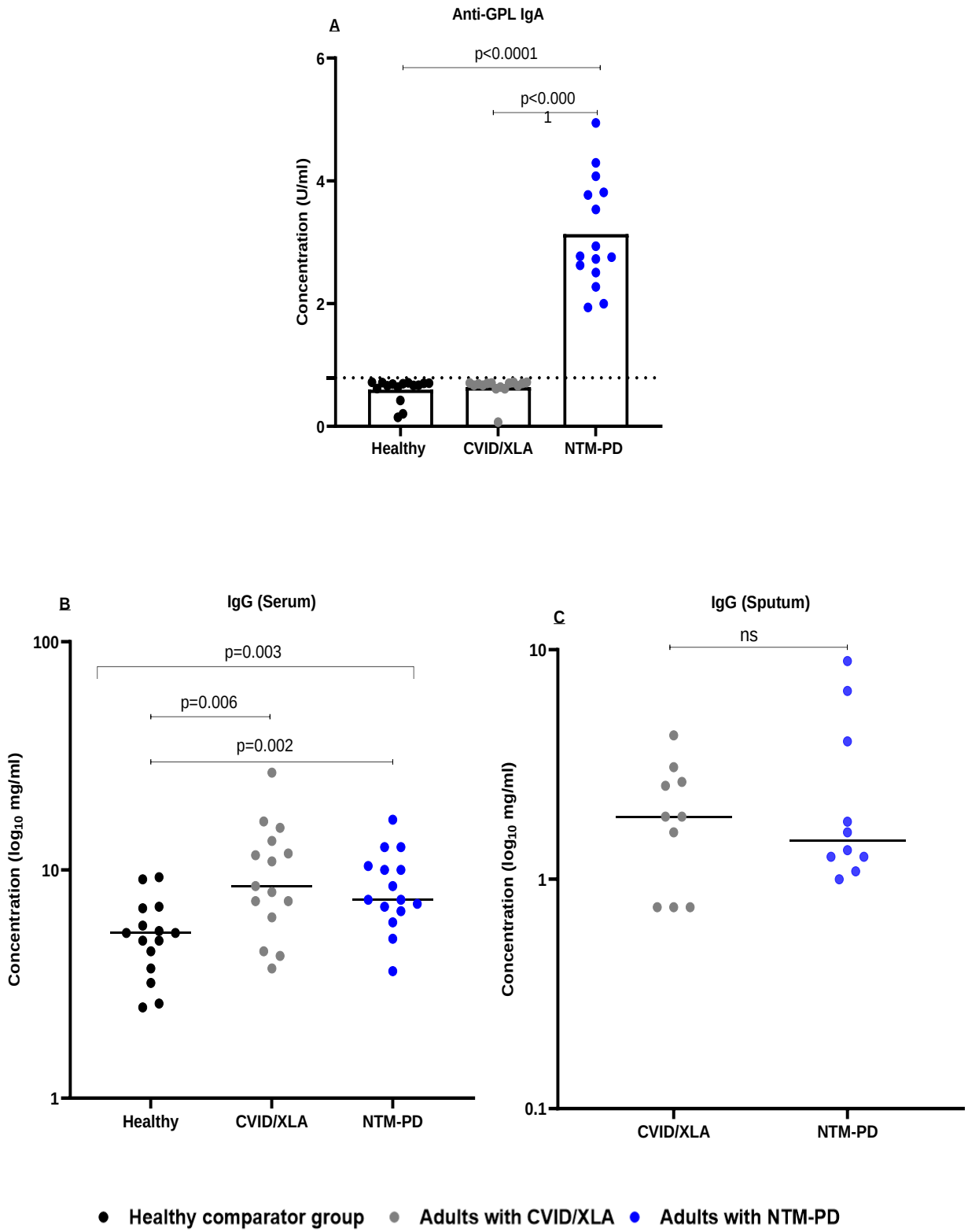


Figure 5. 1: Detection of anti-GPL IgA in serum and total IgG level in serum and sputum in all groups

(15 serum samples from each group and 10 sputum samples from patient groups). **(A)** Results are shown as bar graphs for each group. The dashed line represents the positive cut-off for Anti-GPL core IgA. Statistical differences between the groups were analysed by unpaired t-test; P<0.005. Data in the graph **(B)** and **(C)** represent log total IgG; **(B)** Comparison of total IgG level in serum samples. **(C)** Comparison of total IgG level in sputum samples. All samples were tested in triplicate. At the top of the bars, the lower set of P values shown in the figure are for the second comparison (Post Hoc test), indicating the significance of the differences between the groups.

5.4.2 Adults with NTM-PD exhibit increased Neutrophil Elastase serum concentrations, elevated levels of collagenase-2 (MMP-8) and S100A8/A9 but no notable difference in HNP1-3 levels compared with other groups.

Serum neutrophil elastase concentration was significantly higher among the patient groups, especially NTM-PD, in comparison with healthy donors: (median 9.4 ng/ml, IQR 8.6-11 for NTM-PD) versus (median 9.1 ng/ml, IQR 6.4-10 for CVID/XLA), and (median 5.7 ng/ml, IQR 4.6-6.8 for healthy subjects), p=0.0001. The between group differences are shown in Figure 5.2A.

There was no significant difference in NE sputum concentrations between the patient groups: (median 37 ng/ml, IQR 27-73) and (median 22 ng/ml, IQR 19-162) in CVID/XLA and NTM-PD adults respectively, p =0.2; (Figure 5.2B).

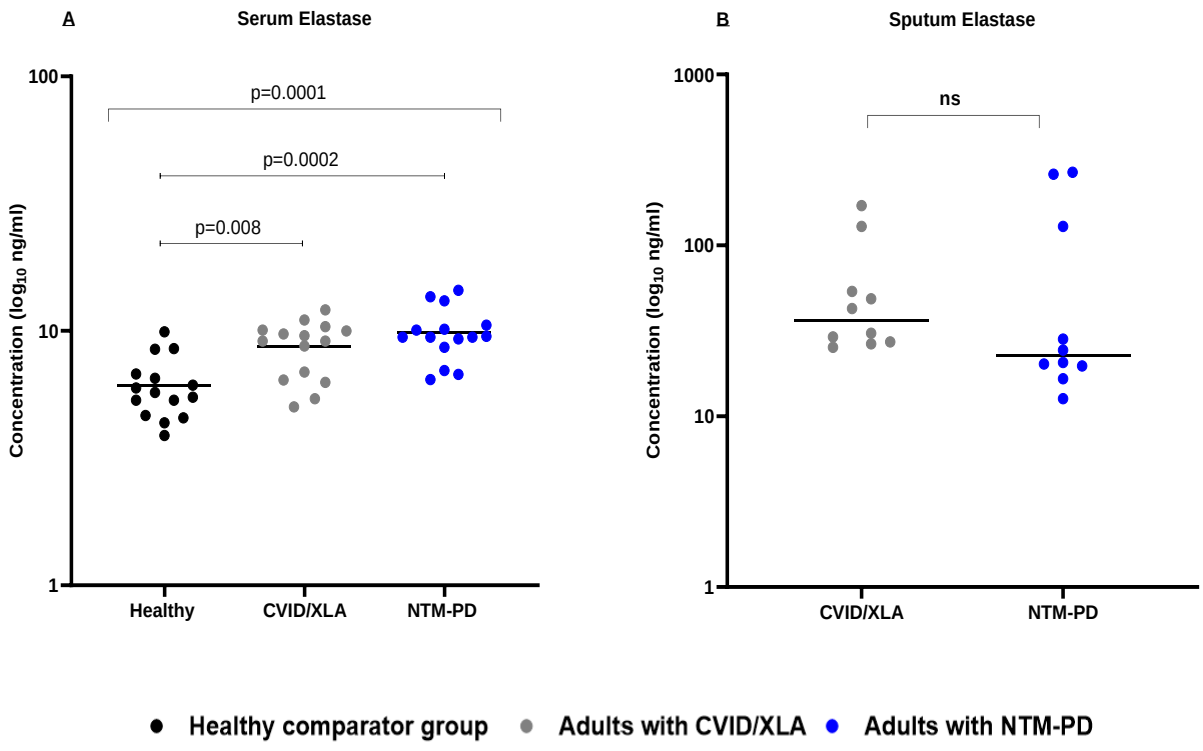


Figure 5. 2: Neutrophil elastase serum and sputum levels in adults with NTM-PD and CVID/XLA and healthy comparators. A. Neutrophil Elastase serum concentrations in NTM-PD (blue, n = 15), CVID/XLA (grey, n = 15) and healthy comparators (black, n =15). **B.** Neutrophil Elastase sputum concentrations in NTM-PD (blue, n = 10) and CVID/XLA (grey, n = 10). At the top of the bars, the lower set of P values shown in the figure are for the second comparison (Post Hoc test), indicating the significance of the differences between the groups.

Serum MMP-8 was significantly higher in NTM-PD (median 346pg/ml, IQR 112-561) than in the CVID/XLA patients (median 80pg/ml, IQR 42-164), p= 0.02. However, serum MMP-8 levels in the healthy comparators (median 185pg/ml, IQR 86-398) lay between the other groups and was not significantly different from either (Figure 5.3A).

There was no difference in sputum MMP-8 concentration between the NTM-PD and CVID/XLA adults: NTM-PD=10, (median 4518pg/ml, IQR 1427-6000) and CVID/XLA n=10, (median 3278pg/ml, IQR 337-6000) (Figure 5.3B).

MMP-8 concentrations in the blood supernatants from all groups were similar (Figure 5.3C).

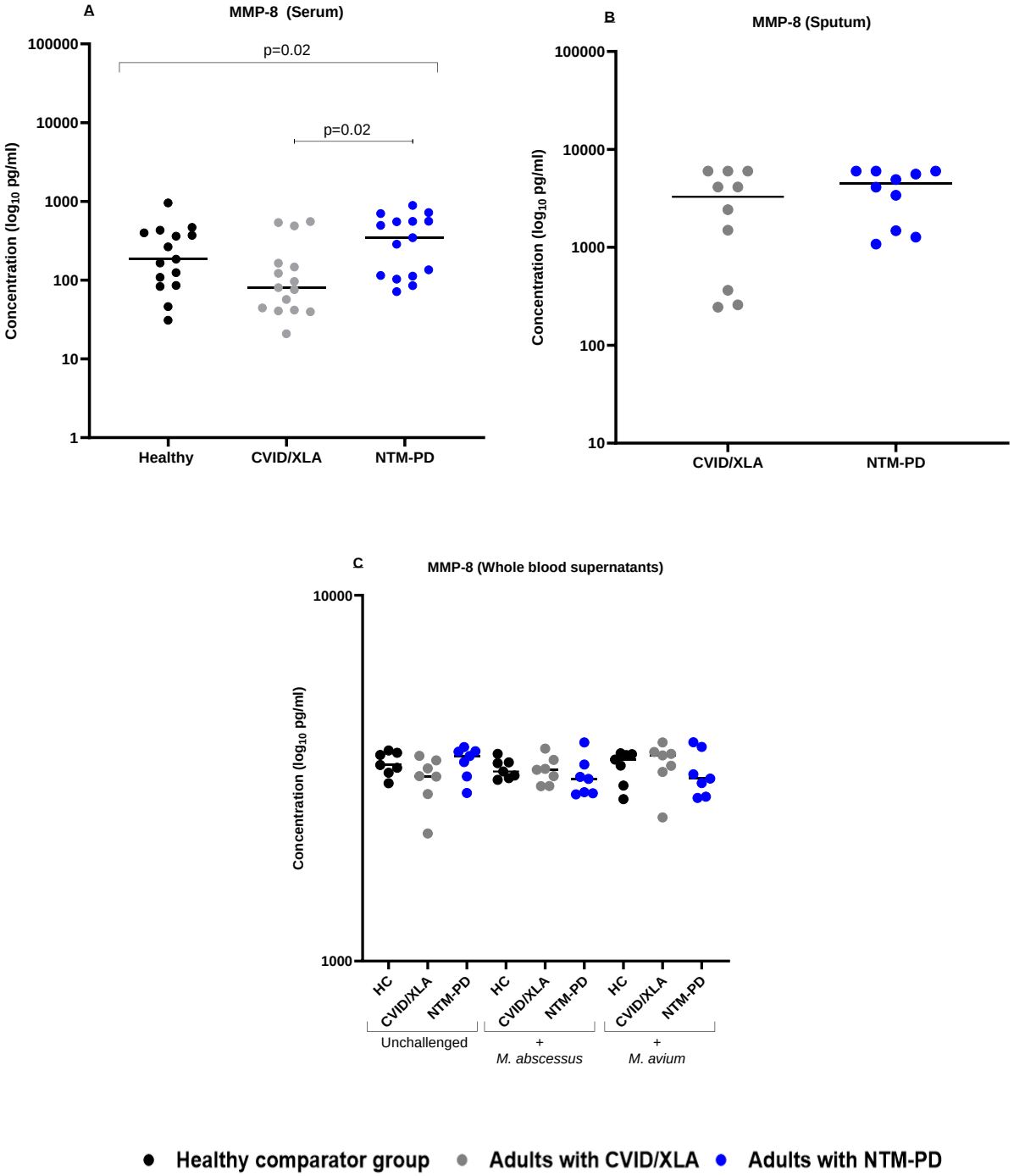


Figure 5. 3: Quantification of MMP-8 in serum, sputum, and whole blood supernatant in all groups.

(A) Serum samples from 15 subjects in each group. **(B)** 10 sputum samples from both patient groups. **(C)** Supernatants of whole blood samples that were incubated on a rocking plate (20rpm) at 37°C for 96 hours were aspirated and stored at -80°C until analysis. Samples analysed from all groups were challenged with *M. abscessus* and *M. avium* or unchallenged, n=7 for each group

Lower levels of S100A8/A9 were identified in CVID/XLA patients' serum (n=15, median 150 pg/ml, IQR 79-183) compared to adults with NTM-PD (n=15, (median 222 pg/ml, IQR 177-299) and the healthy comparator group (n=15, median 241 pg/ml, IQR 175-428) (Figure 5.4A).

Figure 5.4B shows the S100A8/A9 levels in sputum. No differences were seen between the NTM-PD (n=10) and CVID/XLA patients (n=10).

The levels of S100A8/A9 in whole blood supernatants presented in Figure 5.4C. Statistical analysis was performed between the groups at different conditions (unchallenged, challenged with *M. abscessus* and challenged with *M. avium*) and between the two infection states. There were no clear differences between adults with NTM-PD versus those with CVID/XLA and healthy comparators, with or without *in vitro* challenge with mycobacteria.

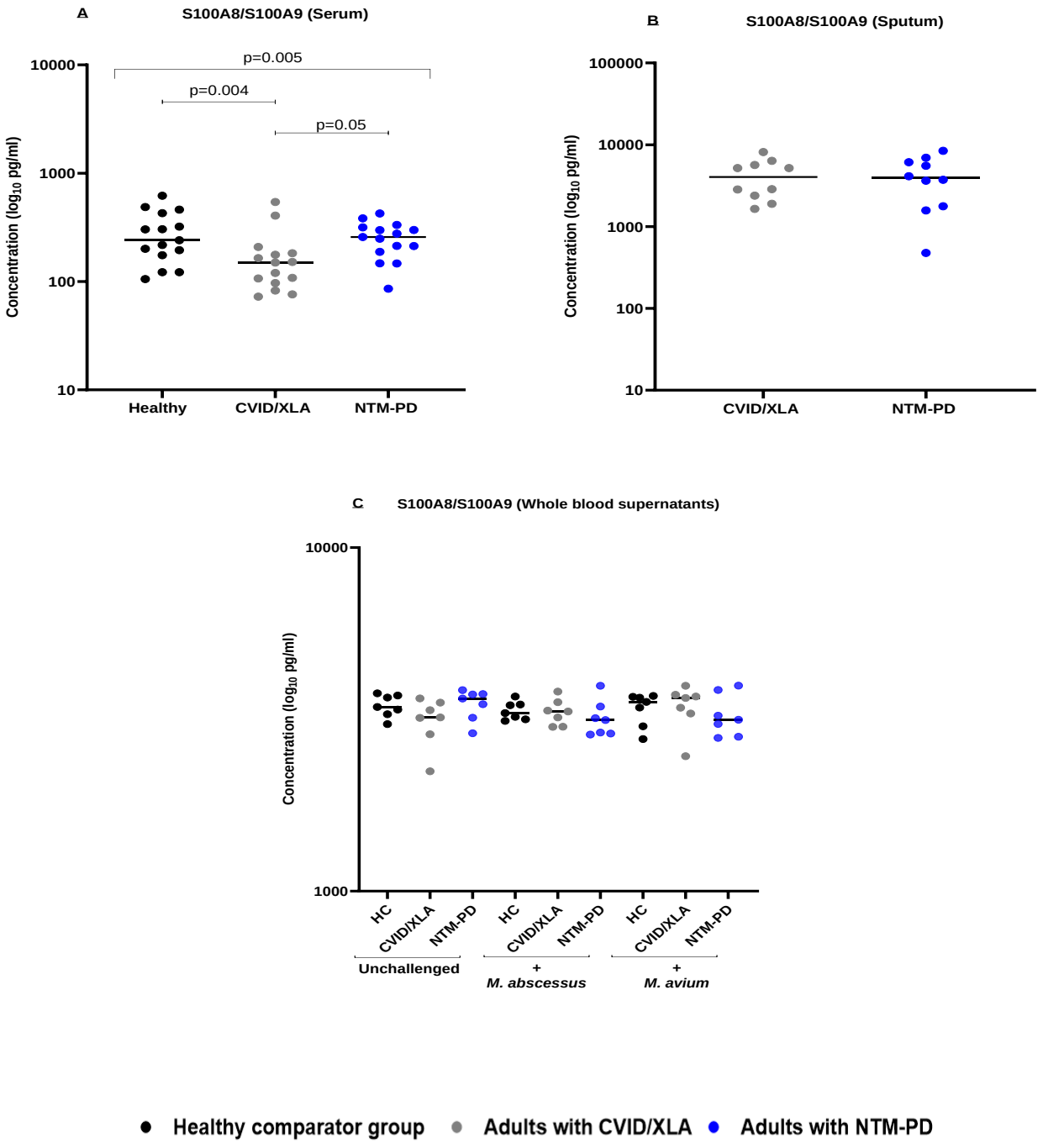


Figure 5. 4: Levels of S100A8/A9 in serum, sputum and whole blood supernatants in healthy comparator group and patient groups. (A) S100A8/A9 levels in CVID/XLA adults' serum are lower compared to NTM-PD adults (p=0.04) and healthy comparators (p=0.04). At the top of the bars, the lower set of P values shown in the figure are for the second comparison (Post Hoc test), indicating the significance of the differences between the groups. **(B)** S100A8/A9 levels in sputum samples did not exhibit any differences between the groups. **(C)** Whole blood supernatant concentrations of S100A8/A9 showed no differences between the groups in any condition.

Quantification of HNP1-3 level in the serum of all cohorts is shown in Figure 5.5A. Serum levels of HNP 1-3 were not significantly different between the three groups: Healthy comparators, n=15, (median 1099pg/ml, IQR 513-2225), CVID/XLA, n=15, (median 890pg/ml, IQR 672-1301), and NTM-PD, n=15, (median 970pg/ml, IQR 260-1760).

There was also no difference between the patient groups in the HNP1-3 levels measured in sputum (CVID/XLA, n=10, median 3601pg/ml, IQR 1444-5171), and NTM-PD, n=10, median 4337pg/ml, IQR 532-5501) (Figure 5.5B).

Concentrations of HNP 1-3 in whole blood supernatants (n=7 for NTM-PD patients, n=15 for CVID/XLA patients and (n=15 for HC) are shown in Figure 5.5C. Between the patient groups there was a significant difference in HNP1-3 levels in the whole blood samples' supernatant challenged with *M. avium*: CVID/XLA (median 7557pg/ml, IQR 5326-9010) versus NTM-PD (median 4995pg/ml, IQR 4882-5649), p=0.02.

