Using Neutrophil-specific biomarkers as a measure of *Mycobacterium tuberculosis* burden, lung damage and treatment response kinetics

By

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Supervisor

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Preface

The study described in this thesis was carried out at the Africa Health Research Institute (AHRI), Nelson R. Mandela School of Medicine, University of KwaZulu-Natal in Durban in South Africa between March 2017 and May 2021 under the supervision of Dr Alasdair Leslie.

The study described in this thesis is original work done and reported by the author. The study has not been used in any form, by any person or submitted to any tertiary institution for award of a degree or diploma. Some of the work has been published in accredited journals in line with the thesis guidelines of UKZN. Due acknowledgements have been accorded where other people's work has been used in the text.

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Declaration 2: Publication

The publication (published, in print and/or submitted) that constitute this thesis and the contribution I made to each of the manuscripts are presented here.

Publication

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Author contributions

AL and LN conceived, designed the experiments, and wrote the paper. AP, TS, FK, and M-YM designed and managed the cohort. FK and MM enrolled patients and collected samples and data. KK and SM supervised laboratory collection of clinical samples and analysis. LN, SM, SN, and AN performed the experiments. TS, YH, FM, and LP made intellectual contributions. All authors contributed to the article and approved the submitted version.

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Author contributions

AL and LN conceived, designed the experiments, and wrote the paper. AP, TS, FK, and M-YM designed and managed the cohort. FK and MM enrolled patients and collected samples and data. KK and SM supervised laboratory collection of clinical samples and analysis. LN, SM, and MP performed the experiments. TS, YH, FM, BA and LP made intellectual contributions. All authors contributed to the article and approved the submitted version.

Dedication

I dedicate this thesis to my Lord and Saviour, Jesus Christ, whose wisdom and guidance has always kept me going. My parents, Dolly and Muzomuhle Mtshali, who were the first to see the scientist in me nurtured my curiosity. And my husband and kids, Tsepang, Oyama and Nhloso Ndlovu for their patience and unfailing support.

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List of Acronyms

ACS	Adolescent Cohort Study
ATB	Active TB Infection
APC	Antigen Presenting Cells
AUC	Area Under the Curve
BCG	Bacillus Calmette–Guérin
BMI	Body Mass Index
CAD	Computer Aided Detection
CDC	Centre for Disease Control
CFP	Culture Filtrate Protein 10
COPD	Chronic Obstructive Pulmonary Disease
CSF	Cerebral Spinal Fluid
CTRC	Catalysis Treatment Response Cohort
CUBS	Collection of Urine, Blood, And Sputum
CXR	Chest X-Ray
DC's	Dendritic Cells
ELISA	Enzyme-Linked Immunosorbent Assay
EMB	Ethambutol
ESAT-6	Early Secreted Antigenic Target 6
ESL-1	E-Selectin Ligand 1
FBC	Full Blood Count
fMLF	N-Formyl-Methionyl-Leucyl-Phenylalanine
FPR-	Formyl Peptide Receptor
G-CSF	Granulocyte Colony-Stimulating
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPCR	G-Protein Coupled Receptors

HbA1c	Haemoglobin A1c
HC	Healthy Controls
IFNg	Interferon Gamma
ICAM-	Intercellular Adhesion Molecule
IGRA	Interferon Gamma Release Assay
IL-	Interleukin
INH	Isoniazid
IP-10	Interferon Gamma-Induced Protein 10
LAM	Lipoarabinomannan
LDN	Low Density Neutrophils
LFA-1	Lymphocyte Function-Associated Antigen 1
LMIC	Low- And Middle-Income Countries
LPS	Lipopolysaccharides
LTB4	Leukotriene B4
LTBI	Latent Tb Infection
MAC-1	Macrophage-1 Antigen
MCP-1	Monocyte Chemoattractant Protein 1
MDR-TB	Multidrug Resistant TB
MFI	Mean Fluorescence Intensity
MGIT	Mycobacterium Growth Indicator Tube
MIP-	Macrophage Inflammatory Protein
MLR	Monocyte-Lymphocyte Ratio
Mtb	*Mycobacterium Tuberculosis*
NAA	Nucleic Acid Amplification
NDN	Normal Density Neutrophils
NET	Neutrophil Extracellular Traps
NLR	Neutrophil-Lymphocyte Ratio
NTM	Non-Tuberculose Mycobacterium

OSM	Oncostatin M
PAF	Platelet Activating Factor
PAMPS	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PCA	Principal Component Analysis
PECAM-1	Platelet/Endothelial Cell Adhesion Molecule
JAM	Junctional Adhesion Molecules
PLWH	People Living with HIV
POC	Point-Of-Care
PRR	Pattern Recognition Receptors
PSGL-1	P-Selectin Ligand 1
PZA	Pyrazinamide
GFT	Quantiferon-TB Gold In-Tube
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
Report	Regional Prospective Observational Research for Tuberculosis
RFI	Final Relative Fluorescence Intensity
RIF	Rifampin
ROC	Receiver Operator Characteristics
RNA	Ribonucleic Acid
SA	South Africa
SDF-1 A	Stromal Cell-Derived Factor 1 A
STI	Sexually Transmitted Diseases
TB	Tuberculosis
TGF B	Transforming Growth Factor Beta
TLR	Toll-Like Receptors
TNF-A	Tumour Necrosis Factor Alpha
TPP	Target Product Profiles
TREM-1	Triggering Receptors Expressed on Myeloid Cells

TST	Tuberculin Skin Test
VCAM-1	Vascular Cell Adhesion Protein 1
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation
XDR-TB	Extensive Drug Resistant TB
ZN	Ziehl-Neelsen

Abstract

The spectrum of TB disease remains poorly characterized impacting case identification and linkage to care. Furthermore, the absence of simple, sensitive and easy to measure biomarkers of disease severity and treatment response makes it difficult to distinguish patients at risk of treatment failure from those who would benefit from shorter treatment regimens. Ultimately, these challenges contribute to perpetuating the global TB epidemic. This thesis explores the potential of blood neutrophils as biomarkers for delineating TB disease, characterizing disease severity and monitoring TB treatment response. Neutrophils are short lived and rapidly responsive phagocytes that have been shown to have a strong TB associated signature. Using longitudinal samples, collected during a 2-year observational TB treatment response study, I analyzed the neutrophil response over the course of standard TB drug therapy. This included the novel assessment of a panel of neutrophil surface markers by flow cytometry. Overall, I show that an active TB infection is associated with elevated blood neutrophil counts that resolved over the course of TB treatment and is associated with disease severity, shown by direct correlation with bacterial load and extent of lung involvement. Importantly, these associations were not impacted by the high background of HIV infection present in this cohort. In addition, I identified the expression level of surface CD15 as a novel, rapid, robust and highly sensitive marker of TB disease. This correlated strongly with clinical characteristics and, uniquely, baseline CD15 expression predicted TB treatment response, as shown by solid culture conversion at 2 months. To extend these findings I measured a panel of inflammatory cytokines and neutrophil specific soluble factors by Luminex and ELISA. As shown in other studies, many plasma cytokines are elevated in subjects with active TB and reduced in response to treatment. Baseline levels of the neutrophil associated markers, S100A8/9 and TREM-1 associated most strongly with disease severity, again supporting the hypothesis that neutrophils are a good sentinel immune cell in TB. Hierarchical clustering indicated a coordinated inflammatory response to TB infection, which resolved over the course of treatment. However, these did not associate with disease severity or predict treatment outcome. Finally, I tested the potential of simple neutrophil characteristics (abundance and neutrophil:lymphocyte ratio) to identify clinical and subclinical TB identified in the community, using blood smears obtained during a recent community

wide TB screen conducted in rural Kwa-Zulu Natal. Despite reasonable performance in the clinic, however, these metrics failed to distinguish symptomatic TB from sub-clinical TB, and either of these from TB uninfected community controls. Unfortunately, it was not possible to evaluate neutrophil phenotype, particularly CD15 expression level, in this setting. Taken together, these data suggest that detail measuring of the neutrophil response to TB in the clinic may provide useful information about disease severity, offer a sensitive measure of treatment response and, potentially, identify patients at baseline who may be at risk of poor treatment outcomes.

Chapter 1: Introduction

1.1 Background

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is one of the top 10 leading causes of death worldwide. In 2018, 10 million people fell ill with TB and a total of 1.5 million people died (WHO 2019). Infection with Mtb is a result of its inhalation as respiratory droplets and deposition into the alveolar space. Once in the lung, Mtb encounters innate immune cells which play a vital role in the outcome of the infection. Activation of the innate immune system is dependent on the recognition of pathogen-associated molecular patterns (PAMPs) of MTB by pattern recognition receptors (PRR) expressed by immune cells (Kleinnijenhuis et al. 2011). Furthermore, these cells, and particularly dendritic cells (DCs), can function as antigen presenting cells (APCs) which carry fragments of the microbe to the lung draining lymph nodes in order the initiate an adaptive immune response (Abrahem et al. 2020; de Martino et al. 2019). This response is characterised by the activation, differentiation and maturation of T cells and B cells which are capable of mounting a specific and targeted response to the infection (de Martino et al. 2019).

Infection with Mtb can results in a spectrum of clinical manifestations, from a latent TB infection (LTBI), incipient, sub-clinical to an active TB (ATB) infection. A latent infection is often considered to be as a result of the containment of the initial infection (O'Garra et al. 2013). It is characterised by persistent bacterial viability, immune control and the absence of clinical symptoms. LTBI is diagnosed by testing an individual's immune response to Mtb antigens, using techniques such as an interferon-γ release assay (IGRAs) (Getahun et al. 2015), with the rationale that adaptive immune responses will only be present following infection. An estimated one-third of the world's population is latently infected with TB and remain asymptomatic and of these latently infected individuals, 5-10% are at risk for developing active TB disease in their lifetime (O'Garra et al. 2013). Rapid progression to active TB disease following infection, termed primary-progressive TB, is less frequent than LTBI and is more likely in children, or in adults with overt immunosuppression or medical comorbidities (Frieden et al.

2003). More commonly, ATB develops following an extended period of asymptomatic infection/latency and is termed post-primary TB. A study by Moran-Mendoza and colleagues following the household contacts of TB cases for 12 years and identified risk factors associated with developing active TB disease. This included malnutrition, no or incomplete treatment of LTBI, being 0-10 years old or having a tuberculin skin test (TST) size ≧5mm (Moran-Mendoza et al. 2010). Other significant risk factors include alcoholism, smoking, diabetes and HIV infection (Lönnroth et al. 2009). Mtb infection is the leading cause of death among people living with HIV. ATB is characterised by a combination of symptoms such as fever, sustained weight loss, night sweats, chronic cough, haemoptysis and radiological abnormalities such as lung cavities and densities (O'Garra et al. 2013). ATB is typically diagnosed through identification of Mtb bacilli in sputum or other relevant samples, by acid-fast staining, culture on solid or in liquid media or by using nucleic acid testing to detect bacteria DNA (Afsar et al. 2018). Sub-clinical and incipient TB largely go undiagnosed and poses a major challenge to the eradication of TB worldwide. They form at least 60% of individuals with bacteriologically positive sputum and do not experience TB associated symptoms that would prompt health-seeking behavior (Hoa et al. 2010; Corbett et al. 2010; Ayles et al. 2009). Furthermore, unclear case definitions and inadequate characterization of the inflammatory states significantly impacts the identification of these 'missing cases' and their linkage to care.

The standard course of TB treatment is six months and consists of two phases, the intensive phase and the continuous phase. The intensive phase occurs during the first 2 months and is typically comprised of 4 anti-mycobacterial drugs; isoniazid (INH), rifampin (RIF), ethambutol (EMB) and pyrazinamide (PZA). The continuation phase is comprised of only INH and RIF taken over the remaining 4 months (Nahid et al. 2016). Treatment may be extended to 9 or up to 24 months due to complications. This may be as a result of inconsistent adherence or the development of multiple and extensive drug resistant TB (MDR-TB and XDR-TB respectively) (Nahid et al. 2019). This extension is determined by a positive sputum culture following 7 weeks of the intensive phase. Poor adherence to TB therapy may also leads to relapse and treatment failure, ultimately increasing the risk of TB transmission. A pressing area of TB research is the development of new drugs that are better tolerated with fewer side effects and

treatment regimens that can shorten therapy to less than 6 months, thereby improving patient adherence and limiting the risk of treatment failure (Gillespie et al. 2014; Lee et al. 2012). A significant challenge in achieving this is the absence of validated point-of-care (POC) biomarkers needed to monitor patient response to TB treatment in real-time and markers that can accurately describe the host immune and disease status. These specific host biomarkers not only need to be simple and sensitive but must also be easily accessible in resource limited settings, where most of the TB disease occurs. The following literature review covers the detection and treatment of TB in more detail.

1.2 Literature Review

1.2.1 TB burden in South Africa

The Sub-Saharan African region has 14% of the world's population yet at least a quarter of all TB related deaths occurred within it (ACDC 2020). South Africa (SA) is among the eight countries that constitute 2 thirds of all new infections worldwide. It is estimated that at least 80% of the population is latently infected with TB, with the highest prevalence among people in the age group of 20-39 (Lawn et al. 2006). In 2018 alone, a total of 301,000 cases were recorded of active TB disease with a mortality rate of 21%. Of these at least 60% were HIV positive, a key driver of the TB epidemic (Kanabus 2020). Other contributors to the TB epidemic, particularly in low- and middle-income countries (LMIC), include poor TB control programmes, diagnostics and treatment (WHO 2011).

Table 1: Annual trends in TB incidence, treatment success and TB related mortality rates in South Africa.

	2009	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**
Incidence rate per 100 000	832	805	762.3	689.3	648.9	593	520	436	398	406
Notified Cases	411724	404929	389974	349594	328896	318193	301000	296603	227244	235652
HIV+TB cases (%)	-	60	65	65	62	61	57	59	60	59
MDR-TB	9070	7386	10085	15419	26023	18734	19613	19073	15986	13199
Treatment Success rate (%)	72,3	70,8	75,4	76,1	77,9	77,2	81,0	81,7	76,3	71%
Mortality rate (%)	7.2%	11.6	10.7	9.9	8.8	8.4	7.2	6.6	-	-

Figures were adapted from the WHO global tuberculosis report (WHO 2020) and the South African district health barometer (HST 2020)

Over the last decade huge gains have made to reduce the rate of TB incidence in SA from 832 infections per 100,000 in 2009 to a rate of 520 per 100,000 (**Table 1**). This has been largely due to the launch of national strategic plan on HIV, STIs and TB in 2012 (HST 2012). The plan introduced better screening and improved diagnostic tools leading to increased testing, an increase in the number of infected people on treatment and improved cure rates. However, more work is needed in order to meet the World Health Organization (WHO) 2035 goal of ending the TB epidemic, with targets to reduce TB deaths by 95% and cut new cases by 90% which South Africa has committed to.

1.2.2 Standard detection and diagnosis of *Mycobacterium tuberculosis*

On 24 March 1882, Dr Robert Koch presented his discovery of *Mycobacterium tuberculosis* as the TB causing bacteria to the society of Physiology in Berlin (Daniel 2006). At the time of his announcement, one in seven people living in the united States and Europe were dying from the disease (CDC 2016). Dr Koch's discovery was a crucial step in the efforts to control and eradicate this deadly disease.

The TST (Mantoux test) was the first test developed for the detection of an Mtb infection, in 1907, by Charles Mantoux. It is performed by injecting a small amount of tuberculin purified protein derivative under skin in the lower arm. This protein contains over 200 antigens shared with the BCG vaccine and environmental non-TB mycobacteria (Huebner, Schein, and Bass 1993). If there is a TB disease, a bump forms that can be measured in millimetres within 48-72 of the test (Brock et al. 2004). This test is widely used even today, is inexpensive and does not require special lab supplies or infrastructure. However, the test requires adequately trained staff, and, importantly, it cannot distinguish people with ATB disease from people individuals with LTBI, who have been BCG vaccinated or exposed to non-tuberculous mycobacteria (NTM). In addition, it has been shown to have reduced sensitivity in HIV positive individuals (Farhat et al. 2006; Cobelens et al. 2006).

1.2.2.1 Screening tools

Symptom screening

Primary evaluation of individuals suspected of having TB typically involves symptom screening followed by a Chest X-ray (CXR). Symptom screening looks for the common symptoms associated with TB, such as persistent cough, night sweats, weight loss, fever or hemoptysis. In South Africa, individuals who present with at least 3 of these symptoms will be referred to the TB clinics for further assessment and a CXR. Symptom screening in non-invasive, rapid and low cost. However, symptom screening may incorrectly indicate TB as many health conditions have similar symptoms. In addition, up to 20-60% of people with prevalent, bacterially confirmed, ATB report having no clinical symptoms (Brennan et al. 2020; Miller et al. 2000). This is referred to as asymptomatic or subclinical TB, and its prevalence highlights the pressing need for non-symptoms based diagnostic tests.

Chest Radiograph

Diagnosis by Chest x-ray (CXR) involves the imaging of the lung to identify characteristic features of pulmonary TB. The CXR is often available in urban areas, its fast and is sensitive enough to rule out an active Mtb infection (van't Hoog, Onozaki, and Lonnroth 2014). Chest X-rays, miss at least 10% of culture positive pulmonary TB cases, increasing up to 30% of people living with HIV (PLWH), in whom cavities are less likely to develop (Cudahy and Shenoi 2016). In addition, they require trained personnel to operate and health care professionals to interpret the results, and have limited availability in LMIC, especially in rural settings in South Africa (Onozaki et al. 2015). A chest X-ray is also unable to detect TB disease in its early stages, incipient TB, as the lung damage might not have occurred, or extra-pulmonary disease with no lung involvement. In addition, scarring from a previous infection or other lung conditions may confound interpretation of the CXR.

Digital CXRs are an advancement and produce digital images that can be interpreted by computer software via 'computer aided detection' (CAD) rather than manual reading of film and slides. The primary goal of this method is to reduce false negative rate due to observational oversight, and thereby increasing the detection of disease (Castellino 2005; Fehr et al. 2021). The advantages of this method

include better image quality, low radiation dose, fewer consumables and results that can be available immediately. Furthermore, this screening tool does not require a healthcare provider on site as images can be sent digitally, an attractive approach for remote areas. However, neither symptom screening or CXR can be used in isolation to diagnose TB disease and they are unable to detect drug resistance. Other radiological screening tools such as computed tomography (CT) (Li et al. 2021) and Magnetic resonance imaging (MRI) (Zeng et al. 2019) have also been evaluated for use in identifying cavities, non-calcified nodules, mass and ground glass opacity and found to be comparable. However, though effective, are not easily available or cost effective for use in resource limited settings.

1.2.2.2 Microbiological confirmation

Confirmation of Mtb infection requires detection of Mtb bacilli in a clinical specimen. The most used specimen for pulmonary TB is sputum, produced either through coughing and induction. A bronchoalveolar lavage or gastric aspiration can also be used to isolate bacteria in the lung. Extra-pulmonary TB can be diagnosed using urine, CFS, pleural fluid or tissue biopsies (Tadesse et al. 2019).

Smear microscopy

The oldest, quickest, simplest and cheapest method available for detecting TB is sputum smear microscopy with a light microscope. It involves direct observation of acid-alcohol resistant bacilli, typically via Ziehl-Neelsen (ZN) staining. This method is widely used in resource limited settings but does have limitations. Firstly, smear microscopy requires a large number (5000 – 10000) of bacilli in the sputum in order to give an accurate result and it is not able to differentiate Mtb from other acid-fast bacilli in sputum. Consequently, only 45-80% of all new cases can be diagnosed this way (Dunlap et al. 2000). Fluorescent microscopy using Auramine O can also be used where available and has been widely accepted as a standard diagnostic (Steingart et al. 2006). This is faster and easier to perform than ZN staining, but there are high costs associated with the equipment and its maintenance (Toman and WHO 2004).

Bacterial culture

Mtb bacilli are generally detected by culture on solid, Löwenstein-Jensen, media plates or using a liquid media system such as the Mycobacterium Growth Indicator Tube (MGIT). Sputum culture is both sensitive (81%) and highly specific (98%) for live mycobacterium - with a detection limit of ~100 organisms, it is simple to perform and widely used for the drug-susceptibility/resistance testing (Van Deun 2004; Hobby et al. 1973). However, this test does have disadvantages, such as the amount of time needed to get a result which ranges from 3-8 weeks; it requires live organisms and cannot reliably detect LTBI or extra-pulmonary TB. Furthermore, it is subject to cross-contamination and personnel need to follow strict safety protocols to prevent exposure (Joh et al. 2007; Chakravorty, Sen, and Tyagi 2005; Muyoyeta et al. 2009). Comparison of the two culture systems shows liquid culture to be superior to solid media due to reduced time to positivity and the ability to detect more mycobacterial isolates (Muyoyeta et al. 2009; Chihota et al. 2010). Importantly, despite generally being considered a gold standard test for detecting Mtb, at least 19% of individuals with Mtb infection cannot be diagnosed using culture (Van Deun 2004).

1.2.2.3 Alternative methods of MTB testing

Nucleic acid amplification (NAA)

NAA involves the amplification of DNA or RNA segments to identify the microorganism in a specimen (Schweitzer and Kingsmore 2001). Guidelines for its use were first published in 1996 by the Centre for disease control and prevention (CDC) and have since been updated in by both the CDC and WHO in 2009 and 2013 respectively (CDC 2009; WHO 2013). It has been established as a routine test in many settings due to the reliable detection of MTB in a specimen and treatment can be initiated within a week of testing (Peralta, Barry, and Pascopella 2016). Early laboratory confirmation of TB can lead to earlier treatment initiation, improved patient outcomes, increased opportunities to interrupt transmission, and more effective public health interventions. In two separate retrospective studies the impact AFB and NAA for the diagnosis of TB was compared. Results showed NAA to be superior in its sensitivity, specificity, positive predictive value and negative predicative value, over 90%, in patients with positive AFB (Wu et al. 2019; Laraque et al. 2009). However, sensitivity and specificity is greatly reduced in

patients with negative AFB and in people living with HIV (Laraque et al. 2009; Davis et al. 2011). Furthermore, NAA does not discriminate between live or dead bacterial DNA potentially increasing the rates of false positives (Costantini, Marando, and Gianella 2020). Therefore, growing bacteria in culture remains the gold standard for the diagnosis of TB and similarly to smear microscopy NAAT results require culture confirmation. Xpert MTB/RIF is the most commonly used NAA test, which uses sputum to detect Mtb infection. Results indicate the presence of mycobacterium or NTM, it is also able to determine whether the Mtb is RIF resistant. Xpert Ultra assay is an advanced form of Xpert MTB/RIF and was designed to overcome its limitations in detecting Mtb in PLWH and paucibacillary disease. This is largely due to a lower limit of detection and higher sensitivity in detecting RIF resistance.

Interferon gamma release assays (IGRAs)

IGRA is an *in vitro* blood test designed to measure cell-mediated immune responses and is commonly used as an alternative to TST were resources are available (Acharya et al. 2020). It measures the amount of Interferon gamma (IFN-γ) produced by T lymphocytes following stimulation with the MTB-specific antigens early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10)(Pai et al. 2014). IGRAs are widely accepted and used in America and Europe but not in LMIC. There are two approved and commercially available IGRAs; the QuantiFERON-TB Gold In-Tube (QFT) assay (Cellestis/Qiagen, Carnegie, Australia) and the T-SPOT.TB assay (Oxford Immunotec, Abingdon, United Kingdom). QFT and T-SPOT.TB are enzyme-linked immunosorbent assays-based tests and while QFT is performed on whole blood, T-SPOT.TB is performed on separated peripheral blood mononuclear cells (PBMCs) (Pai et al. 2014; Al-Zamel 2009).

Recent studies have evaluated the performance of IGRAs in the diagnosis of TB, LTBI and non-TB disease. QFT and T.SPOT.TB were highly sensitive (81-98% and 82-97% respectively) and had variable specificity (60-98% and 63-98% respectively) (Takeda et al. 2020; Wang et al. 2018; Du et al. 2018). Both tests show concordance of at least 92% in diagnosing ATB and were superior to the TST as they require only a single patient visit, results are available within 24hrs, can detect LTBI and can differentiate ATB from NTM (Huo and Peng 2016; Pinto et al. 2012; Pai et al. 2014). However, IGRAs

require specialised handling of equipment and patients' blood, high material costs, results are affected by immune suppression, including HIV related, and there is a lack of data available on the future risk of progressing from LTBI to active disease (Santin, Muñoz, and Rigau 2012; Huo and Peng 2016; Connell et al. 2008; Dewan et al. 2006; Gupta et al. 2020).

The development of more accurate, rapid and low-cost screening tools for the detection of ATB and LTBI is essential to the control of global TB spread. Particularly, tests that can diagnose children, pregnant women, PLWH, extra-pulmonary TB and tests that can predict progression from LTBI to ATB.

1.2.3 TB biomarkers: developments and Challenges

The detection of live bacteria in sputum is the primary method for diagnosing active TB despite its limitations. And as previously mentioned there is a need for new tools and biomarkers that are not only sensitive but broadly applicable and, ideally, do not rely on sputum, which is not always easy to obtain. Biomarkers are defined as characteristics that are indicators of normal biological processes, pathogenic processes or biological responses to an exposure or intervention, including therapeutic interventions (FDA-NIH 2016). Biomarkers are therefore an attractive alternative approach for diagnosing TB, monitoring responses to treatment, characterizing disease severity and, potentially, in predicting disease progression in LTBI. The World Health Organization has defined suitable characteristics for POC biomarker tests in its high-priority target product profiles (TPPs) (Kik et al. 2014). The following section focuses on the most promising non-sputum-based biomarkers to date.

1.2.3.1 Blood based biomarkers

Blood-based biomarkers are by far the most studied form of TB biomarkers and new candidates continue to be discovered (MacLean et al. 2019). However, there is typically a significant gap between biomarker identification and validation, ultimately impacting their application as POC tests. Blood-based biomarkers or biosignatures can be either host- or pathogen-specific and range from full differential blood count (FBCs) counts, plasma cytokines, antibodies, and RNA (MacLean et al. 2019).

Full differential blood counts

Lymphocytes, monocytes and neutrophils are the most studied leukocytes in TB, comprising between 20% – 40%; 2% –10%; and 40% – 60% respectively. Active TB disease is associated with decreased absolute lymphocyte counts and increased monocyte, neutrophil, and total leukocyte count (Jeon et al. 2019) when compared to healthy controls. However, monocyte-lymphocyte ratios (MLR) and neutrophil-lymphocyte ratios (NLR) are often the most useful measures for distinguishing ATB from LTBI and other pulmonary infections, characterizing TB disease severity and monitoring responses to treatment. In a recent quantitative and qualitative study profiling monocytes in TB, elevated MLR distinguished ATB patients from LTBI and healthy controls (La Manna et al. 2017). A receiver operator characteristics (ROC) curve analysis allowed the discrimination of ATB from HC and LTBI with a sensitivity of (91.04% and 85.07% respectively) and a specificity of (93.55% and 85.71%). In addition, MLR is significantly reduced following treatment with anti-TB drugs (La Manna et al. 2017; Wang et al. 2015). In HIV-infected children MLR was also able to distinguish culture confirmed TB from unlikely TB and decreased following treatment, suggesting the potential usefulness of this marker in PLWH and during sputum scarcity (Choudhary et al. 2019).