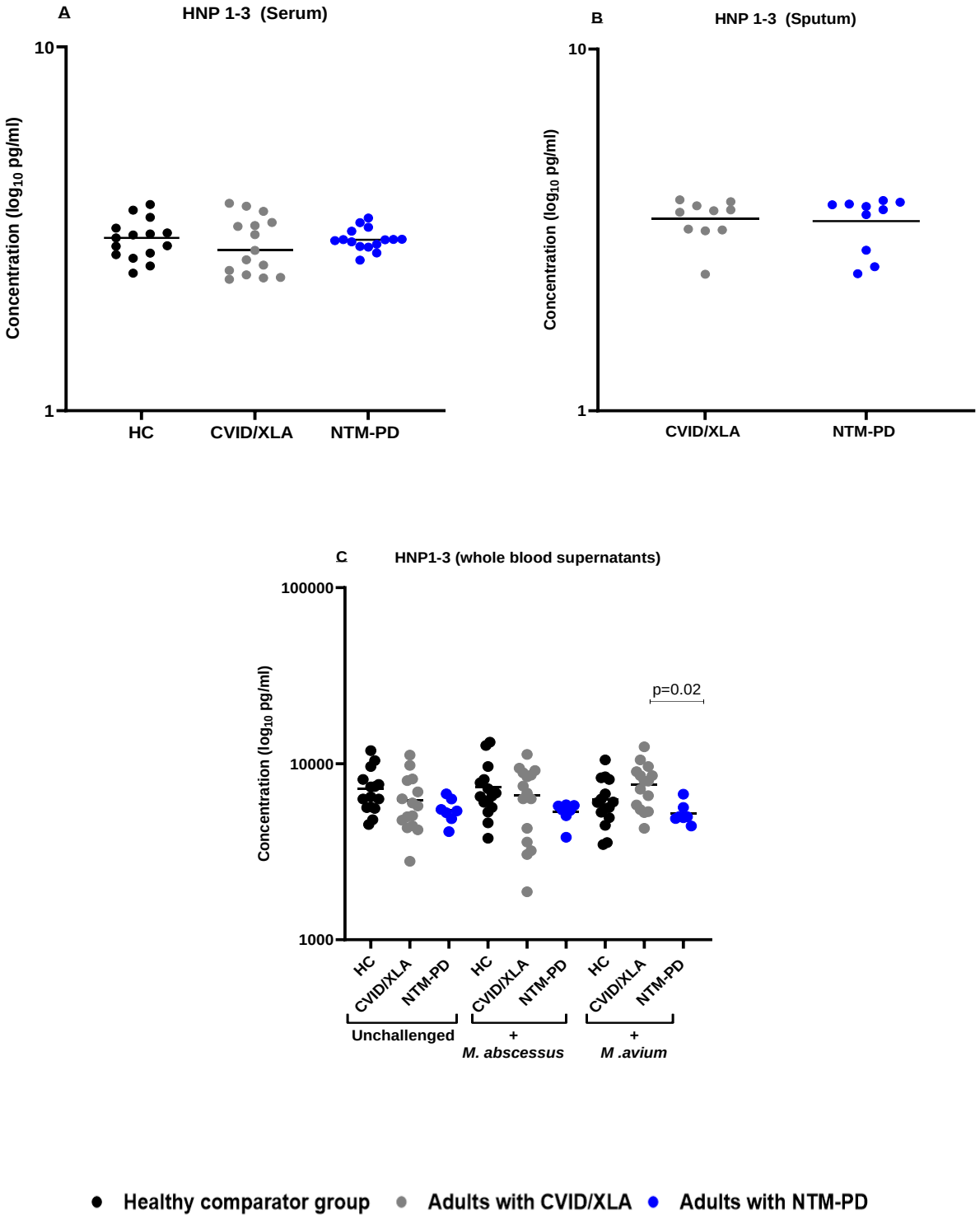


Figure 5. 5: Concentrations of HNP 1–3 in serum, sputum, and whole blood supernatants.

(A) 15 serum samples from each group, **(B)** 10 sputum samples from each patient group, and **(C)** Whole blood supernatants (HC n=15, CVID/XLA n=15, NTM-PD n=7) were analysed by Protease-Tag. No differences in HNP 1-3 were identified in serum and sputum between the groups. Supernatants of whole blood samples from CVID/XLA patients after 96hrs of incubation at 37°C demonstrated a higher concentration of HNP1-3 with *M. avium* compared with NTM-PD (p=0.03). Note: all graphs are plotted on log scales.

5.4.3 Whole blood supernatant cytokine, chemokine and growth factor analysis.

Luminex® assays using a customised panel (IL-10, IL-1, PD-L1, IFN-γ, TNF-α, IL-12, IL-8, IL-17A, G-CSF, and GM-CSF) were performed on 96-hour supernatants of blood challenged with *M. avium*, *M. abscessus*, or without challenge in adults with CVID/XLA (n=18), adults with NTM-PD (n=8) and a healthy comparator group (n=15). All groups showed significant differences between the unchallenged and challenged states for both *M. avium* and *M. abscessus* for all analytes.

5.4.3.1 NTM-PD adults expressed more IL-10 than healthy subjects and CVID/XLA adults in unchallenged samples.

NTM-PD adults expressed higher levels of IL-10 in unchallenged samples compared to healthy comparators and CVID/XLA adults: NTM-PD, (median 2pg/ml, IQR 1.3-5) versus HC, (median 0.9pg/ml, IQR 0.5-1), p=0.01, and versus CVID/XLA, (median 1.3pg/ml, IQR 1-3), p=0.05 (Figure 5.6 Left hand panel). All groups responded to mycobacterial challenge by increasing production of IL-10 in a similar manner (Figure 5.6).

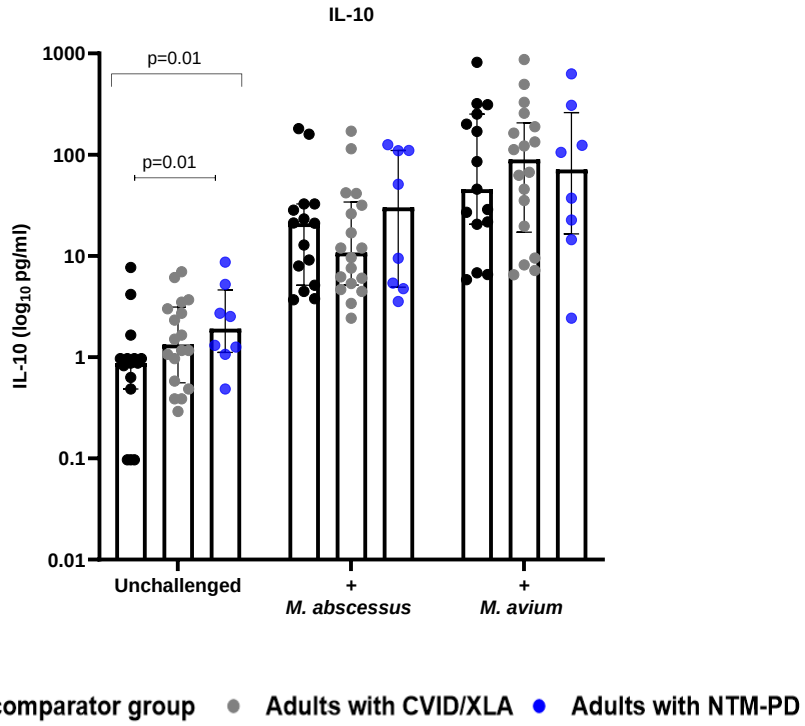


Figure 5. 6: Whole blood Luminex multiplex assay showing results for IL-10 concentrations in healthy comparator group, adults with CVID/XLA and adults with NTM-PD. The lower set of P values shown in the figure are for the second comparison (Post Hoc test), indicating the significance of the differences within and between groups.

5.4.3.2 Mycobacterial challenge-induced production of IFN-γ, TNF-α and IL-12 in blood.

Blood from all participant groups produced IFN-γ, TNF-α, and IL-12 during in vitro challenge with *M. abscessus* and *M. avium* (Figure 5.7):

- IFN-γ: In unchallenged samples, CVID/XLA group levels were higher than the NTM-PD and healthy comparator groups (median 42pg/ml, IQR 21-202, median 32pg/ml, IQR 22-46, and median 8.1pg/ml, IQR 5.8-10) respectively, P=0.0003) (Figure 5.7A). The healthy comparator population had lower levels than both patient groups. Following mycobacterial challenge, IFN-γ expression in healthy subjects markedly increased. A similar response was not seen in the two patient groups (Figure 5.7A).
- TNF-α: The CVID/XLA group had lower levels upon either mycobacterial challenge than the other groups (Figure 5.7B).
- IL-12: There was no observed difference between the patient groups and healthy comparators with and without challenge, however adults with CVID/XLA had higher detectable levels of IL-12 in the non-ed state compared to HC and NTM-PD patients: median 111pg/ml, IQR 76-288, median 61pg/ml, IQR 30-202, and median 35pg/ml, IQR 20-172), respectively (Figure 5.7C).

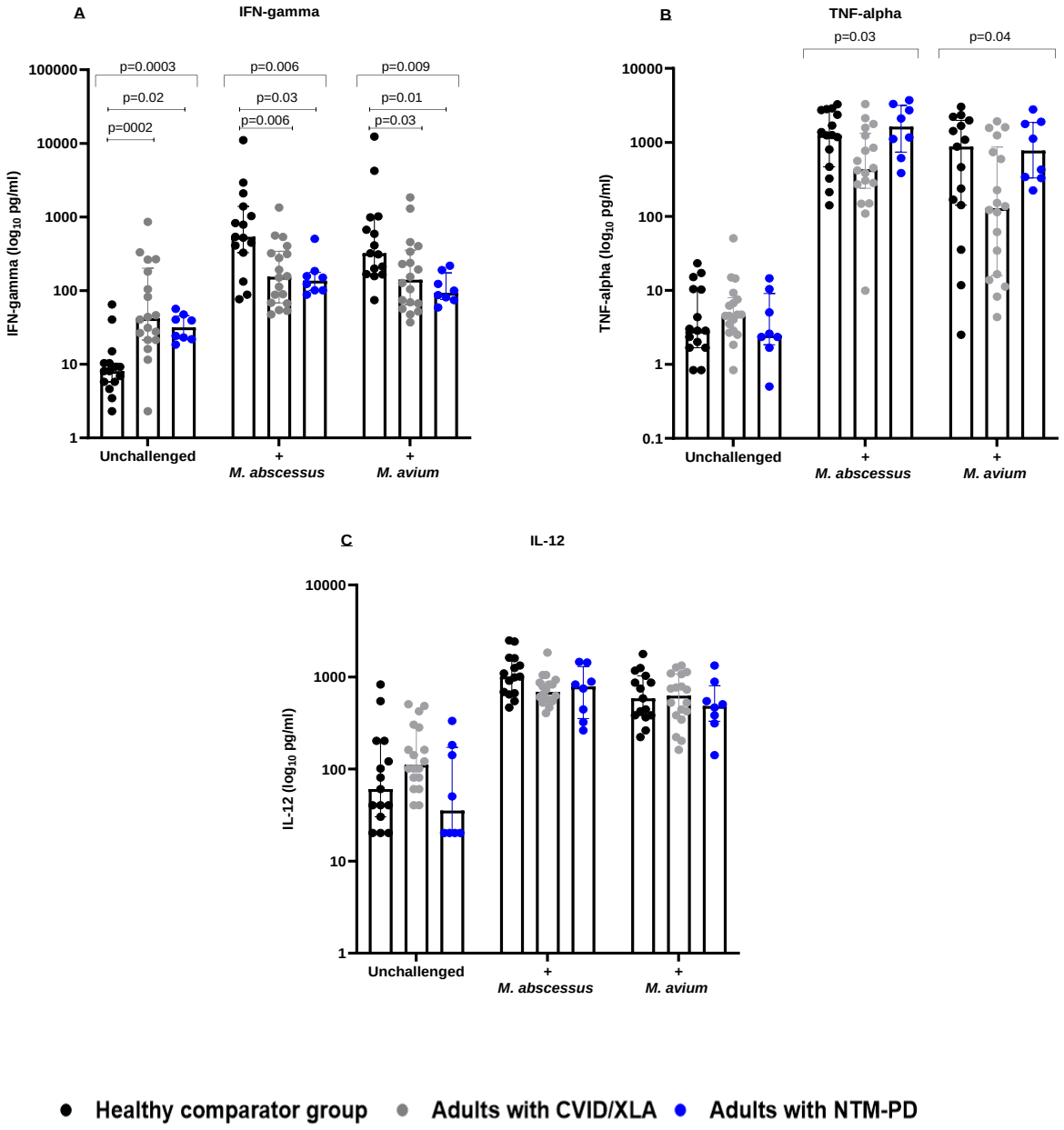


Figure 5. 7: levels of IFN-γ, TNF-α, and IL-12 in blood supernatants of adults with CVID/XLA, adults with NTM-PD and healthy comparator group at 3 different conditions: unchallenged, challenged with *M. abscessus* and challenged with *M. avium*. IFN-γ (A), TNF-α (B), and IL-12 (C) in 96hrs supernatant of fresh blood, aspirated and stored at -80°C until analysis by Luminex™ technology. Significant differences ($p<0.05$) between the groups are indicated. The lower set of P values shown in graph A are for the second comparison (Post Hoc test), indicating the significance of the differences between groups.

5.4.3.3 PD-L1 tended to vary between the patients and comparator groups and adults with NTM-PD produced less proinflammatory cytokines IL-1, IL-8 and IL-17a.

Concentrations of PD-L1 were higher in adults with CVID/XLA and lower in those with NTM-PD compared to the comparator group, both with and without challenge. For example, in unchallenged samples the values were as follows: CVID/XLA n=18, (median 36pg/ml, IQR 27-48), healthy comparators n=15, (median 14pg/ml IQR 6-23), and NTM-PD n=8, (median 5.9pg/ml, IQR 4.4.-6.8) (Figure 5.8A).

IL-1 expression among adults with NTM-PD was significantly lower in the unchallenged state compared with healthy comparators, (p=0.02) and CVID/XLA adults, (p=0.002). Following mycobacterial challenge with either MAC or MAB this did increase in adults with NTM-PD, though remained low following challenge with *M. abscessus* compared with healthy comparators (NTM-PD n=8, (median 242pg/ml, IQR 184-481), HC n=15, (median 525pg/ml, IQR 391-810), p=0.03), (Figure 5.8B).

The NTM-PD group had significantly lower levels of IL-8 in unchallenged 96hrs supernatants compared to the CVID/XLA and healthy comparator groups (Figure 5.8C). On challenge all groups showed an increase in IL-8 to similar detectable levels (Figure 5.8C).

For IL-17a, generally equivalent concentrations were recorded between groups with and without challenge. However, *M. abscessus*-challenged samples and *M. avium*-challenged healthy comparators samples had higher expression than seen in the CVID/XLA group (Figure 5.8D).

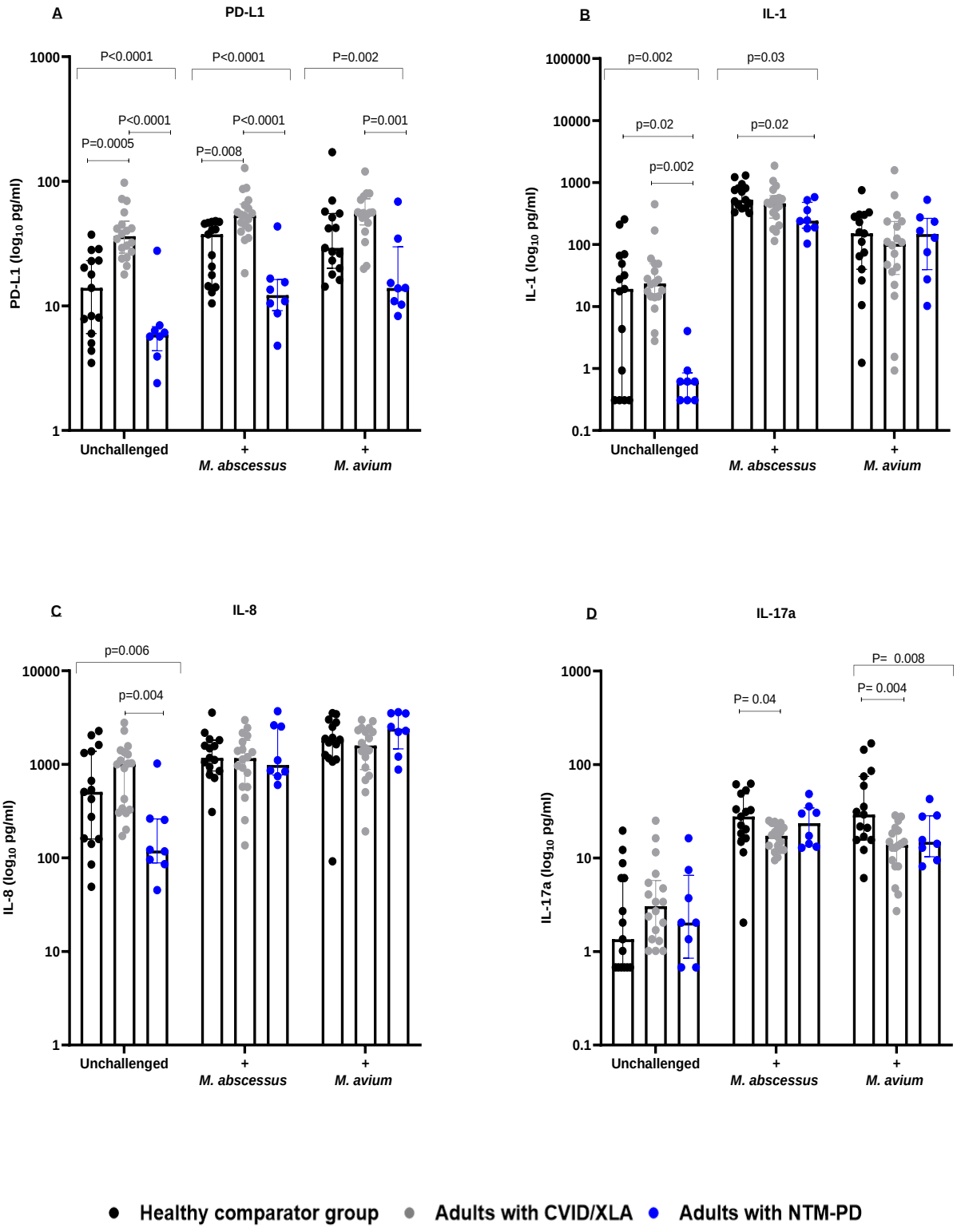


Figure 5.8: Whole blood Luminex multiplex assay showing results for PD-L1, IL-1, IL-8, and IL-17a concentrations in a healthy comparator group (n=15), adults with CVID/XLA (n=18), and adults with NTM-PD (n=8). (A) PD-L1, **(B)** IL-1**, (C)** IL-8, and **(E)** IL-17a. Kruskal-Wallis test were used to compare between the three groups and Dunn's tests for post-hoc comparisons to compare between group changes. The lower set of P values shown in the graphs are for the second comparison (Post Hoc test), indicating the significance of the differences within and between groups.

5.4.3.4 People with CVID/XLA have higher levels of G-CSF and GM-CSF in unchallenged samples.

In unchallenged samples, G-CSF concentration was significantly higher in people with CVID/XLA compared to healthy comparators and lower compared to NTM-PD patients. There was a similar marked response to challenge in all groups (Figure 5.9A).

Greater GM-CSF expression was seen in the CVID/XLA group without challenge compared with the healthy group: (median 6.3pg/ml, IQR 2.2-35) versus (median 0.7pg/ml, IQR 0.5-4), p=0.006. In contrast, the CVID/XLA group expressed less GM-CSF than the healthy group and NTM-PD group in *M. abscessus*-challenged samples (Figure 5.9B).

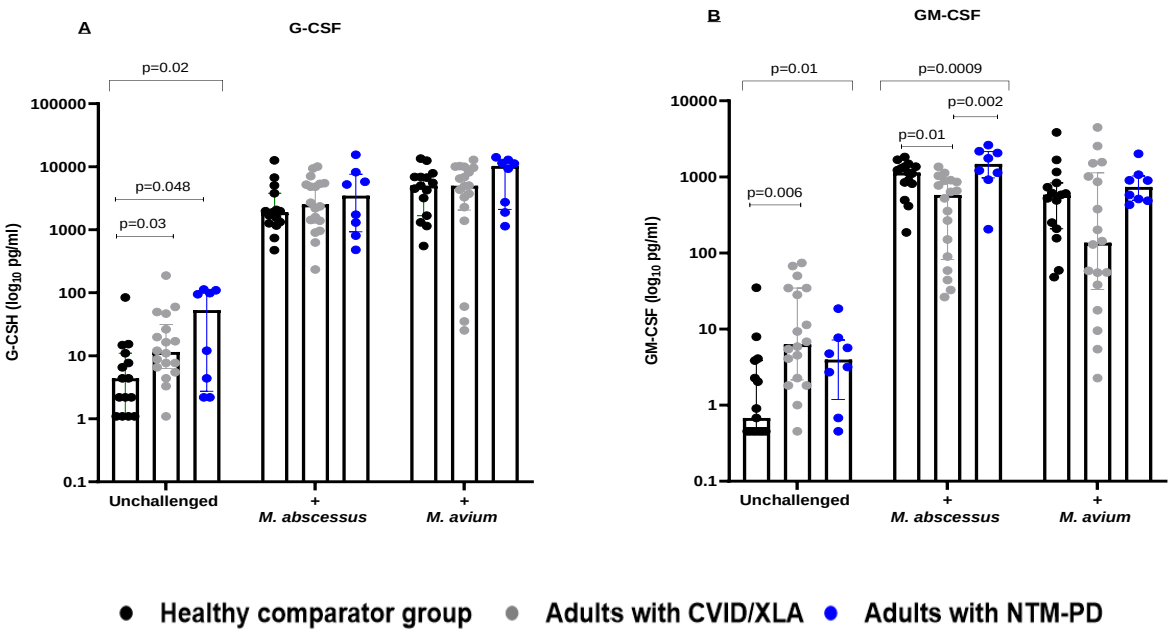


Figure 5. 9: Blood supernatant growth factor levels in a healthy comparator group, CVID/XLA group, and NTM-PD group and at different conditions. (A) G-CSF and **(B)** GM-CSF. The lower set of P values shown in the figure are for the second comparison (Post Hoc test), indicating the significance of the differences within and between groups.

Table 5.1 summarises the data presented in this chapter, and Figure 5.10 is the principal component analysis (PCA) of the results. There was a distinct separation observed between, the healthy comparator group, adults with CVID/XLA, and adults with NTM-PD in terms of the expression of inflammatory cytokines and neutrophilic inflammatory markers. However, the differences were less pronounced between CVID/XLA individuals and healthy comparators regarding neutrophil-derived proteins and antibodies in the serum and the expression of cytokines, chemokines, and growth factors at baseline (without challenge). Table 5.2 summarizes the differences between all cohorts at each condition, with all results displayed as median (IQR).

Table 5. 1: Anti-GPL IgA, Total IgG, NE, MMP-8/9, S100A8/A9, and HNP1-3, results presented as Mean ± SD, Median (IQR), and p-values for intergroup comparisons.

		Healthy comparators Serum (n=15)		CVID/XLA adults Serum (n=15)		NTM-PD adults Serum (n=15)		
1-	**Anti-GPL IgA**	All samples <0.7 U/ml (Negative)		All samples <0.7 U/ml (Negative)		All samples >2 U/ml (Positive)		
		Mean ± SD	**Median (IQR)**	**Mean ± SD**	**Median (IQR)**	**Mean ± SD**	**Median (IQR)**	
2-	**Total IgG** mg/ml	5±2	5.3 (3.7-6.8)	10±6	8.5 (6.2-13)	8.7± 3.4	7.4 (6.6-10)	Healthy vs CVID/XLA **P=0.006** Healthy vs NTM-PD **P=0.01**
3-	**NE** ng/ml	6.1±1.7	5.7 (4.6-6.8)	8.7±2.1	9.1 (6.4-10)	9.9±2.4	9.4 (8.6-11)	Healthy vs CVID/XLA **P=0.008** Healthy vs NTM-PD **P=0.0002**
4-	**MMP-8/9** pg/ml	272±241	185 (86-398)	168±191	80 (42-164)	382±275	346 (112-561)	CVID/XLA vs NTM-PD **P=0.02**
5-	**S100A8/A9** pg/ml	287±153	241 (175-428)	176±131	150 (97-183)	241±91	222 (177-299)	CVID/XLA vs Healthy **P=0.02** CVID/XLA vs NTM-PD **P=0.04**
6-	**HNP1-3** pg/ml	1434±1237	1099pg/ml (513-2225)	1032±555	890 (672-1301)	1385±1501	970 (260-1760)	**ns**

	Non-significant
*	Significantly lower
*	Significantly higher

		CVID/XLA adults Supernatants (n=15)		NTM-PD adults Supernatants (n=7)		
		Mean ± SD	**Median (IQR)**	**Mean ± SD**	**Median (IQR)**	
1-	**HNP1-3** pg/ml challenged with *M.avium*	7359±2329	7557 (5326-9010)	5231±745	4995 (4882-5649)	CVID/XLA vs NTM-PD **P=0.02**

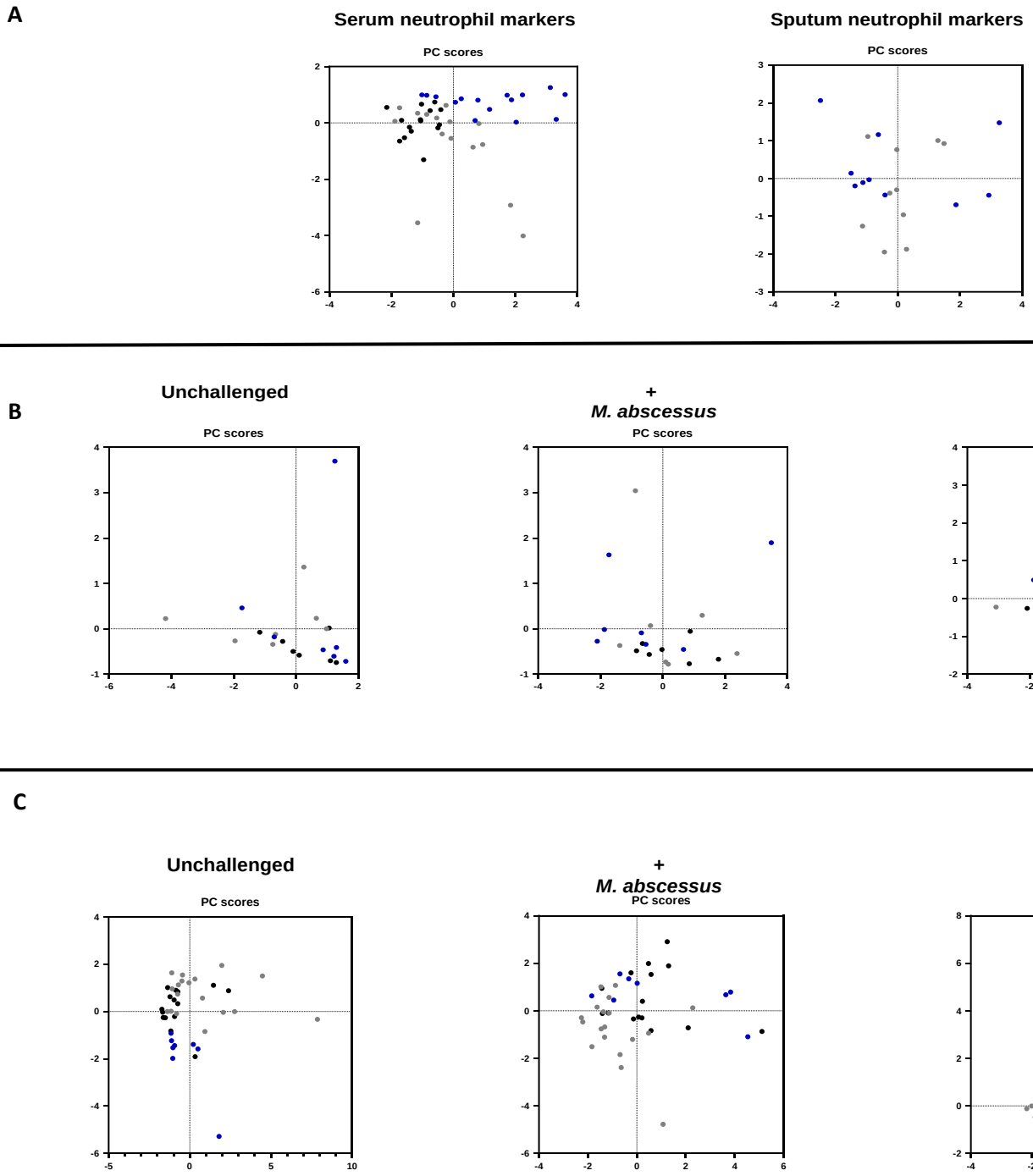


Figure 5. 10: Plots for principal component analysis (PCA). Healthy samples (black circle), CVID/XLA samples (grey circle), and NTM-PD samples (blue circle)**.** PCA of neutrophil-derived proteins and antibodies in serum and sputum **(A)**, and **(B)** whole blood supernatants. **(C)** PCA of cytokine, chemokine, and growth factors in whole blood supernatant samples from the three groups under different conditions: unchallenged, challenged with *M. abscessus*, challenged with *M. avium.*

Table 5. 2: Summary of cytokine, chemokine and growth factor results, including median values and IQR.

Unchallenged				Median (IQR)		
	H vs PID	H vs NTM	PID vs NTM	H	PID	NTM-PD
IL-10 (pg/ml)		↑NTM		0.87 (0.5-1)	1.3 (1-3.1)	2 (1.3-4.6)
IFN-γ (pg/ml)	↑PID	↑NTM		8.1 (5.8-10)	42 (21-202)	32 (22-46)
TNF-α (pg/ml)				3 (1.7-10)	5 (3-8)	2.5 (1.8-9.1)
IL-12 (pg/ml)				61 (30-202)	111 (76-288)	35 (20-172)
IL-1 (pg/ml)		↑HC	↑PID	19 (0.3-66)	24 (14-49)	0.6 (0.3-0.9)
PD-L1 (pg/ml)	↑PID		↑PID	14 (6-23)	36 (27-48)	6 (4.4-7)
IL-8 (pg/ml)			↑PID	509 (159-1375)	1010 (322-1365)	120 (88-261)
IL-17a (pg/ml)				1.4 (0.7-6.1)	3.1 (1.4-5.8)	2 (0.9-6.5)
G-CSF (pg/ml)	↑PID	↑NTM		4.4 (1.1-11)	12 (6.3-31)	53 (2.7-107
GM-CSF (pg/ml)	↑PID			0.74 (0.5-4)	6.34 (2.2-35)	4 (1.2-7.2)

Challenged with *M. abscessus*				Median (IQR)		
	HC vs PID	HC vs NTM	PID vs NTM	HC	PID	NTM-PD
IL-10 (pg/ml)				21 (5.1-33)	11(5.2-34)	30 (5-110)
IFN-γ (pg/ml)	↑HC	↑HC		539 (328-1391)	154 (68-343)	137 (101-178)
TNF-α (pg/ml)				1253 (468-2740)	434 (240-1322)	1634 (739-3163)
IL-12 (pg/ml)				1012 (668-1599)	688 (562-885)	789 (354-1300)
IL-1 (pg/ml)		↑HC		525 (391-810)	461 (266-615)	242 (184-481)
PD-L1 (pg/ml)	↑PID	↑HC	↑PID	37 (14-46)	53 (42-66)	12 (9.2-16)
IL-8 (pg/ml)				1175 (851-1816)	1163 (575-1809)	983 (770-2596)
IL-17a (pg/ml)	↑HC			28 (16-49)	17 (12-22)	24 (13-34)
G-CSF (pg/ml)				1889 (1263-3796)	2517 (1284-5387)	3482 (928-7603)
GM-CSF (pg/ml)	↑HC		↑NTM	1141 (815-1363)	580 (82-892)	1484 (971-2149)

Challenged with *M. avium*				Median (IQR)		
	HC vs PID	HC vs NTM	PID vs NTM	HC	PID	NTM-PD
IL-10 (pg/ml)				46 (21-251)	90 (17-206)	71 (17-261)
IFN-γ (pg/ml)	↑HC	↑HC		322 (168-990)	140 (65-354)	93 (76-174)
TNF-α (pg/ml)				875 (142-1985)	129 (16-866)	775 (332-1864)
IL-12 (pg/ml)				587 (384-1032)	627(374-1078)	486 (331-804)
IL-1 (pg/ml)				151 (40-300)	103 (33-236)	147 (39-264)
PD-L1 (pg/ml)			↑PID	29 (20-55)	55 (45-72)	14 (10-30)
IL-8 (pg/ml)				1837 (1162-2802)	1588 (883-2349)	2402 (1463-3516)
IL-17a (pg/ml)	↑HC			29 (16-75)	14 (8.1-20)	15 (10-28)
G-CSF (pg/ml)				5027 (1671-6883)	4976 (2055-9729)	10261 (2095-12473)
GM-CSF (pg/ml)				581 (209-835)	136 (33-1135)	738 (492-1027)

	No significant difference
	Healthy (H) significantly increased
	CVID/XLA adults (PID) significantly increased
	NTM-PD adults (NTM) significantly increased

5.5 Discussion and conclusion

In this chapter I have investigated the differences in response to NTM challenge in adults with NTM-PD, CVID/XLA and a healthy comparator group. I measured antibodies, neutrophil-derived proteins and cytokine concentrations in serum, sputum, and whole blood supernatants.

I found that a serum anti-glycopeptidolipid (GPL)-core IgA antibody test was only detectable in people who already had a positive sputum culture of slow growing mycobacteria. This is consistent with our study participants' clinical status (and hence reassuring), though doesn't take us much further.

According to a study by Kawasaki et al., compared to the usual diagnostic approach serological tests such as the anti-GPL-core IgA antibody enabled earlier diagnosis of MAC-PD or MAB-PD in individuals with radiologically suspected NTM-PD and a prior positive sputum culture (252). However, work by Jeong et al. suggested that this is more nuanced, as the antibody may help to differentiate NTM-PD from Mtb but not MAC-PD from MAB-PD (253).

The variation in IgA levels between patients with NTM-PD may correlate with disease activity (254). Furthermore, studies from the USA (255) and Taiwan (256) reported a low sensitivity due to cross-reactivity with rapidly growing mycobacteria including *M. abscessus* complex – which has implications for its clinical utility in some settings (253). It should be noted that its value in immune deficient patients such as those with CVID or XLA is also limited by their inability to produce IgA.

It has been reported that total IgG in plasma is greater in people with NTM disease and SGM than healthy controls (257). In line with this, I found considerably higher levels of total IgG in serum samples of NTM-PD patients than the healthy comparator group. However, people with CVID/XLA had the highest detectable levels of all.

Bronchiectasis can occur in people with normal IgG levels but an inability to produce specific antibodies (258). This can be assessed by measuring the response to polysaccharide vaccination and should be performed in all patients with bronchiectasis whether or not there is co-existent NTM infection.

My study's findings, particularly the tendency of lower total IgG levels in the serum of the NTM-PD group compared to the CVID/XLA group, might point towards a subtle antibody deficiency in people with NTM-PD and are intriguing as these findings align with the results discussed in Chapter 4, where the NTM-PD group had a lower opsonisation capacity than CVID/XLA. This prompts further consideration of whether these patients may have specific antibody defects that were not measured in our study. However, the difference in serum IgG between the patient groups is not statistically significant, indicating the need for larger study populations in both groups. On other hand, all CVID/XLA study participants were having therapeutic replacement of immunoglobulins – and this may well have compensated for any immune antibody-related defects that we would otherwise have detected.

Moreover, a study measuring the immunological changes in patients with bronchiectasis found that whilst the total IgG did not differ from that of controls some patients with radiologically-defined severe disease had higher total IgG concentrations (259), suggesting that IgG levels may vary between individuals with bronchiectasis.

My finding of increased serum S100A8/A9 in adults with NTM-PD reflects its documented contribution to the inflammatory response (240). However, S100A8/A9 was only clearly raised in serum and not sputum. Whilst this may underline the importance of serum-related components as well as local lung immune responses in disease pathogenesis and control, it may also highlight the technical issues that arise when trying to standardise assays on respiratory samples.

It has been suggested that S100A8/A9 has a role in leukocyte recruitment and is known to be a useful biomarker for a number of inflammatory disorders (260). Neutrophils release S100A8/A9, and through TLR2/TLR4-mediated signalling pathways, this results in the release of cytokines and chemokines that

promote inflammation. As such it has been proposed as a potential marker of disease severity in several autoimmune conditions (261). S100A8 and S100A9 expression have also been linked to neutrophilic inflammation in Mtb, with S100A9 expression being proposed as a possible disease biomarker here too (76). Ndlovu et al found that S100A9 was one of six markers that returned to healthy control levels following treatment for Mtb, and was significantly correlated with bacterial load, severity of chest X-Ray (CXR), blood neutrophil counts, and CD15 expression (262). Moreover, it has been also reported that S100A8/A9 proteins play a major pathologic role in mediating the local neutrophil accumulation and inflammation associated with TB; and hence targeting specific molecules, such as S100A8/A9 proteins, might reduce lung tissue damage without affecting protective immunity against TB (241). Of interest, the same study demonstrated the potential utility of S100A8/A9 proteins and neutrophil-attracting chemokines in serum as possible indicators of inflammation and disease severity in human TB (241).

In my study CVID/XLA adults had the lowest level of S100A8/A9 compared with NTM adults and healthy comparator group. One potential explanation for this could be that this reflects the therapeutic interventions administered to CVID/XLA patients, including the long-term use of antibodies and various medications.

I also found patient groups (NTM-PD and CVID/XLA) had significantly higher levels of serum NE than the healthy comparator population. Even though NE is primarily involved in anti-bacterial responses, it can have a number of negative effects, such as the destruction of extracellular matrix, hyperplasia of the mucus glands and an increase in mucus production, a decrease in ciliary beating rate, and direct damage to the airway epithelium (263).

Of note, neutrophils and neutrophil elastase in particular are recognised to play a significant role in the development of bronchiectasis, a common radiological feature of NTM-PD (264).

In adults with bronchiectasis, sputum neutrophil elastase activity can indicate disease severity and future risk of exacerbations, and predict declining lung function (236).

Whilst some current treatment trials in people with bronchiectasis focus on NE as their therapeutic target (265); a small randomised study of oral neutrophil elastase inhibitors in bronchiectasis found spirometry (as FEV1) was the only measure that improved with treatment (266).

Recently, Dente and colleagues reported an association between sputum neutrophils and lower pulmonary function, bacterial colonisation, and severe disease in patients with bronchiectasis (267). In addition, sputum NE activity evaluated in a study including a large group of adults with bronchiectasis showed an correlation between higher activity and a future risk of exacerbation (236, 268). Although sputum NE has been used for some time to measure the severity and development of disease (269), sputum biomarker tests are challenging to perform reliably and are not in routine use. In my study, I could detect not difference in sputum NE between the patient groups.

Serum MMP-8 and MMP-9 levels were highest in the NTM-PD group. This is consistent with other work which suggested that pathological changes in the lungs during MAC infection are driven by MMPs released from neutrophils, themselves enhanced by NET formation (270).

In addition, a correlation between sputum MMP-8 and MMP-9 and bronchiectasis severity was reported in a Chinese cohort; and the authors suggested that these molecules might be useful for predicting the future risk of exacerbations, and also supported the argument for MMP inhibitors' evaluation in clinical studies (271).

MMP-9 activity has been also observed to be induced by mycobacterial infection both in vitro and in vivo; and this activity is controlled by TNF-alpha, IL-18, and IFN-gamma. These findings suggest that MMPs may play a role in the tissue remodelling processes that take place during mycobacterial infections (272). In line with this, MMP-8 and MMP-9 were identified in lung tissue samples taken from individuals with pulmonary tuberculosis, and were associated with lung pathogenesis such including cavitation, and bronchiectasis (270, 273).

Activated neutrophils during infection and inflammation release defensins as antibacterial molecules which also regulate the innate and the adaptive immune system (274). For example, HNP 1-3 has been shown to induce bronchial epithelial cells to increase the production of the powerful neutrophil chemotactic factor, IL-8 (275). Although differences between the patient groups and healthy comparators in the expression of α-defensins (HNP 1-3), therefore might be expected, I found no difference in either serum, sputum, or whole blood supernatant.