The NLR has been extensively studied in both ATB and other infections. Neutrophils are the most abundant white blood cell and often the first to respond to infectious pathogens (Rosales et al. 2016). Elevated neutrophils are therefore considered a hallmark of infection. Similar to MLR, elevated NLR was significantly higher in ATB compared to HC and distinguished ATB from non-TB disease such as sarcoidosis and pneumonia (Jeon et al. 2019; Iliaz et al. 2014; Yoon, Son, and Um 2013). NLR sensitivity and specificity in discriminating ATB from HC or non-TB ranged from 79% - 91% and 73% - 81.6% respectively. However, neutrophil responses are non-specific and therefore NLR should be considered together with clinical data for the diagnosis of TB. NLR can also be used to determine TB disease severity and monitoring responses to treatment. In two separate studies by Abakay et al. and Panteleev et al., elevated neutrophil counts and NLR were associated with advanced TB disease, in particular pulmonary destruction, chest X-Ray score and bacterial burden (Panteleev et al. 2017;

Abakay et al. 2015). Neutrophils show great promise as candidate biomarkers for TB disease, their usefulness will be covered more extensively in the subsequent sections.

Plasma cytokines

Plasma cytokines have also emerged as leading TB biomarker candidates. Cytokines are small, soluble proteins that are produced and released by cells to influence the behavior of other cells (paracrine), or themselves (autocrine). A recent systemic review identified Interferon (IFN)-γ, Tumour necrosis factor (TNF)-α, Interferon gamma-induced protein (IP)-10, Interleukin (IL)-2, IL-10 and IL-13 as the most frequently studied cytokines biomarkers to distinguish ATB from LTBI (Sudbury et al. 2020). IFNγ, IL-2, TNFα and IP-10 are involved in the pro-inflammatory responses associated with active TB disease. These include, but are not limited to, enhancing bacterial killing by macrophages, granuloma formation, organizing leukocyte recruitment and activation (Domingo-Gonzalez et al. 2017). These markers, along with IL-8, Vascular endothelial growth factor (VEGF), IL-2Ra, Monocyte chemoattractant protein (MCP)-1, Granulocyte colony-stimulating factor (G-CSF), and Granulocyte-macrophage colony-stimulating factor (GM-CSF) to name a few, have shown potential in discriminating active TB from LTBI and HC in other studies (Yao et al. 2017; Luo et al. 2019).

In addition, using a small number of markers in combination has been shown to have superior discriminatory power than individual markers alone. Kumar et al. analysed 7 pro-inflammatory cytokines IFNγ, TNFα, IL-17A and IL-17F, IL-6, IL-12, and IL-1β in individuals with pulmonary ATB, LTBI or HC (Kumar et al. 2019). All seven markers were elevated in ATB compared to HCs, four of the markers, IFNγ, TNFα, IL-17A and IL-1β, were elevated in patients who presented with cavities or bilateral disease, and cytokine levels were significantly lower after standard TB treatment. Similar data has been presented in numerous studies which have also shown the potential of cytokines to distinguish ATB from non-TB disease (La Manna et al. 2018), to identify TB patients regardless of HIV status (Mihret et al. 2013; Mihret et al. 2014), and potential in evaluating extra-pulmonary TB (Ranaivomanana et al. 2018; Jamil et al. 2007).

In addition to the widely reported plasma cytokines, acute phase proteins such as CRP, ferritin and serum albumin have also emerged as a potentially strong TB biomarkers. Studies evaluating CRP both in community and hospital settings show a pooled sensitivity of 89% and specificity of 57%. (Choi et al. 2007; Wilson, Badri, and Maartens 2011; Yoon et al. 2017; Kang et al. 2009). CRP has been found to useful in distinguishing ATB from non-TB in irrespective of HIV co-infection and to be more sensitive in areas with high TB burden. Thus making it a prime POC test candidate.

Ribonucleic acid (RNA) sequencing

The use of transcriptomics for biomarker discovery is an emerging field, gaining popularity as platforms becoming increasingly available and affordable (Gierahn et al. 2017). Several studies have evaluated the use of transcriptional biosignatures as candidates for TB diagnosis, correlates of tuberculosis risk and as predictive biomarkers for treatment outcomes. In a longitudinal adolescent cohort study (ACS), healthy South African adolescents were followed for 2 years to assess gene signatures associated with risk of developing active TB disease (Zak et al. 2016). A 16-gene signature was identified from TB progressors, which discriminated from controls with 71% sensitivity and 80% specificity within 6 months of diagnosis. In a validation data set, sensitivity was reduced to 53.7% but specificity was relatively unchanged at 82.8%. Berry et al., identified a 393-gene signature dominated by a neutrophil driven interferon inducible gene profile (Berry et al. 2010). The gene signatures' sensitivity and specificity in the UK test data set comparing ATB, LTBI and HC was 61.7% and 93.75% respectively and, was 94.12% and 96.67% respectively in the SA validation set. Importantly, this signature correlated with the extent of lung involvement and resolved to healthy controls signatures following treatment. Furthermore, an 86-gene signature discriminating ATB from other bacterial and inflammatory disease was also identified. Sensitivity in the training set was 92% and 90% in the validation set. A similar study by Kaforou et al. aimed at discriminating ATB from other diseases in HIV positive and negative patients, identified a 27-gene signature distinguishing culture confirmed ATB from LTBI with 95% sensitivity and 90% - 94% specificity (Kaforou et al. 2013). And a 44-gene signature distinguishing ATB from other diseases with 93% - 100% sensitivity and 88% - 96% specificity.

A recent study by Thompson et al. explored the usefulness of gene signatures in predicting TB treatment outcomes in the Catalysis treatment response cohort (CTRC) (Thompson et al. 2017). This study identified a 9-gene 'DISEASE' signature to be strongly discriminative and predictive of treatment failure and cure in patients within a month of treatment commencement, AUC = 0.70-0.72, $p<0.05$ and AUC = 0.99, $p<0.05$ respectively. It also identified 13 'FAILURE' gene signature of differentially expressed genes at baseline (pre-treatment) between treatment failures and cure. Enrichment analysis revealed an abundance of genes that are enriched in neutrophils and for suppression of mitochondrial genes. Suggesting that patients who present with enhanced neutrophil inflammation pre-treatment are at risk of treatment failure.

At least 3 of the 9 'DISEASE' genes were present in the ACS COR, indicating the reproducibility and transferability of gene signatures. The enrichment of genes associated with neutrophils during ATB in blood warrants further investigation into the role of neutrophils in TB pathogenesis and their potential as POC biomarkers for TB disease infection and host immune responses.

1.2.3.2 Urine based biomarkers

The detection of Mtb-specific and biologically active components (ie. MTB antigens) in urine is potentially advantages over the use the sputum. The most widely reported mycobacterial antigen is lipoarabinomannan (LAM), a 17.5kD glycolipid and major component of the MTB cell wall (Besra et al. 1997). LAM is released in its soluble form by metabolically active or degrading mycobacteria during an infection and theoretically can be associated with bacterial burden (Chan et al. 1991; Hunter, Gaylord, and Brennan 1986). The feasibility and sensitivity of urine-LAM based tests has been evaluated in numerous studies over the years. These studies were predominantly conducted in LMIC with high TB and HIV prevalence. Commercially available LAM-ELISAs show overall high specificity (87% - 99%) and reduced sensitivity among HIV-uninfected (20% - 60%) adults and children, compared to PLWH (67%-85%) (Shah et al. 2009; Reither et al. 2009; Mutetwa et al. 2009; Daley et al. 2009; Shah et al. 2010; Dheda et al. 2010; LaCourse et al. 2018; Iskandar et al. 2017). When compare

to NAA tests such as Xpert-MTB/RIF or smear microscopy, LAM did not significantly improve diagnostic yield (Peter et al. 2015). However, studies also show LAM to be highly sensitive (88%) in patients with extrapulmonary TB (Kerkhoff et al. 2017) and a useful prognostic marker for mortality in PLWH (Peter et al. 2015; Kerkhoff et al. 2017; Suwanpimolkul et al. 2017). Other sample types that have been investigated for TB diagnosis include pleural fluid, cerebral spinal fluid and saliva (Jacobs et al. 2016; Liu et al. 2016; Sutherland et al. 2012; Sharma et al. 2015).

1.2.4 Neutrophils in health and inflammation

The first line of defense against invading pathogens is the innate immune system, of which neutrophils are in the vanguard. They develop in the bone marrow in a process called granulopoiesis and are released at a rate of 10^9 neutrophils/kg body weight each day during homeostasis (Cartwright, Athens, and Wintrobe 1964). Typically characterized by the presence of large cytoplasmic granules, circulating neutrophils have a half-life of ~7 h due to the cytotoxic nature of their contents (Mauer et al. 1960). However, under inflammatory conditions, the half-life of neutrophils is greatly increased and is dependent on the disease (Tak et al. 2013). These granulocytes patrol the tissue environment and are poised to initiate an aggressive immune response should the need arise. In the event of inflammatory stimuli, neutrophil release from the bone marrow is accelerated (emergency granulopoiesis) and circulating neutrophils migrate into the site of infection. Upon arrival, they are able to ingest, kill and digest the invading pathogen(Nauseef and Borregaard 2014). Granules form the neutrophil armory, and consist of antimicrobial proteins, proteases and reactive oxygen metabolites which are delivered to the phagosome during phagocytosis or the cell exterior following degranulation or during the release of networks of chromatin and granular contents (NETosis) (Faurschou and Borregaard 2003). Senescent or apoptotic neutrophils are then subsequently cleared in the spleen, liver or bone marrow (Furze and Rankin 2008; Greenlee-Wacker 2016). Due to the highly reactive nature and their cytotoxic contents, neutrophil recruitment, activation and clearance is tightly regulated. The following sections will follow neutrophils from the site of production to the site of infection and thus highlight their potential as biomarkers of disease.

1.2.4.1 Neutrophils in bone marrow

As previously mentioned, neutrophils are bone marrow derived immune cells produced in a process termed granulopoiesis (Cartwright, Athens, and Wintrobe 1964). Under normal conditions a large storage pool of mature neutrophils accumulates within the bone marrow and is released in order to maintain homeostatic levels. However, excessive release of neutrophils during inflammation may contribute to tissue damage and exacerbate disease (Weiss 1989; Bratton and Henson 2011), and neutrophil depletion or neutropenia is an independent risk factor for infections likes TB (Martineau et al. 2007; Pedrosa et al. 2000). Neutrophil mobilization is regulated by several key molecules, including the growth factor, granulocyte colony stimulating factor (G-CSF) and stem cells factor, stromal cell-derived factor 1α (SDF-1α). G-CSF plays a critical role in the differentiation, proliferation, survival and function of mature neutrophils and their progenitors. This is highlighted in mouse models with chronic neutropenia as a result of G-CSF gene disruptions (Lieschke et al. 1994) and neutrophilia in mice with excess G-CSF (Pojda, Molineux, and Dexter 1990). In addition, a randomized clinical trial showed that G-CSF treatment prevented infection in patients with cyclic neutropenia (Dale et al. 1993) and in a subsequent study showed a reduction in the number of sepsis episodes in treated versus untreated patients (Dale et al. 2017). SDF-1α on the other hand is a key chemokine for retention of hematopoietic cells in the bone marrow and has also been implicated in senescent/pre-apoptotic neutrophil homing (Martin et al. 2003). Semerad et al. showed that treatment with G-CSF in wild type mice resulted in a significant decrease in SDF-1α in the bone marrow and that this decrease was strongly directly correlated with neutrophil mobilization (Semerad et al. 2002).

These cytokines and chemokines however do not function in isolation and neutrophil phenotype plays a significant role in the mobilization of neutrophils. Chemokine receptors, chemokine receptor type 2 (CXCR2) and -4 (CXCR4) work antagonistically to mediate neutrophil migration. CXCR2 is the ligand for inflammatory chemokines CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8 (also known as IL-8), and is involved in the recruitment of mature neutrophils from blood into tissue during infection (Eash et al. 2010). SDF-1α binds exclusively to CXCR4, therefore, mature neutrophils express higher

levels of CXCR2 and low levels of CXCR4 to facilitate bone marrow egress (Bachelerie et al. 2013; Martin et al. 2003). CXCR2 antagonists has been evaluated as therapeutic targets in pulmonary diseases with neutrophil driven lung pathology such as chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis (Chapman et al. 2009; Zhang et al. 2019). Other key regulators of neutrophil recruitment are IL-23 and IL-17A. IL-23 is produced by macrophages and dendritic cells and is able to stimulate the production of IL-17A in specialized T cells (Ley, Smith, and Stark 2006). The release of IL-17A can also influence neutrophil transmigration by stimulating G-CSF production (Hirai et al. 2012; Schwarzenberger et al. 2000; Ye et al. 2001).

1.2.4.2 Neutrophils in blood

Granulopoiesis under normal conditions will result in the release of only mature neutrophils. However, during inflammation the rapid release of neutrophils from the bone marrow includes immature granulocyte with a distinct morphology and phenotype. Immature neutrophils, also called band cells, are characterized by a curved nucleus and express a $CD66b^{+}CD13^{dim}CD15^{+}CD11b^{dim}CD16^{dim}$ $CD62L^{high}$ and $CD10^{-}$ phenotype (Orr et al. 2005; van Lochem et al. 2004; Pillay et al. 2012). Mature neutrophils on the other hand have a segmented or lobular nucleus and are typically $CD66b^{+}CD13^{high}CD15^{+}CD11b^{high}CD16^{high}CD62L^{high}$ and $CD10^{+}$. In addition, 45 – 65% of circulating neutrophils express CD177 an important glycoprotein-anchored receptor involved in neutrophil transmigration into tissue (Sachs et al. 2007).

Neutrophils can be further differentiated into low density neutrophils (LDNs) and normal density neutrophils (NDNs). During density gradient separation, LDNs are collected from the peripheral blood mononuclear cell (PBMC) layer whilst NDNs are isolated from granulocyte-erythrocyte pellet. Although both subsets contain mature and immature neutrophils, the LDNs layer contains a higher percentage of immature ($CD16^{dim}/CD62L^{high}$) neutrophils during emergency granulopoiesis (Hong 2017). Although the precise mechanisms are not clear, LDNs are thought to be derived from NDNs due to degranulation, which would reduce cell density (Hassani et al. 2020; Li et al. 2019). In addition, LDNs typically express higher levels of activation markers compared to NDNs and are associated with

severe inflammation (Ssemaganda et al. 2014; Li et al. 2019; van den Akker et al. 2008) and, interestingly, display enhanced T-cell suppressor functions (Darcy et al. 2014; La Manna et al. 2019). NDN exist in resting, primed or activated states (Nauseef and Borregaard 2014), and perform effector functions such as phagocytosis, NETosis and make an oxidative burst, where LDNs cannot (La Manna et al. 2019). The balance of these neutrophil subsets is likely to be important in the outcomes of infection; however, extensive characterization of the phenotype and associated functions of these subsets is lacking to date.

1.2.4.3 Neutrophils in tissue

Neutrophil extravasation from blood into tissue follows a 5-step process involving tethering, rolling, adhesion, crawling and transmigration through the endothelial barrier. This process is initiated during an inflammatory response to invading pathogens or tissue damage, triggering the release of pro-inflammatory mediators by tissue resident cells such as macrophages, dendritic cells, mast cells and epithelial cells (Kim and Luster 2015). Mediators such as IL-8, complement factor C5a, platelet activating factor (PAF), Leukotriene B4 (LTB4) and bacterial peptide, N-formyl-methionyl-leucyl-phenylalanine (fMLF), form a chemoattractant gradient that neutrophils migrate towards (Soehnlein, Lindbom, and Weber 2009). Briefly, neutrophil tethering is driven by the binding of P-selectin on endothelial cells to the cell surface glycoprotein P-selectin ligand (PSGL-1). E-selectin and PSGL-1, also expressed by endothelial cells bind to E-selectin ligand 1 (ESL-1) and L-selectin respectively resulting in the slowing of neutrophil rolling speed (Ley et al. 2007; Hidalgo et al. 2007). This is then followed by firm adhesion which is mediated by β_2 integrins, lymphocyte function-associated antigen 1 (LFA-1) and macrophage-1 antigen (MAC-1 or CD11b/CD18) and their endothelial surface ligands intercellular adhesion molecule (ICAM)-1 and ICAM-2. Conformational changes of the integrins results in the formation of tight binding junctions responsible for neutrophil arrest (Kolaczkowska and Kubes 2013). In order to transmigrate, neutrophils need to locate the endothelial cell-cell junctions by actively crawling. This process occurs under shear blood flow and is dependent on the integrin-ICAM interactions. Once the transmigration sites have been identified, neutrophils can cross the endothelium

layer into tissue either between cell walls (paracellularly) or through the cell (transcellularly), although the latter is less efficient (Phillipson et al. 2008). Transmigration requires integrins, CAMs (ICAM-1, ICAM-2, vascular cell adhesion protein (VCAM)-1) and junctional proteins such as platelet/endothelial cell adhesion molecule (PECAM)-1) and junctional adhesion molecules (JAMs) (Kolaczkowska and Kubes 2013). PECAM-1 is a key mediator of trans-endothelial migration of neutrophils by binding to glycoprotein CD177. Studies blocking PECAM-1 or CD177 not only inhibited adhesion but also inhibited transmigration (Sachs et al. 2007). Once in tissue neutrophils are able to continue following the chemoattractant gradient to the site of disease. There they become more active as phagocytic cells and activate a transcriptional program resulting in the production of cytokines such as IL-8, S100A9 and CXCL1 which recruit more neutrophils and other inflammatory cells (Scapini et al. 2000; Ryckman et al. 2003).

1.2.4.4 Neutrophil-pathogen interaction

The interaction between neutrophils and the pathogen can be opsonin-dependent or opsonin-independent. The opsonin dependent pathway is mediated by complement components composed of more than 30 proteins that can distinguish host from microbe resulting in opsonisation or bacterial killing by membrane attack complexes (Dunkelberger and Song 2010). Immunoglobulins (Igs) are also an important component of this pathway and can either activate the complement system or directly neutralize toxins and/or virulence factors and subsequently mediate phagocytosis of opsonised particles using Fcγ receptors (FcγRs) on the surface of neutrophils (Bredius et al. 1994). The opsonin-independent pathway involves the use of pattern recognition receptors (PRRs), G-protein coupled receptors (GPCRs) and opsonin receptors on the surface of neutrophils to initiate phagocytosis. PRRs such as Dectin-1, triggering receptors expressed on myeloid cells (TREM)-1 and toll-like receptors (TLRs) recognise pathogen-associated molecular patterns (PAMPS) such as bacterial DNA, lipopolysaccharides (LPS) and peptidoglycans (Thomas and Schroder 2013). GPCRs such as formyl peptide receptor (FPR)-1 and -2 , recognise bacterial antigens such as fMLP (Subramanian, Moissoglu, and Parent 2018). Activation of the opsonin pathway and it's receptors is critical for enhancing

phagocytosis and neutrophil-mediated pathogen killing. However, pathogens can manipulate/subvert neutrophil mechanisms for infection control which can alter outcomes.

Once an infection has been cleared macrophages and dendritic cells play a key role in clearing dead or dying neutrophils from the infection site and suppressing the inflammatory response. The uptake of senescent/apoptotic neutrophils has been shown to result in the production of anti-inflammatory cytokines, IL-10 and transforming growth factor (TGF)-β, as well as reduced expression of TNF-α and IL-23 (Lucas et al. 2003; Stark et al. 2005). Deregulation of neutrophil apoptosis and clearance or the initiation of different forms of cells death may drive chronic inflammation and contribute to tissue damage (Brostjan and Oehler 2020).

This section highlighted the complex nature of neutrophils in health and disease and their role in driving chronic inflammation during disease or suppressing it. Numerous studies report on the complex nature/roles of neutrophils in TB disease in either clearing infection or driving pathogensis (Lowe et al. 2012; Kroon et al. 2018). This thesis will focus on further understanding this complex relationship and how it can be exploited for diagnostic purposes.

1.3. Research Problem and significance

1.3.1 Problem Statement

TB remains among the leading causes of death worldwide. Challenges faced in controlling the spread of infection can in part be attributed to case identification and poor adherence to the standard six months of treatment. Ultimately, contributing to TB spread due to missed cases, treatment failure, relapse or the development of drug resistance. There is sufficient data to suggest that reducing treatment duration may improve adherence. However, there remains an urgent need for simple, sensitive and broadly applicable biomarkers of early TB treatment response, as the current gold standard of evaluating culture conversion at two months has proved poorly predictive of TB cure. Neutrophils are abundant and short lived immune cells that are rapidly recruited to the site of infection, which in turn have been linked to

disease severity and poor outcomes in a number of studies. These Inherent characteristics point to their potential at providing a strong and universal disease signature that rapidly responds to changing disease status. However, the detailed characterization of the of early TB treatment response has not been extensively evaluated for its potential to yield useful biomarkers.

1.3.2 Hypothesis

Active TB is associated with measurable changes in blood neutrophil counts, phenotype and neutrophil associated cytokine/chemokine expression which correlate with severity of TB disease and can rapidly respond to changing disease status.

1.3.3 Research aims and objectives

The overarching aim of this work was to evaluate blood neutrophil counts, phenotypic markers and neutrophil-associated chemokines/cytokines as a biomarker of mycobacterial burden, lung damage and treatment response kinetics. The objectives were to:

1. Measure blood neutrophil phenotypic changes at TB diagnosis and during the course of treatment by flow cytometry.
2. Investigate neutrophil-associated cytokines/chemokines and other soluble factors as potential drivers of the neutrophil response and their impact on the immunopathology of TB disease.
3. Evaluate the use of blood neutrophil counts in characterising sub-clinical TB using blood smears.
4. Understand the correlation between neutrophil associated responses to Mtb infection and clinical markers of disease severity
5. Evaluate the potential use of baseline neutrophil responses to predict TB treatment outcomes

Chapter 2: Research Methodology

2.1 Study population

Results in chapters 3 and 4 were obtained from analysing study participants from the RePORT and CUBS cohorts whilst chapter 5 study participants were recruited from the Vukuzazi TB screening cohort.

2.1.1 TB cases

Study participants were recruited from the Regional Prospective Observational Research for Tuberculosis (RePORT)-South Africa, Durban study between December 2016 and December 2019. All participants were recruited from the Kwadebeka TB clinic in the Durban metropolitan area, as TB symptomatic and referred for TB testing by attending clinician. Enrolled subjects were GeneXpert and culture positive (solid or liquid media), 18 years or older and provided written informed consent. All RePORT study participants received standard 6-month treatment for TB. Study participants were followed for the duration of TB treatment and up to 24 months post-enrolment. Enrolees found to have baseline drug resistance to any standard anti-TB drug (isoniazid, rifampicin, ethambutol, or pyrazinamide), who developed resistance during treatment, or who had concomitant extrapulmonary TB were excluded from the study.

Additional study participants (n=176) aged 15 years and older were recruited from the Vukuzazi TB screening cohort at the Umkhayakude community in Durban, South Africa. Baseline assessment involved a health screen including a health questionnaire, anthropometric measurements, blood pressure measurements, followed by collection of a venepuncture blood sample, a urine sample and two rectal swabs. A digital chest X-ray was performed and participants with an abnormal chest X-ray provided sputum samples for Mtb testing by GeneXpert and liquid culture. Follow-up visits were scheduled 2-4 weeks after the initial visit for patients with abnormal test results or ATB. Symptoms were assessed and repeat blood pressure and haemoglobin A1c measurements were made. TB cases were identified based on positive sputum test and/or radiologically suggestive TB.

2.1.2 Controls

TB symptomatic participants initially recruited for RePORT as GeneXpert positive, but subsequently found to be culture negative at baseline were included as Xpert-positive/culture-negative controls (n=14) and were followed for 2 months from enrolment and found persistently culture negative. An additional control group consisting of healthy asymptomatic individuals (n=23) were recruited from the Collection of Urine, Blood, and Sputum (CUBS) cohort in Durban between January and December 2018. This group is referred to as healthy controls and not recruited in the clinic. In the Vukuzazi cohort, study participants (n=34) screened at baseline and found to have normal lung fields and did not present with TB symptoms were classified as healthy controls. All studies received approval from the UKZN ethics board (BE285/16).

Descriptive statistics

For RePORT TB cases and Xpert-positive/culture-negative controls age, sex, race, body mass index (BMI), self-reported alcohol use, tobacco use, and employment status were recorded. For PLWH, CD4 cell counts, HIV-1 RNA (viral load), and timing of antiretroviral therapy were also collected. For RePORT participants , extent of lung disease at enrolment was determined from CXR, evaluated by a radiologist blinded to all study participant information, using a CXR score (% area of lung infected + 40 for the presence of cavities; giving a scale of 0-140 (Ralph et al. 2010). Bacterial burden was estimated from liquid culture using the BD BACTEC MGIT 960 mycobacterial detection system (Becton Dickinson Microbiology Systems, Sparks, Md.) and full blood counts were measured independently by a commercial laboratory. For the healthy controls age, sex, and race were recorded. For Vukuzazi participants, CXR was immediately categorised as normal or abnormal by computer aided detection (CAD) software, an automated artificial intelligence algorithm for identifying TB (Murphy et al. 2020). CXRs with a score of >60 was considered to be abnormal (Fehr et al. 2021).

2.2 Sample collection and processing

Samples

Blood samples were collected from healthy controls at one time-point only, and blood, plasma and sputum samples from RePORT TB cases were collected at baseline (pre-treatment) and at post-treatment initiation study visits: week 1, week 3, month 2, and month 6. For all participants, fresh blood samples were collected in EDTA vacutainers (BD Biosciences) for cell phenotyping. Blood smears and sputum from Vukuzazi participants were collected at baseline only. Sample collection and preparation occurs within a ~6hr window.

Sample preparation

Neutrophil isolation and staining: A 100 µl of whole blood was stained with 13 surface markers and incubated for 30 min at 4°C in the dark. The phenotyping panel comprised of markers V500 conjugated mAb anti-CD45 (clone HI30), BUV496 conjugated mAb anti-CD16 (clone 3G8), BUV395 conjugated mAb anti-CD11b (clone ICRF44), V450 conjugated mAb anti-CD66b (clone G10F5) all from BD Bioscience; BUV785 conjugated mAb anti-CD3 (clone OKT3) and BV711 conjugated mAb anti-CD14 (clone M5E2) from Biolegend. The following phenotype markers were used, PE conjugated mAb anti-CD177 (clone MEM-166) and BV605 conjugated mAb anti-HLA-DR (clone G46-6) from BD Bioscience; FITC conjugated mAb anti-CD15 (clone HI98) and PE-Cy7 conjugated mAb anti-CXCR4 (clone 12G5) from Biolegend; APC conjugated mAb anti-CD32 (clone FLI8.28) and PE-Cy5 conjugated mAb anti-CD10 (clone HI10a) from BD Pharmigen and Live dead stain (ThermoFischer Scientific). Phenotype markers were chosen based on preliminary data from our lab indicating differential expression between TB cases and HIV-TB- controls. Red blood cells were removed, and leukocytes fixed by adding 2 mls of FacsLyse solution (BD Bioscience) and incubating for 10 min in the dark at room temperature. Samples were then centrifuged at 500 g for 5 min and the supernatant decanted. Cells were washed with 2 mls of 1X PBS (Sigma Aldrich) for 5 min at 500 g and the cell pellet resuspended in 2% PFA (Sigma Aldrich). Cells were acquired on a 17 colour FACSAria Fusion (BD Bioscience), with calibrating beads run before each acquisition to ensure consistent performance.