I also analysed the concentrations of cytokines, chemokines, and growth factors. Although the specific association between neutrophils and cytokine expression is unclear, there is likely to be a bidirectional relationship. In Chapter 4, I identified there to be lowered levels of CD15 (indicating neutrophil immaturity) and higher CD66b (indicating neutrophil activation), in the CVID/XLA and NTM-PD populations. Here, I found also higher baseline IFN-γ levels plus increased expression of cytokines and neutrophilic inflammatory markers: all of which suggests ongoing or chronic infection and the presence of chronic inflammation.

Interestingly, patient groups expressed high levels of IFN-γ at 96 hours without challenge; though, in contrast to healthy comparators, who had a dramatic increase in this inflammatory cytokine following challenge with MAC or MAB, this was rather attenuated in the two patient groups – a finding that suggests patient's cells are already in a state of activation pre- challenge but are then unable to respond as a healthy individual would when exposed to infection. Given that this was seen in both patient groups, yet those with NTM-PD are more susceptible to NTM infection and disease than people with CVID/XLA, it seems likely that there are a number of steps which lead to NTM infection and possible disease in people with bronchiectasis.

Pre-mycobacterial challenge IL-10 expression by patient groups was higher than in healthy comparators, and significantly so for NTM-PD patients. However, following challenge, IL-10 levels were similar. This could be interpreted as an attempt to control hyperinflammation (and hence potentially lung tissue

damage) in the baseline state. However, high IL-10 expression may also suppress protective immune responses, enabling chronic mycobacterial infection to develop in the host.

In contrast to IL-10, IL-1 was expressed at significantly lower levels in NTM-PD patients in the unchallenged state compared with the healthy and CVID/XLA groups. This is in agreement with Jung et al where, in response to MAC antigens, NTM-PD patients produced less IL-1 from PBMCs compared with a healthy comparator population (276).

Taken together, these findings suggest that cytokine responses in people with NTM-PD reflect a state of chronic infection, and this may both make them more prone to mycobacterial infection and result in an impaired ability to clear infections should they occur.

The expression of PD-L1 in patients with CVID/XLA was significantly higher than observed in healthy comparators and NTM-PD. The results are similar to previous work with immunodeficient patients with the cutaneous T-cell lymphoma mycosis fungoides, where elevated PD-L1 was seen in an immunodeficient population compared to people with apparently normal immunity. However, this does not explicitly establish a direct link to bronchiectasis. Further investigation is needed to explore the specific implications of PD-L1 expression in immunodeficient patients and whether the high expression seen in a population who appear to be at low risk of NTM disease may be relevant when managing people with established NTM-PD or at high risk of the disease, such as people with established chronic lung disease.

G-CSF and GM-CSF expression were both elevated in CVID/XLA patients' samples without challenge. These findings may indicate the potential phagocytic role of neutrophils in this group. However, the response to challenge in all groups showed no differences other than GM-CSF expression was lower in CVID/XLA patients in *M. abscessus*-challenged samples.

In summary, neutrophil-related products were generally found at higher concentrations in the serum of NTM-PD patients, and at relatively lower levels in those with primary immunodeficiency. The latter finding is interesting, especially given the markers of neutrophil activation demonstrated in Chapter 4 and the elevated baseline cytokine levels observed in this chapter, and possibly point towards an immunomodulatory effect of immunoglobulin replacement therapy. Also, CVID/XLA patients were all taking long-term antibacterial therapies (including macrolides in 7 cases); and these may have further contributed to changes in cytokines and neutrophil-related products through both a suppression of infection and potentially further immune-modulatory effects.

Additionally, the observed alterations in neutrophil behaviour, along with the presence of chronic inflammation, raise the possibility of immune exhaustion or dysregulation. Further investigation should explore this issue so as to gain an understanding of whether this may also be relevant to the acquisition of infection and development of disease in people who are not obviously immunocompromised.

In the current study, a major limitation is the relatively poor predictability of in vitro immune assays to assess actual changes in host response to NTM infection in the lungs. Moreover, the analysis of the customized cytokine panel designed for this study in stimulated whole blood do not provide information on neutrophils specifically as the association between neutrophils and cytokine release is unclear. However, the whole blood samples tested provide valuable insights into the role of neutrophils, which is a critical aspect of understanding the immune response in both blood and tissue.

In addition, the quantity and quality of sputum samples from patient groups varied and some could not produce sputum. For logistical reasons, I did not collect sputum from the healthy comparator population. This means that any interpretation of the sputum data must be considered, and future work should look to perform all assessments on all study populations.

I also tried to mitigate the potential issue of not recruiting a sufficient number of older healthy comparators (which might compromise the statistical power and also introduce unrecognised bias) by recruiting over half of them within the age band 50-64 years.

In conclusion, I found that neutrophil-associated markers, NE, S100A8/A9, MMP-8/9, IL-1, IL-10, IFN-γ, and TNF-α could discriminate people with NTM-PD and also those with CVID/XLA from healthy comparators. Although there were between-group differences in these markers, I could not calculate the diagnostic sensitivity and specificity. To achieve this would require ROC curves analysis together with sensitivity analyses using data from a prospective longitudinal study.

Anti-GPL core IgA and IgG levels are elevated among NTM patients, and these could be related to opsonization. NTM-PD patients have a neutrophilic inflammatory process, evidenced by high levels of elastase, MMP8/9, and S100A8/A9. However, although CVID/XLA patients have a lot of inflammation (indicated by increased inflammatory cytokines) in comparison with the other subjects, they have less apparent specific neutrophil inflammation than NTM patients. How this relates to the apparent difference in the two group's risk for NTM disease requires further investigation.

Conclusion:

- People with NTM-PD had detectable levels of IgA antibodies against mycobacteria GPL-core, whereas people with CVID/XLA and healthy comparator group had concentrations that were considered negative.
- Total IgG levels in serum were higher in NTM-PD adults than in healthy comparators, and CVID/XLA adults' concentrations were the highest (presumably reflecting therapeutic immunoglobulin administration).
- NTM-PD group have elevated levels of collagenase-2 (MMP-8) and S100A8/A9, as well as increased NE serum concentrations, but no notable difference in HNP1-3 levels compared with other groups in serum, sputum, and whole blood supernatant.

- *M. avium* and *M. abscessus* induce the release of specific cytokines which could be associated with the infection.
- Patient groups exhibited high levels of IFN-γ at 96 hours in the absence of mycobacteria, while the expression change was minimal upon challenge, in contrast to healthy comparators who expressed IFN-γ significantly with the challenge and low levels without.
- Patient groups expressed higher baseline levels of IL-10 in comparison with healthy comparator groups although following challenge IL-10 production was similar in all groups.
- People with CVID/XLA have significant inflammation indicated by high concentrations of inflammatory cytokines, but they do not have the same degree of neutrophilic inflammation as the NTM population. This may be due to the immunomodulatory effects of IVIG or the absence of NTM lung infection as a result of long-term antibacterial preventive therapy.

Chapter 6: Correlating immunological markers, blood transcriptomic markers and clinical measures to predict disease severity in NTM-PD patients.

6.1 Introduction

The assessment of the severity of NTM-PD often uses a combination of the patient's symptoms and biomarkers (such as blood CRP), an assessment of mycobacterial sputum concentration (eg smear status) and extent/type of radiological change. Unfortunately, these criteria are not particularly sensitive, given the inevitable subjectivity and hence variability of reported symptoms plus the wide range of other parameters seen in otherwise clinically similar cases (277). Radiology documenting the extent of disease (such as bronchiectasis, cavitation, nodules, and consolidation on a CT chest scan) has been used in NTM-PD research studies to assess severity (55, 278).

As, for safety reasons, CT scans should not be performed too frequently, it would be useful to have a simple and safe test that can predict the severity of disease, response to treatment or disease progression if untreated.

In this chapter I assessed validated questionnaires, including those for NTM-PD patients, respiratory patients and quality of life, and evaluated several laboratory parameters to investigate their relationship with NTM-PD disease severity as assessed by CT scans.

I selected six questionnaires that have been shown to be both valid and reliable indicators of health-related quality of life in lung disease: QOL-B- NTM module, Modified MRC Breathlessness Scale, COPD Assessment Test™ (CAT), GAD-7 Anxiety Scale, PHQ-9 Depression, and EQ-5D-5L Health score. The modified Quality of Life Questionnaire–Bronchiectasis (QOL-B) was validated in patients with pulmonary NTM disease due to *Mycobacterium avium* complex and measures respiratory and general symptoms as well as functional domains (279).

Anxiety and depression measures were included as these are both common in NTM-PD patients (280), and are associated with other patient-reported symptoms (281).

Serum anti-glycopeptidolipid (GPL)-core IgA antibody is a specific test for infection with *M. avium* complex, *M. abscessus*, and some other mycobacterial species. When evaluated in patients with radiologically suspected MAC-PD or MAB-PD who already had a sputum culture positive for mycobacteria it was found to provide an early and accurate identification of MAC-PD or MAB-PD (252). However, whether the concentration of anti-GPL-core IgA correlates with disease severity is unknown.

Neutrophil-related inflammatory markers can indicate the extent of inflammation and may provide information on disease severity. Therefore, I specifically investigated the correlation between neutrophil activity or dysfunction, as indicated by these markers, and disease severity, defined by CT scan scores.

The use of a host RNA transcriptomic signature as a biomarker for NTM-PD would offer several advantages over conventional methods, including faster and more accurate diagnosis, improved prognosis prediction, and more effective treatment monitoring. While it is important to note that gene expression in peripheral blood can be influenced by a range of genetic and environmental factors, identifying a transcriptomic signature would still provide valuable information for future research.

In this chapter, I investigated whether a whole blood RNA transcriptomic signature in NTM-PD patients correlates with markers of disease severity including CT scan scores and concentrations of host neutrophil markers measured in serum.

No single common genetic or immunological defect has been identified in individuals with NTM-PD (248) and it is likely that multiple pathways determine host susceptibility to NTM-PD. These interact with environmental and microbiological factors which ultimately results in clinical disease (248). However, once disease is established it is possible that gene expression could reflect NTM-PD severity, as defined by CT scores. Therefore whole-blood transcriptomics may help indicate disease severity and progression, and so assist patients and clinicians in decisions about treatment.

To date it has been shown that an abnormal innate or adaptive immune response is linked to the development and severity of NTM-PD (76). However, the same has not been done for host gene expression.

6.2 Aim

I aimed to:

1) Explore the relationship between neutrophil markers in serum or sputum (including neutrophil elastase concentration, MMP-8/9, S100A8/A9, and HNP1-3) and the severity of lung disease in adults with NTM-PD as defined by a radiological score.
2) Investigate the correlation between indicators of humoral immune response (serum and sputum IgG concentrations and specific IgA antibody concentrations) and radiological severity of lung disease in adults with NTM-PD.
3) Determine whether the questionnaire-assessed health status of people with NTM-PD correlated with CT chest radiological severity.
4) Investigate whether a host transcriptomic signature in blood could be identified that differentiates the severity and extent of radiological changes, or the concentration of neutrophil-associated proteins, in people with untreated NTM disease.

6.3 Materials and Methods

The methodology used in this chapter is described in Chapter 3 sections 3.14 and 3.15. Patients were identified through recruitment at the Grove Centre Mycobacterial Clinic at Royal Free Hospital (2021–2022). At enrolment, each participant completed questionnaires that assessed breathlessness, quality of life (NTM Module), anxiety and depression and severity of airways disease.

6.3.1 Data collected from adults with NTM-PD

Demographic and clinical data were collected, including age, gender, treatment status, laboratory results, and chest CT results. The validity of all data was checked by physicians (Marc Lipman and David Lowe). Patients' characteristics and ethical permissions are detailed in Chapter 3.

6.3.2 Questionnaires

The questionnaires used in this study were QOL-B- NTM module, Modified MRC Breathlessness Scale, COPD Assessment Test™ (CAT), GAD-7 Anxiety Scale, PHQ-9 Depression, and EQ-5D-5L Health score.
Since most of the questionnaires have four possible responses for each question, the results of all surveys were categorised into four levels to stratify patients' results:

- Grade 1 - none (or only minimal) symptoms.
- Grade 2 - mild symptoms,
- Grade 3 - moderate symptoms,
- Grade 4 - severe symptoms.

For instance, the COPD Assessment Test (CAT) scale is from 0 to 40, with scores ranging from 0–10 representing Grade 1, 11–20 Grade 2, 21–30 Grade 3, and 31–40 Grade 4.

The EQ-5D-5L questionnaire has 5 components with 5 responses for each. Here the outcome was calculated as the total of all components (minimum score 5 and maximum score 25) and was then categorized into a 4-grade scale.

The Modified MRC Breathlessness Scale questionnaire consists of five statements, but patients' responses for this questionnaire did not include grade 1 ("I only get breathless with strenuous exercise"), and the scale was therefore used as a 4 point-scale.

Further information about these questionnaires can be found in Chapter 3, Section 3.14, and the Appendices.

6.3.3 Chest CT imaging data

Radiological results from spiral CT scans for all recruited NTM-PD patients were scored by specialist radiologists (Dr Charlotte Cash and Dr Joanne Cleverley) according to accepted protocols (53) (Appendix 15, CT scan scoring system). Patients were subdivided into two groups ('low' and 'high') based on their disease severity as defined by chest CT score; I evaluated bronchiectasis scores (Bronchiectasis extent and Bronchiectasis severity), and signs of active infection (tree-in-bud, nodules, cavitating nodules, severe cavitation, and consolidation, in addition to presence of mycetoma).

According to the patients' scores ranging from 0-23 for bronchiectasis scores (with a maximum score of 30) and from 0-18 for signs of active disease scores (with a maximum score of 72), I divided them into two groups using a threshold score of 10 on the CT scan. Scores above 10 were categorized as more severe ("high") and scores of 10 or below were categorized as less severe ("low").

The Total CT scan score includes bronchiectasis scores, such as "Bronchiectasis extent" and "Bronchiectasis severity," as well as signs of active infection (e.g "tree-in-bud," nodules, cavitating nodules, severe cavitation, consolidation, and the presence of mycetoma).

This chapter aims to determine the relationship between disease severity (defined by the CT scan score) and neutrophil-associated markers. For several reasons I focused predominantly on bronchiectasis CT scan scores: (1) bronchiectasis is the most common sign of NTM-PD and all patients have confirmed bronchiectasis on the CT scan and symptoms of bronchiectasis (2) neutrophils are implicated in the pathology of bronchiectasis (3) when investigating the correlation with total CT scan scores, I found there to be similar results to those from my analysis using just bronchiectasis scores.

6.3.4 RNA extraction, RNA-seq and transcriptomic analysis

RNA extraction, RNA-seq and transcriptomic analysis were carried out according to the methods described in Chapter 3, Section 3.15.

The RNA sequencing was done in collaboration with the UCL Pathogen Genomics Unit. The transcriptomic analysis was performed by Professor of Infectious Diseases Mahdad Noursadeghi and his bioinformatics team.

For transcriptomic analysis, I first divided the dataset in two ways: first, based on the CT severity scores of bronchiectasis, and second by applying principal component analysis (PCA) to the markers measured in serum (neutrophil markers and mycobacteria-specific/total immunoglobulins). I split the group based on the median of the CT bronchiectasis score, with one group having a score greater than 10 (high) and the other group having a score equal or less than 10 (low). In the PCA, I separated the groups at zero along PC1, which is mostly composed of neutrophil-related proteins and accounts for about 70% of the variance, (group 1 and group 2).

6.4 Results

6.4.1 Demographic and clinical data

Fifteen adult patients with confirmed NTM-PD participated (Table 6.1). Five (33.3%) were <70 years-old and 10 (66.7%) 70 years-old and older; the median age was 77 years (interquartile range [IQR]: 68 to 80). Eight of the participants were women (53.3%) and 7 (46.7%) men.

Table 6. 1: Demographic and baseline characteristics of adults with NTM-PD (n = 15)

Variable	Median (IQR) or n (%)
Age, years	
Median	77 (68-80)
Range	63-94
< 70	5 (33.3%)
≥ 70	10 (66.7%)
Sex	
Female	8 (53.3%)
Male	7 (46.7%)
Ethnicity	
White British	8 (53.33%)
White (other background)	5 (33.3%)
Other Asian Background	2 (13.3%)

6.4.1.1 Correlation analysis of participants' demographic and clinical data with bronchiectasis CT score

By identifying the species of slow-growing mycobacteria present in each patient and determining which patients underwent treatment shortly after recruitment through a review of their medical records, I investigated whether there was a correlation between mycobacterial species or the timing of treatment initiation and the CT score of bronchiectasis (extent and severity) or CT score of the features of active disease: (Tree-in-bud+ Nodules+ Cavitating nodules+ Severe cavitation+ Consolidation+ Mycetoma). No significant correlation was identified between disease severity CT score of bronchiectasis (median score 17) or CT score of active disease signs (median score 10), and the subsequent need for treatment within 6 months (n=5) using Mann Whitney test, to compare mean scores in those who needed treatment versus those who did not. Additionally, no significant differences were observed between patients who were challenged with RGM and experienced mixed infection (n=4) versus those who only had SGM (n=11). However, of the four patients challenged with *M. abscessus* (RGM), three had a high bronchiectasis CT score (Table 6.2).

Table 6. 2: Characteristics of participants with NTM-PD ranked according to date of recruitment for the study.

	Age	Gender	*M* species (SGM)	RGM*?*	Bronchiectasis CT score (0-23) *	Signs of active disease CT score (0-18) *	Post-Recruitment Treatment*
1	94	F	*M. chimaera*	No	9	9	No
2	83	M	*M. avium*	No	2	0	No
3	82	F	*M. avium-intracellulare*	No	9	2	No
4	80	F	*M. kansasii*	No	21	7	Yes
5	78	M	*M. kansasii*	No	0	10	Yes
6	78	F	*M. avium*	No	2	6	No
7	77	M	*M. chimaera*	No	13	2	No
8	77	F	*M. avium-intracellulare*	*M. abscessus*	17	16	Yes
9	71	M	*M. avium*	*M. abscessus*	2	14	Yes
10	70	M	*M. chimaera*	No	20	7	No
11	69	M	*M. kansasii*	No	5	18	No
12	68	F	*M. avium*	No	20	5	No
13	66	F	*M. avium & M. kansasii*	No	23	8	Yes
14	66	F	*M. avium*	*M. abscessus*	13	14	No
15	63	M	*M. avium*	*M. fortuitum*	13	14	No

*Bronchiectasis CT score = (Bronchiectasis extent score + Bronchiectasis severity score)

*Signs of active disease CT score = (Tree-in-bud+nodules+cavitating nodules+severe cavitation+ consolidation+ aspergilloma)

*Post-Recruitment Treatment = Participants began treatment within 6 months after being recruited for the study.

Demographic data of recruited patients did not show correlation with severity of disease, as measured by bronchiectasis CT score using Pearson correlation coefficient test and Mann Whitney test (Figure 6.1).

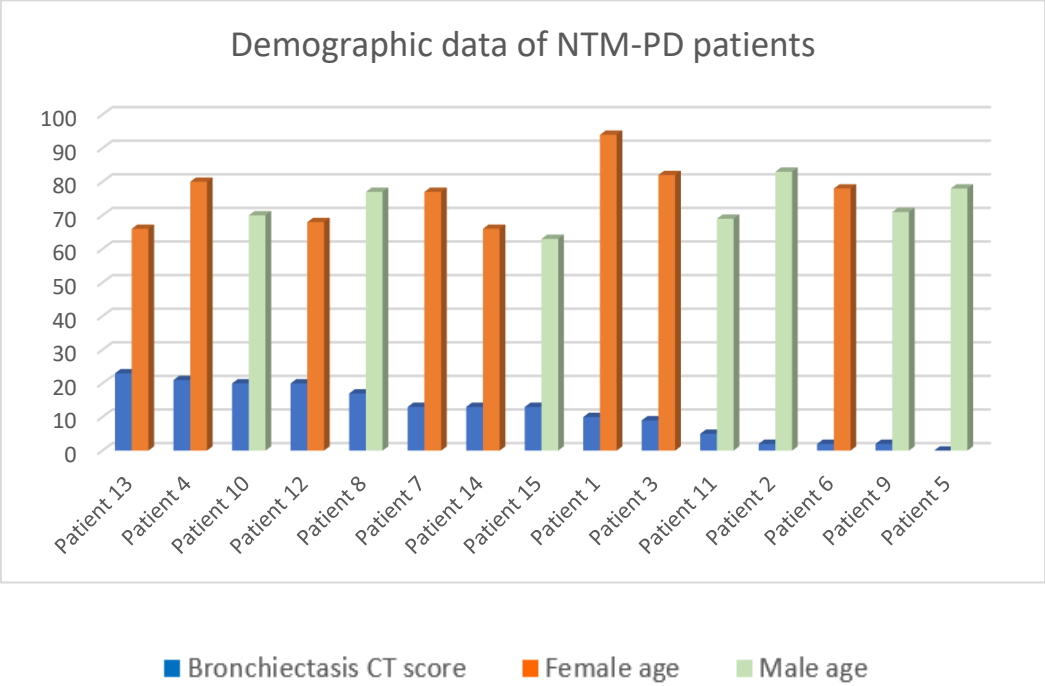


Figure 6. 1: Bar graph illustrates the baseline demographic data (age and gender) in relation to bronchiectasis CT scores. The CT scores are represented by blue bars, while female and male age are shown by orange and green bars respectively. Patient numbers according to their bronchiectasis CT scores.

6.4.2 Questionnaires

The questionnaire outcomes from 15 recruited patients, scored out of 4 (4 indicating severe symptoms and 1 very mild or none), reveal varying symptom severities. 46.7% rated their respiratory and vitality symptoms as moderate, with the same percentage reporting severe symptoms in physical function. Nearly 53.3% of the patients rarely experienced eating problems or had concerns about their body and 80% had mild digestive issues. Patients experienced moderate breathlessness and COPD-related symptoms. Additionally, over half of the participants reported mild or no general health symptoms (median score of 1 or 2), and more than 70% had mild or no depression or anxiety symptoms, highlighting a range of symptom experiences and mental health concerns among the patients (Table 6.2).

No positive correlations were found between the CT score disease severity and health status, symptoms, or anxiety and depression assessments.

A heat map was generated to display the reported questionnaires for all patients, including the QOL-B-NTM module, EQ-5D-5L, breathlessness scale with NTM-PD, Modified MRC Breathlessness Scale, COPD Assessment Test™ (CAT), GAD-7 Anxiety Scale, PHQ-9 Depression Scale, and health score (Figure 6.2 and Figure 6.3).

Table 6. 3: Questionnaire analysis for adults with NTM-PD (n=15) (maximum score= 4 indicating severe symptoms and 1= very mild / no symptoms).

Questionnaire		Median	IQR	Scale [score -- n (%)]
QOL-B- NTM module	Respiratory symptoms	3	(2-3)	1 -- 0 (0%) 2 -- 6 (40%) 3 – 7 (46.7%) 4 – 2 (13.3%)
	Physical function	3	(3-4)	1 -- 3 (20%) 2 -- 0 (0%) 3 -- 5 (33.3%) 4 – 7 (46.7%)
	Vitality	3	(2-3)	1 -- 0 (0%) 2 – 6 (40%) 3 – 7 (46.7%) 4 – 2 (13.3%)
	Body image	1	(1-3)	1 -- 8 (53.3%) 2 – 3 (20%) 3 -- 4 (26.7%) 4 – 0 (0%)
	Digestive Symptoms	2	(1-2)	1 -- 6 (40%) 2 -- 6 (40%) 3 – 3 (20%) 4 -- 0 (0%)
	Eating problem	1	(1-3)	1 – 8 (53.3%) 2 – 2 (13.3%) 3 – 4 (26.7%) 4 – 1 (6.7%)
	NTM symptoms	2	(1-2)	1 -- 6 (40%) 2 – 6 (40%) 3 – 3 (20%) 4 – 0 (0%)
General Health	EQ-5D-5L	1	(1-2)	1 – 9 (60%) 2 – 3 (20%) 3 – 2 (13.3%) 4 – 1 (6.7%)
	Health score	2	(2-4)	1 – 1 (6.7%) 2 – 7 (46.7%) 3 – 2 (13.3%) 4 – 5 (33.3%)
Respiratory measures	Modified MRC Breathlessness Scale	3	(2-4)	1 – 1 (6.7%) 2 – 6 (40%) 3 – 2 (13.3%) 4 – 6 (40%)
	COPD Assessment Test™ (CAT)	3	(3-4)	1 – 0 (0%) 2 – 2 (13.3%) 3 – 7 (46.7%) 4 – 6 (40%)
Mental health	GAD-7 Anxiety Scale	2	(1-3)	1 – 7 (46.7%) 2 – 4 (26.7%) 3 – 2 (13.3%) 4 – 2 (13.3%)
	PHQ-9 Depression	2	(1-2)	1 – 5 (33.3%) 2 – 7 (46.7%) 3 – 2 (13.3%) 4 – 1 (6.7%)

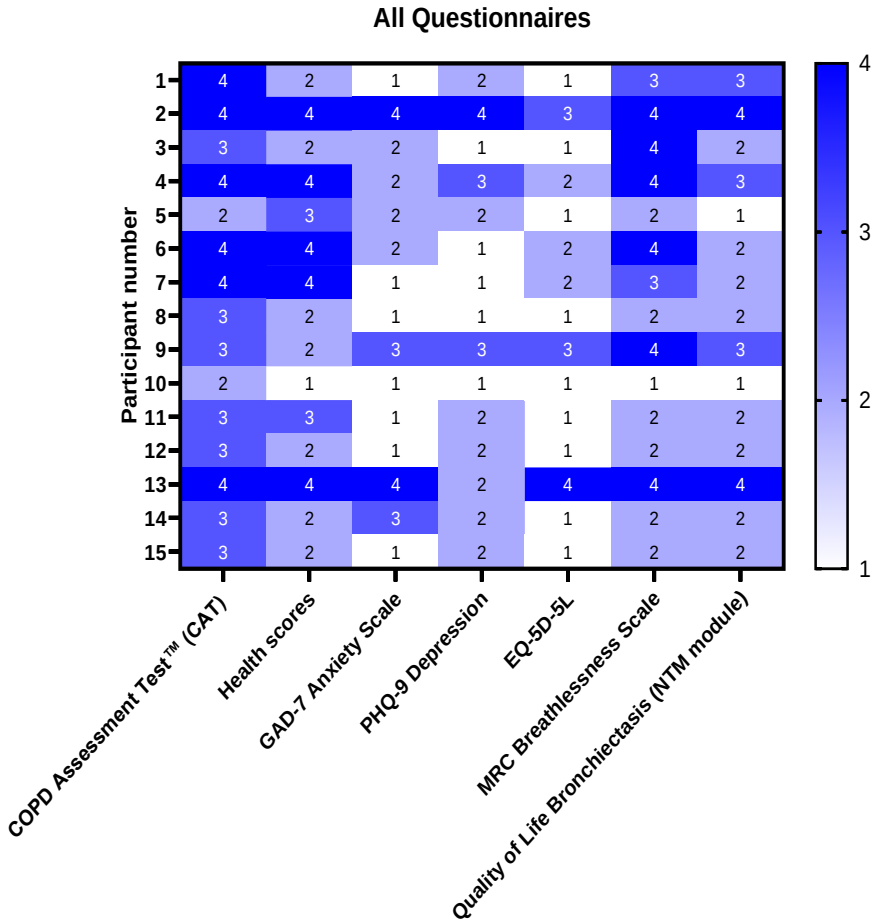


Figure 6. 2: Heatmap of the questionnaire scaled scores (score out of 4; 4 severe, 3 moderate, 2 mild, 1 no symptoms or not considerable). All questionnaires; PHQ-9: Patient Health Questionnaire, Generalized Anxiety Disorder Scale-7, COPD Assessment Test™ (CAT), Quality of Life Questionnaire-Bronchiectasis (QOL-B)-NTM module, EQ-5D-5L, and Modified Medical Research Council (MRC) Breathlessness Scale (scale out of 4). *The health score questionnaires range from 0-100%. They are categorized on a scale of 1 to 4: 0-25 = grade 4, 26-50 = grade 3, 51-75 = grade 2, and 76-100 = grade 1.

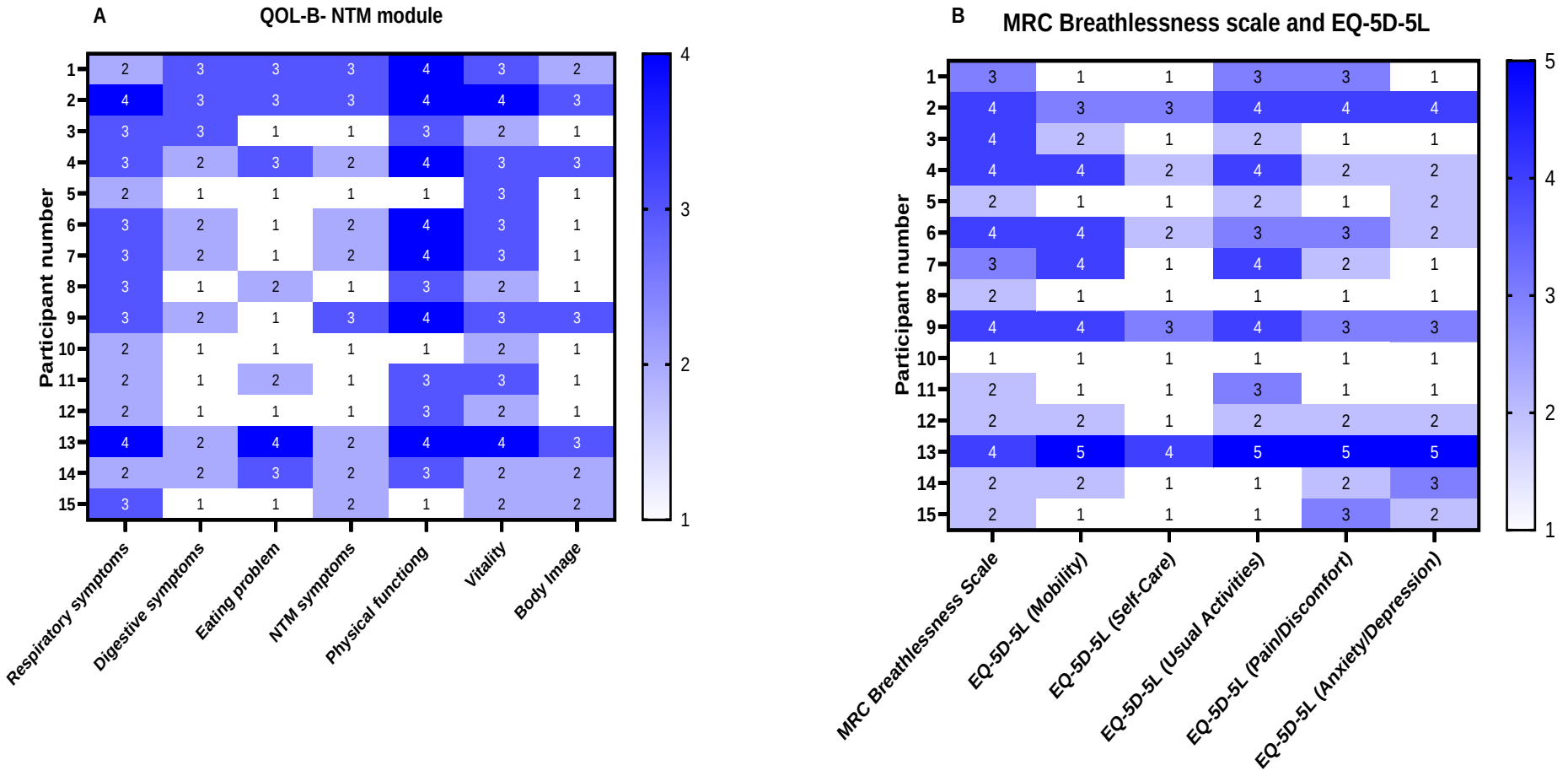


Figure 6. 3: Heatmap of the questionnaire scaled scores (score out of 4; 4 severe, 3 moderate, 2 mild, 1 no symptoms or not considerable).
(A) Quality of Life Questionnaire-Bronchiectasis (QOL-B)-NTM module, and **(B)** The EQ-5D-5L (European Quality of Life 5 Dimensions 5 Level Version) and Modified Medical Research Council (MRC) Breathlessness Scale (scale out of 5).

6.4.3 CT scoring system to evaluate severity of NTM-PD

53.3% of patients (n=8) had high severity bronchiectasis (median score 13 [IQR 13-19]), and 46.7% (n=7) had low bronchiectasis scores (median score 2 [IQR 2-9]) (Figure 6.4A). In contrast, on the scale of active infection signs, the majority (n=10, 66.7%) had low severity (median score 6.5 [IQR 2-8.3]), and only n=5 (33.3%) of NTM-PD patients had high severity (median 14 [IQR 14-17]), (Figure 6.4B).

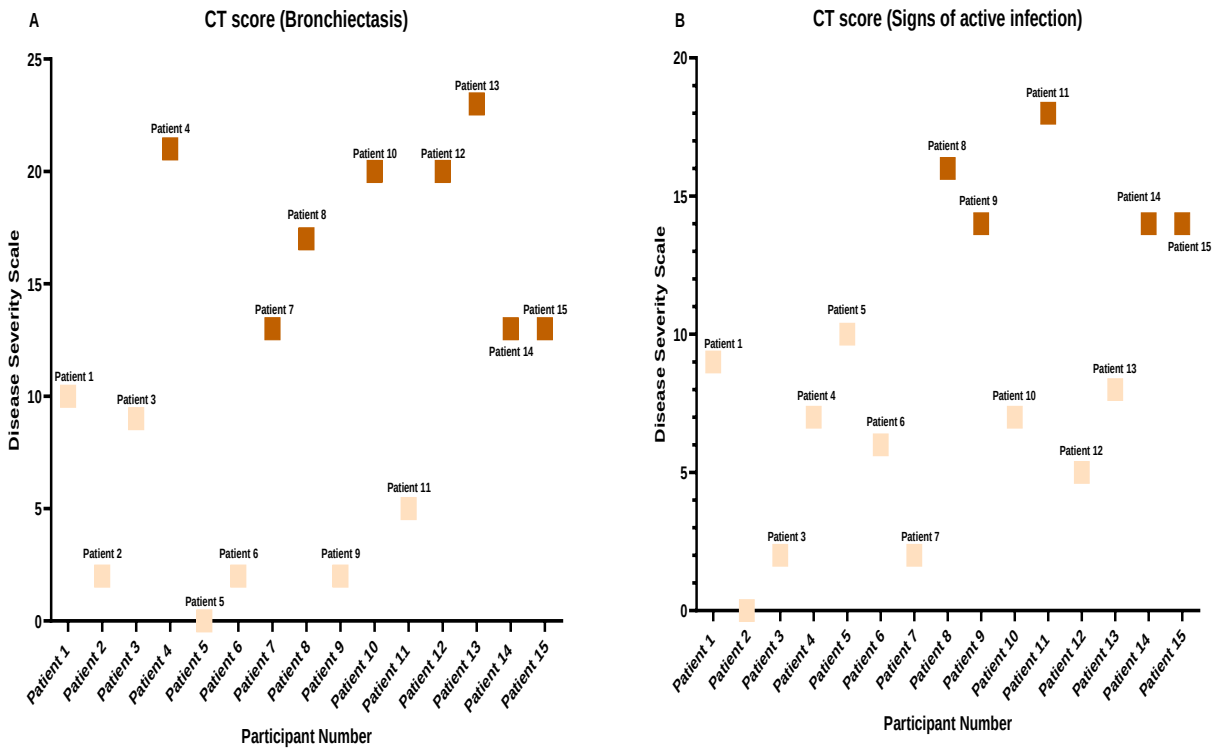


Figure 6. 4: Chest CT score of people with NTM-PD.
(A) Bronchiectasis scores, **(B)** Signs of active infection. The group was divided into two based on severity of disease, low severity cases (scores ≤10) and severe cases (score>10).

6.4.4 Relationship between clinical and biological outcome measures and NTM-PD severity based on bronchiectasis CT score

There was no clear relationship between bronchiectasis CT scores and the various questionnaires and tools such as NTM-PD symptoms, anxiety and depression assessments. CT scores indicating other signs of active lung infection also showed no correlation with bronchiectasis scores (Figure 6.5).

Significant correlations were observed between bronchiectasis CT scores and serum Anti-GPL core IgA, total IgG, NE, MMP-8/MMP-9, and S100A8/A9 ($r= 0.95$, $p<0.0001$), ($r= 0.74$, $p=0.002$), ($r= 0.91$, $p<0.0001$), ($r= 0.82$, $p=0.0001$), ($r= 0.83$, $p=0.0001$) respectively). HNP1-3 showed a positive correlation, although the p-value was not significant (Figure 6.6). While HNP1-3 and MMP-8/MMP-9 in sputum also exhibited a non-significant positive correlation with bronchiectasis CT scores (Figure 6.7).

Additionally, there were 9 patients with nodular bronchiectasis, 1 with fibrocavitary bronchiectasis, and 5 with mixed features. The patient with fibrocavitary bronchiectasis had the lowest Anti-GPL core IgA level.

There were generally weak correlations between the “active disease” chest CT scan scores and the questionnaire results or the levels of measured products in serum, sputum, and whole blood supernatant. A strong negative correlation was observed between the S100A8/A9 levels in supernatants of unchallenged whole blood and the active disease CT scores ($r=-0.89$, $p=0.006$).

Table 6.4 shows the distribution of values and the correlation between bronchiectasis CT scores and the signs of active disease CT scores with questionnaire data, while Table 6.5 provides a summary of the correlation between bronchiectasis CT scores and the signs of active disease CT scores with immunological markers.

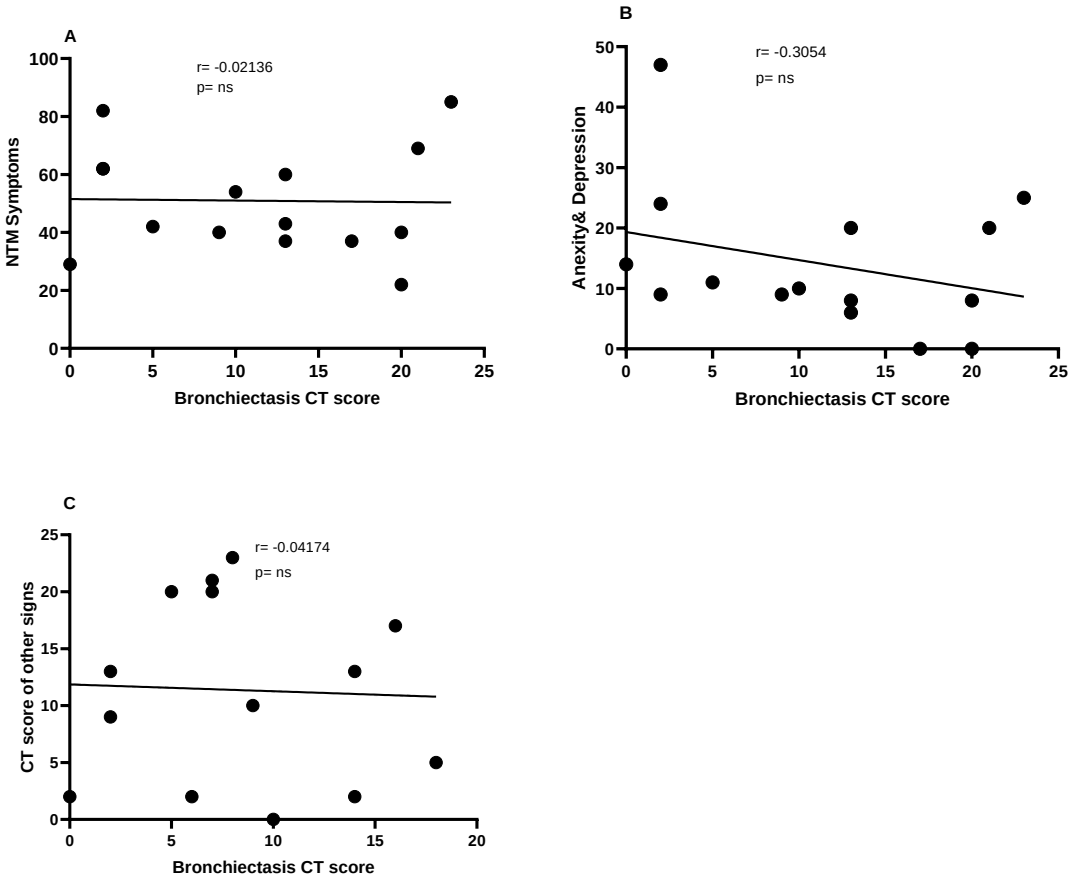


Figure 6. 5: The correlation between (A) NTM-PD symptoms (from questionnaire), **(B)** Anxiety and depression scores (from questionnaires) and **(C)** CT score of other signs of active infection with bronchiectasis CT scores.