Due to the longitudinal nature of the study, we normalized the data based on a published method by Upreti et al. (31). Data normalization was performed by transforming the Mean Fluorescence Intensity (MFI) values of the test samples (TB cases, Xpert-positive/culture-negative, and healthy controls) to a common scale using the following equation: Final relative fluorescence intensity (RFI) = MFI of the test sample/MFI of the internal control (rainbow beads). Phenotypic data are presented in two ways: (1) In a form of a publication which is attached and, (2) as additional data that were not included in the paper and will be discussed separately. Markers which produce two distinct cell populations (positive or negative) were recorded as a percentage and markers who

Neutrophil-associated cytokine panel: Stored plasma samples from RePORT participants were thawed at room temperature for 45min and then centrifuged for 15min at $1000_{gmax.}$ Levels of Basic fibroblast growth factor (FGF2/BasicFGF), Eotaxin, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), Interleukin (IL)-10, IL-12p70, IL-13, IL-15, IL-17A, IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, interferon (IFN)-γ, interferon gamma-induced protein (IP)-10, monocyte chemoattractant Protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, Platelet-derived growth factor (PDGF.bb), regulated on activation, normal T cell expressed and secreted (RANTES), tumour necrosis factor (TNF)- α and vascular endothelial growth factor (VEGF) were measured using a premixed human magnetic Luminex kit according to the manufacturer's instructions (R&D Systems, MBL International Corporation, Woburn, MA). In a neutrophil-associated Luminex panel analytes IL-6, IL-10, IP-10, IL-8, G-CSF, IL-18, TREM-1, IL-6Ra, S100 calcium binding protein 9 (S100A9) and Oncostatin M (OSM) were also measured using a premixed human magnetic Luminex kit according to the manufacturer's instructions (R&D Systems, MBL International Corporation, Woburn, MA). Plates were read using the Bio-Plex 200 system (Bio-rad laboratories Inc). Lastly, a duoset ELISA kit (R&D Systems, MBL International Corporation, Woburn, MA) for human was also used to measure S100A8/A9 in plasma from TB cases according to the manufacturer's instructions. ELISA plates' results were read using the GlowMax-Multi detection system (Promega) at 450nm.

Blood smears: A drop of blood (5-7ul) was applied onto a clean slide and using a slide spreader smeared at a 30° angle to cover at least two-thirds of the slide. Slides was then air dried for 10 minutes in the hood and then immersed in Rapi-Diff fixative (Atom Scientific) 5 times for 1 second each time. Fixed slides were air dried overnight and were ready for storage, shipment or staining. To stain the slide, immersion in Rapi-Diff reagent 1 (Atom Scientific) was performed. Slides were dunked five times for 1 sec each and excess reagent blotted out using a paper towel. This was followed by immersion in Rapi-Diff reagent 2 (Atom Scientific) five times at 1 sec each and excess reagent blotted out. Slides were then washed by dunking in deionised water 10 times at 1 sec each time. The water was replaced, wash step was repeated, and slides dried for 2-24hrs. Lastly, a 2mm drop of DPX mountant (Sigma-Adrich) was then spread onto a cover slip, gently mounted onto the slide smear and left to dry overnight. Slide edges were sealed with clear nail polish and scanned using the NanoZoomer (Hamamatsu Photonics K.K). A digital scanner which converts glass slides into digital data.

2.3 Statistical analysis

Baseline characteristics were compared between TB cases, Xpert-positive/culture-negative, and healthy controls using Chi-square or Fisher's exact test for categorical variables and Kruskal-Wallis test for continuous variable. Correlations between CXR score, bacterial burden, blood counts, neutrophil phenotype, plasma cytokine expression, and demographic data were determined using the Mann-Whitney test and Spearman's correlation analysis. Differences in distributions and median values of neutrophil blood counts, phenotype and cytokine expression were compared between cases and controls and also examined for changes from baseline during the course of TB treatment using the Wilcoxon rank sum test and Kruskal-Wallis test followed by Dunn's multiple comparison test as data was observed to not be normally distributed. Impact of pre-treatment immune measures on patient response to treatment, defined by early (within a week or 1 month), medium (from 2 months), slow (at 6 months) culture conversion as well as treatment failure responses were graphically evaluated. The association between baseline immune markers and culture status at month 2 (positive vs. negative) and end of

treatment outcome (successful vs. unsuccessful treatment) using univariable and HIV-adjusted logistic regression was also examined.

Cytokine data were further analysed to identify dominant and co-expressed cytokines, and evaluate their capacity to distinguish; (i) ATB from GeneXpert+/Culture- and healthy controls, (ii) inflammatory profiles of TB cases at baseline and during the course of TB treatment and (iii) the impact of HIV co-infection on the inflammatory condition of TB cases. Hierarchical clustering was performed using Euclidean distancing with ward method of cytokine concentrations z-score standardised by rescaling each analyte to have a mean of 0. Participant cytokine concentrations are presented as a standard deviation of the mean where 1=upregulation and -1=down-regulation. Statistical analysis was performed using StataIC, version 16 (StataCorp, College Station, TX, USA), GraphPad PRISM, version 8.3.1 (GraphPad Software, San Diego, CA, USA) and BioVinvi, version 3.0.9 (BioTurig Inc., San Diego, CA, USA).

Chapter 3: Increased neutrophil count and decreased neutrophil CD15 expression correlate with TB disease severity and treatment response irrespective of HIV co-infection.

3.1 Published work

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Increased Neutrophil Count and Decreased Neutrophil CD15 Expression Correlate With TB Disease Severity and Treatment Response Irrespective of HIV Co-infection

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Tuberculosis remains a leading cause of death globally despite curative treatment, partly due to the difficulty of identifying patients who will not respond to therapy. Simple host biomarkers that correlate with response to drug treatment would facilitate improvement in outcomes and the evaluation of novel therapies. In a prospective longitudinal cohort study, we evaluated neutrophil count and phenotype at baseline, as well as during TB treatment in 79 patients [50 (63%) HIV-positive] with microbiologically confirmed drug susceptible TB undergoing standard treatment. At time of diagnosis, blood neutrophils were highly expanded and surface expression of the neutrophil marker CD15 greatly reduced compared to controls. Both measures changed rapidly with the commencement of drug treatment and returned to levels seen in healthy control by treatment completion. Additionally, at the time of diagnosis, high neutrophil count, and low CD15 expression was associated with higher sputum bacterial load and more severe lung damage on chest x-ray, two clinically relevant markers of disease severity. Furthermore, CD15 expression level at diagnosis was associated with TB culture conversion after 2 months of therapy (OR: 0.14, 95% CI: 0.02, 0.89), a standard measure of early TB treatment success. Importantly, our data was not significantly impacted by HIV co-infection. These data suggest that blood neutrophil metrics could potentially be exploited to develop a simple and rapid test to help determine TB disease severity, monitor drug treatment response, and identify subjects at diagnosis who may respond poorly to treatment.

Keywords: tuberculosis biomarker, neutrophil phenotype, blood neutrophil count, tuberculosis treatment outcome, tuberculosis disease severity

INTRODUCTION

Though global tuberculosis (TB) disease incidence is falling at a rate of 1.5–2% per year, it remains one of the leading causes of death from a single infectious agent, and much greater declines in incidence are required to meet global TB targets (1). TB is a curable disease, but better tools to evaluate early TB treatment response would facilitate optimization of TB treatment, and cure rates (2, 3). Shorter TB treatment regimens are also needed as poor adherence to the current standard 6-months regimen can lead to treatment failure, relapse, or the development of drug resistant TB (4). Likewise, tools for identifying patients at the point of diagnosis who are at risk of responding poorly to treatment could help with clinical management. In addition, novel methods of evaluating response to treatment could expedite testing of new TB treatment regimens in clinical trials. Host immune responses are potential biomarkers for predicting response to anti-TB treatment (5, 6) as the current approach of evaluating culture conversion at 2 months has proved poorly predictive of cure (7–9). Typically, these biomarkers are measured in blood or urine, which also makes them an attractive option for groups that are inherently difficult to diagnose and monitor with conventional sputum samples, such as children, pregnant women, and people living with HIV (10–12).

As proof of this concept, Adekambi et al. showed that the expression of cellular activation markers on *Mycobacterium tuberculosis* (Mtb)-specific CD4+ T-cells was highly effective at distinguishing individuals with asymptomatic latent TB infection (LTBI) from those with untreated active TB (13). Furthermore, decreasing expression of these markers correlated with decreasing mycobacterial load during treatment. These observations were subsequently confirmed in HIV/TB co-infected individuals (14). The advantage of this biomarker is that it is highly TB disease specific. However, the assay requires an extended period of cell stimulation and also, by definition, is only useful in individuals with detectable Mtb responses. This last point is important, as up to 30% of people with active TB infection do not have a detectable Mtb-specific CD4 response at the time of diagnosis (15), and this proportion can be higher in individuals co-infected with HIV (16, 17).

Neutrophils are the most abundant immune cells in the blood, accounting for ∼60% of circulating leukocytes, and are rapidly activated and recruited to sites of infection or tissue damage (18). Moreover, neutrophils have a lifespan in circulation of ∼7 h, increasing on activation to 2–4 days. Consequently, the large pool of neutrophils in blood and tissue is continuously being replenished from precursors in the bone marrow (19–21). Therefore, neutrophil based biosignatures may be expected to change rapidly. The role neutrophils play during active TB is complex and depends on timing. Initially, following arrival at the site of infection, neutrophils can recognize Mtb through both opsonin-dependent and -independent pathways, resulting in phagocytosis (22, 23). This probably contributes to early immune control, as experimental depletion of neutrophils during initial infection in mice has a negative outcome for the host (24). In addition, low blood neutrophil count in household contacts of TB patients is an independent predictor of TB infection (25). However, if infection fails to resolve, neutrophils appear to be one of the main drivers of lung destruction (6, 26, 27). A detailed investigation of the neutrophil response to TB infection in humans may also, therefore, improve our understanding of the immunopathology of this disease.

Transcriptomic analysis of whole-blood from TB patients has revealed a 393-transcript signature for active TB disease that is dominated by a neutrophil-driven IFN-inducible gene profile, consisting of both IFN-γ and type I IFN-αβ signaling (28). This signature was subsequently confirmed in sorted neutrophils and found to correlate with radiographic extent of disease and could distinguish active TB disease from other respiratory diseases. In addition, this signature significantly diminished following 2 months of TB treatment (29). We, therefore, hypothesized that (1) active TB is associated with both increased blood neutrophil count and phenotypic changes that could be easily measured by flow cytometry, (2) neutrophil count and phenotype is correlated with disease severity and, (3) due to the lifespan of neutrophils, these changes rapidly respond to changing disease status.

MATERIALS AND METHODS

Study Population

TB Cases

Study participants were recruited from the Regional Prospective Observational Research for Tuberculosis (RePORT)-South Africa, Durban study between December 2016 and December 2019. All participants were TB symptomatic and referred for TB testing by attending clinician. Enrolled subjects were GeneXpert and culture positive (solid or liquid media), had evidence of pulmonary involvement by chest X-ray (CXR), were 18 years or older and provided written informed consent. All RePORT study participants received standard 6-months treatment for TB, consisting of isoniazid, rifampicin, pyrazinamide, and ethambutol. Study participants were followed for the duration of TB treatment and up to 24 months post-enrolment. Enrolees found to have baseline drug resistance to any standard anti-TB drug (isoniazid, rifampicin, ethambutol, or pyrazinamide), who developed resistance during treatment, or who had concomitant extrapulmonary TB were excluded from the study.

Controls

TB symptomatic participants initially recruited for RePORT as GeneXpert positive, but subsequently found to be culture negative at baseline were included as Xpert-positive/culture-negative controls ($n = 14$) and were followed for 2 months from enrolment. An additional control group consisting of healthy asymptomatic individuals ($n = 23$) were recruited from the Collection of Urine, Blood, and Sputum (CUBS) cohort in Durban between January and December 2018. This group is referred to as healthy controls.

Descriptive Statistics

For TB cases and Xpert-positive/culture-negative controls age, sex, race, body mass index (BMI), self-reported alcohol use, tobacco use, and employment status were recorded. For persons

living with HIV (PLWH), CD4 cell counts, HIV-1 RNA (viral load), and timing of antiretroviral therapy were also collected. Extent of lung disease at enrolment was determined from CXR, evaluated by a radiologist blinded to all study participant information, using a CXR score [% area of lung infected + 40 for the presence of cavities; giving a scale of 0–140 (30)] see **Table S1**. Bacterial burden was estimated from liquid culture using the BD BACTEC MGIT 960 mycobacterial detection system (Becton Dickinson Microbiology Systems, Sparks, Md.) and full blood counts were measured independently by a commercial laboratory. For the healthy controls age, sex, and race were recorded.

Sample Collection, Processing, and Analysis

Samples

Blood samples were collected from healthy controls at one time-point only, and blood and sputum samples from TB cases were collected at baseline (pre-treatment) and at post-treatment initiation study visits: week 1, week 3, month 2, and month 6. For all participants, fresh blood samples were collected in EDTA vacutainers (BD Biosciences) for cell phenotyping.

Neutrophil Isolation and Staining

A 100 μl of whole blood was stained with 13 surface markers and incubated for 30 min at 4°C in the dark. The phenotyping panel comprised of standard lineage markers V500 conjugated mAb anti-CD45 (clone HI30), BUV496 conjugated mAb anti-CD16 (clone 3G8), BUV395 conjugated mAb anti-CD11b (clone ICRF44), V450 conjugated mAb anti-CD66b (clone G10F5) all from BD Bioscience; BUV785 conjugated mAb anti-CD3 (clone OKT3) and BV711 conjugated mAb anti-CD14 (clone M5E2) from Biolegend. The following phenotype markers were used, PE conjugated mAb anti-CD177 (clone MEM-166) and BV605 conjugated mAb anti-HLA-DR (clone G46-6) from BD Bioscience; FITC conjugated mAb anti-CD15 (clone HI98) and PE-Cy7 conjugated mAb anti-CXCR4 (clone 12G5) from Biolegend; APC conjugated mAb anti-CD32 (clone FLI8.28) and PE-Cy5 conjugated mAb anti-CD10 (clone HI10a) from BD Pharmigen and Live dead stain (ThermoFischer Scientific). Phenotype markers were chosen based on preliminary data from our lab indicating differential expression between TB cases and HIV-TB- controls. Red blood cells were removed, and leukocytes fixed by adding 2 mls of FacsLyse solution (BD Bioscience) and incubating for 10 min in the dark at room temperature. Samples were then centrifuged at 500 g for 5 min and the supernatant decanted. Cells were washed with 2 mls of 1X PBS (Sigma Aldrich) for 5 min at 500 g and the cell pellet resuspended in 2% PFA (Sigma Aldrich). Cells were acquired on a 17 color FACSAria Fusion (BD Bioscience), with calibrating beads run before each acquisition to ensure consistent performance. Due to the longitudinal nature of the study we normalized the data based on a published method by Upreti et al. (31). Data normalization was performed by transforming the Mean Fluorescence Intensity (MFI) values of the test samples (TB cases, Xpert-positive/culture-negative, and healthy controls) to a common scale using the following equation: Final relative fluorescence intensity (RFI) = MFI of the test sample/MFI of the internal control. Gating strategy shown in **Figure S1**.

TB Treatment Outcomes

We examined two TB treatment outcomes; (1) culture status on solid media 2 months after initiation of anti-TB therapy; and (2) end of TB treatment outcome, as defined by World Health Organization (WHO) into two mutually-exclusive categories—treatment success (cure or treatment completion) vs. unsuccessful outcome (death, failure, loss to follow-up, or not evaluated) (32). Cure was defined as completing treatment with a negative culture result at the end of treatment and on at least one previous occasion. Treatment completion was based on completion of standard anti-TB therapy without evidence of failure, and without documentation of a negative culture in the last month of TB treatment and/or on at least one previous occasion (either because it was not done, or results are unavailable). Death included mortality from any cause during TB treatment. Treatment failure was defined as a positive culture at month 5 of treatment or later. Loss to follow-up was any participant who interrupted treatment for 2 consecutive months or more. Not evaluated included any participant for whom treatment outcome was unassigned, including cases who transferred out of care or whom treatment outcome was unknown.

In addition, for exploratory analyses, we categorized TB cases into four groups based on time to culture negativity. Individuals who were culture negative within 1 month of treatment initiation were defined as early responders, those who converted to culture-negative by 2 months as medium responders, those who converted to culture-negative at 6 months were slow responders, and treatment failures were those who remained culture positive during the full 6 months of treatment.

Statistical Analysis

Baseline characteristics were compared between TB cases, Xpert-positive/culture-negative, and healthy controls using Chi-square or Fisher's exact test for categorical variables and Kruskal-Wallis test for continuous variable. Correlations between CXR score, bacterial burden, blood counts, neutrophil phenotype, and demographic data were determined using the Mann-Whitney test and Spearman's correlation analysis. Differences in distributions and median values of neutrophil blood counts and CD15 expression were compared between cases and controls and also examined for changes from baseline during the course of TB treatment using the Wilcoxon rank sum test and Kruskal-Wallis test followed by Dunn's multiple comparison test. We graphically evaluated the impact of pre-treatment immune measures on patient response to treatment, defined by early, medium, slow and treatment failure responses. Additionally, we examined the association between baseline immune markers and culture status at month 2 (positive vs. negative) and end of treatment outcome (successful vs. unsuccessful treatment) using univariable and HIV-adjusted logistic regression. Confounders, including age, sex, baseline BMI, and HIV were considered *a priori* based on clinical expertise. Due to a limited number of outcome events, adjusted models controlled only for HIV co-infection, as it was

believed to be most clinically relevant confounder. We also examined the association between the aforementioned exposures with treatment failure/death and with loss to follow-up/not evaluated. The referent group for these analyses was successful outcome (i.e., in analyses with the outcome of failure/death), persons who were lost to follow-up/not evaluated were excluded, and in analysis with the outcome of lost to follow-up/not evaluated, persons with failure/death were excluded. Statistical analysis was performed using StataIC, version 16 (StataCorp, College Station, TX, USA) and GraphPad PRISM, version 8.3.1 (GraphPad Software, San Diego, CA, USA).

RESULTS

The characteristics of the study population are listed in **Table 1**. Of note, HIV co-infection was 63%, the majority of whom had not started antiretroviral therapy (ART) at baseline (67%). As expected, smoking was significantly higher in the TB subjects (22%) compared to healthy controls (4%). HIV co-infected individuals had significantly lower CXR scores, primarily due to fewer cavities ($P = 0.0028$); they also had lower bacterial burden than HIV uninfected individuals (median HIV- = 34.54 and HIV+ = 31.88; $P = 0.0083$) (**Figure S2A**). Consistent with these results, we observed a weak correlation between CD4 count and CXR score ($r = 0.2370$; $P = 0.0268$) and bacterial load ($r = 0.2478$; $P = 0.0143$, **Figures S2D,E**).

Neutrophil Blood Count and CD15 Expression Level Are Altered at Baseline and Associated With Disease Severity

Full blood counts were measured at baseline and during the course of treatment. Neutrophil count and neutrophil/lymphocyte ratio (NLR) were significantly higher in TB cases at diagnosis compared to healthy controls (HC) (**Figures 1A,B**). Interestingly, symptomatic Xpert-positive/culture-negative controls also had slightly higher neutrophil counts and NLR compared to healthy controls, but this did not reach statistical significance. Of the surface markers measured in this study, CD15 expression level showed the most consistent difference between TB cases and controls at baseline. As shown in **Figure 1C**, surface expression of CD15, a canonical neutrophil marker, was found to be highly down regulated at baseline in TB patients compared to healthy controls ($P < 0.0001$). In addition, TB symptomatic Xpert-positive/culture-negative controls displayed significantly higher CD15 expression levels than active TB cases ($P = 0.01$).

To explore the relationship between baseline neutrophils and disease severity, we performed univariate correlations with sputum bacterial load and CXR score, clinically important measures of TB disease severity. Despite the relatively small sample size, we observed significant correlations between neutrophil count and both bacterial load ($r = 0.25$, $P = 0.034$; **Figure 1D**) and CXR score ($r = 0.41$, $P = 0.0004$; **Figure 1E**), and CD15 expression and bacterial load ($r = -0.3187$; $P = 0.01$; **Figure 1F**). Together these data support the existence of a relationship between the blood neutrophil response to TB and disease pathogenesis in the lung, as suggested by several recent publications (6, 33, 34). Finally, we found CD15 expression level was inversely correlated with neutrophil count ($r = -0.5222$, $P < 0.0001$; **Figure 1H**) but had no correlation with CXR score (**Figure 1G**). Importantly these measurements were taken independently by our in-house flow cytometry assay and by an accredited commercial laboratory, thereby adding support to the observed phenotypic changes.

Neutrophil Count and CD15 Expression Resolved With TB Treatment

Neutrophil characteristics were examined longitudinally to determine how they respond to TB drug treatment. Baseline neutrophil count and NLR both significantly improved within the 1st month of TB treatment, and reached the level seen in healthy controls by month 6 (**Figures 2A,B**), irrespective of HIV status (**Figures 3A,B**). Additionally, surface expression of CD15 increased during TB treatment and expression level was no longer significantly different from healthy controls by 2 months of treatment (**Figure 2C** and **Figure S1**). CD15 expression was already elevated by week 1 (for which complete blood counts were not available). This supports our hypothesis that rapid turnover of blood neutrophils makes them extremely sensitive to changes in the lung environment. This is also supported by the fact that, following 3 weeks of drug treatment, neutrophil count and CD15 expression remain correlated with falling sputum bacterial load ($r = 0.3929$, $P = 0.0006$ and $r = -0.3498$, $P = 0.0046$, respectively; median bacterial load baseline vs. month 1 = 33 vs. 21 $P < 0.0001$), and each other ($r = -0.5751$, $P < 0.0001$) (**Figures S4A–C**). As with neutrophil count, changes in CD15 expression were not affected by HIV co-infection (**Figure S3C**). Interestingly, no changes in neutrophil count, NLR and CD15 expression were detected among the Xpert positive/culture negative controls following 2 months of treatment with anti-TB drugs (**Figures 2D–F**).

Pre-treatment Characteristics Are Associated With Rate of Culture Conversion

Given the correlation between neutrophil characteristics and clinical measures of disease severity at baseline, we sought to determine whether the baseline neutrophil characteristics, bacterial load, or chest X-ray changes correlated with treatment response kinetics. Initially, participants were grouped as being early, medium or slow responders as well as treatment failure, based on achieving and maintaining TB culture negative status at month 1, month 2, month 6, or never, respectively. As shown in **Figure 3**, there is a trend for individuals with a lower blood neutrophil counts and higher CD15 surface expression at baseline to become culture negative early compared to those who culture converted later or not at all (**Figures 3A,B**). These differences did not reach statistical significance by univariate analysis. Bacterial load at baseline, however, was significantly associated with treatment response, with early responders having significantly lower bacterial burden than all others (**Figure 3C**).

TABLE 1 | Baseline clinical and demographic characteristics of culture positive, drug susceptible TB cases; Xpert-positive/culture-negative controls and healthy controls.

Characteristics *N* (%) or Median [IQR]	TB cases ($n = 79$)	Xpert-positive/culture-negative controls ($n = 15$)	Healthy controls (HC) ($n = 23$)	*P*-value[§]
Age	34 (7–43)	37 (30–40)	32 (25–38)	0.37
Male sex	53 (67)	9 (60)	12 (52)	0.41
HIV status (positive)	50 (63)	11 (73)	0 (0)	**<0.01***
On antiretroviral therapy	18 (23)	6 (40)	-	0.16
CD4 count	350 [156–608]	386 [111–536]	-	0.87
Current smoker	17 (22)	4 (27)	1 (4)	**<0.01***
BMI (kg/m^2)	20.7 [18.6–22.8]	20.2 [19.0–22.4]	-	0.90
Employed	35 (44)	7 (47)	-	0.87
Previous TB	16 (20)	5 (33)	-	0.26
Bacterial load (MGIT[a])	32.8 [28.7–35.3]	-	-	-
Cavitation present	55 (74)	3 (25)	-	**<0.01***
Chest-X ray score (CXR)	70 (35–80)	8.5 [3.5–56]	-	**0.02***

[§]*P-values determined by Fisher's exact, chi square, or Wilcoxon rank sum tests.*
[*]$P < 0.05$.
[a]*MGIT, Mycobacteria Growth Indicator Tube.*
Values are bold to highlight statistical significance compared to the other values.

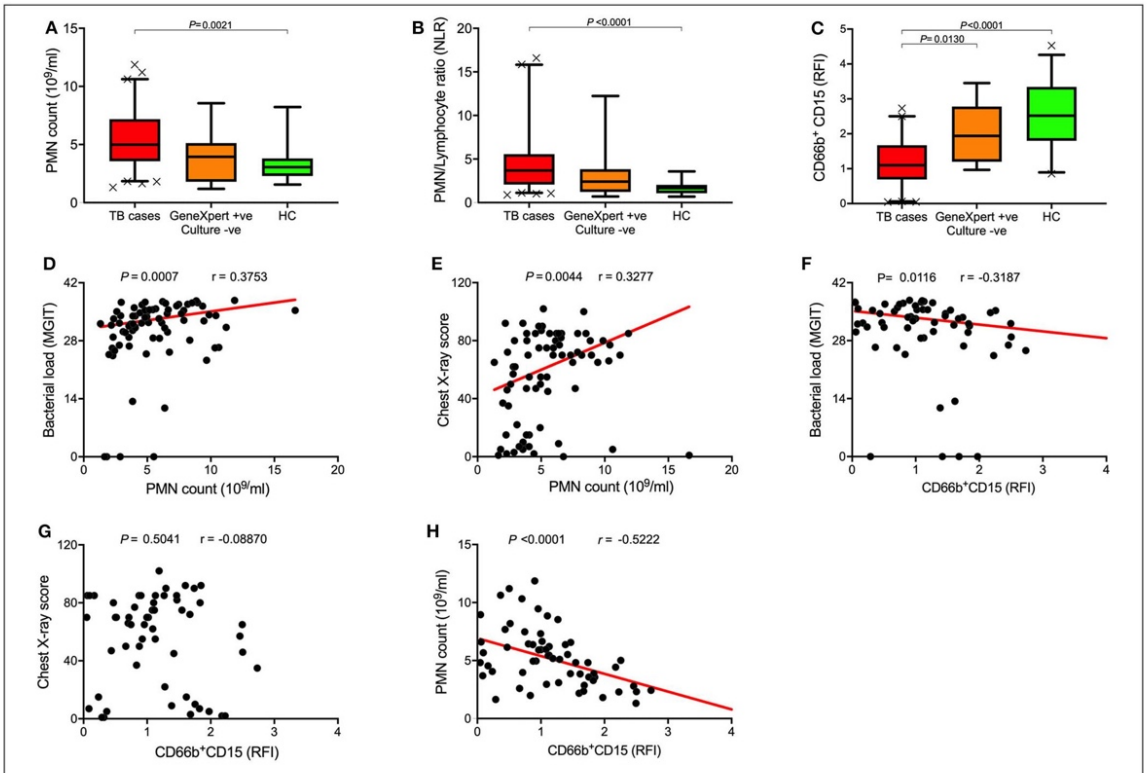

FIGURE 1 | Baseline neutrophil blood counts and phenotypic changes in active TB disease. **(A–C)** Box plots of **(A)** baseline neutrophil blood count (PMN count), **(B)** PMN/Lymphocyte ratios, and **(C)** Surface CD15 expression levels of TB cases ($n = 79$), Xpert-positive/culture-negative controls ($n = 15$), and healthy controls (HC; $n = 23$), Data represents median values and was analyzed using Kruskal-Wallis test for Dunn's multiple comparisons. **(D–H)** Spearman's rank correlation analysis of neutrophil blood counts and CD15 expression with bacterial burden and chest X-ray score.