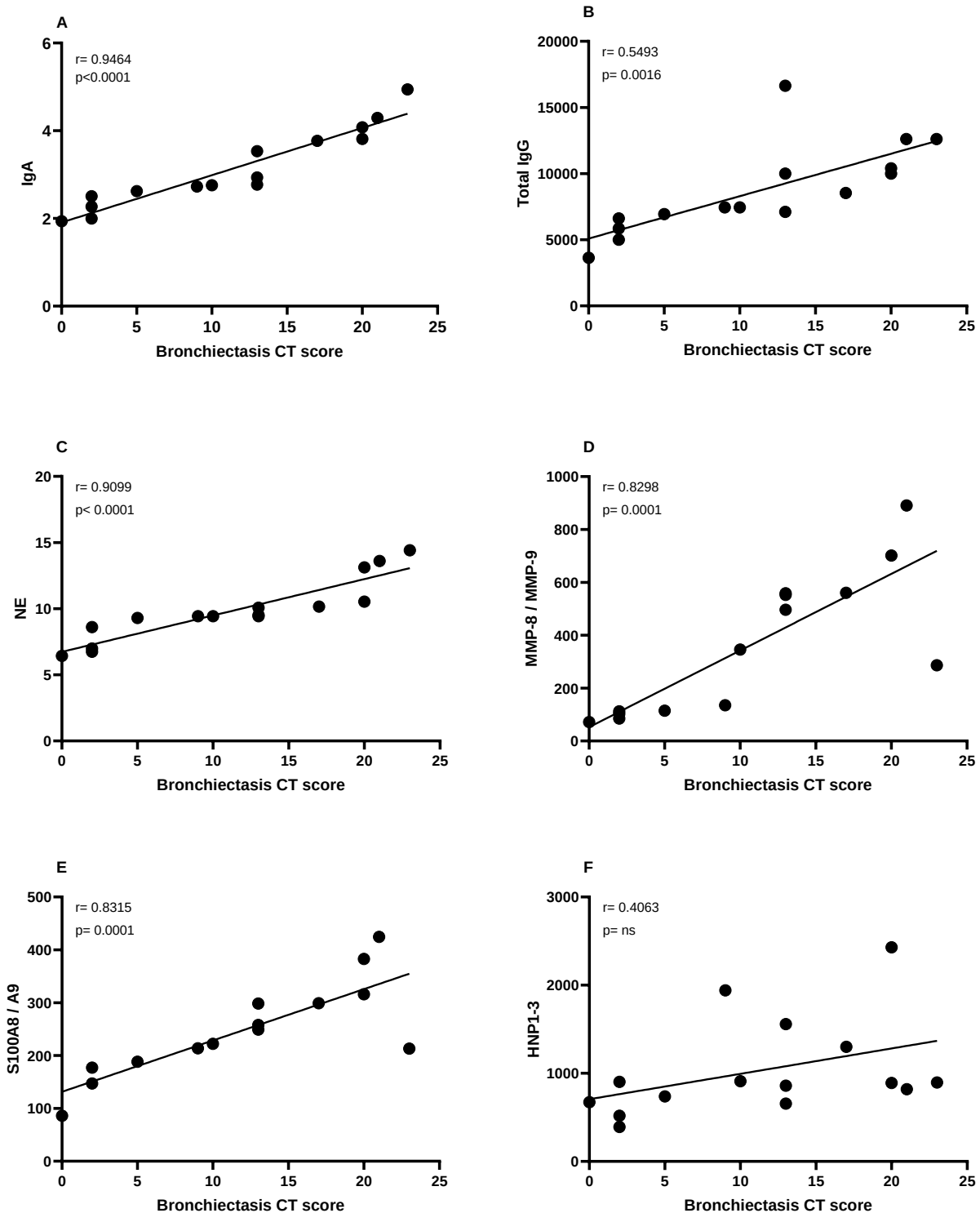


Figure 6. 6: The relationship between neutrophil-derived proteins and antibodies in serum with bronchiectasis CT scores: (A) Anti-GPL core IgA (U/ml), **(B)** Total IgG (mg/ml)**, (C)** NE (ng/ml)**, (D)** MMP-8/MMP-9 (pg/ml)**, (E)** S100A8/A9 (pg/ml)**, and (F)** HNP1-3 (pg/ml).

(Total IgG); Total immunoglobulin G, (NE); Neutrophil elastase, (MMP-8/MMP-9); Matrix metalloproteinase-8/9, (S100A8/A9); S100A8 and S100A9, proteins, (HNP1-3); Human neutrophil peptides 1-3.

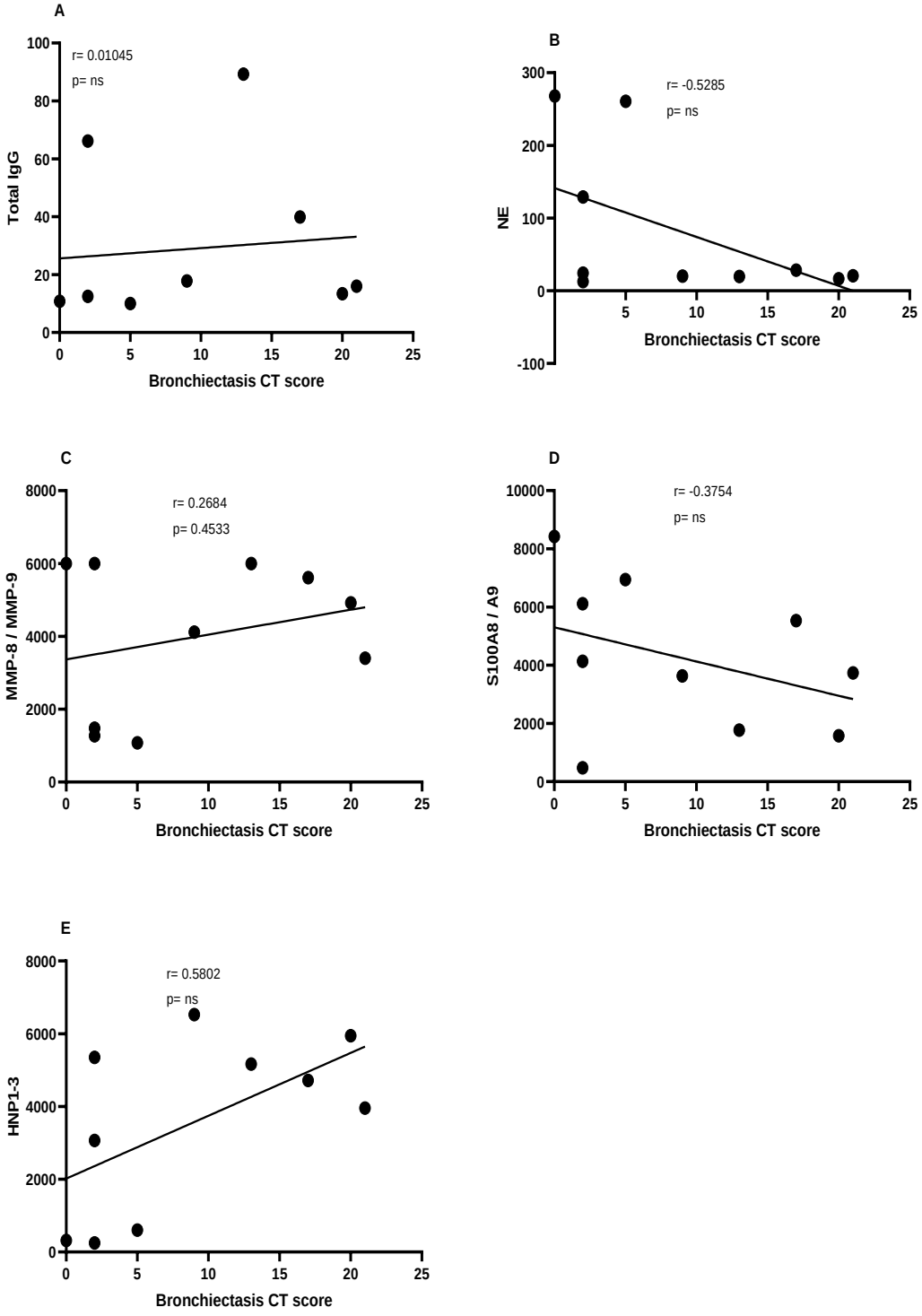


Figure 6. 7: The relationship between neutrophil-derived proteins and antibodies in sputum with bronchiectasis CT scores: (A) Total IgG (mg/ml)**, (B)** NE (ng/ml)**, (C)** MMP-8/MMP-9 (pg/ml)**, (D)** S100A8/A9 (pg/ml)**, and (E)** HNP1-3 (pg/ml).

(Total IgG); Total immunoglobulin G, (NE); Neutrophil elastase, (MMP-8/MMP-9); Matrix metalloproteinase-8/9, (S100A8/A9); S100A8 and S100A9, proteins, (HNP1-3); Human neutrophil peptides 1-3.

Table 6. 4: The comparative evaluation of chest CT scores in relation to various signs and symptoms using Pearson Correlations Coefficients

Signs and Symptoms	Bronchiectasis CT score		Signs of active disease CT score	
	r	P-value	r	P-value
NTM-PD symptoms	-0.0214	0.94	-0.37	0.18
Anxiety and Depression	-0.306	0.268	-0.26	0.35
Modified MRC Breathlessness Scale	-0.17	0.51	-0.48	0.07
COPD Assessment Test™ (CAT)	0.068	0.8	-0.14	0.13
EQ-5D-5L	0.01	0.79	-0.31	0.29
Health score	0.18	0.53	0.33	0.24
CT score Bronchiectasis	-	-	-0.04174	0.88
CT score of other signs of active disease	-0.04174	0.88	-	-

Table 6. 5: The comparative evaluation of CT scores in relation to various host immunological markers using Pearson Correlation Coefficients

Markers	Bronchiectasis CT score								Signs of active disease CT score							
	Serum samples (n=15)		Sputum samples (n=10)		Whole blood supernatant Unchallenged (n=7)		Whole blood supernatant challenged with *M. avium* (n=7)		Serum samples (n=15)		Sputum samples (n=10)		Whole blood supernatant Unchallenged (n=7)		Whole blood supernatant challenged with *M. avium* (n=7)	
	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value	r	P-value
Anti-GPL core IgA	0.9464	<0.0001	-	-	-	-	-	-	-0.15	0.59	-	-	-	-	-	-
Total IgG	0.7412	0.002	0.105	0.77	-	-	-	-	-0.11	0.69	-0.02	0.97	-	-	-	-
NE	0.9099	<0.0001	-0.53	0.12	-	-	-	-	-0.14	0.61	0.03	0.94	-	-	-	-
S100A8/A9	0.8315	0.0001	-0.375	0.29	0.028	0.95	0.498	0.256	-0.12	0.67	-0.13	0.73	-0.89	0.006	0.118	0.801
MMP-8/ MMP-9	0.8298	0.0001	0.268	0.45	0.596	0.16	0.532	0.376	-0.03	0.92	-0.15	0.7	0.037	0.93	0.002	0.99
HNP1-3	0.4063	0.13	0.58	0.08	0.427	0.34	-0.399	0.22	-0.16	0.58	-0.15	0.68	-0.67	0.102	-0.29	0.52

6.4.5 Transcriptomic analysis

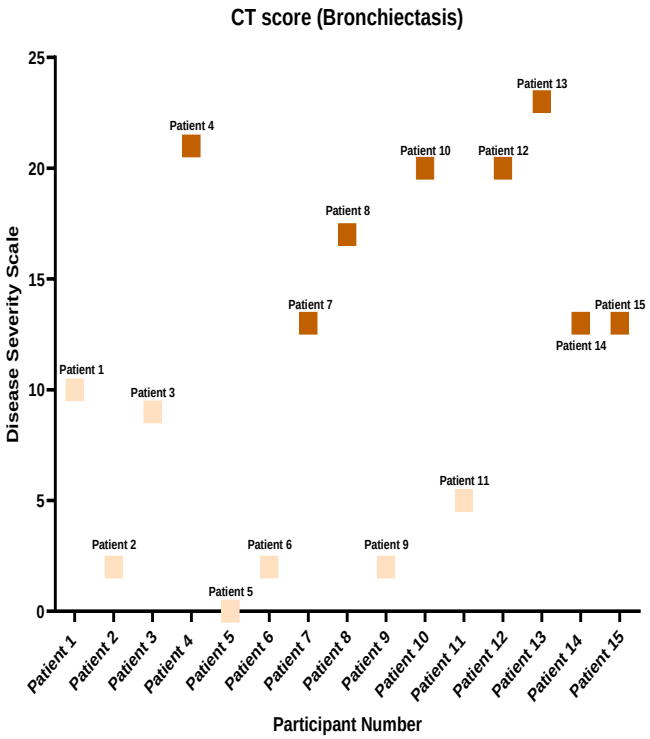
Study participant classifications are listed in Table 6.6; the 'high' score group consists of NTM-PD patients with the most severe disease as defined by bronchiectasis CT scores, while the 'low' score group consists of NTM-PD patients with the least severe disease. Group 1 is comprised of NTM-PD patients with high expression of neutrophil-related markers and serum immunoglobulins, while Group 2 is comprised of NTM-PD patients with low expression of neutrophil-related markers and serum immunoglobulins.

Interestingly, but consistent with the close correlation between these serum markers and the bronchiectasis CT scores (Figure 6.6), the groups were similar whether I used CT score or PCA of serum markers, other than Patient 15. Patient 15's PCA value was just below zero, and the CT score was at the median, which resulted in the patient being classified as 'low severity' by PCA of serum markers and 'high severity' using the CT score (Figure 6.8).

Table 6. 6: Participants classification according to bronchiectasis CT score and PCA classification on measurements of serum markers

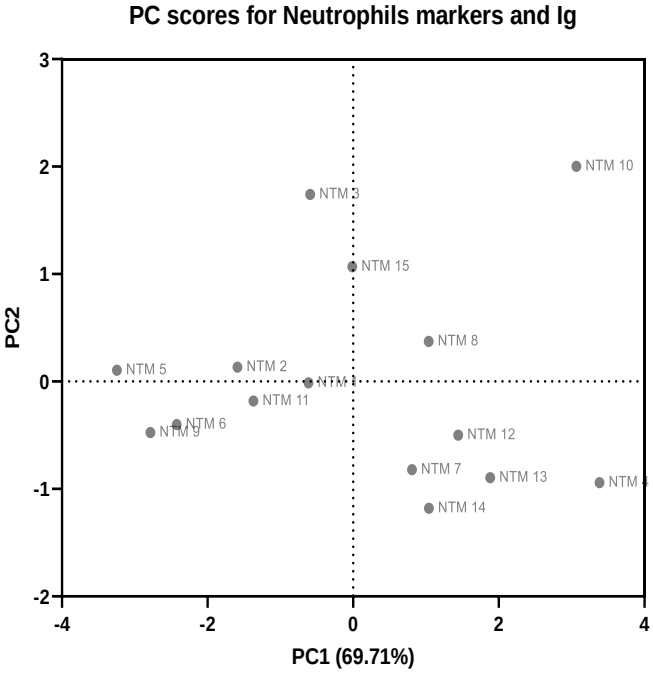
Participant number	Bronchiectasis CT score (Severity)	Neutrophil Phenotype Cluster
1	Low	group2
2	Low	group2
3	Low	group2
5	Low	group2
6	Low	group2
9	Low	group2
11	Low	group2
4	High	group1
7	High	group1
8	High	group1
10	High	group1
12	High	group1
13	High	group1
14	High	group1
15	High	group2

A



Participant number	Bronchiectasis CT score	
Patient 13	23	High
Patient 4	21	
Patient 12	20	
Patient 10	20	
Patient 8	17	
Patient 7	13	
Patient 14	13	
Patient 15	13	
Patient 1	9	Low
Patient 3	9	
Patient 11	5	
Patient 2	2	
Patient 6	2	
Patient 9	2	
Patient 5	0	

B



Participant number	
Patient 10	Group 1
Patient 8	
Patient 12	
Patient 7	
Patient 13	
Patient 14	
Patient 4	
Patient 3	Group 2
Patient 15	
Patient 2	
Patient 5	
Patient 1	
Patient 11	
Patient 6	
Patient 9	

Figure 6. 8: NTM-PD patient Grouping. (A) Participants were divided into two groups based on their Bronchiectasis CT scores: more severe ('High') cases (patients 4,7,8,10,12,13,14, and 15) and less severe ('Low') cases (patient 1,2,3,5,6,9, and 11). **(B)** Participants were divided into two groups based on the PCA scores calculated for neutrophil markers and immunoglobulins (Ig): Group 1 (patient 4,7,8,10,12,13, and 14) and Group 2 (patient 1,2,3,5,6,9, and 15).

6.4.5.1 Whole-blood transcriptomic signatures show gene upregulation in more severe NTM-PD (using either bronchiectasis CT scores or neutrophil-related marker criteria)

Patients in Group 1, with high concentrations of neutrophil-related products and serum immunoglobulins, and the group with high bronchiectasis CT scores, had upregulation of KAT8 Regulatory NSL Complex Subunit 1 (*KANSL1*) and Pleckstrin Homology And RUN Domain Containing M1 (*PLEKHM1*).

There was a 5.1-fold increase in *KANSL1* expression in patients with high CT scores compared to those with low CT scores ($p<0.0001$). Similarly, Group 1 patients with high Ig and neutrophil markers exhibited a 4.8-fold increase in *KANSL1* expression compared to patients with lower levels of Ig and neutrophil markers ($p<0.0001$).

Additionally, I found that patients with high CT scores expressed 3.5-fold more *PLEKHM1* than patients with low CT scores ($p=0.001$). The expression of *PLEKHM1* was also increased 3.6-fold in Group 1 patients with high Ig and neutrophil markers compared to those with lower levels ($p=0.0001$).

Immediate Early Response 3 (*IER3*) gene expression was significantly higher in Group 2 and the low CT score group compared to their respective comparator groups, with an approximate 23.8-fold increase ($p < 0.0001$) and 22.7-fold increase ($p < 0.0001$), respectively. Differentially expressed genes for both conditions are listed in Table 6.7 and Table 6.8.

6.4.5.2 NTM-PD patients with high bronchiectasis CT scan score exhibit upregulation of *HSP1L* expression.

Heat Shock Protein Family A (*HSP1L*) gene was significantly more expressed (approximately 22.3-fold, $p<0.0001$) in NTM-PD patients with high bronchiectasis CT scores.

6.4.5.3 Differentially expressed genes in NTM-PD patients according to Ig and neutrophil-related markers.

TBC1 Domain Family Member 3K (*TBC1D3K*) was significantly upregulated with a 23.7-fold change in Group 1 (NTM-PD patients with higher levels of Ig and neutrophils-markers) compared to Group 2.

1-Acylglycerol-3-Phosphate O-Acyltransferase 1 (*AGPAT1*) and Leukocyte Immunoglobulin Like Receptor B2 (*LILRB2*) were both observed to have higher expression in Group 1 patients (high level of Ig and neutrophils-markers) in comparison to Group 2 (lower levels of Ig and neutrophils-markers), the changes respectively being 7.6-fold (p=0.0003), and 2.3-fold (p=0.04).

On the other hand, Mastermind Like Transcriptional Coactivator 1 (*MAML1*) expression showed increased expression (3.9-fold) in NTM-PD patients Group 2, who have lower levels of Ig and neutrophils-related markers, in comparison with NTM-PD patients in Group 1 (Table 6.8).

Table 6. 7: Differentially expressed genes in NTM-PD patients according to the Bronchiectasis CT scan score. Using FDR <0.05.

Name	Full name	Symbol	Log_2 Fold Change	p-value	Upregulated/ Downregulated
ENSG00000234258	Heat Shock Protein Family A	HSP1L	22.297	1.02E-09	**Upregulated in High score group**
ENSG00000275867	KAT8 Regulatory NSL Complex Subunit 1	KANSL1	5.076	3.04E-06	**Upregulated in High score group**
ENSG00000277111	Pleckstrin Homology and RUN Domain Containing M1	PLEKHM1	3.464	0.001065	**Upregulated in High score group**
ENSG00000137331	Immediate Early Response 3	IER3	-22.904	3.30E-10	**Upregulated in Low score group**

High score group= NTM-PD patients with more severe disease as defined by Bronchiectasis CT score
Low score group= NTM-PD patients with less severe disease as defined by Bronchiectasis CT score

Table 6. 8: Differentially expressed genes in NTM-PD patients according to the variation of neutrophils marker expression between the patients. Using FDR <0.05

Name	Full name	Symbol	Log_2 Fold Change	p-value	Upregulated
ENSG00000275867	KAT8 Regulatory NSL Complex Subunit 1	KANSL1	4.781	2.27E-05	**Upregulated in Group 1**
ENSG00000277111	Pleckstrin Homology and RUN Domain Containing M1	PLEKHM1	3.586	2.69E-05	**Upregulated in Group 1**
ENSG00000275153	TBC1 Domain Family Member 3K	TBC1D3K	23.695	2.09E-11	**Upregulated in Group 1**
ENSG00000227642	Acylglycerol-3-Phosphate O-Acyltransferase 1	AGPAT1	7.612	0.000289	**Upregulated in Group 1**
ENSG00000131042	Leukocyte Immunoglobulin Like Receptor B2	LILRB2	2.899	0.04367	**Upregulated in Group 1**
ENSG00000137331	Immediate Early Response 3	IER3	23.779	2.09E-11	**Upregulated in Group 2**
ENSG00000161021	Mastermind Like Transcriptional Coactivator 1	MAML1	3.928	0.016042	**Upregulated in Group 2**

Group 1= NTM-PD patients with high expression of neutrophil-related products and serum Immunoglobulins
Group 2= NTM-PD patients with low expression of neutrophil-related products and serum Immunoglobulins

6.4.5.4 Whole blood transcriptomes reveal a diversity of NTM-PD patients, as explained by the clustering of patients based on their bronchiectasis CT score or neutrophil-related markers in PCA.

As shown in Figure 6.9, the PCA plot revealed that NTM-PD patients with low bronchiectasis CT scan scores tended to cluster together, with the exception of one sample (patient 3) that was located lower along the PC1 axis. This axis accounted for 15.2% of the total variability in the data, while the PC2 axis accounted for 11.9%. In contrast, NTM-PD patients with high bronchiectasis CT scan scores tended to overlap, but most of them were located further along PC1. Additionally, clustering was observed in PC1 vs PC3.

Using serum concentrations of Ig and neutrophil-related proteins, I plotted these for each NTM-PD patient on two axes, (Figure 6.10). Those in Group 1 (red) had high levels of Ig and neutrophil-related markers, while patients in Group 2 (blue) had lower levels. The patients overlapped mainly along the PC1 axis, particularly in PC1 vs PC2.

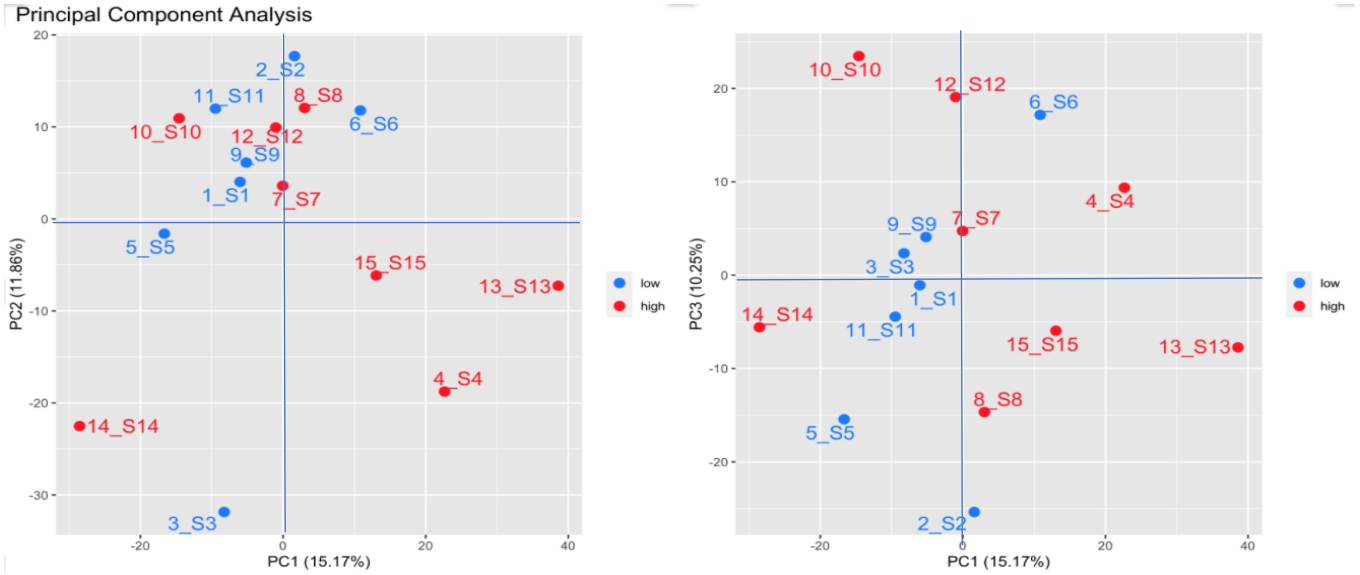


Figure 6. 9: PCA plots for NTM-PD patients coloured according to high bronchiectasis CT scores (red) and low bronchiectasis CT scores (blue).
The principal components (PC) with percentages of variance associated with each component are shown in the plots.

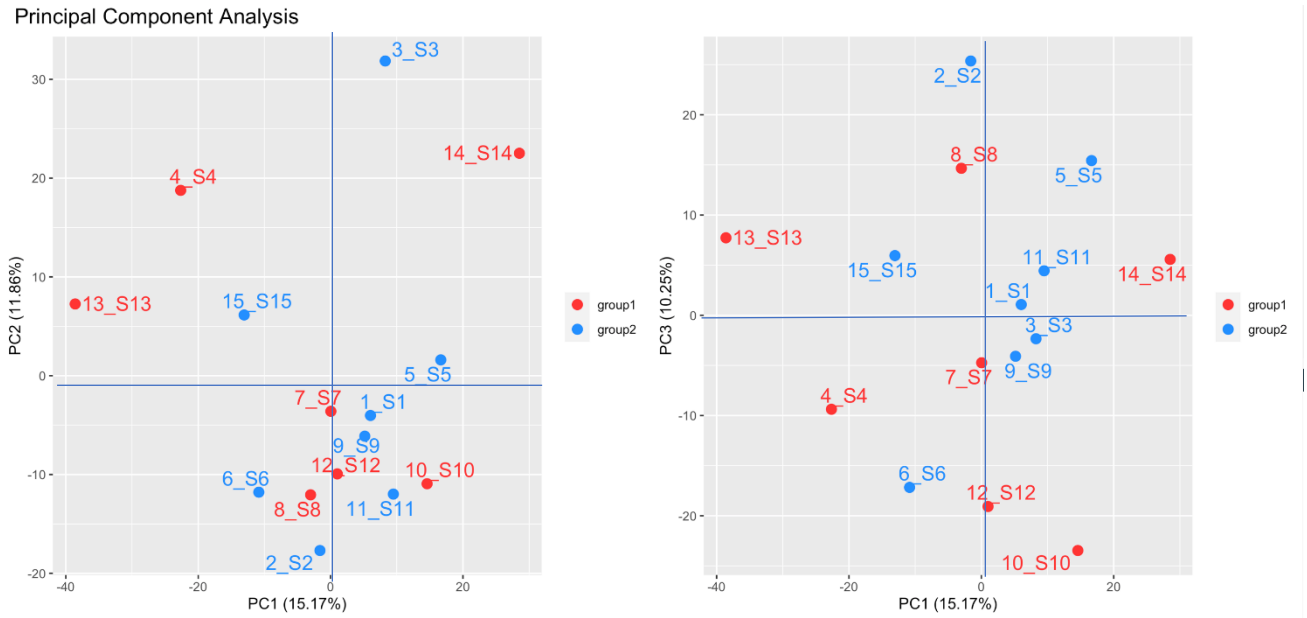


Figure 6. 10: PCA plots for NTM-PD patients coloured according to higher levels of Ig and neutrophils markers 'Group1' (red) and lower levels of Ig and neutrophils markers 'Group2' (blue).
The principal components (PC) with percentages of variance associated with each component are shown in the plots.

6.5 Discussion and conclusion

This chapter focuses on the relationship between various laboratory markers and questionnaire assessment tools and the severity of NTM-PD, as determined by chest CT scan scores.

Although previous work has suggested that there may be differences in the clinical characteristics of NTM-PD based on sex and age (282), I found no obvious associations between the severity of disease and participants' age or gender.

NTM-PD has a multi-dimensional impact on patients' health and well-being. This includes anxiety and depression, which in turn is related to patient outcomes (280).

Common symptoms include food and weight issues, sensory abnormalities, mouth and body temperature changes, and persistent discomfort. However, my study found no correlation between the severity of NTM-PD disease (when defined radiologically) and the symptoms reported by participants using validated NTM-PD symptoms, breathlessness, quality of life and depression or anxiety questionnaires.

This lack of association might result from the complex nature of the condition. Despite utilizing validated questionnaires assessing respiratory symptoms and general health, I found no strong relationship between the radiologically-defined severity of NTM-PD and patient-reported symptoms. This somewhat unexpected finding may be attributed to the inherent limitations of the selected questionnaires, which might lack the sensitivity needed for subtle variations in NTM-PD symptoms. Moreover, the relatively small sample size in our study could have influenced our ability to detect this signal. The described limitations underscore the complex nature of NTM-PD and highlight the challenges in capturing its diverse clinical manifestations through currently available assessment tools.

The timing of symptom assessments and the fluctuating nature of NTM-PD symptoms can also impact on the relationship between severity and symptoms (283, 284). The presentation of anxiety and

depression varies in severity and duration, and subjective factors such as personal perceptions, coping mechanisms, as well as psychosocial issues like fear and social isolation can influence symptom perception independent of disease severity. However, a prospective study reported that anxiety and depression were associated with NTM-PD patients' reported physical symptoms. Though, despite those people with anxiety or depression requiring more frequent antibiotic treatment, its effectiveness was unrelated to the initial severity of a patient's anxiety or depression (281).

Several biomarkers have been evaluated as NTM-PD diagnostic tools and indicators of disease activity. However, none are robust enough yet to support adoption within clinical practice (285).

I found a positive and significant correlation between the serum level of IgG in NTM-PD and the radiographic score of bronchiectasis. However, there was no correlation between the sputum levels of total IgG and the bronchiectasis radiographic scores. Measuring IgG levels can provide insights into the host's immune response and inflammatory processes related to NTM infection. This can help us understand host mechanisms for NTM control.

Anti-GPL-core IgA antibody has been considered to be a useful diagnostic indicator of MAC-LD (255). However, anti-GPL-core IgA levels do not differ between SGM and RGM, and the marker could not distinguish between MAC-PD and MAB-PD (251, 253).

It has been proposed that the antibody level may reflect MAC-LD progression, correlating with *Mycobacterium avium* complex lung disease chest CT findings (286). In line with this, I found that anti-GPL-core IgA serum levels were positively correlated with the radiological bronchiectasis score. This is also consistent with a previous study conducted by Kitada and colleagues using the commercial Anti-GPL IgA kit, who found that patients with MAC-PD had elevated levels of antibodies compared to Mtb patients. Further, those with more extensive disease on chest CT had higher antibody levels, regardless of whether they had nodular bronchiectatic or fibrocavitary disease (287). This raises the question of whether this blood antibody may have value as a marker of disease activity.

My work supports this concept, in that I found the participant with the highest bronchiectasis radiological score, which was positively related to Anti-GPL IgA serum levels, was treated shortly after recruitment. This has also been reported by Matsuda et al, where a positive anti-GPL-core IgA antibody result in the presence of cavitary lesions was related to disease progression requiring treatment, whilst patients negative for anti-GPL-core IgA antibody had a lower disease severity based on the radiological score (288).

The GPL core antibody levels in patients with the most common radiologic types of NTM-PD (nodular-bronchiectatic) are reported to be significantly higher than in fibrocavitary disease (142). Interestingly, I observed that patients with fibrocavitary disease had the lowest IgA levels. However, it is challenging to draw meaningful conclusions or make statistical comparisons due to the small sample size (9 patients with nodular bronchiectasis, 1 with fibrocavitary, and 5 with a mixed picture).

Unexpectedly, IgA antibodies against GPL-core were detected in 3 NTM-PD patients challenged with *M. kansasii*, even though *M. kansasii* itself lacks GPL-core. The presence of positive serum IgA antibody against GPL-core could be explained by undetected mixed infections, a phenomenon reported in previous studies (289). This raises the concern that latent subclinical MAC infections could potentially lead to false-positive results. It is plausible to consider that patients with NTM-PD might not be exclusively challenged by a single NTM species (in the case described above, not just *M. kansasii*, but also possibly *M. avium* or *M. abscessus*). Moreover, given the complexity of immune responses in NTM-PD and the potential cross-reactivity of antibodies, further investigation is required to understand the reasons for these unexpected IgA antibody results. Considering the duplicate testing and the small sample size of 15 patients, it is less likely that these results are due to artifact.

The serum levels of neutrophil elastase were positively and significantly correlated with the radiographic score of bronchiectasis. This suggests that serum elastase may have clinical value as an indicator of NTM disease severity and associated bronchiectasis.

It has been found that NE levels in sputum are potentially valuable as a biomarker for the classification of disease severity, prediction of exacerbations, and determination of long-term clinical outcomes in bronchiectasis (263). Contrary to this, in my study sputum elastase did not correlate with disease severity using the bronchiectasis CT scores. Possible explanations include the relatively small sample size and the variation in quality and quantity of sputum I obtained from participants.

Although there is still a need for interventional studies to evaluate whether elastase reduction can be used as a surrogate of efficacy in clinical trials, Chalmers, et al. reported elastase to be a marker of disease progression in bronchiectasis that may be useful in addition to clinical assessment (236).

S100A8/A9 and MMP-8 and -9 are highly abundant proinflammatory proteins released by neutrophils. The association between serum levels of calprotectin (S100A8/A9) and inflammation has been noted previously (290). The presence of the S100A8/A9 complex is a reliable indicator of some inflammatory conditions including chronic inflammatory lung disease (291) and also attracts more neutrophils to the lungs (241). In my study, serum S100A8/A9 levels were significantly positively correlated with the CT bronchiectasis scores. However, as for elastase, the same relationship was not seen with sputum or blood supernatant S100A8/A9 concentration. The sputum and supernatant samples are likely to be subject to more variability due to patient and experimental factors and fewer participants. Further evaluation is needed to comprehensively assess the existing findings, but the strong correlation between serum levels and CT bronchiectasis scores is interesting.

MMP-8 and MMP-9 genes are highly expressed in PBMCs and granulocytes during viral respiratory infections, and in this setting have been proposed as possible indicators of disease severity (292). Furthermore, data from Tezera et al demonstrate that neutrophil-derived MMP-8 plays a significant role in the immunopathology of Mtb, and targeting this to decrease tissue destruction may improve outcomes in TB and other inflammatory disorders (293). Bronchiectasis, which is largely neutrophilic in terms of pathophysiology, is also characterised by upregulation of MMP-8 and MMP-9. As with the

other neutrophil-associated proteins, I observed a strong correlation between the serum MMP8/MMP9 concentration and the CT bronchiectasis score. This finding suggests the need to understand the role of MMP8 and MMP9 in the pathogenesis of bronchiectasis in NTM-PD and may have clinical implications, such as the development of diagnostic or prognostic markers for disease severity.

Several previous studies have reported a relationship between the activity and severity of inflammatory diseases and alpha-defensin levels: for example PMN function is inhibited by high concentrations of HNP in bronchiectatic airways (294). However, I found that serum and sputum levels of HNP1-3 were only weakly positively correlated with the radiographic bronchiectasis score.

The differences reported between my results and others may be due to a number of factors, including the study methodology employed as well as the samples analysed. I chose to look at both blood and lung derived samples, acknowledging that serum and sputum samples lack the cellular interactions and signalling networks found in whole blood supernatants. This might explain why a negative correlation between S100A8/A9 and CT scores of active disease was only observed in unchallenged whole blood supernatant.

This also highlights the importance of sample size, as for logistical reasons my experiments on whole blood supernatants included only small numbers of participants (often 7) – meaning that some statistical results need to be interpreted with care, in particular when multiple comparisons were performed.

In general, I saw worse correlations between laboratory-measured parameters (including neutrophil elastase concentration, MMP-8/9, S100A8/A9, and HNP1-3), and the CT chest scores that indicate 'active disease' (as opposed to the bronchiectasis scores). This could be because neutrophils are more directly involved in bronchiectasis development. Collectively, the correlations between neutrophil-

related products and the bronchiectasis score were strong and consistent across different but related serum markers.

The analysis finds there to be a robust correlation between CT scan-derived bronchiectasis severity in NTM-PD patients and elevated neutrophil markers, particularly NE, supporting the concept of neutrophil involvement in disease pathology. However, it's noteworthy that this may also reflect the underlying bronchiectasis process (that is independent of NTM infection). Results such as this highlight the complex interplay between NTM-PD and bronchiectasis. Future prospective longitudinal studies with larger cohorts, including bronchiectasis patients with and also without NTM-PD as well as appropriate comparator groups are needed if we are to better understand these relationships. Such investigations would provide deeper insights into the specific contributions of neutrophil-associated inflammation to NTM infection, bronchiectasis and disease progression.

I identified 8 genes that were differentially expressed in NTM-PD patients depending on their bronchiectasis CT scan scores or the levels of serum Ig and neutrophil-related markers: *HSP1L*, *KANSL1, PLEKHM1*, *IER3, TBC1D3K, AGPAT1, LILRB2,* and *MAML1*. Two genes out of 8 were commonly upregulated in both states (high bronchiectasis CT score and high neutrophil-related markers/immunoglobulins), including *KANSL1 and PLEKHM1*, while *IER3* was upregulated in those with low bronchiectasis CT score and low neutrophil-related markers.

PLEKHM1 controls autophagosome-lysosome fusion through the proteins GTPase and Rab7. Several intracellular bacteria, such as *Coxiella burnetti* and *Helicobacter pylori*, are targeted by Rab7 (295), while Mtb (296) actively blocks its action.

It has been suggested that when certain proteins like Rab7 are excluded, the mycobacterial phagosome may be prevented from fusing with the lysosomal compartment (296). This could have significant consequences for microbial infection, tissue inflammation, and the onset of disease.

KANSL1 is a crucial regulator of various genes that are important for the development and function of diverse body systems according to the data available in the BioGPS database (297).

IER3 gene, on the other hand, provides protection against TNF-α induced apoptosis (298). Consistent with this, the current study found higher expression of IER3 in patients with low bronchiectasis CT scores compared to those with high CT scores.

Modulation of LILR activity is believed to be associated with the inflammatory response triggered by bacterial infection. LILRB2 is part of a family of receptors that regulate immune responses and inflammation. There are two types of LILRs: inhibitory LILRs that help reduce inflammation and activating LILRs that trigger immune reactions against infections. However, when activating LILRs are overly active, they can lead to excessive immune responses and persistent inflammation, which can have an impact on disease outcomes. (299). LILRB2 in neutrophils rapidly moves to the cell surface when neutrophils are stimulated, and upregulated LILRB2 enhances the inhibitory signals of HLA-G, leading to suppression of neutrophil degranulation and phagocytosis (300, 301). This may explain the upregulation of LILRB2 in NTM-PD patients who have high levels of neutrophil-related markers.