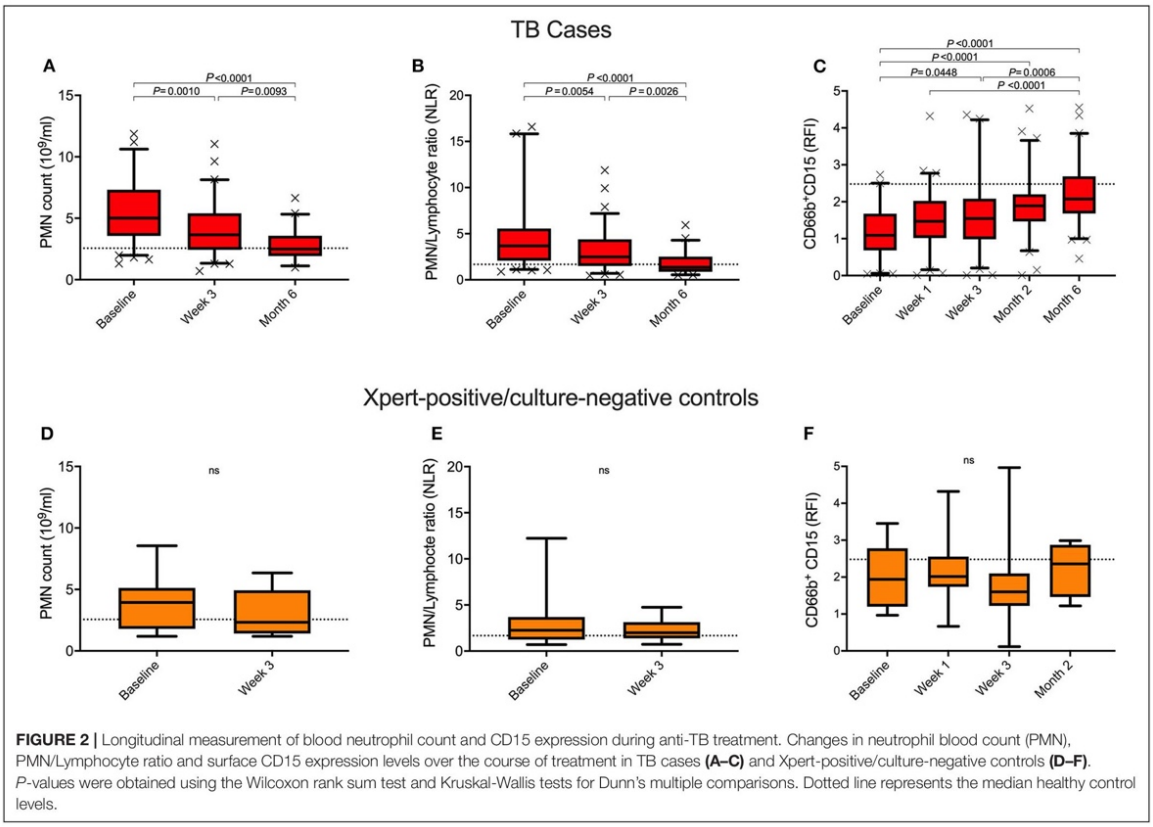

FIGURE 2 | Longitudinal measurement of blood neutrophil count and CD15 expression during anti-TB treatment. Changes in neutrophil blood count (PMN), PMN/Lymphocyte ratio and surface CD15 expression levels over the course of treatment in TB cases **(A–C)** and Xpert-positive/culture-negative controls **(D–F)**. *P*-values were obtained using the Wilcoxon rank sum test and Kruskal-Wallis tests for Dunn's multiple comparisons. Dotted line represents the median healthy control levels.

CXR score, on the other hand, was not predictive of treatment response (**Figure 3D**).

Next, we examined the association between baseline neutrophil characteristics, CXR score, and bacterial load with sputum culture conversion at 2 months as an endpoint, a clinically relevant measure of TB treatment response (35). For this analysis, we excluded four participants whose baseline culture was positive on liquid media, but not solid media, and 8 participants who were missing culture results at month 2. Among 67 remaining participants with a solid culture at baseline, 7 (10%) remained culture positive after 2 months of anti-TB treatment. Results of this analysis are shown in **Table 2**. Interestingly, in this reduced dataset, the only baseline characteristic associated with remaining culture positive at month 2 was CD15 expression level (Odds Ratio (OR): 0.17, 95% Confidence Interval (CI): 0.03, 0.97). In other words, for every unit increase in baseline CD15, the odds of remaining culture positive after 2 months of TB treatment was reduced by 83%. After adjusting for HIV-status, the relationship appears slightly stronger (adjusted OR: 0.14, 95% CI: 0.02, 0.89).

TB Treatment Outcome

We also examined the association between bacterial load, CXR score, neutrophil count, and CD15 with TB treatment outcome, defined according to the WHO definitions. Among 75 TB cases who were positive for Mtb on solid culture, 53 (71%) were cured, 2 (3%) completed treatment, 2 (3%) failed treatment), 2 died (3%), 8 (11%) were lost to follow-up (LTF), and 8 (11%) were not evaluated (NE). Combining death, failure, loss to follow-up, and not evaluated, the unsuccessful outcome rate was 27%. However, none of baseline bacterial load, CXR score, neutrophil count, or CD15 were associated with TB treatment outcomes (**Table 2**).

DISCUSSION

In this study we investigated changes in the frequency and phenotype of blood neutrophils associated with active TB at diagnosis and patient response to standard TB drug therapy. Overall, we find that active TB is associated with both an increase in neutrophil count and down-regulation of the canonical neutrophil marker CD15, irrespective of HIV co-infection. In addition, changes in neutrophil count and CD15 expression correlate with clinical markers of disease severity—sputum bacterial load, as measured by time to positivity in liquid culture, and extent of lung involvement, as determined by an independent radiology examination of the CXR. This suggests a potential direct relationship between the blood neutrophil response and the severity of lung disease. As neutrophils are short-lived and

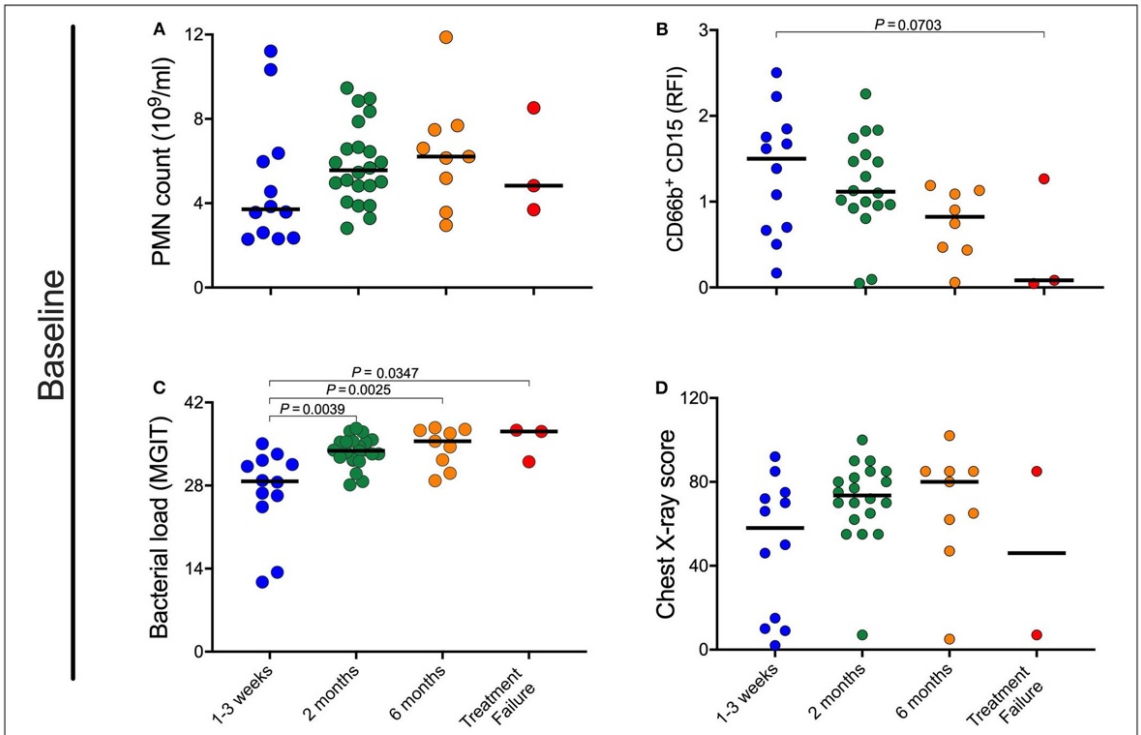

FIGURE 3 | Impact of pre-treatment characteristics on rate of culture conversion. **(A–D)** Baseline neutrophil blood (PMN) count, CD15 expression, bacterial load, and chest X-ray scores were retrospectively separated into four groups based on time to MGIT culture negativity. Early responders became and remained culture negative within the 1st month, medium responders by 2 months, and slow responders by month 6 post-treatment initiation. In this analysis treatment failure are individuals who were culture positive at all time points. *P*-values were obtained using the Mann-Whitney test.

rapidly recruited to the site of infection, we hypothesized that any blood neutrophil signature would respond rapidly to clearance of the bacteria. Our data support that hypothesis, as both neutrophil count and CD15 expression level significantly increased by month 2. Moreover, CD15 expression, which was measured more frequently, was higher after only 1 week of drug treatment. The fact that the correlation between neutrophils and bacterial load is maintained after 1 month of treatment, when bacterial load has greatly reduced, also highlights the direct relationship between the blood neutrophil response and the kinetics of the response to drug therapy. Finally, in a multivariate analysis, baseline CD15 expression level was the only variable that predicted sputum culture conversion at 2 months.

The potential role of neutrophils in the immune response to TB is somewhat conflicting and has been well-reviewed elsewhere (36, 37). However, targeting these cells as potential biomarkers for diagnosing active TB disease and monitoring treatment outcomes has been less well-studied (38, 39). Abakay et al. found that an elevated neutrophil count and NLR were both associated with advanced TB disease; with increased NLR, in particular, associated with the most severe lung involvement (40). Disease severity was estimated from CXR and thus our findings are comparable, although in our study, NLR was less informative than neutrophil count alone or CD15 expression level. Furthermore, our data are consistent with clinical studies and experimental infections showing that acute pulmonary tuberculosis is accompanied by an influx of neutrophils (26, 41). In mice, for example, neutrophil influx is associated with mycobacterial burden and lung pathology, and their depletion in chronic infection leads to decreased CFU and improved survival (26, 42–44). It is important to note, however, that the influx of neutrophils is not unique to TB disease and has been well-described in other inflammatory conditions such as asthma, influenza and bacterial or viral pneumonia (45–47). Therefore, neutrophil characteristics alone would not be appropriate as a diagnostic test. None the less, studies have shown the usefulness of neutrophil phenotyping in distinguishing bacterial infection from other inflammatory conditions (48, 49). Therefore, together with other diagnostic tests, neutrophil monitoring could be valuable as a rule our test and for identifying patients at base line with the most severe disease. Such metrics could prove useful for testing treatment shortening interventions, for example.

Unlike other immune subsets, it is not possible to freeze neutrophils for future analysis. Consequently, few studies have

TABLE 2 | Crude and HIV-adjusted effect measures for association between baseline bacterial load, chest x-ray score, neutrophil (PMN) count, and CD15 with TB treatment outcomes (M2 culture conversion, unsuccessful outcome, death/failure, and losses to follow-up/not evaluated), using solid culture.

Exposure	Outcome	Events/*N* (%)[a]	Unadjusted OR 95% CI)	HIV adjusted OR (95% CI)
Bacterial load	M2 culture positive	7/67 (10%)	0.98 (0.90, 1.07)	0.98 (0.90, 1.06)
	Unsuccessful treatment	20/75 (27%)	0.99 (0.93, 1.06)	0.99 (0.93, 1.06)
	Death/failure	4/59 (7%)	0.98 (0.87, 1.10)	0.98 (0.85, 1.13)
	LTF/NE	16/71 (23%)	0.99 (0.92, 1.07)	0.99 (0.92, 1.06)
CXR score[b]	M2 culture positive	6/64 (9%)	1.02 (0.98, 1.06)	1.03 (0.98, 1.07)
	Unsuccessful treatment	18/71 (23%)	1.00 (0.98, 1.02)	1.00 (0.98, 1.02)
	Death/failure	3/56 (5%)	0.99 (0.96, 1.03)	1.00 (0.96, 1.04)
	LTF/NE	15/68 (22%)	1.00 (0.98, 1.02)	1.00 (0.98, 1.02)
PMN count[c]	M2 culture positive	7/67 (10%)	1.06 (0.82, 1.37)	1.06 (0.81, 1.37)
	Unsuccessful treatment	19/74 (26%)	0.90 (0.73, 1.11)	0.90 (0.73, 1.11)
	Death/failure	3/58 (5%)	0.68 (0.35, 1.30)	0.79 (0.44, 1.40)
	LTF/NE	16/71 (23%)	0.94 (0.76, 1.15)	0.92 (0.74, 1.15)
CD15[d]	M2 culture positive	6/56 (11%)	**0.17 (0.03, 0.97)**	**0.14 (0.02, 0.89)**
	Unsuccessful treatment	10/58 (17%)	0.57 (0.21, 1.57)	0.58 (0.21, 1.58)
	Death/failure	3/51 (6%)	0.54 (0.09, 3.21)	0.68 (0.15, 3.09)
	LTF/NE	7/55 (13%)	0.58 (0.18, 1.87)	0.57 (0.17, 1.89)

[a] *Complete case analysis. The referent group for M2 culture positive is M2 culture negative (n = 8 people missing culture results at M2 were excluded). The referent group for unsuccessful outcome, death/failure, and loss to follow-up/not evaluated was successful outcome. In analyses with the outcome of failure/death, persons who were lost to follow-up/not evaluated were excluded, and in analysis with the outcome of lost to follow-up/not evaluated, persons who with failure/death were excluded.*

[b] *Missing: n = 4.*

[c] *Missing: n = 1.*

[d] *Missing: n = 17.*

Values are bold to highlight statistical significance compared to the other values.

examined neutrophil phenotypic changes in human TB cohorts. The phenotyping of blood neutrophils by flow cytometry in this study was undertaken with the aim of potentially identifying sensitive markers of the neutrophil response. Initially, this included eight neutrophil surface markers based on preliminary data from our lab showing differential expression between healthy individuals and TB cases. However, in this longitudinal study, only CD15 expression showed a consistent response during the course of TB treatment. CD15 is a carbohydrate antigen associated with cell surface of glycoproteins or glycolipids, including CD11b/CD18 (Mac-1) and CR3 β-chain (50, 51). It is highly expressed by mature neutrophils (52) and has been shown to affect a number of neutrophil functions including adhesion to endothelium, phagocytosis, degranulation, and the respiratory burst (50, 53–55). However, there are very few studies that have examined CD15 expression level *ex vivo*. CD15 expression is elevated on low density neutrophils (56), which can arise from activation both *in vitro* and *in vivo* (57). Conversely, expression of CD15 was found to dramatically decreases in asthmatic patients following acute allergen challenge (58), suggesting that activation *per se* does not elevate CD15 in general. Interestingly, treatment of healthy donors with Granulocyte-Colony Stimulating Factor (G-CSF) to induce neutrophil release from the bone marrow (59, 60), also leads to decreased CD15 expression on blood neutrophils (61). Therefore, both the elevated blood neutrophil numbers and low CD15 expression observed in active TB may relate to sustained recruitment of neutrophils from the bone marrow in response to cytokine release from Mtb infection. As neutrophils mature in the bone marrow they upregulate CD15 (62, 63), implying that the CD15 low neutrophils we observed in blood are not fully mature. In addition, when G-CSF treatment was ceased in the above studies, CD15 expression level in returned to normal, consistent with the rapid response kinetics observed in our study. Whether the influx of CD15 low neutrophils observed in TB directly relates to G-CSF, alone or in combination with other neutrophil related cytokines, is an interesting area for future study and might lead to alternative and potentially more sensitive measures of on-going lung involvement.

In the analysis we included individuals who were TB symptomatic and GeneXpert positive at diagnosis, but turned out to be culture negative. Discordant gene-expert and culture results have previously been linked, at least in part, to residual DNA in the lung from killed bacteria (64), poor sputum quality and low bacterial load as determined by CT cycle number (65). One third of these subjects reported previous TB episodes, slightly higher than the culture positive group (20%), but detail on sputum quality and GeneXpert quantitation data was not recorded. Nonetheless, the neutrophil data on this group were interesting and potentially informative. First, neutrophil counts and CD15 expression in this group were not significantly higher than healthy controls, and, second, neither neutrophil count nor CD15 expression level were changed by 2 months of TB treatment. This suggests that monitoring blood neutrophils during TB screening could help to inform classification and management of discordant subjects. In addition, to identify novel

prognostic factors, we sought to determine whether neutrophil characteristics at TB diagnosis were predictive of response to treatment. We found that baseline CD15 expression level was predictive of culture positivity at month 2, suggesting potential value in helping triage subjects into those likely to respond well to treatment. In the univariate analysis, sputum bacterial load also appeared to distinguish subjects who became culture negative rapidly compared to those with a slower response to treatment, consistent with other studies (66, 67). However, neutrophil phenotype can be determined much faster than sputum bacterial load, and thus could be more relevant in the clinical setting.

There were some limitations of this study. First, sample size was relatively small. However, it was sufficiently large to detect significant correlations between neutrophil count, CD15 expression level, and TB treatment outcomes. Second, study participants were from one city in South Africa, and may not be generalizable to other settings. However, there was a high proportion of HIV-positive participants, and we were able to demonstrate that findings did not differ according to HIV status. Further work is planned to further explore the potential utility of neutrophil signatures in larger cohorts and with additional measures of neutrophil activity and neutrophil related cytokines.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Biomedical Research Ethics Committee of the University of Kwa-Zulu Natal. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AL and LN conceived, designed the experiments, and wrote the paper. AP, TS, FK, and M-YM designed and managed the cohort. FK and MM enrolled patients and collected samples and data. KK and SM supervised laboratory collection of clinical samples and analysis. LN, SM, SN, and AN performed the experiments. TS, YH, FM, and LP made intellectual contributions. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01872/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2 Supplementary figures

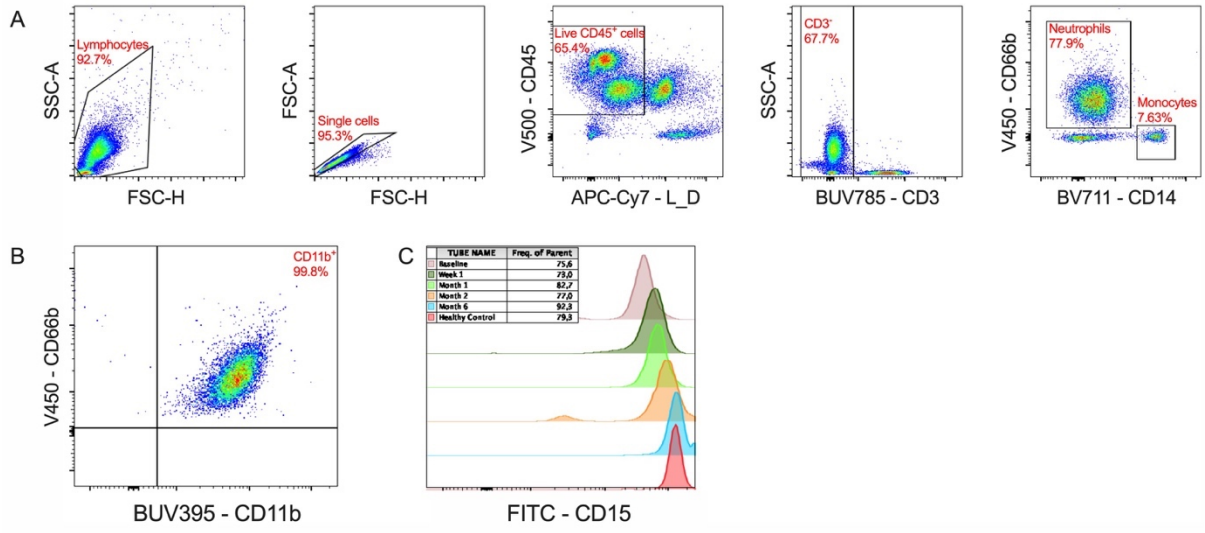


Figure S1. FASC analysis of neutrophils. Neutrophils were stained for flow cytometry and analyzed on the FACSAria Fusion. Figure **A** represents that standard gating strategy with neutrophils identified as $CD66b^{+}$ cells. The numbers are percentages of cells falling in each gate. **(B)** All neutrophils are $CD11b^{+}$. **(C)** Histograms representing change in CD15 MFI observed in TB cases from baseline/pre-treatment to treatment completion compared to healthy controls (HC).

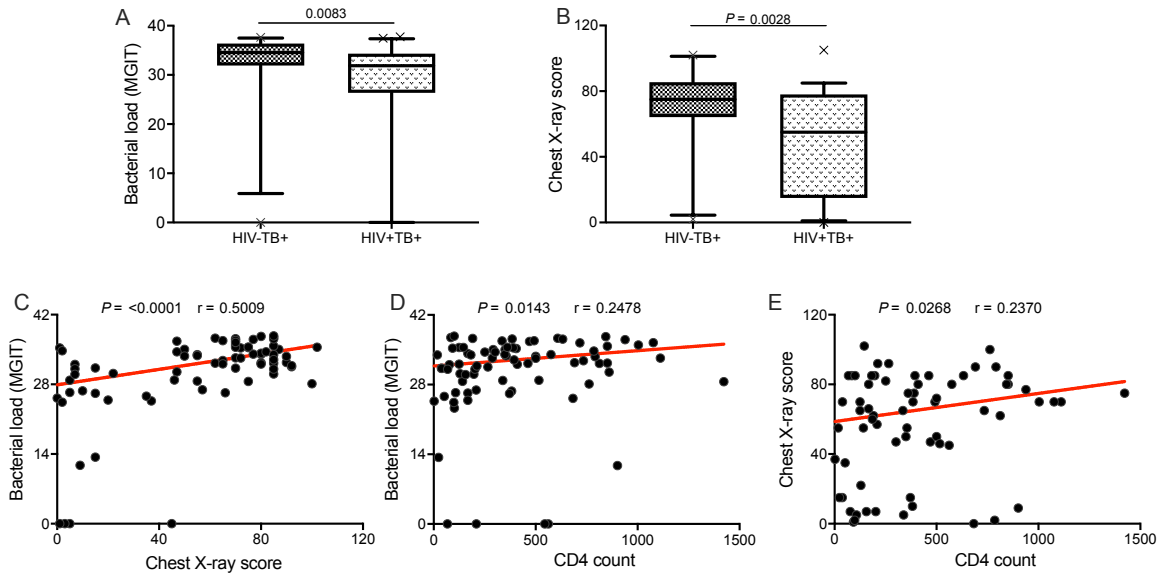


Figure S2: Effect of HIV status on baseline bacterial load and Chest X-ray scores. Box plots of **(A)** baseline bacterial load and **(B)** CXR scores among HIV negative and positive participants. Data represents median values and was analysed using Mann-Whitney t-test. **(C-E)** Spearman's rank correlation analysis of CD4 T cell counts with bacterial burden and CXR scores.

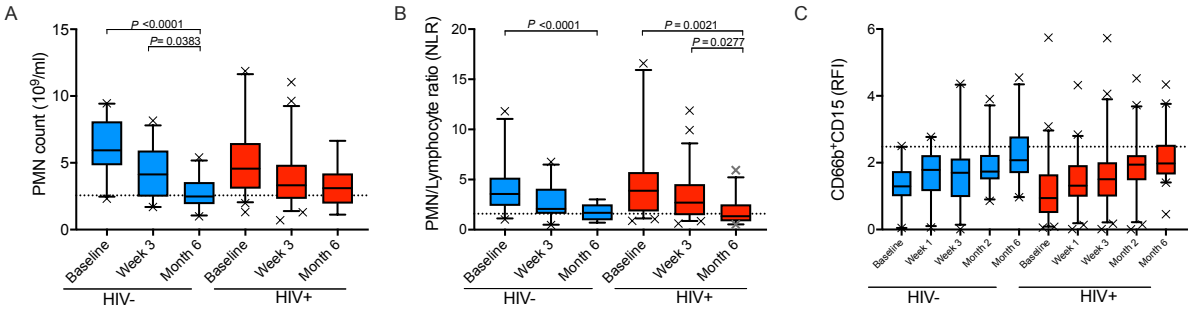


Figure S3: Longitudinal measurement of blood neutrophil count, NLR ratio and CD15 expression during anti-TB treatment. Changes in neutrophil blood (PMN) count **(A),** PMN/Lymphocyte ratio **(B)** and surface CD15 expression **(C)** levels over the course of treatment in TB cases stratified by HIV infection. P-values were obtained using the Wilcoxon rank sum test and Kruskal-Wallis tests for multiple comparisons. Dotted line represents the median healthy control levels.

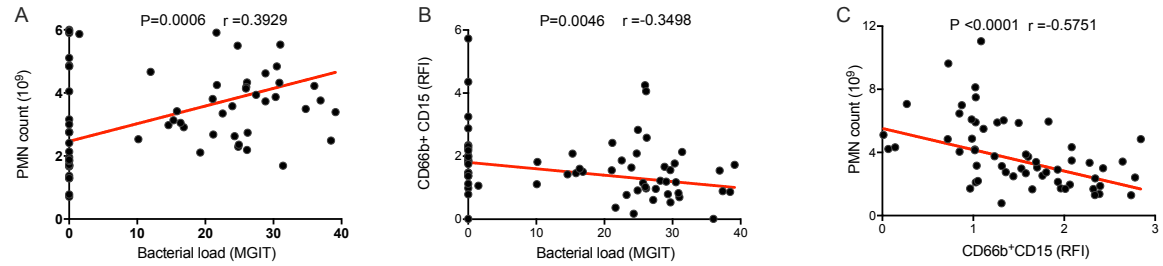


Figure S4: Neutrophil blood counts and CD15 expression correlate with bacterial burden at week 3 post-treatment initiation. Spearman's rank correlation analysis of neutrophil blood (PMN) counts and CD15 expression with bacterial burden **(A-C).**

3.3 Additional results and discussion

Active TB disease is associated with changes in monocyte counts, MLR, immature PMN count and platelet counts

In addition to the neutrophil blood counts and NLR, analysis of full blood counts, not included in the above manuscript (**Figure 4A-F**) revealed that baseline monocyte counts, MLR, immature PMN count and platelet counts were significantly elevated in TB cases compared to healthy controls. Monocyte counts and MLR in TB have been widely reported and evaluated as a TB biomarker (Wang et al. 2015; Choudhary et al. 2019; Wang et al. 2019). Elevated MLR distinguished ATB patients from LTBI and healthy controls (La Manna et al. 2017), is significantly reduced following treatment with anti-TB drugs (La Manna et al. 2017; Wang et al. 2015) and can be associated with risk of TB incidence (Naranbhai et al. 2013). Monocytes counts alone have been arguably less useful as a diagnostic biomarker, however, our data suggest the potential use of monocyte counts in distinguishing active TB cases from individuals who are referred to the TB clinic but have no evidence of an active TB infection. Furthermore, our findings show monocyte counts to be the only measure that significantly correlates with both bacterial load (r=0.4134, P=0.0003; **Figure 5A**) and extent lung involvement (r=0.3276, P=0.0064; **Figure 5E**).

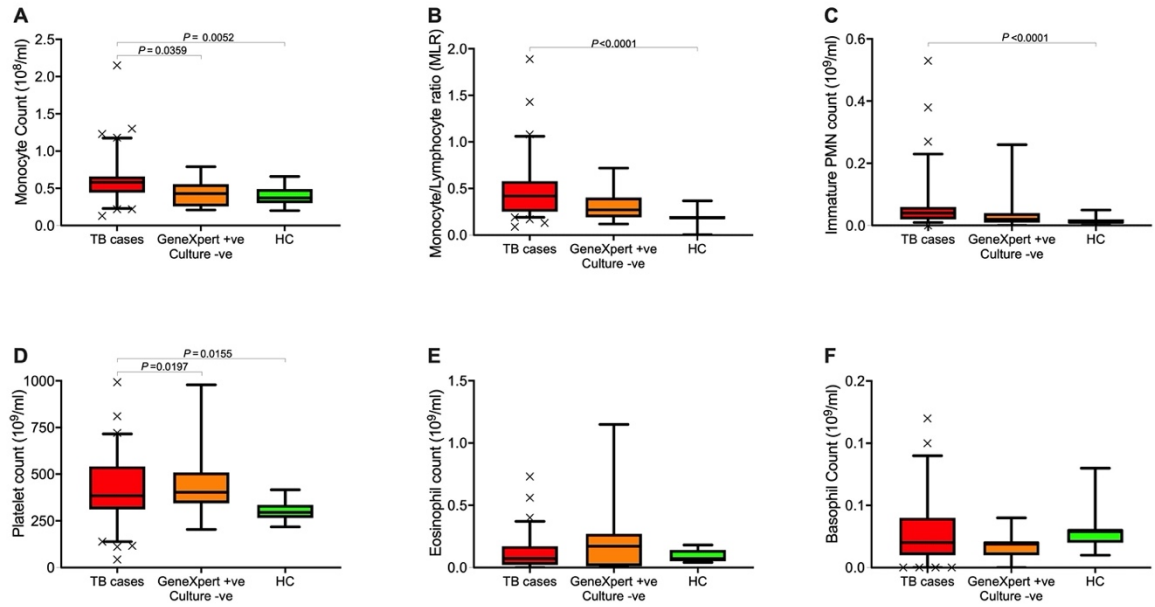


Figure 4: Baseline white blood cells counts in active TB disease. Box plots of (A) Monocyte counts, (B) Monocyte/Lymphocyte ratio, (C) Immature granulocyte count, (D) Platelet count, (E) Eosinophil

count and (F) Basophil count of TB cases (n=), Xpert-positive/culture-negative controls (n = 15), and healthy controls (HC; n = 23), Data represents median values and was analyzed using Kruskal-Wallis test for Dunn's multiple comparisons.

Immature PMN count was also significantly elevated in TB cases compared to healthy controls (P<0.001; **Figure 4C**), this is most likely reflective of emergency granulopoiesis initiated by active TB disease (Orr et al. 2005; Kolaczkowska and Kubes 2013). However, unlike the neutrophil counts, it did not correlate to bacterial burden or extent of lung involvement. Platelets have received increased attention over the years for their potential role in TB disease (Cox and Keane 2018). Most notably, significantly elevated platelet counts were observed in ATB compared to non-TB controls which decreased following treatment (Tozkoparan et al. 2007). In addition, higher platelet counts were found to be significantly associated with increased CXR score and has been implicated as a potential driver of chronic inflammation (Fox et al. 2018). These observations are in line with our findings of elevated counts in TB cases compared to healthy controls (P=0.0155; **Figure 4D**) and Xpert-positive/culture-negative controls (P=0.0197; **Figure 4D**). A significant but weak correlations was also observed between platelet counts and bacterial load (r=0.2964, P=0.0180; **Figure 5D**). These findings, though interesting, were not included in the paper as they did not provide information which superseded that observed in neutrophil counts and NLR. Furthermore, neutrophil responses were the focus of my research. Nonetheless, there is mounting evidence pointing to the potential usefulness of FBCs as potential markers of Mtb infection and disease severity which may provide potential avenues for future work.

Baseline neutrophil CD10, CD11b and CD32 expression levels are altered in active TB disease

Using flow cytometry, neutrophils were phenotyped using 8 surface markers chosen based on preliminary data from our lab showing differential expression between TB cases and healthy controls. In this study, CD15 expression data was the most informative and was therefore included in the manuscript. However, several of the other phenotypic markers analysed were also differentially expressed between ATB cases and controls. **Figure 6** shows baseline CD10 and CD32 expression levels

to be significantly down-regulated (P=0.0432 and P<0.0001 respectively; **Figure 6A,C**) whilst CD11b expression is up-regulated (P= 0.0475; **Figure 6B**) in ATB compared to healthy controls. Surprisingly, no detectable changes were observed in CD177 (**Figure 6E**), CD16 (**Figure 6D**) and CXCR4 (**Figure 6F)** expression levels in active TB despite significant roles in neutrophil activation and mobilization.

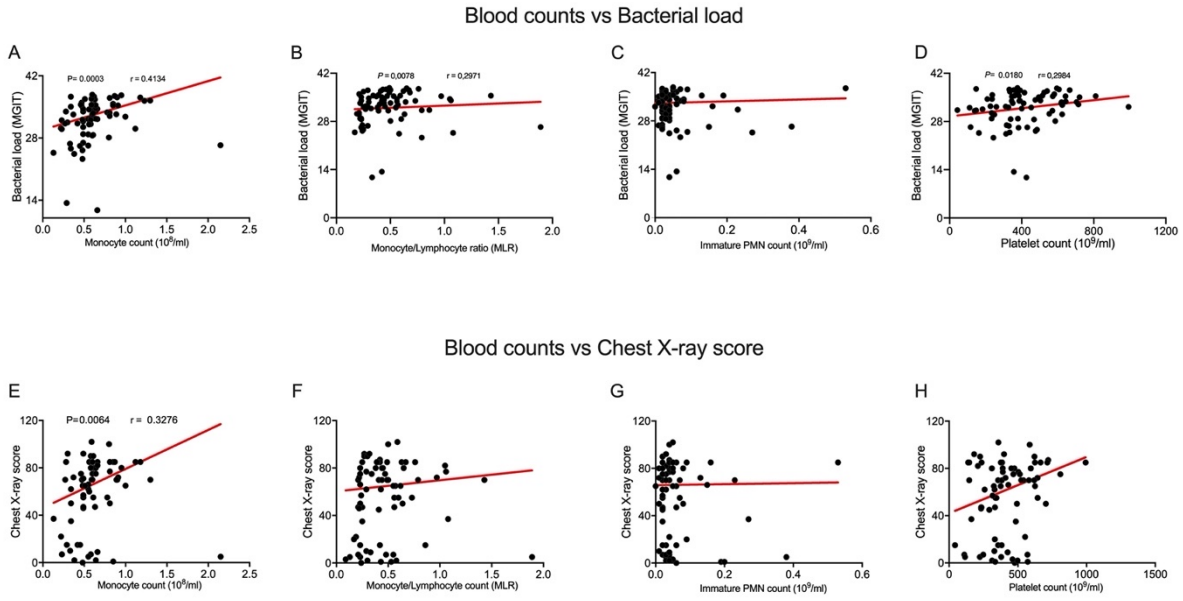


Figure 5: Baseline correlation of blood counts with clinical markers of disease severity. Spearman's rank correlation analysis of monocyte counts, MLR, Immature PMN count and platelet count with bacterial burden **(A-D)** and chest X-ray score **(E-H)**. R values between 0.7-1 are considered strong; 0,5-0.7 medium; 0.3-0.5 weak and 0-0.3 not correlated.

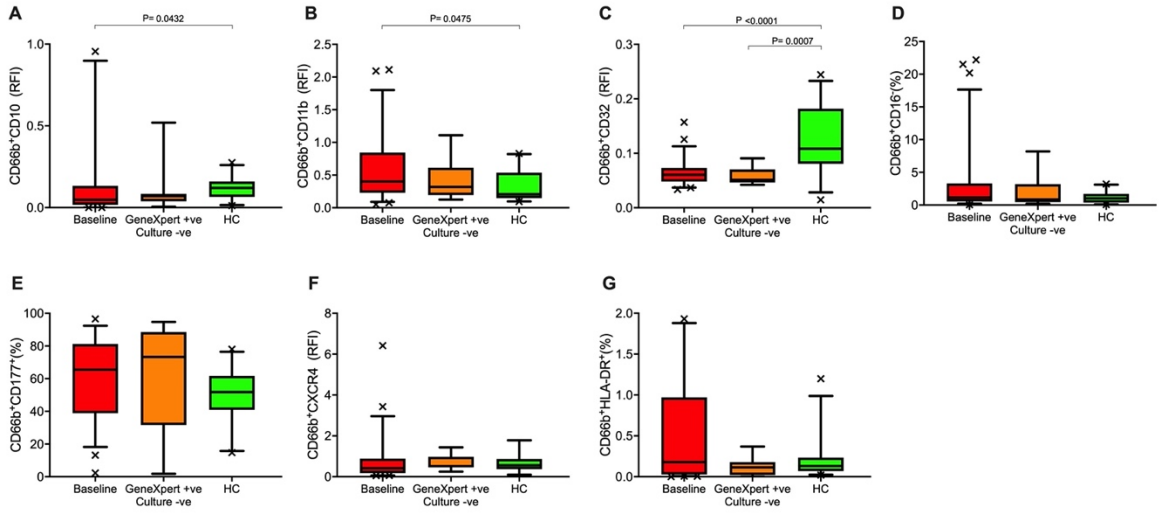


Figure 6: Changes in blood neutrophil surface marker expression in active TB disease. Box plots of CD66b+ neutrophils expressing (**A**) CD10, (**B**) CD11b, (**C**) CD32, (**D**) CD16, (**E**) CD177, (**F**) CXCR4 and (**G**) HLA-DR from TB cases (n=52), Xpert-positive/culture-negative controls (n = 15), and healthy controls (HC; n = 23), Data represents median values and was analysed using Kruskal-Wallis test for Dunn's multiple comparisons.

Immature neutrophils express a $CD10^{-}/CD10^{low}$ phenotype. Reduced expression in ATB point to an influx of immature neutrophils as a result of chronic inflammation. This is supported by the increase in immature granulocytes (**Figure 4C**), neutrophil counts and reduced CD15 expression (**Figure 1A,C**). In addition, recent published works by Marini et al, involving G-CSF treated stem cell donors and cancer patients further highlights this relationship. In the study, G-CSF, a potent neutrophil growth factor, treatment resulted in an increased of immature $CD66b^{+}CD10^{-}$ neutrophils which predominantly fell within the LDNs fraction during gradient centrifugation (Marini et al. 2017). CD32 is an FcγR that is up-regulated in disease and involved in neutrophil activation, directs killing of opsonized target cells and triggers the release of inflammatory mediators (Anania et al. 2019; Skilbeck et al. 2006).

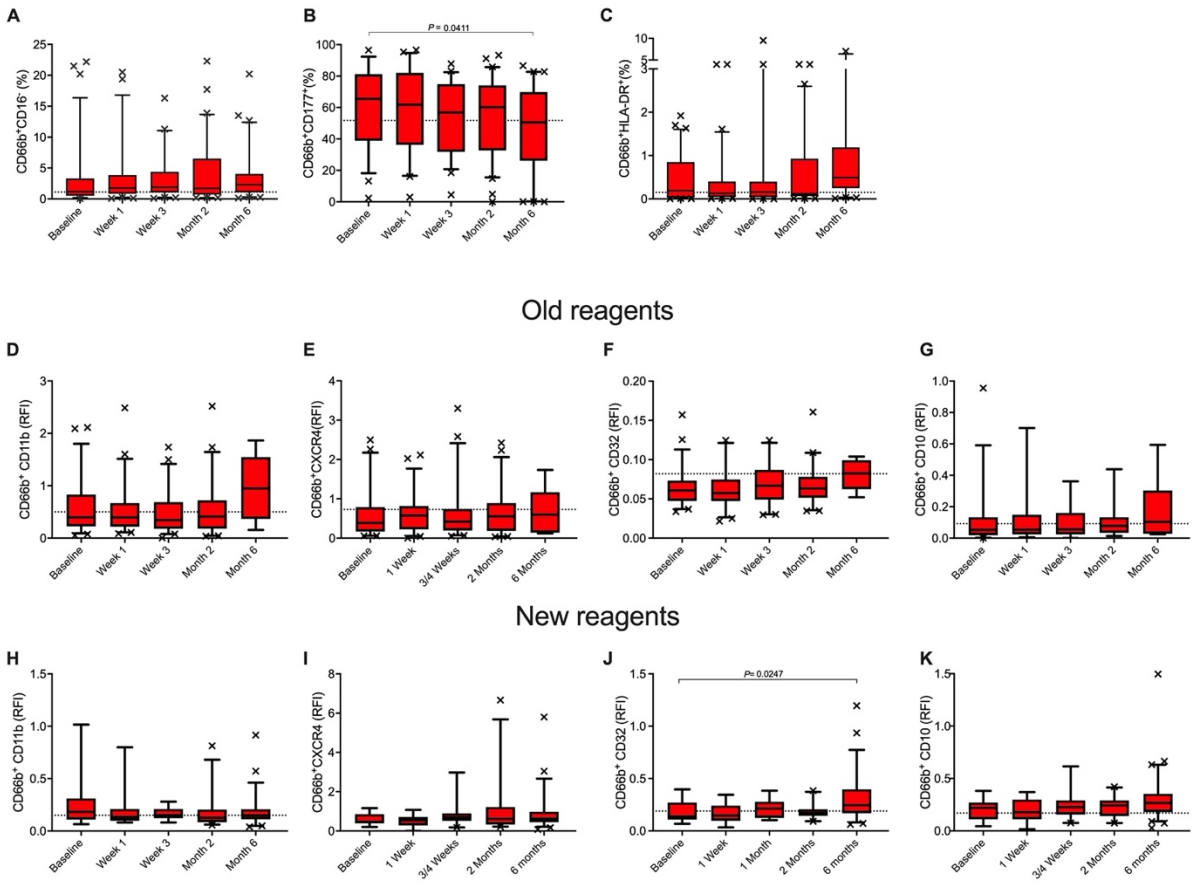


Figure 7: Longitudinal measurement of neutrophil surface marker expression levels during anti-TB treatment. Changes in CD16, CD177 and HLA-DR percent expression **(A-C)** and, CD11b, CXCR4, CD32 and CD10 expression levels on the neutrophil surface using old reagents **(D-G)** and new reagents **(H-K)**. P-values were obtained using the Wilcoxon rank sum test and Kruskal-Wallis tests for Dunn's multiple comparisons. Dotted line represents the median healthy control levels.

Down-regulation of this marker could be associated with increased binding of immune complexes on apoptotic cells thus reducing responsiveness to stimuli resulting in effector functions (Hart et al. 2000) whilst increasing phagocytosis by macrophages (Hart, Alexander, and Dransfield 2004). However, the increase in neutrophil phagocytosis was linked to the release of inflammatory cytokines that could potentially drive the development of chronic inflammation. CD11b is an adhesion marker commonly associated with neutrophil activation and transmigration into tissue. Fortunati et al. reported CD11b expression levels to be significantly upregulated on neutrophils from bronchiolar lavage fluid when compared to blood (Fortunati et al. 2009). And Scott et al. showed, using TB mouse models, that

increased CD11b expression on the neutrophil surface was associated with neutrophil accumulation in the lung (Scott et al. 2020). Therefore, Increased CD11b expression could potentially indicate neutrophil homing to the lung in TB.

Longitudinal analysis of the surface markers (**Figure 7A-K**) revealed no significant changes in neutrophil surface marker expression over the course of TB treatment with the exception of CD177. Expression levels were significantly reduced (P=0.0411; **Figure 7B**) following 6 months of treatment with anti-TB drugs. However, CD10, CD11b, CD32 and CD177 were excluded as potential TB biomarkers for a number of reasons. Firstly, CD177 was not able to distinguish ATB from healthy controls and longitudinal changes did not occur early enough to make it useful as a potential predictive marker of treatment outcome. However, CD177 may be useful as an indicator of reduced neutrophil recruitment to the lung during TB treatment (Sachs et al. 2007).

The lack of clear regulation of these markers during treatment was highly unexpected, given strong preliminary data in the differential expression between TB cases and controls, which was somewhat confirmed here. When attempting to uncover a potential explanation behind this it was discovered that the batch of reagents used to calibrate the FACS machine before each run were changed during the course of the study. This was found to impact the mean fluorescent intensity of the antibodies used to detect changes in phenotypic expression. As described in the methods section, fluctuations in machine performance were accounted for by normalising against the calibration beads for each run. As shown in **Figure 7D-K**, despite this normalisation, a clear distinction between the new and old reagents persisted and could not be controlled for. Of note this did not significantly affect baseline measurements presented in **Figure 6** as all healthy and Xpert-positive/culture-negative controls as well as 66% (n=52) of TB cases used the same reagents. In addition, as CD16, CD177 and HLA-DR expression was measured in term of +ve and -ve populations rather than MFI, these metrics were not affected (**Figure 7A-C).** It is highly likely, however, that this technical issue has severely limited my ability to objectively evaluate the use of CD11b, CXCR4, CD32 and CD10 markers to monitor TB treatment

response. None-the-less, and despite the technical noise in the system, CD15 expression levels remained robust enough to not be impacted by this, further validating its value as a potential marker.

Neutrophil CD15s expression is not inversely related to CD15 expression levels in ATB

CD15 is related to another marker sialyl-CD15 or CD15s on the surface of neutrophils with distinct functions (Elghetany 2002). Both molecules are synthesised by fucosyltransferases, however CD15 is primarily formed through sialidase activity that results in the shedding of sialyl acid from CD15s to generate CD15 (Gadhoum and Sackstein 2008; Nakayama et al. 2001). CD15s is recognized as a ligand by endothelial selectins, initiating cell adhesion and transmigration as part of an inflammatory response (Lowe 2001). CD15 is also able to bind selectins, but it's primary function appears to be binding to Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN/CD209) found on the surface of DCs or macrophages (van Gisbergen et al. 2005). As CD15 is generated from CD15s, I hypothesized that decreased CD15 expression on neutrophils during infection might be associated with an increase in CD15s expression and vice versa. This was an attractive idea, as the ratio of CD15/CD15s might potentially be a more sensitive measure of neutrophil state, and overcome the issues of technical assay variation that arose in this longitudinal study. Upon testing the hypothesis, using additional baseline samples obtain from TB clinics under the CUBS protocol and healthy controls, I found that CD15 expression was significantly lower than CD15s among healthy controls (P=0.0192) (**Figure 8A**). However, during infection both CD15 and CD15s are significant reduced compared to control expression levels, and a decrease in CD15 is not associated with an increase in CD15s expression. Interestingly, **Figure 8B** shows higher median CD15/CD15s ratios among active TB cases compared to controls which warrant further exploration.

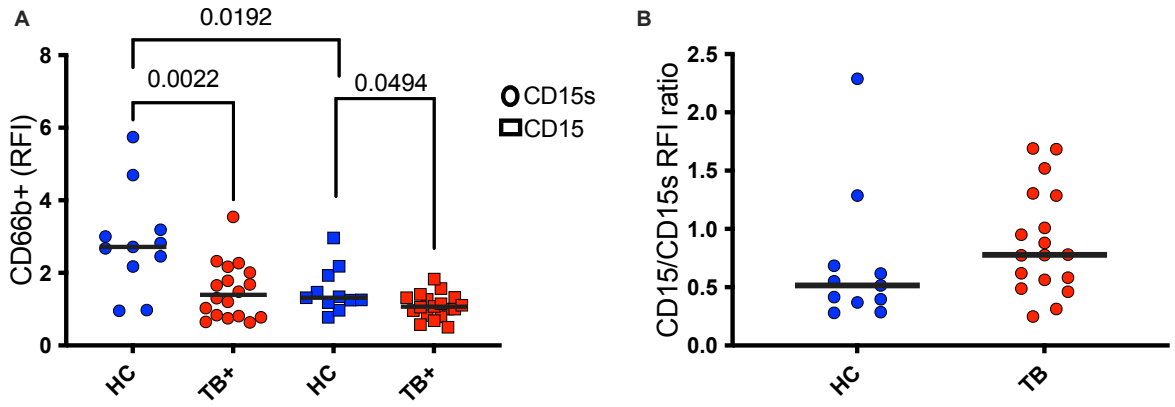


Figure 8: Comparison of CD15 and CD15s surface marker expression on neutrophils: Relative fluorescent intensity of neutrophil surface markers (A) CD15 and CD15s expression and (B) CD15/CD15s ratios in active TB cases (n=18) and healthy controls (n=11). P-values were obtained using the Mann Whitney t-test.

Chapter 4: Neutrophil associated cytokines correlate with the immune response to TB but do not predict treatment outcome

4.1 Introduction

Cytokines have been extensively evaluated as potential biomarkers of TB disease diagnosis, progression and severity (Sudbury et al. 2020; Qiu et al. 2020). Neutrophilia is also been linked to severe TB disease however few studies have looked at the mechanisms driving this response in ATB disease or the relationship between cytokine levels and neutrophil counts (Muefong and Sutherland 2020). In the previous chapter I showed that decreased neutrophil CD15 expression correlated with both increased neutrophil counts and sputum bacterial load. We hypothesized that the observed reduction in this neutrophil maturation marker may be related to continual recruitment of immature neutrophils from the bone marrow due to ongoing lung inflammation. Therefore, to investigate this relationship further, we measured plasma levels of cytokines, chemokines and other soluble factors involved in neutrophil activation, recruitment and immune modulation. we hypothesised that these neutrophil related cytokines would (1) be significantly elevated at baseline, (2) would correlate with clinical markers of TB disease severity as well as neutrophil counts and CD15 expression and (3) would resolve to levels seen in healthy controls following 6 months of anti-TB treatment. Using stored plasma samples from the RePORT cohort we first analysed a panel of 27 analytes associated with immune activation and modulation by luminex immunoassays in TB cases (n=77) and Xpert-positive/culture negative controls (n=13). We then analysed a panel of neutrophil-associated markers using a subset of TB cases (n=40) and confirmed neutrophil associated observations using an ELISA.

4.2 Results

Neutrophil associated cytokines/chemokines and soluble factors are elevated in active TB disease and distinguished from healthy controls.

To determine if plasma cytokine levels were elevated in TB disease, we compared measures baseline levels from ATB cases with symptomatic Xpert-positive/culture-negative and healthy controls. Among the 27 analytes measured, 23 were significantly increased at baseline in ATB disease compared to the healthy controls **(Figure 9)**. Of these analytes nine (IL-8, IFNγ, MCP-1, IL-4, IL-1Ra, IL-10, IL-13, G-CSF and GM-CSF) were able to distinguish symptomatic TB patients from symptomatic Xpert-positive/culture-negative patients **(Figure 9A-C)**. In addition, six analytes (IL-6, IL-1β, IL-2, IL-15, VEGF-A and IL-7) distinguished Xpert-positive/culture-negative controls from healthy individuals **(Figure 9A, C)**. The neutrophils specific panel revealed highly significant increases in baseline S100A9, S100A8/A9, TREM1, IL-18, IL-6Ra in TB cases compared to healthy controls and no difference in OSM levels **(Figure 10A,B)**.

Baseline data from the 27 analyte Luminex panel was then standardised by rescaling each analyte to have a mean of 0 and a standard deviation of 1 or -1 and analysed using hierarchical clustering, which revealed four main clusters (**Figure 11**). Plasma cytokines levels were significantly elevated in clusters 1 and 2, and significantly reduced in clusters 3 and 4. The highest proportion (91%) of TB cases were found in cluster 1 which also presented with the most significantly elevated cytokines and likely represents patients with the most severe TB disease. Interestingly, TB cases were present in all four clusters, potentially reflecting differences in disease severity at baseline, The Xpert-positive/culture-negative controls were also present in all four clusters suggesting a spectrum of inflammation experienced by patients from this group. Expectedly, most healthy controls (n=15, 83%) were present in cluster 4 where the lowest levels of cytokine expression were observed. The presence of TB cases in this cluster may represent an interesting group of patients with an active infection but with mild clinical manifestation. We tested this hypothesis by comparing baseline CXR scores, bacterial loads, neutrophil counts and neutrophil CD15 expression levels between each cytokine cluster for both TB cases and Xpert-positive/culture-negative controls. No clear relationship between cytokine cluster and

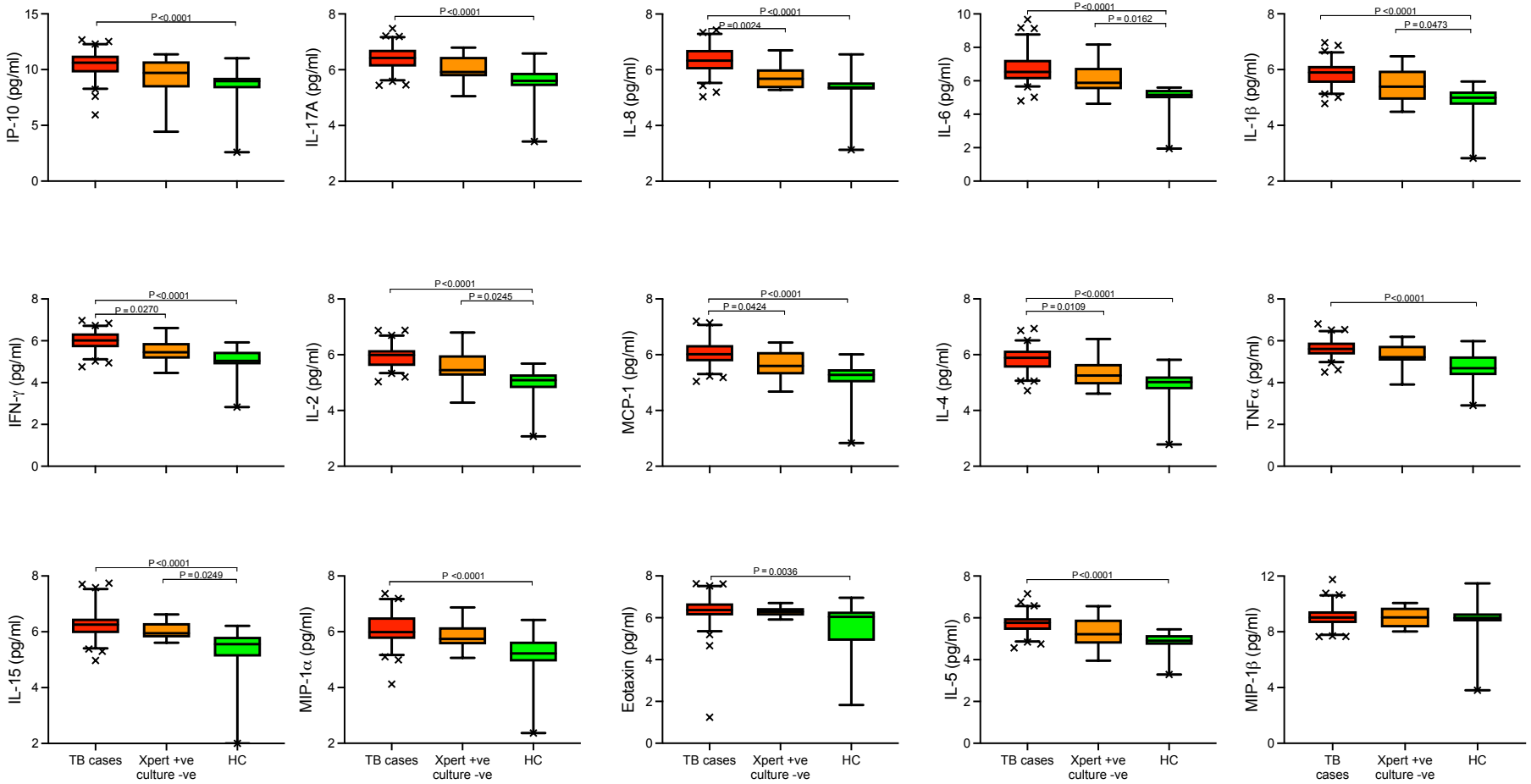
these clinical measures was apparent for either confirmed TB cases **(Figure 11B)** or Xpert-positive/culture-negative controls **(Figure 11C)**. Interestingly, the heatmap suggests a differential regulation of the analytes measured, of which the group represented in the bottom part of the heatmap (IL-2, IL-13, IL-5, IL-7, TNFα, IL-1β, IL-4, IFNγ, GM-CSF, IL-17A, IL-12p70, IL-10, G-CSF, MCP-1, IL-8 and IL-6) show a more distinct pattern of expression across the 4 clusters **(Figure 11A)**.