The intracellular heat shock protein 70 (HSP70) is encoded by HSPA1L gene (302). A pro-inflammatory response to foreign particles, including mycobacteria, is induced by Hsp70 released from exosomes (303). HSPs protect proteins from stress factors such as temperature, pH, and low levels of oxygen, and are clinically significant by facilitating protein folding and enhancing cell survival. Anand et al observed upregulation of Hsp70 that was significantly released through exosomes into the extracellular media in response to mycobacterial infection (303). Consistent with this, my study has identified upregulation of the HSPA1L gene among the NTM-PD patients with high bronchiectasis CT scan scores. One possible explanation for this is that it represents the body's attempt to protect itself from further injury.

TBC1D3K is predicted to be involved in activation of GTPase activity and intracellular protein transport. The TBC1D3 family gene has been proposed as a prognostic biomarker for Kidney renal clear cell carcinoma (*KIRC*) (304). I found the gene to be upregulated in NTM-PD patients with high serum Ig and neutrophil-related products. This contrasts with a study by Prieto who found higher TBC1D3H expression in CF patients who didn't develop NTM-PD (n=30) compared to those who did (n=12) (305).

Cowman and colleagues suggested that altered immune responses may contribute to both the development and severity of pulmonary nontuberculous mycobacterial disease. They also identified genes that could be potential markers for disease activity and therapeutic intervention (76).

It is worth noting that no common genes were found in my study and the differentially expressed genes between the NTM-PD cases and the control subjects in Cowman's study. This suggests that the differentially expressed genes identified in this study may be specific to NTM-PD and could potentially be used to predict the severity of disease.

Overall, my study provides novel insights into the genes that are expressed differently in adult patients with NTM-PD that could be correlated to the severity of disease depending on radiological or immunological markers. This exploratory cohort's findings could be used as a validation dataset and assist in progress to identifying future biomarkers of different health states. It would be important to include a larger, more diverse group of NTM-PD patients; whilst applying strict inclusion criteria could help identify genes specifically related to bronchiectasis in patients with NTM-PD and distinguish them from bronchiectasis in people without NTM-PD.

My study has some limitations that could affect the accuracy and generalizability of its findings. The research was conducted in a single facility, and the number of recruited participants was quite small (n=15). Hence, results needed to be interpreted carefully in the context of multiple comparisons as the potential exists for both false-positive and false-negative results plus an over/under-estimation of the magnitude of associations. While the transcriptomic analysis showed promising results, there are

several issues that need to be acknowledged. Again, the sample size was small, and I did not include comparator groups (with or without other lung diseases). Additionally, some of the apparently differentially expressed genes were only observed in a subset of patients, while others did not express the gene at all. Lastly, the time between the onset of NTM-PD disease in each case and the start of this study could have an impact on the findings. To address these limitations, future studies should consider conducting large, multi-centre prospective studies involving individuals at risk or with NTM-PD who have regular assessments and systematic sampling over time. Through this we will build a rich dataset that can provide insights into risk and protection from mycobacterial disease progression.

Conclusion

- In the future, monitoring the progression of NTM-PD could utilise a panel of blood markers as well (and possibly instead of) serial, regular chest CT scans. However, additional research employing prospective longitudinal designs and diverse cohorts is essential.
- Neutrophil-associated markers in blood are promising candidates, giving clinicians a novel additional option for diagnosing and managing people with NTM-. The strong correlations between neutrophil proteins and chest CT severity scores also supports the concept that neutrophils or their products are valid targets for host-directed treatment. It should be noted though that total IgG and NE may lack specificity and could be elevated in other respiratory conditions: highlighting the need for continued research to refine their utility and specificity in clinical practice.
- Whole blood RNA transcriptomic signatures identified in people with NTM-PD could be useful as markers for predicting disease severity. Further validation through longitudinal studies involving large cohorts is recommended.

Chapter 7: Conclusions and Future Direction

7.1 The role of neutrophils in NTM

This thesis has investigated the potential roles of neutrophils in the response to mycobacterial infection (NTM) in three adult groups: people with CVID/XLA, people with NTM-PD and a healthy comparator population.

I employed flow cytometry-based WBA to assess neutrophil activation and phagocytosis. Confocal microscopy confirmed these findings. I also evaluated mycobacterial restriction in whole blood samples using MGIT cultures. Opsonophagocytosis was analysed in serum and sputum samples using isolated neutrophils from healthy donors. Additionally, I measured neutrophil-associated markers and proteins in serum, sputum, and whole blood supernatant. Lastly, I assessed the health status and radiological severity of 15 NTM-PD patients.

First, neutrophils from people with CVID/XLA or NTM-PD appear to differ in certain important characteristics when compared to a healthy comparator population (Chapter4); my results demonstrated that neutrophils in patient groups phagocytose NTM similarly but showed greater activation and immaturity, as indicated by higher expression of CD66b and consistent reduction in CD15 expression. However, the shedding of CD62L during mycobacterial challenge occurred to the same extent in all groups. Additionally, the study did not find any differences in the ability of whole blood obtained from all study subjects to restrict the slow-growing *M. avium* and the rapid-growing *M. abscessus'* growth, even in CVID/XLA patients using macrolide antibiotics. Confocal microscopy confirmed that isolated neutrophils from healthy donors internalized *M. abscessus* more than *M. avium*.

One of the approaches tested in this thesis was the opsonisation capacity of serum or sputum samples in patient groups to mediate internalisation into neutrophils: CVID/XLA patients exhibited better serum opsonic capacity compared to NTM-PD patients and healthy donors which may reflect the use of therapeutic immunoglobulin in the CVID/XLA group (Chapter 4).

I measured humoral immune response indicators, neutrophil-related products, in addition to the expression of cytokines, chemokines, and growth factors in response to NTM challenge. My findings suggested that *M. avium* and *M. abscessus* induce the release of specific cytokines that could drive further infection.

Healthy comparators had low levels of IFN-γ expression without challenge, this increased significantly following exposure to mycobacteria. In contrast both groups of patients had high levels of IFN-γ secretion at 96 hours in the absence of mycobacteria, which did not increase to the same extent following challenge. Moreover, IL-10 expression in both patient group's samples without challenge was higher compared to the healthy comparator group, although following challenge this was similar in all groups (Chapter 5).

Additionally, I found that NTM-PD patients had detectable levels of IgA antibodies against mycobacteria GPL-core, whereas CVID/XLA patients and healthy controls had concentrations that were considered negative. Furthermore, serum total IgG levels were higher in NTM-PD patients than in healthy comparators, with CVID/XLA patients' concentrations being the highest. This may arise from the use of therapeutic immunoglobulin by all CVID/XLA patients within the study – though it would be difficult to find a CVID/XLA population not on treatment with immunoglobulin or long-term preventive antibacterials (Chapter 5).

Neutrophils and their related products and proteins may be even more important in predicting the severity of NTM-PD, particularly in patients challenged with SGM. I found distinct patterns of neutrophil biomarker expression in NTM-PD (Chapter 5).

Patients with NTM-PD have elevated levels of collagenase-2 (MMP-8) and S100A8/A9, as well as increased NE serum concentrations, but no notable difference of HNP1-3 levels in comparison with other groups in serum, sputum, and whole blood supernatant, It's important to note that sputum samples were exclusively collected from the patient groups (CVID/XLA and NTM-PD), while serum and whole blood supernatant samples were obtained from all groups.

Overall, my findings demonstrated that CVID/XLA patients have significant inflammation indicated by high concentrations of inflammatory cytokines, but do not have the same level of neutrophilic inflammation as NTM patients. Possible explanations for this include the immunomodulatory effects of IVIG or the absence of active lung infections in these patients as they are also receiving additional macrolide prophylaxis or other antibiotics as bacterial preventative therapy (Chapter 5).

However, the data currently do not fully support the use of neutrophil markers for monitoring the disease, and further research, especially longitudinal studies, is needed.

I proceeded to investigate whether the measurement of neutrophil products may help guide NTM-PD management. I found that radiological scores of bronchiectasis positively and strongly correlated with neutrophilic inflammatory markers in serum such as NE, S100A8/A9, MMP-8/MMP-9, and HNP1-3 as well as indicators of humoral immune response (IgG and anti-GPL-core IgA antibodies) (Chapter 6). However, no correlations were observed between these markers and NTM-PD symptoms, anxiety and depression, or demographic data.

I then examined the host transcriptomic response in patients with NTM-PD to NTM infection. This has the potential to provide valuable insights regarding the genes that are differentially expressed in patients with NTM-PD, and which may be correlated with disease severity based on radiological or immunological markers. After correction for the false discovery rate, I identified 8 genes (*HSP1L*, *KANSL1*, *PLEKHM1*, *IER3*, *TBC1D3K*, *AGPAT1*, *LILRB2*, and *MAML1*) that are significantly upregulated depending on the bronchiectasis CT scan scores or the levels of serum Ig and neutrophil-related

markers. Additionally, two of these genes (*KANSL1* and *PLEKHM1*) were commonly upregulated in both states (i.e., high bronchiectasis CT score and high neutrophil-related markers and Ig). *IER3* was upregulated in those with low bronchiectasis CT score and low neutrophil-related markers and Ig (Chapter 6).

PLEKHM1 and *KANSL1* showed upregulation in patients with elevated neutrophil-related markers and high bronchiectasis CT scores. This aligns with PLEKHM1's role in bolstering control over autophagosome-lysosome fusion and KANSL1's involvement in gene regulation through chromatin remodelling. Furthermore, *HSPA1L*, responsible for *Hsp70*, exhibited upregulation, which corresponds to its activation in response to mycobacterial infection, indicative of a pro-inflammatory response. *IER3*, associated with apoptosis protection, demonstrated higher expression in patients with low bronchiectasis CT scores, suggesting a potential link to milder disease. *LILRB2*, implicated in inhibitory signals, possibly explains the suppression of neutrophil degranulation and phagocytosis, especially in patients with high neutrophil-related markers. Lastly, *TBC1D3K*, upregulated in patients with high serum Ig and neutrophil-related products, may have distinct roles in these individuals.

Overall, clinical decisions for monitoring the progression of NTM-PD could depend on a variety of blood markers instead of frequent CT scans, which would be less invasive and more cost-effective. This suggestion is based on the observed associations between blood markers, such as neutrophil-related products and gene expression, with bronchiectasis CT scores, indicating that these markers may serve as indicators of disease severity. However, further research and validation are necessary to establish these blood markers as reliable indicators of disease progression and even treatment response, which could ultimately lead to more efficient monitoring and treatment strategies for NTM-PD. It would also have been intriguing to evaluate the fluctuations over time in NTM patients, both those receiving treatment and those without.

7.2 Potential therapeutic and clinical application

What are the implications of my findings? The better serum opsonisation capacity (with or without heat inactivation) in CVID/XLA compared to NTM-PD patients and healthy comparators may indicate that using therapeutic immunoglobulins, such as nebulized Ig or giving IVIG, could have an immunomodulatory effect that helps prevent or control mycobacterial infections.

Nevertheless, it's important to acknowledge that this remains a theoretical concept waiting to be further tested rather than something backed up with robust clinical and laboratory evidence. On other hand, I found a potential link between elevated Ig levels (specifically IgA against GPL-core and total IgG) and the severity of NTM-PD, which is consistent with a study by Schnell, et al. Their study on airway inflammation reported that simply replacing IgG systemically to increase levels in the respiratory tract is not enough to prevent lung disease.

In fact, higher levels of IgG detected in the sputum were associated with more inflammation and dysbiosis (306). This raises the question of whether mycobacteria are effectively internalized and phagocytosed by neutrophils, or if they can instead survive within the phagocytes?

These findings highlight the complex nature of antibodies, as they can both defend against infections and potentially contribute to the severity of disease.

This study has also identified new neutrophilic inflammation markers that are associated with a bronchiectasis CT score, which may be useful in predicting disease severity, monitoring disease progression, and determining the need for treatment. In order to establish the predictive and monitoring capabilities of these neutrophilic inflammation markers, further work is required. This could include expanding the study to encompass a larger and more diverse patient cohort, as well as considering longitudinal studies conducted across multiple centres. Also, the development of an experimental model where these markers could be inhibited and then the subsequent impact on disease progression followed would be an important and necessary step.

Additionally, RNA transcriptomic analysis has identified some new candidate genes, such as *KANSL1*, *PLEKHM1*, and *IER3* which are associated with bronchiectasis severity and/or the levels of neutrophil products and could be investigated further in relation to NTM-PD severity.

These findings also suggest that targeting neutrophils or their products may be effective as adjuvant therapy to stop lung damage, alongside antibiotics or used by themselves in people with NTM-PD unable to tolerate antimicrobials. The results add to a number of current proposed anti-neutrophil treatments for airway disorders (Table 7.1 – and also discussed in more detail in my review article, Appendix 16). However, further longitudinal studies should involve in-depth in *vitro* and in *vivo* work to explore in detail the underlying mechanisms and evaluate their clinical relevance, with a focus on larger patient cohorts and multi-centre investigations to gain more comprehensive insights into the molecular mechanisms of NTM-PD and how we might improve its clinical management.

In summary, I identified neutrophil markers, IVIG treatment and gene expression profiles that could be targeted in future diagnostic or therapeutic approaches. To determine their value in clinical practice requires both further basic science work to identify potential mechanisms of action and hypothesis-driven clinical studies that can help determine the value of specific treatments and biomarkers that have as their focus the neutrophil and its products.

Table 7. 1: Therapies directly targeting neutrophils currently being assessed in human chronic airway diseases.

Drug	Target	Indication	Mechanism of action	Reference
AZD9668	NE	COPD CF	Selective NE inhibitor that elevates FEV1 and reduces inflammatory biomarkers (IL-6 and IL-8).	(237, 266)
BAY 85-8501		Bronchiectasis	Selective NE inhibitor that suppresses inflammation	(307, 308)
MK-7123	CXCR2	COPD	Reduces neutrophil chemotaxis and airway inflammation using a cytokine receptor CXCR2 antagonist (MK-7123)	(309)
AZD7986	DPP1	COPD	Blocks protease activation (reduces NE activity in the blood) via DPP1 inhibition.	(310)
AZD1236	MMP	COPD	MMP-9, -12 inhibitor	(311)
GSK2269557	PI3K	COPD/Asthma	Suppression of IL-8 and IL-6 levels in sputum, airway anti-inflammatory activity	(312)
GSK2292767		Asthma	Inhibits neutrophil migration and degranulation	(313)

7.3 Future work and recommendation

My current work investigating the roles of neutrophils in NTM-PD has several recommendations for future work, summarised in Figure 7.1. One of the most important areas to explore is the relationship between neutrophil markers and CT scores in patients with NTM-PD.

Future research will provide valuable insights into the potential use of neutrophils as biomarkers for disease severity and progression. Additionally, conducting preclinical and clinical trials to evaluate the efficacy of neutrophil-targeting therapies in the treatment of NTM-PD is another important area of research. Moreover, exploring the transcriptomic profile of neutrophils in NTM-PD patients and identifying key genes and pathways associated with disease progression and response to treatment is another important area.

On the other hand, understanding the mechanism of how CVID/XLA patients, who receive Ig therapy and prophylactic antibiotics, can prevent or control NTM infections, will provide valuable insights into the effectiveness of these preventive measures.

Investigating how IVIG replacement therapy might improve opsonophagocytosis in patients is also important, including *in vitro* experiments to understand the interactions between IVIG and neutrophils and how this enhances the immune response against mycobacterial infections. By focusing on this, we can further explore the potential therapeutic value of immunoglobulin therapy in people with NTM-PD who are not obviously immunoglobulin deficient, and so determine if there is enough experimental evidence to develop a clinical trial around this.

Finally, by investigating the impact of modulating neutrophil activity on disease outcomes, including local and systemic inflammation, bacterial clearance, and patient quality of life, we may be able to develop safe host-directed therapies that improve the treatment options for NTM-PD patients who are currently poorly-served by our pharmacological approaches to management of their chronic illness.

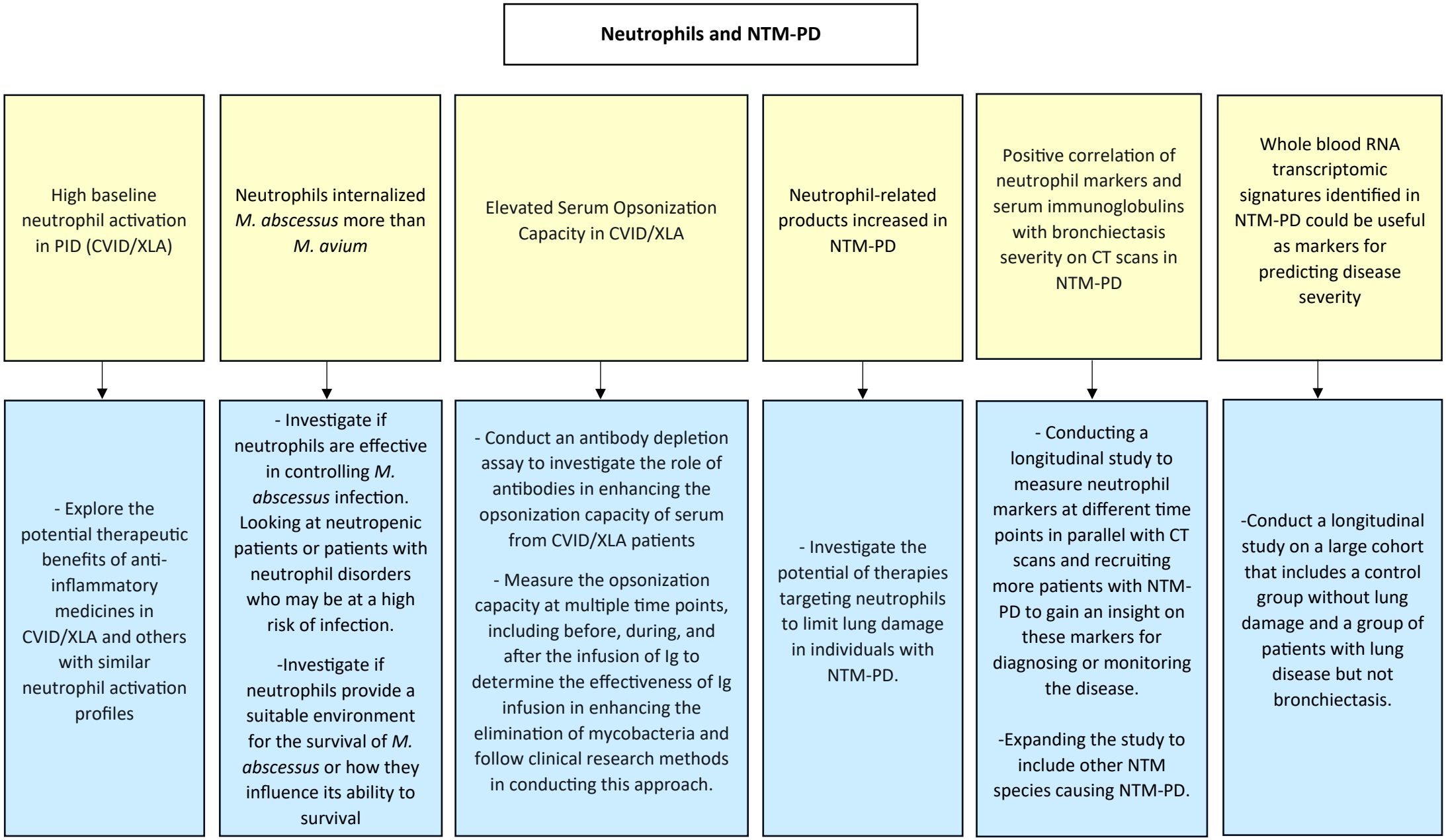


Figure 7. 1: Flow chart of potential findings and their impact on planning future research directions in neutrophils and NTM-PD. Boxes in (Yellow) for the findings and (Blue) for the future research direction

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Appendices

Appendices _
Neutrophils and Non·