Spectrum of inflammation among TB cases is dominated by neutrophil associated analytes.

A principal component analysis was then performed to investigate which analytes drive the variability observed among TB cases, Xpert-positve/culture-negative controls and healthy controls **(Figure 12)**. PC1 which explains 56.36% of the variability was dominated by analytes TNFα, IL-13, IL-5, IL-12p70, IL-7, IFNγ, GM-CSF, IL-10, IL-1β, G-CSF, IL-4, IL-17A, MCP-1, IL-6 and IL-8 **(Figure 12A).** PC2 which accounts for 6.37% of the variance was dominated by TNFα, IL-13, IL-5, IL-12p70, IL-7, IFNγ, GM-CSF, IL-10 and BasicBFG. Similarly, to the heatmap in **Figure 11A** these markers were able to cluster the patient groups though complete separation was not achieved **(Figure 12B)**. We then endeavoured to identify how baseline measures would cluster on the PCA. TB cases (cluster 1) were positively associated with the analytes while healthy controls had a negative association (cluster 4) **(Figure 12C)**. Lastly, we assessed the impact of co-infection with HIV **(Figure 12D)** among TB cases and found that there was no impact of HIV on the clustering of baseline samples.

To assess the relationship between baseline cytokine levels and further explore the association with TB disease severity, a correlation analysis was performed **(Figure 13A-C)**. A Spearman R correlation matrix analysis revealed highly significant and strong correlations ($r>0.7$, $P<0.0001$) among 15 analytes (IL-6, IFNγ, IL-5, GM-CFS, TNFα, IL-2, IL-1β, IL-4, MCP-1, IL-8, IL-10, G-CSF, IL-15, IL-7, IL-12p70 and IL-17A) **(Figure 13A)**. Notably, IL-8, a neutrophil specific chemotactic factor was significantly increased along with inflammatory markers, IL-6, IFNγ, GM-CFS, IL-2, IL-1β, IL-4, MCP-1, G-CSF, IL-7 and IL-17A, which are associated with the differentiation, recruitment and activation of immune cell subsets. The neutrophil-associated panel revealed strong correlations between OSM and IL-6Ra ($r=0.73$, $P<0.0001$) as well as medium correlations between TREM-1, IL-18, S100A8/A9 and S100A9 **(Figure 13B)**.

A: Pro-inflammatory cytokines



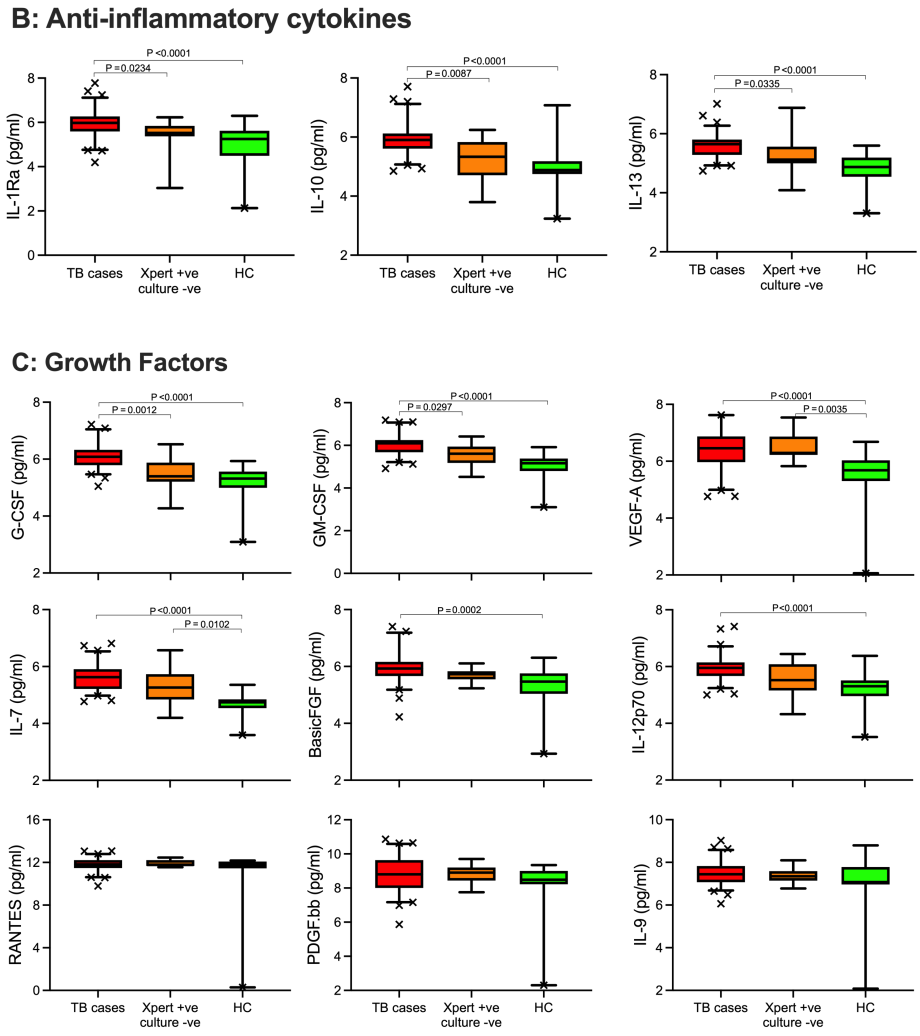


Figure 9: Baseline plasma cytokine levels in active TB disease. Box plots of 17 pro-inflammatory cytokine **(A)**, 3 anti-inflammatory cytokine **(B)** and 9 growth factor **(C)** measures in ATB cases (n=77), Xpert-positive/culture-negative controls (n = 13), and healthy controls (HC; n = 19). Data represents median values and was analyzed using Kruskal-Wallis test for Dunn's multiple comparisons.

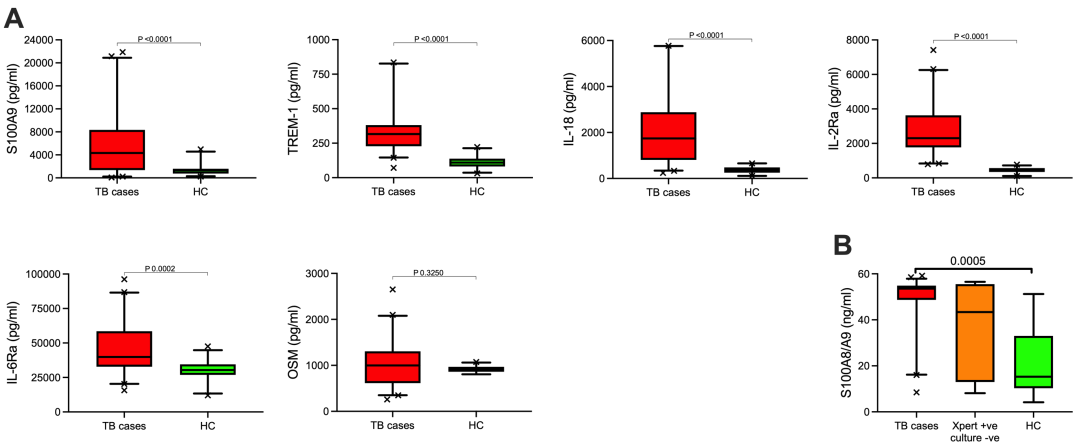
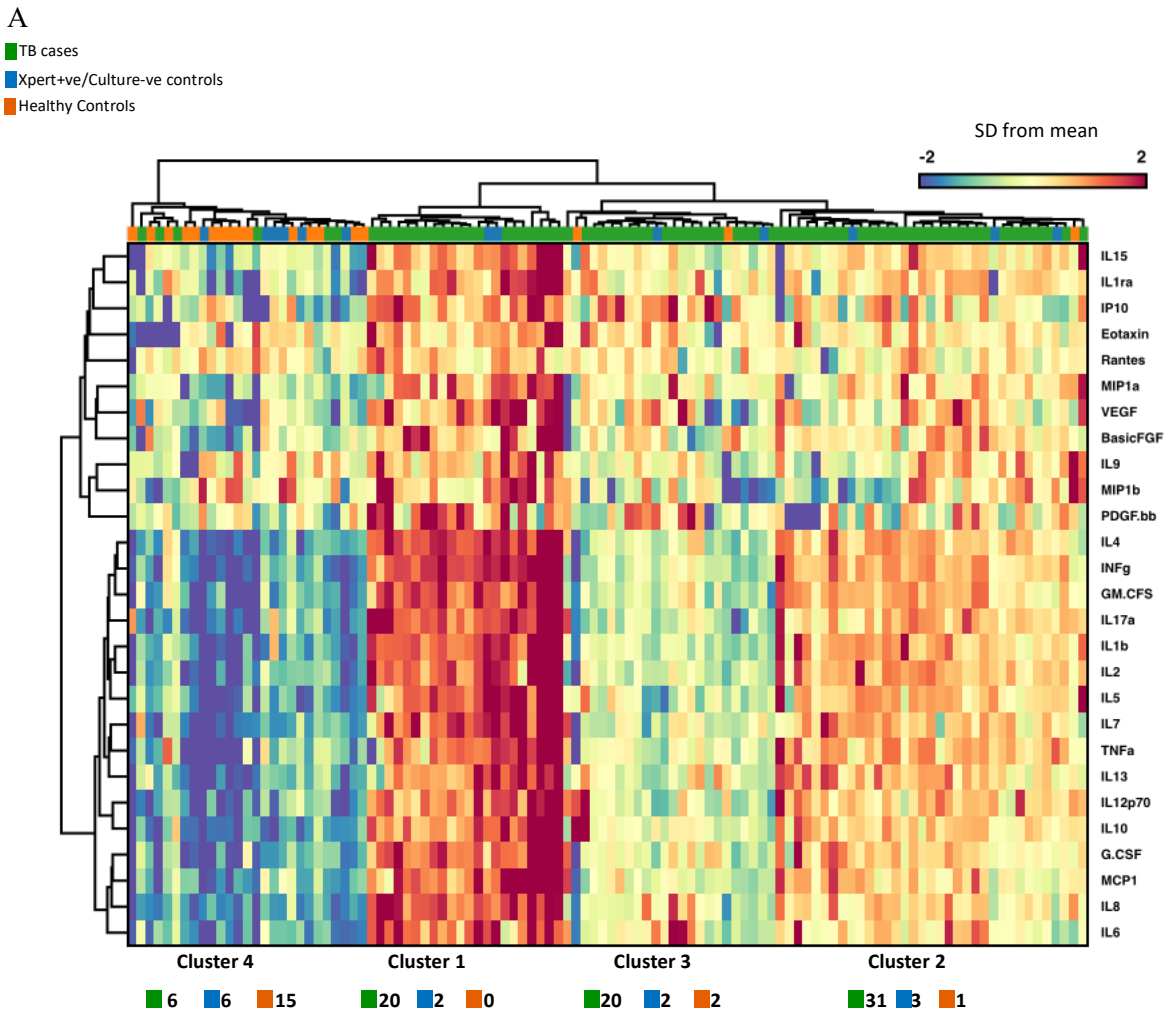


Figure 10: Neutrophil-associated baseline plasma cytokine measures in active TB disease. Box plots of neutrophil-associated cytokine levels quantified by luminex **(A)** and ELISA **(B)** in ATB cases (n=40) and healthy controls (HC; n=24). Data represents median values and was analysed by Mann-Whitney t-test



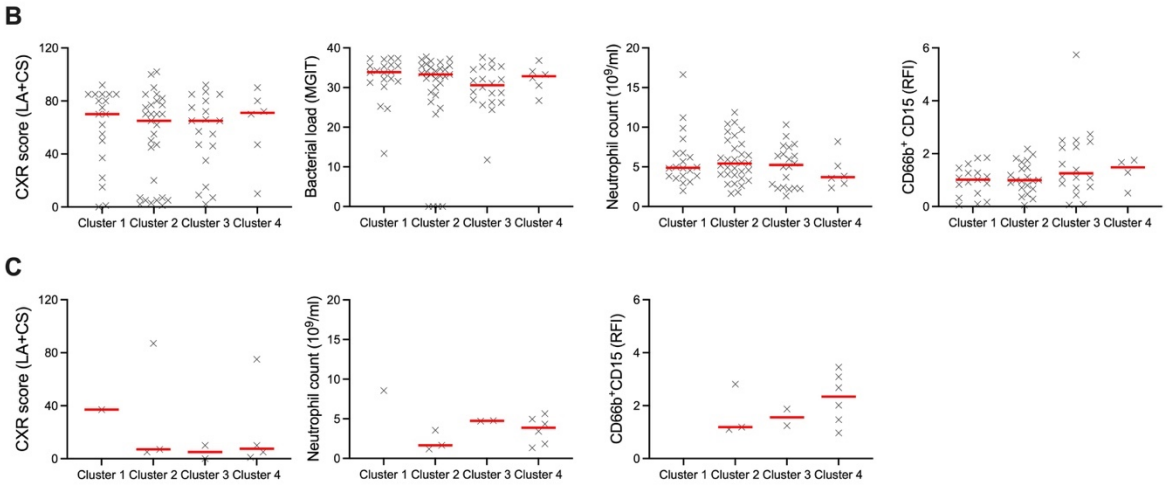


Figure 11: Profiling plasma cytokine levels in patients with active TB disease. (A) Hierarchical clustering using Euclidean distancing with ward method of cytokine concentrations z-score standardised by rescaling each analyte to have a mean of 0. Participant cytokine concentrations are presented as a standard deviation of the mean where 1=upregulation and -1=down-regulation. Each column represents a participant from each group and the number of participants in each cluster is indicated in the figure. Each row represents an analyte. (B-C) Baseline measures of CXR score, bacterial load, neutrophil counts and CD15 expression in TB cases (B) and Xpert-positive/culture-negative controls (C) grouped by clusters. Data represents median values and was analyzed using Kruskal-Wallis test for Dunn's multiple comparisons.

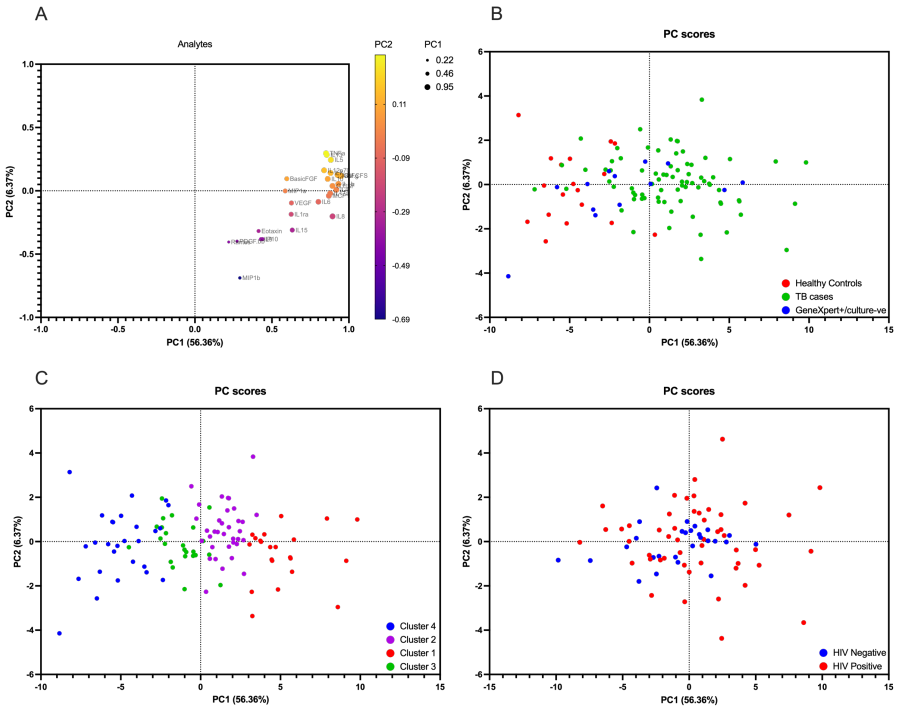


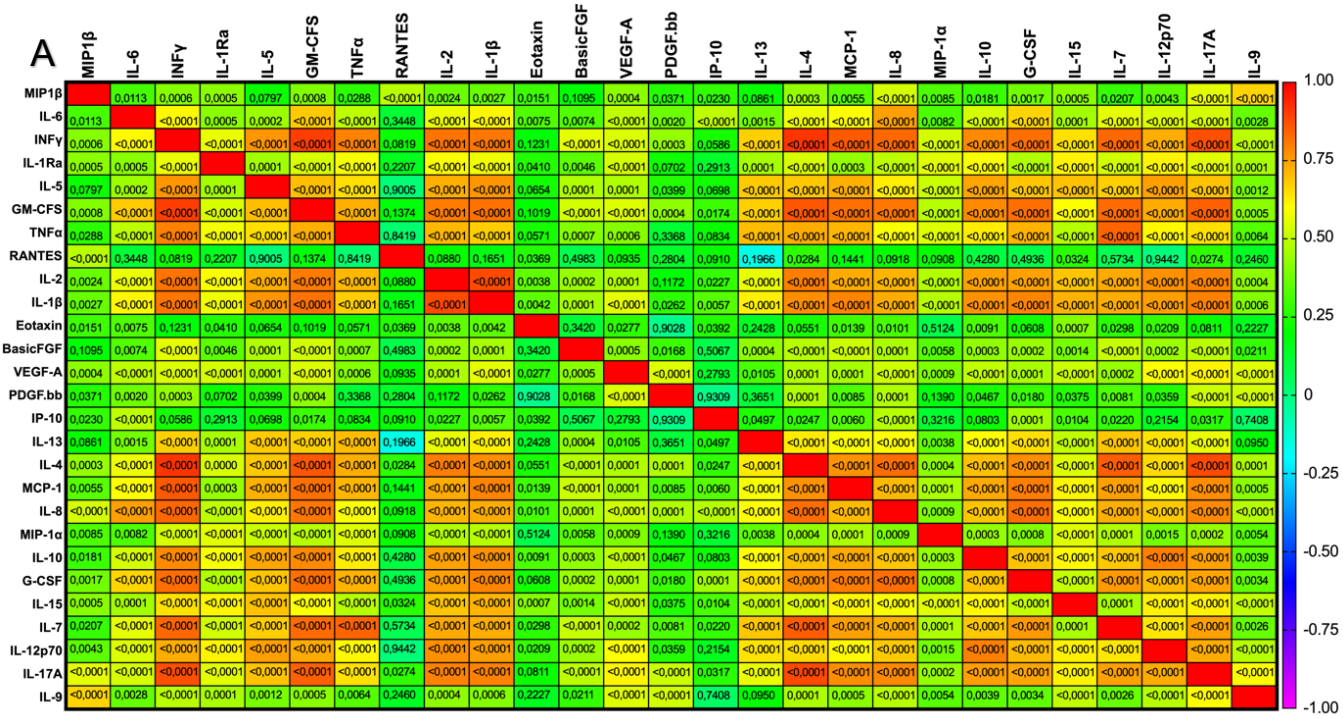
Figure 12: Principal component analysis of baseline measures in active TB disease. Projections of variability and clustering of RePORT study participants based on cytokine expression levels (**A**), patient group (**B**), hierarchical clustering used in **Figure 10A** (**C**) and HIV status.

To determine concordance between the two Luminex panels we included G-CSF, IL-8, IL-6, IL-10 and IP-10 in both immunoassays. Data from both panels showed IP-10 to have weak correlations with IL-8 and G-CSF and no correlation to IL-10. Increased IP-10 was also associated with an increase in neutrophil-associated markers such as IL-18 (r=0.62, P=0.0001), TREM-1 (r=0.5, P=0.0043) and S100A8/A9 (r=0.51, P=0.0033). Highly significant correlations were observed between S100A8/A9 vs S100A9 (r=0.68, P<0.0001) and IL-6Ra (r=0.6, P=0.0004). A correlation analysis of baseline cytokine levels with makers of TB disease severity revealed S100A9, a potent neutrophil-specific chemotactic factor, to have the strongest association with bacterial load (r=0.6506, P<0.0001), CXR score (r=0.6368, P<0.0001), Neutrophil count (r=0.5847, P<0.0001), and was significantly correlated with CD15 expression (r=0.4733, P=0.0054) (**Figure 13C**).

Other analytes generally had weaker (r value between 0.3-0.5) correlations with markers of TB disease severity. PDGF.bb correlated with both bacterial burden (r=0.3442, P=0.0054) and CXR score (r=0.3257, P=0.0092) and TREM-1 with bacterial load (r=0.4563, P=0.0040) and neutrophil counts (r=0.4060, P=0.0114). And in addition to S100A9, IL-6 correlated with both neutrophil counts (r=0.4032, P=0.0007) and CD15 expression (r=-0.3417, P=0.0123). CD15 expression also correlated with G-CSF (r=0.3767, P=0.0054), a neutrophil-specific growth factor and inflammatory markers, MCP-1 (r=0.3609, P=0.0092), IL-1β (r=0.3151, P=0.0216) and IP-10 (r=0.3039, P=0.0269). Interestingly, only CD15 expression was associated the level of plasma OSM (r=-0.5174, P=0.0020). Together, these data support the hypothesis that neutrophils are involved in the immunopathology of TB and that neutrophil specific plasma markers may serve as useful biomarkers of disease severity.

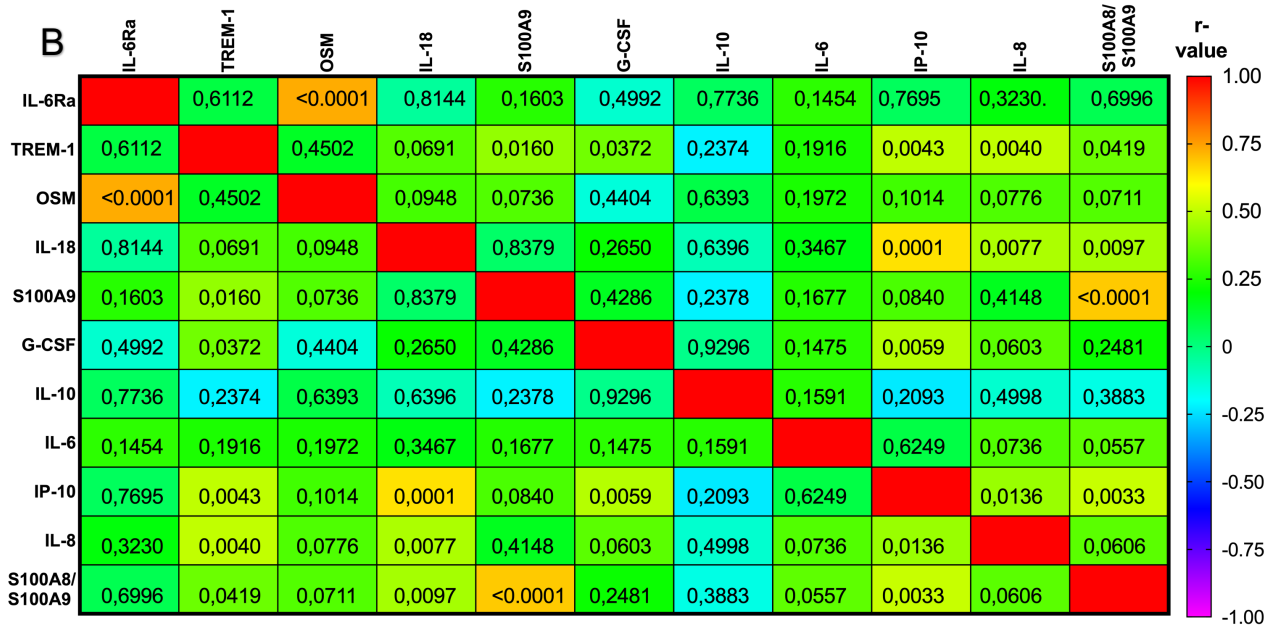
r-value

A



	MIP1β	IL-6	INFγ	IL-1Ra	IL-5	GM-CFS	TNFα	RANTES	IL-2	IL-1β	Eotaxin	BasicFGF	VEGF-A	PDGF.bb	IP-10	IL-13	IL-4	MCP-1	IL-8	MIP-1α	IL-10	G-CSF	IL-15	IL-7	IL-12p70	IL-17A	IL-9
MIP1β		0,0113	0,0006	0,0005	0,0797	0,0008	0,0288	<0,0001	0,0024	0,0027	0,0151	0,1095	0,0004	0,0371	0,0230	0,0861	0,0003	0,0055	<0,0001	0,0085	0,0181	0,0017	0,0005	0,0207	0,0043	<0,0001	<0,0001
IL-6	0,0113		<0,0001	0,0005	0,0002	<0,0001	<0,0001	0,3448	<0,0001	<0,0001	0,0075	0,0074	<0,0001	0,0020	<0,0001	0,0015	<0,0001	<0,0001	<0,0001	0,0082	<0,0001	<0,0001	0,0001	<0,0001	<0,0001	<0,0001	0,0028
INFγ	0,0006	<0,0001		<0,0001	<0,0001	<0,0001	<0,0001	0,0819	<0,0001	<0,0001	0,1231	<0,0001	<0,0001	0,0003	0,0586	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
IL-1Ra	0,0005	0,0005	<0,0001		0,0001	<0,0001	<0,0001	0,2207	<0,0001	<0,0001	0,0410	0,0046	<0,0001	0,0702	0,2913	0,0001	<0,0001	0,0003	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0001
IL-5	0,0797	0,0002	<0,0001	0,0001		<0,0001	<0,0001	0,9005	<0,0001	<0,0001	0,0654	0,0001	0,0001	0,0399	0,0698	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0012
GM-CFS	0,0008	<0,0001	<0,0001	<0,0001	<0,0001		<0,0001	0,1374	<0,0001	<0,0001	0,1019	<0,0001	<0,0001	0,0004	0,0174	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0005
TNFα	0,0288	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001		0,8419	<0,0001	<0,0001	0,0571	0,0007	0,0006	0,3368	0,0834	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0064
RANTES	<0,0001	0,3448	0,0819	0,2207	0,9005	0,1374	0,8419		0,0880	0,1651	0,0369	0,4983	0,0935	0,2804	0,0910	0,1966	0,0284	0,1441	0,0918	0,0908	0,4280	0,4936	0,0324	0,5734	0,9442	0,0274	0,2460
IL-2	0,0024	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0880		<0,0001	0,0038	0,0002	0,0001	0,1172	0,0227	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0004
IL-1β	0,0027	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,1651	<0,0001		0,0042	0,0001	<0,0001	0,0262	0,0057	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0006
Eotaxin	0,0151	0,0075	0,1231	0,0410	0,0654	0,1019	0,0571	0,0369	0,0038	0,0042		0,3420	0,0277	0,9028	0,0392	0,2428	0,0551	0,0139	0,0101	0,5124	0,0091	0,0608	0,0007	0,0298	0,0209	0,0811	0,2227
BasicFGF	0,1095	0,0074	<0,0001	0,0046	0,0001	<0,0001	0,0007	0,4983	0,0002	0,0001	0,3420		0,0005	0,0168	0,5067	0,0004	<0,0001	<0,0001	0,0001	0,0058	0,0003	0,0002	0,0014	<0,0001	0,0002	<0,0001	0,0211
VEGF-A	0,0004	<0,0001	<0,0001	<0,0001	0,0001	<0,0001	0,0006	0,0935	0,0001	<0,0001	0,0277	0,0005		<0,0001	0,2793	0,0105	0,0001	0,0001	<0,0001	0,0009	<0,0001	0,0001	<0,0001	0,0002	<0,0001	<0,0001	<0,0001
PDGF.bb	0,0371	0,0020	0,0003	0,0702	0,0399	0,0004	0,3368	0,2804	0,1172	0,0262	0,9028	0,0168	<0,0001		0,9309	0,3651	0,0001	0,0085	0,0001	0,1390	0,0467	0,0180	0,0375	0,0081	0,0359	<0,0001	<0,0001
IP-10	0,0230	<0,0001	0,0586	0,2913	0,0698	0,0174	0,0834	0,0910	0,0227	0,0057	0,0392	0,5067	0,2793	0,9309		0,0497	0,0247	0,0060	<0,0001	0,3216	0,0803	0,0001	0,0104	0,0220	0,2154	0,0317	0,7408
IL-13	0,0861	0,0015	<0,0001	0,0001	<0,0001	<0,0001	<0,0001	0,1966	<0,0001	<0,0001	0,2428	0,0004	0,0105	0,3651	0,0497		<0,0001	<0,0001	<0,0001	0,0038	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0950
IL-4	0,0003	<0,0001	<0,0001	0,0000	<0,0001	<0,0001	<0,0001	0,0284	<0,0001	<0,0001	0,0551	<0,0001	0,0001	0,0001	0,0247	<0,0001		<0,0001	<0,0001	0,0004	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0001
MCP-1	0,0055	<0,0001	<0,0001	0,0003	<0,0001	<0,0001	<0,0001	0,1441	<0,0001	<0,0001	0,0139	<0,0001	0,0001	0,0085	0,0060	<0,0001	<0,0001		<0,0001	0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0005
IL-8	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0918	<0,0001	<0,0001	0,0101	0,0001	<0,0001	0,0001	<0,0001	<0,0001	<0,0001	<0,0001		0,0009	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
MIP-1α	0,0085	0,0082	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0908	<0,0001	<0,0001	0,5124	0,0058	0,0009	0,1390	0,3216	0,0038	0,0004	0,0001	0,0009		0,0003	0,0008	<0,0001	0,0001	0,0015	0,0002	0,0054
IL-10	0,0181	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,4280	<0,0001	<0,0001	0,0091	0,0003	<0,0001	0,0467	0,0803	<0,0001	<0,0001	<0,0001	<0,0001	0,0003		<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0039
G-CSF	0,0017	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,4936	<0,0001	<0,0001	0,0608	0,0002	0,0001	0,0180	0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0008	<0,0001		<0,0001	<0,0001	<0,0001	<0,0001	0,0034
IL-15	0,0005	0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0324	<0,0001	<0,0001	0,0007	0,0014	<0,0001	0,0375	0,0104	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001		0,0001	<0,0001	<0,0001	<0,0001
IL-7	0,0207	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,5734	<0,0001	<0,0001	0,0298	<0,0001	0,0002	0,0081	0,0220	<0,0001	<0,0001	<0,0001	<0,0001	0,0001	<0,0001	<0,0001	0,0001		<0,0001	<0,0001	0,0026
IL-12p70	0,0043	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,9442	<0,0001	<0,0001	0,0209	0,0002	<0,0001	0,0359	0,2154	<0,0001	<0,0001	<0,0001	<0,0001	0,0015	<0,0001	<0,0001	<0,0001	<0,0001		<0,0001	<0,0001
IL-17A	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001	0,0274	<0,0001	<0,0001	0,0811	<0,0001	<0,0001	<0,0001	0,0317	<0,0001	<0,0001	<0,0001	<0,0001	0,0002	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001		<0,0001
IL-9	<0,0001	0,0028	<0,0001	0,0001	0,0012	0,0005	0,0064	0,2460	0,0004	0,0006	0,2227	0,0211	<0,0001	<0,0001	0,7408	0,0950	0,0001	0,0005	<0,0001	0,0054	0,0039	0,0034	<0,0001	0,0026	<0,0001	<0,0001	

B



	IL-6Ra	TREM-1	OSM	IL-18	S100A9	G-CSF	IL-10	IL-6	IP-10	IL-8	S100A8/ S100A9
IL-6Ra		0,6112	<0.0001	0,8144	0,1603	0,4992	0,7736	0,1454	0,7695	0,3230.	0,6996
TREM-1	0,6112		0,4502	0,0691	0,0160	0,0372	0,2374	0,1916	0,0043	0,0040	0,0419
OSM	<0.0001	0,4502		0,0948	0,0736	0,4404	0,6393	0,1972	0,1014	0,0776	0,0711
IL-18	0,8144	0,0691	0,0948		0,8379	0,2650	0,6396	0,3467	0,0001	0,0077	0,0097
S100A9	0,1603	0,0160	0,0736	0,8379		0,4286	0,2378	0,1677	0,0840	0,4148	<0.0001
G-CSF	0,4992	0,0372	0,4404	0,2650	0,4286		0,9296	0,1475	0,0059	0,0603	0,2481
IL-10	0,7736	0,2374	0,6393	0,6396	0,2378	0,9296		0,1591	0,2093	0,4998	0,3883
IL-6	0,1454	0,1916	0,1972	0,3467	0,1677	0,1475	0,1591		0,6249	0,0736	0,0557
IP-10	0,7695	0,0043	0,1014	0,0001	0,0840	0,0059	0,2093	0,6249		0,0136	0,0033
IL-8	0,3230	0,0040	0,0776	0,0077	0,4148	0,0603	0,4998	0,0736	0,0136		0,0606
S100A8/ S100A9	0,6996	0,0419	0,0711	0,0097	<0.0001	0,2481	0,3883	0,0557	0,0033	0,0606	

C

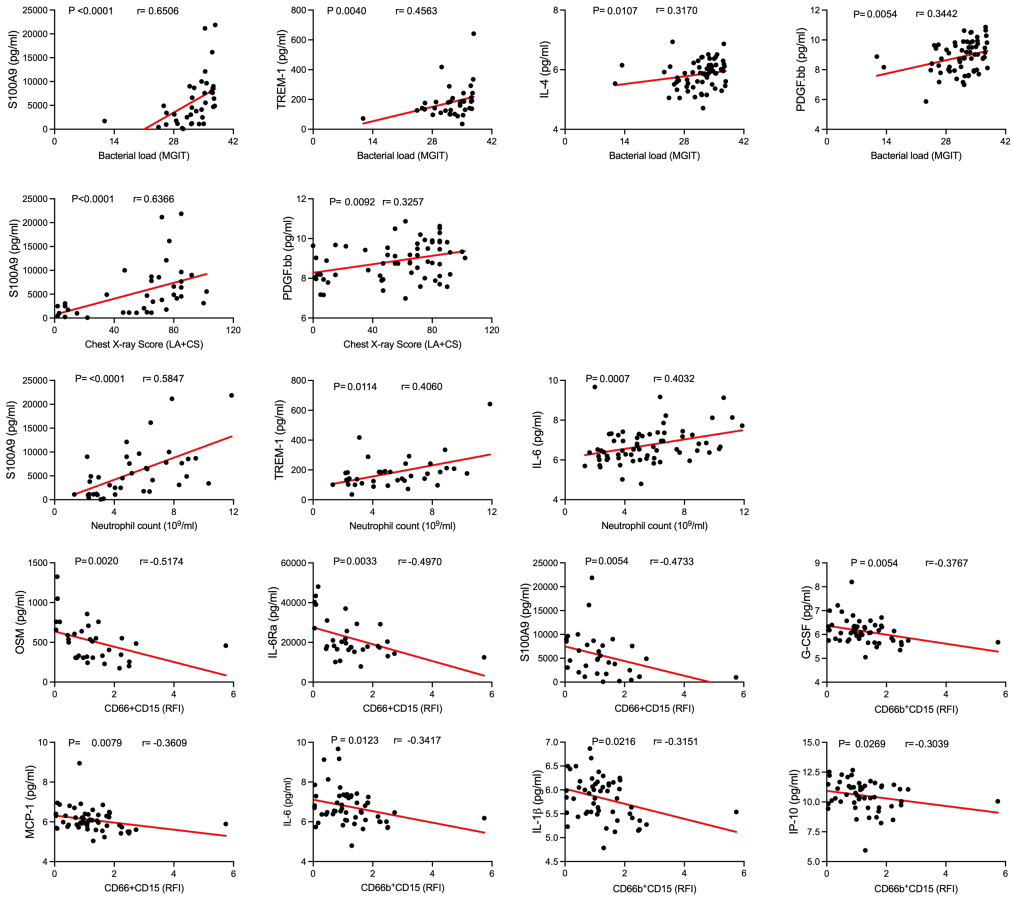


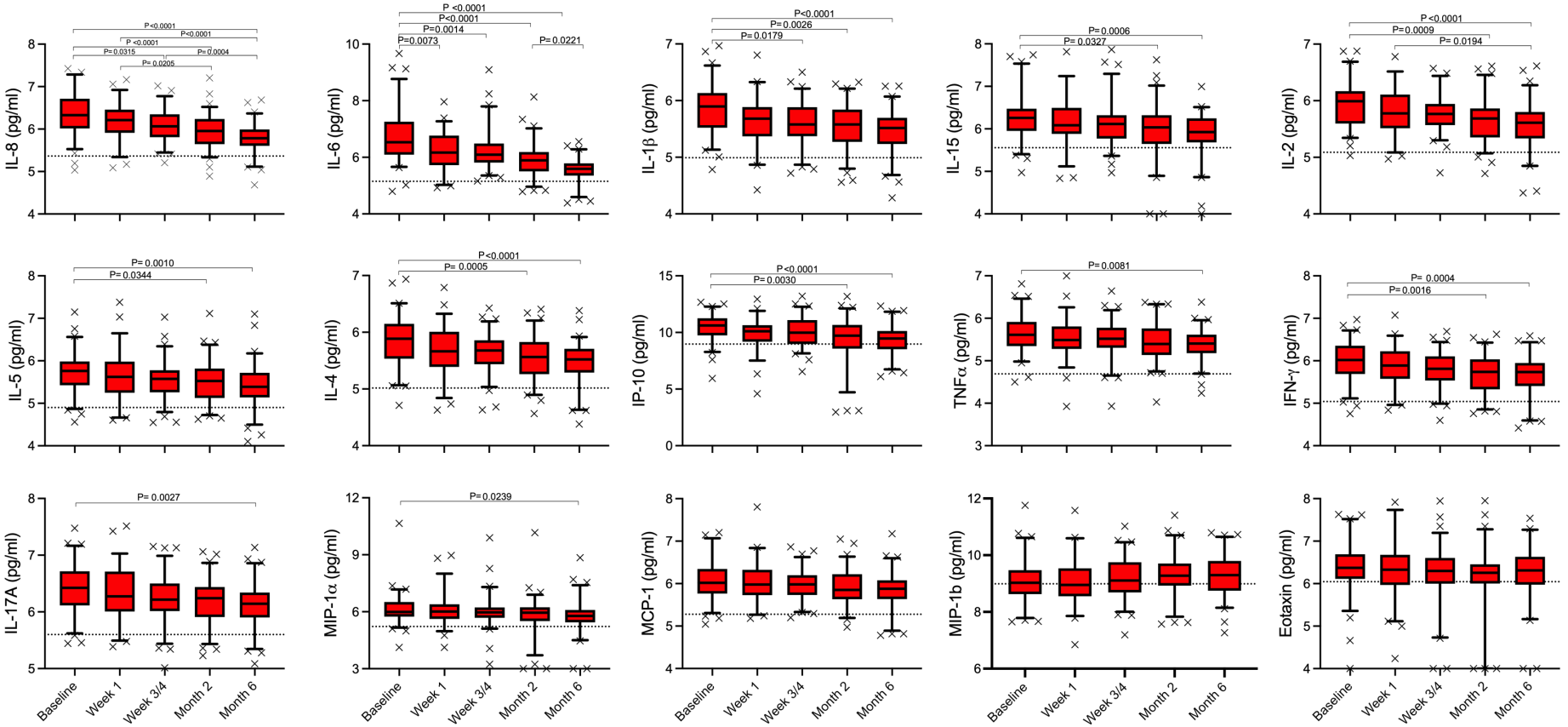
Figure 13: Neutrophil-associated plasma cytokine levels correlate with markers of Active TB disease. (A,B) Spearman's rank correlation analysis of baseline plasma cytokine levels from active TB cases (n=77) and their relationship with bacterial load, chest X-ray scores, neutrophil counts and neutrophil CD15 expression **(C)**. R values between 0.7-1 are considered strong; 0,5-0.7 medium; 0.3-0.5 weak and 0-0.3 not correlated.

Plasma IL_6 levels show detectable decrease within one week of treatment initiation – potential marker of early treatment response.

We then evaluated changes in plasma cytokine levels measured using the 27-plex Luminex kit over the course of treatment to determine how they change over the course of anti-TB drug therapy **(Figure 14)**. Among the analytes tested, IL-6 was the only marker to show a significant (P=0.0073) change in plasma levels in the first week of TB treatment **(Figure 14A)**; while IL-8, IL-1β and G-CSF responded within

3-4 weeks of TB treatment initiation **(Figure 14A,C).** Several other cytokines did decrease over the course of treatment, but not until the 2 months or 6 month time point, suggesting IL-6, IL-8, IL-1β and G-CSF might be most promising as biomarkers of early treatment response. As G-CSF and IL-8 are central players in the release of neutrophils from the bone marrow and their recruitment to inflamed tissue, respectively, these observations again support the investigation of neutrophil specific markers as potential sensitive biomarkers of TB disease.

A: Pro-inflammatory cytokines



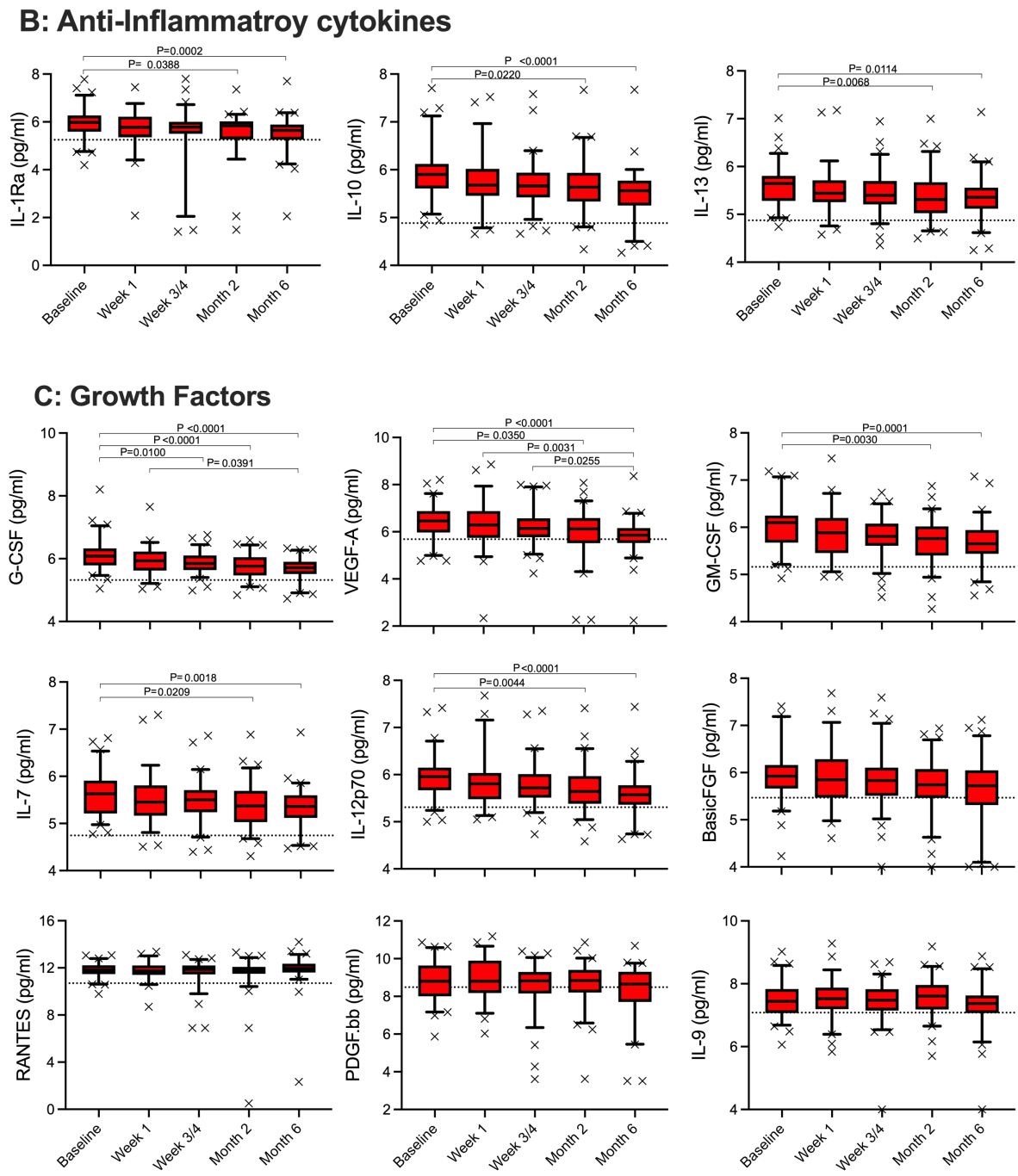


Figure 14: Longitudinal measures of plasma cytokine levels in active TB disease. Box plots of changes in pro-inflammatory cytokine **(A)**, anti-inflammatory cytokine **(B)** and growth factor **(C)** measures in ATB cases (n=77) over the course of TB drug treatment. Data represents median values and was analyzed using Kruskal-Wallis test for Dunn's multiple comparisons. Dotted lines represent the median healthy control levels.

Surprisingly, however, in this study, none of the neutrophil-associated markers measured decreased significantly within the first month of treatment initiation, and only IL-18 (P=0.0269) showed significant differences from 2 months **(**P=0.0269; **Figure 15)**. Despite correlating strongly with disease severity, S100A9 levels were only significantly reduced by month 6, as were TREM-1.

Of all the 23 cytokines with significantly elevated plasma cytokine levels at baseline compared to month 6, only five (IL-1Ra, IP-10, VEGF-A, S100A9 and IL-18) reached healthy control levels. Applying the same hierarchical clustering approach used on baseline samples to baseline (pre-treatment), month two (continuation phase initiation) and month six (end of treatment), revealed 3 distinct clusters characterised by high, medium and low general expression of cytokines **(Figure 16)**. However, there was no clear distinction between the inflammatory response of patients at pre-treatment from those on treatment for two or six months and samples from each group were found in each cluster.

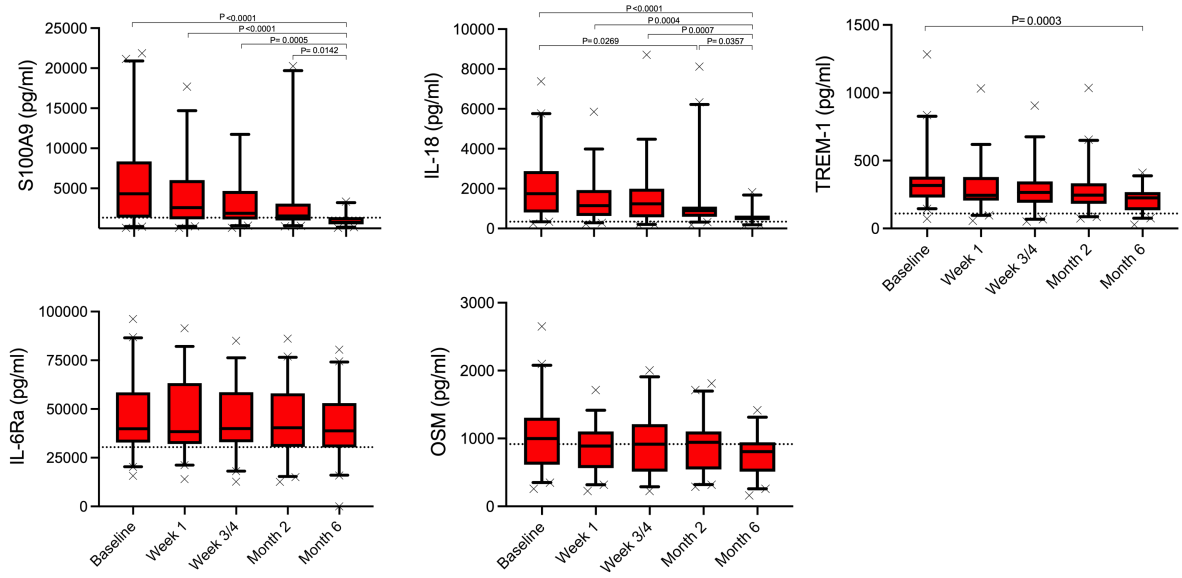


Figure 15: Longitudinal measures of neutrophil-associated plasma cytokine levels in active TB disease. Box plots of changes in plasma cytokine levels in active TB cases (n=40) over the course of standard TB therapy. Data represents median values and was analyzed using Kruskal-Wallis test for Dunn's multiple comparisons. Dotted lines represent the median healthy control levels.

Plasma cytokine levels are not predictive of solid culture conversion at two months.

Adherence to the six month TB treatment regimen is a significant barrier in controlling the spread of TB (Alipanah et al. 2018). However ensuring adherence and improving cure rates requires a means for triaging patients at risk for treatment failure. As in the previous chapter, we therefore evaluated the use of baseline plasma cytokines levels as markers of solid culture conversion at two months and treatment success at 6 months as endpoints. There was no significant risk association between each analyte and either culture conversion at two months or successful treatment **(Table 4)**. Thus, although plasma cytokines do correlate with TB disease severity ay baseline and change in response to TB treatment, albeit with varying kinetics, baseline CD15 expression levels on blood neutrophils was more predictive of treatment outcome.

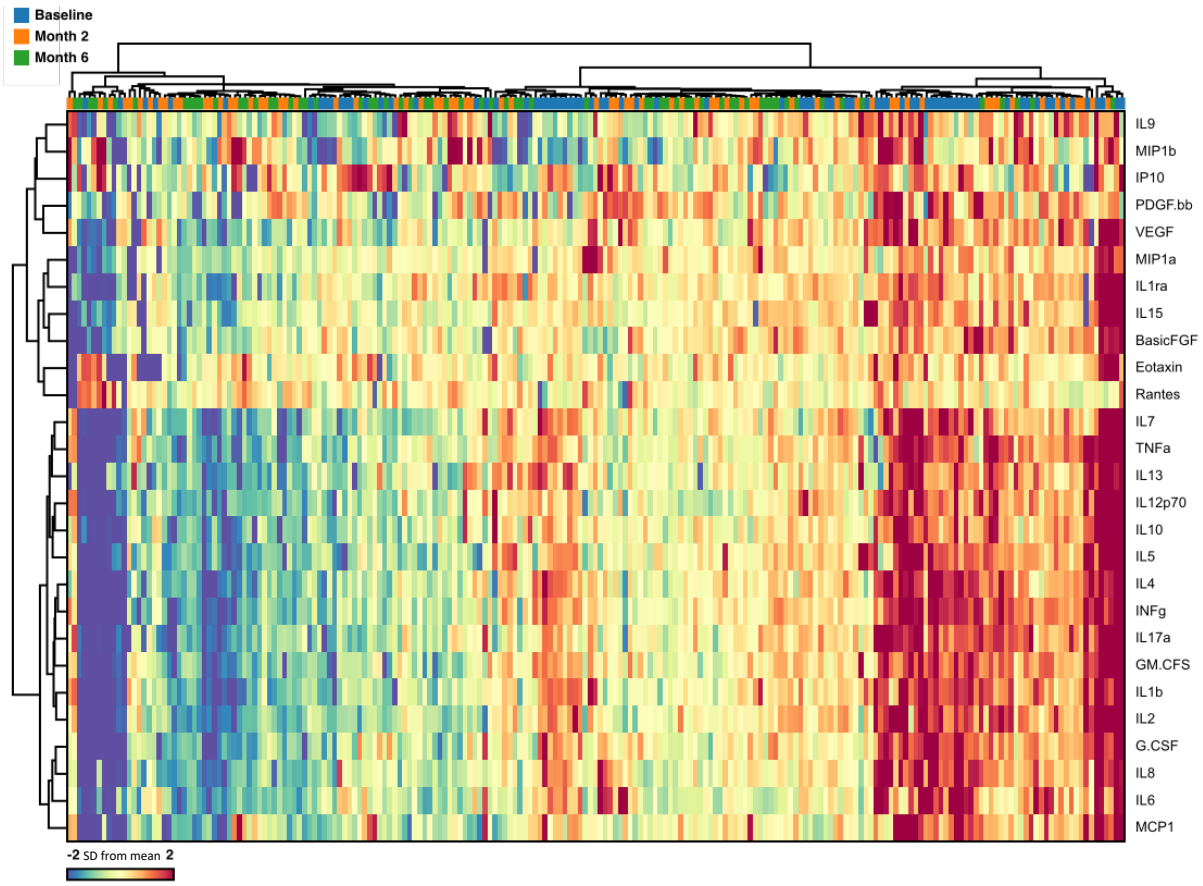


Figure 16: Profiling of longitudinal plasma cytokine levels in patients with active TB disease. Hierarchical clustering using Euclidean distancing with ward method of cytokine concentrations standardised by rescaling each analyte to have a mean of 0. Participant cytokine concentrations are

presented as a standard deviation of the mean where 1=upregulation and -1=down-regulation. Each column represents a participant from each group. Each row represents an analyte.

Table 4: Unadjusted measures for risk association between plasma cytokine levels with TB treatment outcomes, month 2 culture conversion and unsuccessful treatment, using solid culture.

	Month 2 solid culture positive		unsuccessful treatment	
Exposure	**Estimates**	**Unadjusted OR95%CI**	**Estimates**	**Unadjusted OR95%CI**
MIP-1β	0,8932	0.2002 to 3.083	1,13	0,4832 to 2,515
IL-6	1,023	0,2753 to 2,838	0,948	0,4359 to 1,877
INFγ	1,253	0,1432 to 13,77	2,873	0,7046 to 14,28
IL-1Ra	0,3867	0,06626 to 2,172	1,243	0,4290 to 3,654
IL-5	0,7238	0,08092 to 5,835	2,346	0,6353 to 9,630
GM-CFS	0,9691	0,09940 to 8,278	3,516	0,8899 to 16,01
TNFα	0,3975	0,03216 to 4,401	2,221	0,4923 to 10,88
RANTES	0,7713	0,1642 to 4,392	0,7003	0,2455 to 2,014
IL-2	0,3402	0,01618 to 4,819	2,339	0,4663 to 12,62
IL-1β	0,505	0,04181 to 4,930	2,241	0,5490 to 10,07
Eotaxin	0,9107	0,3932 to 4,227	0,9851	0,4939 to 2,552
BasicFGF	2,016	0,4044 to 8,508	1,686	0,5980 to 4,774
VEGF-A	1,651	0,4092 to 7,524	0,8681	0,3716 to 2,021
PDGF.bb	1,319	0,4521 to 4,101	0,9791	0,5293 to 1,831
IP-10	0,6956	0,3338 to 1,544	0,7856	0,4796 to 1,295
IL-13	0,678	0,03786 to 7,690	4,669	0,9918 to 27,53
IL-4	0,7265	0,07021 to 7,279	3,218	0,7505 to 16,42
MCP-1	0,5496	0,04420 to 3,024	1,331	0,4385 to 3,639
IL-8	0,871	0,1059 to 4,204	1,28	0,4133 to 3,659
MIP-1α	0,7661	0,1534 to 2,304	0,7934	0,2920 to 1,711
IL-10	0,6411	0,05165 to 4,299	1,743	0,5289 to 5,548
G-CSF	0,3881	0,02465 to 3,308	1,424	0,3798 to 4,853
IL-15	0,4447	0,03489 to 3,266	1,422	0,4352 to 4,456
IL-7	0,6272	0,05166 to 5,272	1,584	0,4120 to 6,068
IL-12p70	1,516	0,1336 to 11,53	3,449	0,8666 to 15,91
IL-17A	0,6311	0,05646 to 6,462	3,695	0,8376 to 19,49
IL-9	1,318	0,1908 to 8,006	1,597	0,5022 to 5,162
S100A9	1,000	0,9999 to 1,000	1,000	0,9998 to 1,000
IL-18	1,000	0,9995 to 1,001	0,9992	0,9974 to 1,000
TREM-1	1,000	0,9902 to 1,005	0,9978	0,9883 to 1,003
IL-6Ra	1,000	0,9999 to 1,000	1,000	0,9999 to 1,000
OSM	1,001	0,9979 to 1,003	0,9999	0,9974 to 1,002

4.3 Discussion

Studies of plasma cytokine levels in active TB have largely focused on identifying novel biomarkers of TB disease diagnosis, risk, progression and treatment outcome (Goletti, Lee, et al. 2018; Kumar et al. 2020; Sudbury et al. 2020). However, although the link between elevated neutrophil counts and TB disease severity has been demonstrated, very few studies have looked at neutrophil-associated plasma cytokines as potential drivers of TB disease severity or treatment outcomes (Prada-Medina et al. 2017; Muefong and Sutherland 2020). In this study we sought to understand the relationship between plasma cytokine levels associated with neutrophil responses, TB disease severity and treatment outcomes.

Briefly, we found that neutrophil-associated markers, S100A9, S100A8/A9, IL-8, IL-6, IL-1β, IL-17A, GM-CSF, TREM-1 and G-CSF were among the analytes distinguishing symptomatic active TB cases from healthy controls. Specifically, IL-8 and G-CSF distinguished ATB from symptomatic Xpert-positive/culture-negative controls while IL-6 and IL-1β distinguished Xpert-positive/culture-negative controls from healthy participants. Hierarchical clustering produced 4 distinct clusters reflective of the spectrum of inflammation associated with TB disease. These clusters were largely driven by the upregulation of cytokines associated with innate immune responses of which neutrophil-associated analytes dominated. However, the evaluation of TB cases from each cluster showed no significant differences in clinical sign of TB disease severity as measured by bacterial burden, CXR score, neutrophil counts and CD15 expression. Despite this, S100A9 was strongly associated with bacterial load, CXR scores, neutrophil counts and CD15 expression, and is one of six markers that reached healthy control levels upon treatment completion. Surprisingly, and despite significant correlations, none of the baseline analyte measures were associated with solid culture conversion at 2 months or treatment success.

Elevated plasma cytokine levels and blood neutrophil count are a hallmark of infection, and in TB disease the most commonly studied cytokines are IFNγ, TNFα, IP-10, IL-2 and IL-13 (Sudbury et al. 2020), however these markers alone are not reflective of the complex interaction between bacterial loads, extent of lung involvement and immune responses. As shown in chapter three, we found that

elevated neutrophil count was associated with increased bacterial burden and lung pathology and hypothesized that studying neutrophil related cytokines would strengthen these findings and may shed light on the underlying biological processes. S100A8, S100A9 and S100A8/A9 are known as damage-associated molecular pattern (DAMPs) or alarmins, comprise ~40% of total neutrophil proteins, are releases upon activation and induce neutrophil chemotaxis and adhesion (Chan et al. 2012; Ryckman et al. 2003). Gopal et al. recently demonstrated that the heterodimer S100A8/A9 accumulated in TB granulomas and that this was driven by neutrophils (Gopal et al. 2013). Scott et al. further showed that the resultant influx of neutrophils to the site of disease was due to integrin CD11b upregulation on the cell surface (Scott et al. 2020). This is in line with the correlations observed involving S100A9 as being reflective of the prominent role neutrophils play during active TB disease. Furthermore, elevated levels of IL-8, IL-6, IL-1β, IL-17A, GM-CSF, TREM-1 and G-CSF in active TB disease have been widely reported (Kumar et al. 2019; Yao et al. 2017; Feng et al. 2021) and their link to neutrophils is established (Cassatella et al. 2019). These data, therefore, highlight the need to expand or focus panels on neutrophil-specific cytokines and chemokines.

The strong relationship between increased blood neutrophils and decreased CD15 expression is described in chapter three. Furthermore, we found that a unit increase in CD15 expression was associated with 83% reduced risk of solid culture positivity at 2 months post treatment initiation (Ndlovu et al. 2020). We hypothesized this reduced CD15 expression might relate to difference in neutrophil recruitment from the bone marrow and/or chemotaxis to the site of disease and, therefore, that plasma molecules involved with these processes might have improved sensitivity at predicting TB treatment outcome. However, none of the markers were associated with either culture conversion at 2 months or treatment success at 6 months. Other studies have found associations between IL-12p40, IP-10, RANTES, IL-6, IL-1Ra, VEGF are associated with culture conversion and TB cure (Riou et al. 2012; Goletti, Lindestam Arlehamn, et al. 2018; Ronacher et al. 2019; Lee et al. 2015), which might be due to greater sample sizes or different patient populations. None-the-less, an increase in CD15 expression was associated with a decrease in 8 markers, most notably, the IL-6, IL-1β and G-CSF, suggestion a potential mechanistic link. IL-6 and IL-1β are potent inducers of acute phase responses

and regulate neutrophil chemotaxis by enhancing signal transducer and activator of transcription 3 (STAT3) activation and neutrophil migration towards IL-8, CXCL1 and CXCL2 (Wright et al. 2014; Biondo et al. 2014). G-CSF regulates neutrophil differentiation and mobilization from bone marrow and has been linked to the influx of immature, $CD15^{low}$, neutrophils during emergency granulopoiesis (Zarco et al. 1999). In addition, increased IL-6 was also significantly correlated with increased neutrophil counts and has been linked to . Taken together this data suggests that chronic inflammation as a result of Mtb infection drives the influx of immature neutrophils in the blood. However, it is unclear if immature neutrophils are also present at the site of infection and whether this impacts their function.

Hierarchical clustering of TB cases at baseline **(Figure 11A)** and over the course of treatment **(Figure 16)** suggest a co-ordinated spectrum of plasma cytokine response in TB infected subjects. Cluster 1 was associated with the highest level of inflammation and, as expected, had the highest proportion of ATB cases and the least healthy controls or the month 6 samples, depending on the analysis, and vice versa in clusters 3 and 4. These data are in line with published works showing reduced expression of cytokine levels following TB therapy and significant difference of cytokine levels in ATB patients and healthy controls (Moideen et al. 2020; Chowdhury et al. 2014; Prada-Medina et al. 2017). A correlation matrix analysis revealed co-linearity among these analytes thus therapeutic interventions designed to reduce chronic inflammation should target these dominant markers and specifically those linked to neutrophils. Surprisingly, however, in the analysis of baseline samples alone, these distinct clusters did not correlate with clinical measures of TB severity (CXR and bacterial load). Together with the lack of association between plasma cytokines treatment success, which does correlate with CD15 expression, these data suggest that neutrophil phenotype maybe a more sensitive measure of disease severity at baseline.

Finally, we assessed the impact of HIV co-infection on cytokine production on TB infected individuals. The pathogenesis of TB/HIV co-infection has not been completely delineated, but it is characterised by dysregulation of the immune responses to infection often leading to viral or bacterial replication and premature death if untreated (Sharma, Mohan, and Kadhiravan 2005). Mihret et al. recently reported no significant difference in plasma cytokine levels before treatment in HIV- or HIV+ TB patients (Mihret

et al. 2014). Similarly, our data show no clustering among TB cases at baseline based on HIV status which indicates that these analytes do not appear to be influenced by HIV co-infection. This is an important factor in biomarker discovery as it influences how informative or useful that biomarker would be in countries with a high burden of HIV and TB.

This study is not without its limitations. Importantly, the neutrophil-associated panel used only a subset of TB cases (n=40) and therefore there was less statistical power for these comparisons. Given the general support of the concept that neutrophils maybe a useful "sentinel" of TB disease severity and treatment response, it will be important to test the neutrophil specific markers in a larger data set, particularly focusing on those markers that are not typically measured. TB cases from the S100A8/A9 ELISA had cytokine levels which neared the maximum limit of detection resulting in the potential loss of analyte resolution. S100A8/A9 could therefore not be used in any downstream analysis.

Chapter 5: Use of neutrophil biomarkers to characterize and distinguish overt TB disease from subclinical TB

5.1 Introduction

Current TB screening methods rely on symptom manifestation to trigger a referral of suspected TB cases for radiological and/or microbiological testing. Recent data from TB prevalence surveys conducted in Africa and Asia, however, have reported that at least 60% of individuals with bacteriologically positive sputum do not experience TB associated symptoms that would prompt health-seeking behavior (Hoa et al. 2010; Corbett et al. 2010; Ayles et al. 2009). This phenomenon, termed sub-clinical or incipient TB, exists between two ends of a spectrum of Mtb infection from latent infection to overt active TB disease. Case definitions have remained unclear in the past; however, a more recent and applicable definition have been proposed: Incipient TB is an infection with viable mycobacteria that is likely to progress to active, symptomatic disease in the absence of further intervention but has not yet induced clinical symptoms, radiographic abnormalities, or microbiologic evidence; while subclinical TB is disease due to viable mycobacteria that does not cause clinical TB-related symptoms but causes other abnormalities that can be detected using existing radiological or microbiologic assays (Drain et al. 2018). Sub-clinical and incipient TB largely go undiagnosed, and the existence of these 'missing' TB cases greatly hampers efforts to eradicate TB. In addition, the inflammatory states of these individuals remain poorly understood. Prioritizing strategies to identify and distinguish these groups is critically important as people with active, subclinical and incipient TB will most likely require a full course of anti-TB therapy whilst those with latent infection would not.

Studies using differential blood counts as biomarkers of Mtb infection have largely focused on patients with active TB disease from the clinic with very few, if any, evaluating their use in the community. In pursuit of this, we characterized subclinical and potentially incipient TB among 18000 participants in a recent community health screen conducted in the rural Northern KwaZulu natal called "Vukuzazi". All

subjects were screened for classic TB symptoms, received a digital CXR and blood smears were obtained. Individuals reporting any TB symptom, or with evidence of abnormal CXR, as indicated by an automated artificial intelligence algorithm for identifying TB, CAD4 TB (Murphy et al. 2020), were tested for the presence of Mtb in sputum by GeneXpert and liquid culture. This identified 176 microbiologically confirmed TB cases by either gene expert and/or culture, the majority (~80%) of which were asymptomatic.

Based on the data presented in chapter three, we hypothesized that neutrophil counts would be significantly elevated in subjects with microbiologically confirmed TB compared to age and sex matched community controls. Moreover, because they correlated with disease severity in our clinic-based study, we hypothesised they would also distinguish participants with subclinical TB from overt TB cases, based on the assumption that subclinical disease represents an earlier and less severe form of TB. To quantify blood neutrophil counts and NLR, blood smears were selected from age and sex matched Vukuzazi participants (n=170) from one of three categories. Participants in group 1 (n=66) had microbiologically proven baseline TB, group 2 (n= 70) had radiologically suggested baseline TB without microbiological confirmation and participants in group 3 (n=34) were healthy controls with normal baseline chest X-ray. Each group was subdivided into symptomatic and asymptomatic, based on TB specific clinical questionnaires collected at the time of sampling.

5.2 Results and Discussion

Clinical and demographic parameters do not distinguish active from sub-clinical TB disease

The characteristics of the study participants are summarised in **Table 5**. More than 70% of participants from all groups were over 45 years of age and at least 55% were HIV positive. Result from our study show significant differences in BMI across all groups (P=0.0055), with culture positive patients and those with CXR suggestive TB having the lowest BMI when compared to healthy controls. In addition, within groups 1 and 2, ~50% of all participants experienced at least one TB associated symptom, with a cough being the most common. Our data is consistent with findings by Bregani et al. (Bregani et al. 2013) who identified a long-lasting cough (>1 month), low BMI, weight loss, fever and night sweats to

be among the most suggestive indicators of pulmonary TB. Individuals with HbA1c levels above 6.5% are classified here as diabetic. A total of only 20 participants in this study fell into this group, and no significant differences in levels between groups were observed. Participants in group 2 had significantly higher CXR scores compared to group 1 and healthy controls ($P<0.0001$). This is expected as participants in group 1 were categorised based on the presence of Mtb is sputum regardless of CXR score or the presence of abnormalities, whilst group 2 were identified on the basis of CXRs. Symptomatic participants with radiological or microbiological evidence of TB are commonly classified as active TB cases based on the current WHO guidelines (WHO 2010) and non-symptomatic cases classified as either subclinical or incipient TB (Achkar and Jenny-Avital 2011; Drain et al. 2018). Although historically and in clinical settings, CXRs suggestive of TB have been considered as ATB and thus should fall under the same group. From a research perspective, group 1 and group 2 are clearly distinct and should be interrogated seperately.

Initially, we evaluated the impact of symptom presentation on clinical measures among study participants. **Figure 17A** shows no significant effect of symptom presentation within the groups for bacterial load, CXR score, BMI or HbA1c. Indeed, for all parameters, there appears to be an equal degree of variability among symptomatic and non-symptomatic participants. It is important to note that not all individuals in the TB infected group are positive by liquid culture, indicated by the fraction (n=24) in the figure that had negative bacterial load values. In our clinic-based cohort, these individuals were not followed up longitudinally and were considered a separate group, for which a clear diagnosis was not made. As symptom screening is the primary method for identifying potential TB cases this data highlights the need for more accurate and objective methods for identifying TB cases, as many individuals in this cohort have both a high bacterial burden and a high degree of lung involvement. The fact that self-reported symptom presentation is not well associated with disease severity in community TB screening, even at the highest end of the spectrum is somewhat surprising and does raise several questions as to whether people are truly “asymptomatic” or just fail to identify or report the symptoms under study conditions. Never-the-less, taken at face value, these data certainly appear to suggest that symptom screening is a very poor tool indeed for identifying community TB cases.

HIV infection is a known risk factor of active TB disease and in South Africa, at least 50% of all TB cases are co-infected with HIV (Letang et al. 2020). Advanced HIV infection is associated with altered immunopathology contributing to bacterial replication and disease severity (Bell and Noursadeghi 2018). In chapter three we reported increased bacterial burden and CXR scores among HIV-ve patients compared to PLWH. Surprisingly, in this community-based screen, we see no significant differences in bacterial load, CXR score, BMI and HbA1c% based on HIV status (**Figure 17B**). Again, this points to potential differences in the presentation of TB at clinics compared to in community health screens.

Table 5: Baseline clinical and demographic characteristics of microbiologically confirmed (Group 1) and radiologically suggested TB cases (Group 2), and healthy controls (Group 3).

Characteristics N (%) or Median [IQR]	**Group 1 (n=66)**	**Group 2 (n=70)**	**Group 3 (n=34)**	**P-value[a]**
Age (45+)	47 (71,2)	51 (72,9)	24 (70,6)	0,9637
Male sex	36 (54,5)	38 (54,3)	18 (52,9)	0,9878
HIV status (+)	37 (56)	40 (57)	19 (55,9)	0,9890
BMI (kg/m^2)	22,9 [15,6 – 40,06]	22 [14,12 – 43,43]	26 [18,82 – 39,29]	**0,0055[b]**
HBA1c	5,7 [4,6 – 12,5]	5,7 [4,4 – 11,4]	5,9 [4,7 – 11,6]	0,2550
TB symptomatic (yes)	31 (47)	35 (50)	-	0,7349
Fever	12 (18,2)	12 (17)	-	>0,9999
Cough	23 (35)	32 (45,7)	-	0,2237
Night sweats	9 (13,6)	7 (10)	-	0,5989
Weight loss (>6m)	10 (15)	6 (8,6)	-	0,2913
Bacterial load	23,8 [0 – 37,6]	-	-	-
CXR score	63,5 [19 -100]	88 [46 – 100]	22,5 [16 – 63]	**<0,0001[b]**

[a] P-values were determined by Kruskal-Wallis test for Dunn's multiple comparison and Mann-Whitney t tests.
[b] P< 0.05.
Values are bold to highlight statistical significance

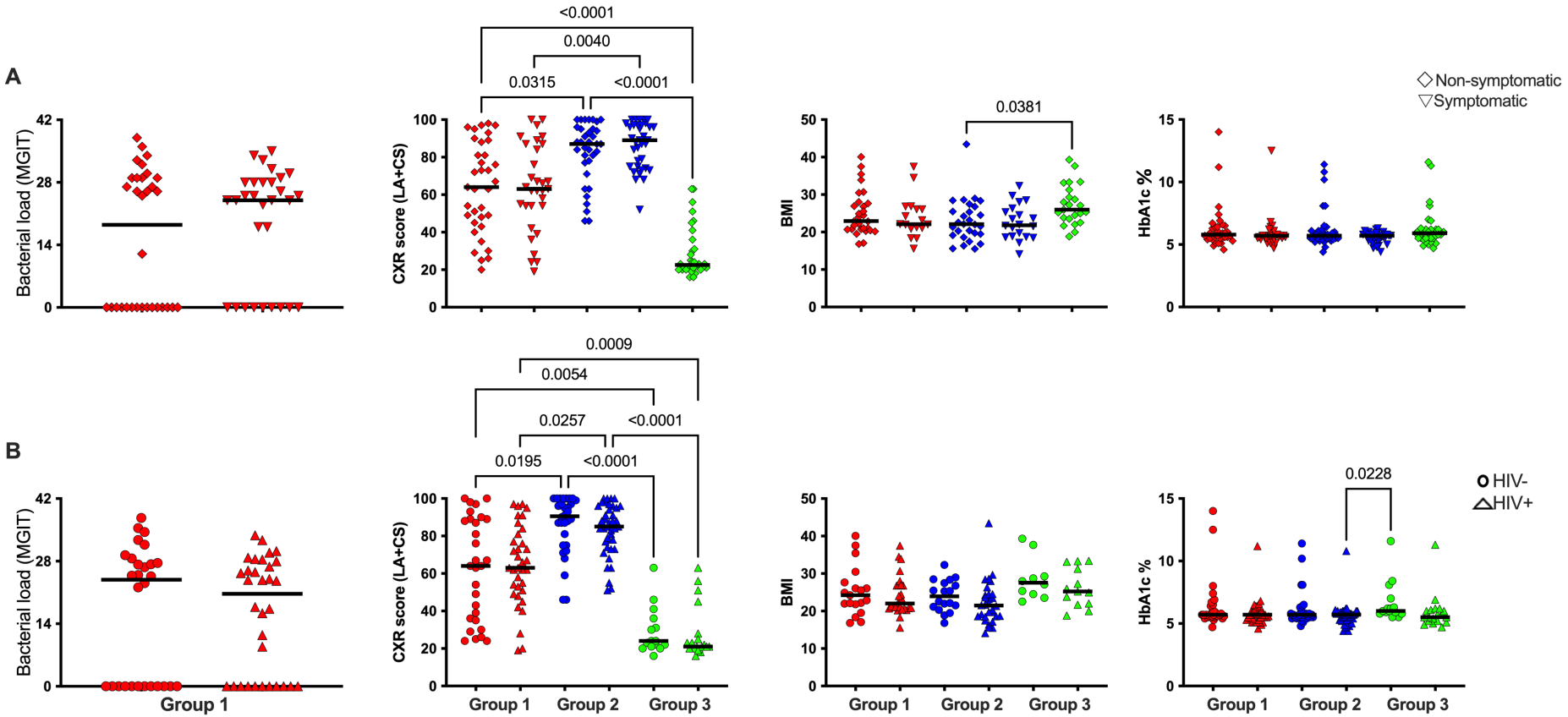


Figure 17: Impact of symptom presentation and HIV co-infection on baseline clinical parameters. Scatter plots of bacterial burden as measured by time to detection (TTD), chest X-ray score, BMI and percent HbA1c of microbiologically confirmed TB cases (group 1), radiologically suggestive TB (group 2) and healthy controls (group 3) separated by symptom presentation **(A)** and HIV status **(B)**. P-values were obtained using the Kruskal-Wallis for Dunn's multiple tests for multiple comparisons.

Neutrophil counts from blood smear are not a sensitive measure of TB disease severity

To assess the sensitivity of neutrophils in distinguishing the groups, differential blood counts were measured at baseline from blood smears using a commercial pathology lab. No significant changes were detected in neutrophil, monocyte and lymphocyte counts, or in the NLR and MLR among the three groups (**Figure 18A-E**). This data is inconsistent with our finding in chapter three and other studies showing elevated neutrophils and NLR to be associated with active TB disease (Yin et al. 2017; Ndlovu et al. 2020; Kerkhoff et al. 2013) when compared to healthy controls. This inconsistency may be largely explained by differences in recruitment protocols. Studies evaluating TB biomarkers use participants recruited from TB clinics who are most likely chronically infected and exhibit clinical TB symptom prompting the need to seek healthcare. In contract, participants identified in Vukuzazi were recruited from the community, only half presented with any symptoms and less than 20% presented with two symptoms or more (**Table 5)**. Perhaps more importantly, these symptoms had not prompted health care seeking behaviour, which might suggest they have less severe or chronic TB infection.

Similarly, to clinical markers, separating groups according to symptom presentation and HIV co-infection showed no significant differences within and between groups (**Figure 19**). This observation remained even when participants in group one was further divided into Xpert-positive and/or culture-negative groups. This result is expected and is consistent with data reported in chapter three. Interestingly, neutrophil phenotype (CD15 expression) and neutrophil specific cytokines (IL-8 and G-CSF) were able to distinguish ATB cases from Xpert-positive/culture-negative controls. Although neutrophil counts are commonly used as markers of inflammation, these data suggest that neutrophil counts are not a sensitive measure of TB disease severity within the community setting. In addition, they suggest that the inflammatory states as measured by neutrophil counts of individuals with sub-clinical and clinical TB do not differ significantly, at least in this setting. As discussed in the introduction, there are many different approaches in TB biomarker discovery, and some may perform better than others at identifying subclinical and/or community TB. However, the fact that neutrophil blood counts, which appear sensitive in the clinic, perform so poorly here, prompts the need to re-

evaluate TB biomarkers through the lens of community-based screening to ensure their viability outside of TB clinics, particularly when the aim is to identify 'missing' cases and eradicate TB disease.

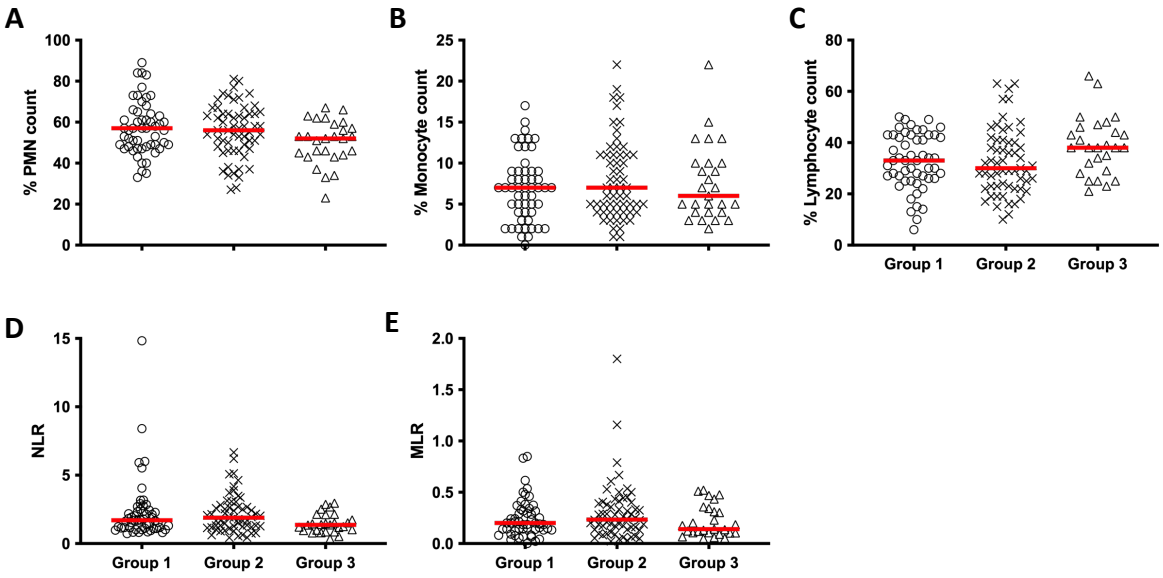


Figure 18: Baseline differential blood counts of Vukuzazi study participants. Scatter plots of percent neutrophil counts **(A**), monocyte counts **(B),** lymphocyte counts **(C),** neutrophil lymphocyte ratio (NLR) **(D)** and monocyte/lymphocyte ratio (MLR) **(E**).

I then regrouped the study participants based on the published case definitions for overt, sub-clinical and incipient TB. Briefly, overt TB is characterized by symptom presentation with microbiological or radiologic evidence while sub-clinical TB is non-symptomatic with microbiological or radiologic evidence. Of the four parameters, no significant differences were detected between TB cases and sub-clinical TB (**Figure 20**). Interestingly, significance was observed in NLR between healthy controls and sub-clinical TB cases (**Figure 20B**).

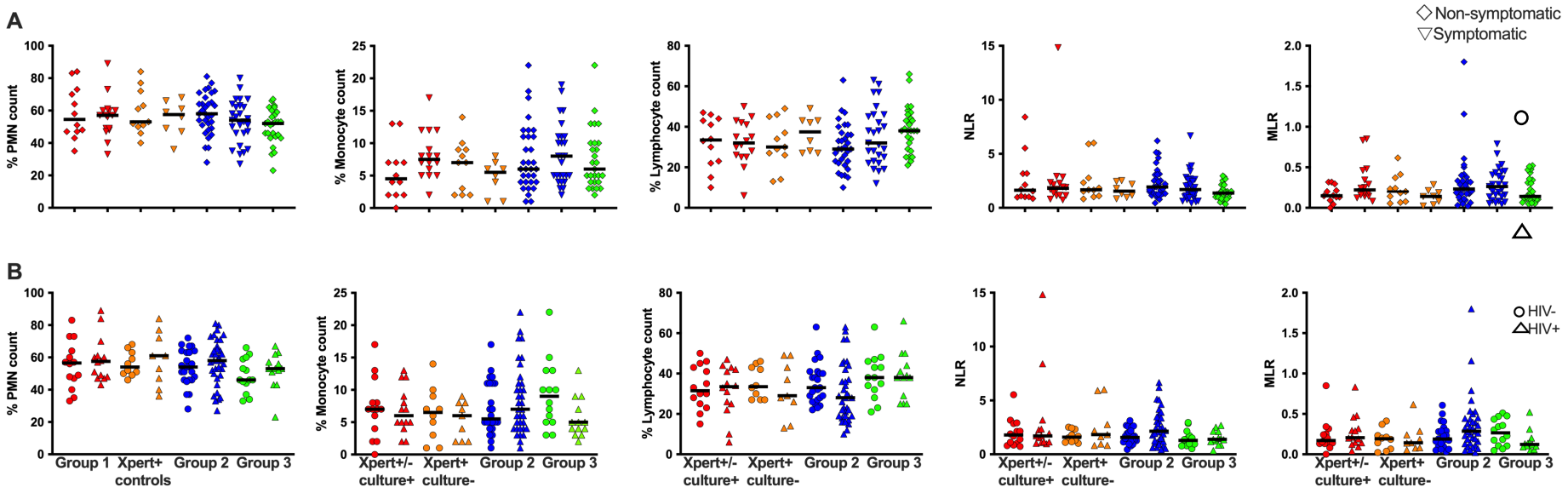


Figure 19: Effect of symptom presentation and HIV co-infection on differential blood counts. Differences in neutrophil counts, monocyte counts lymphocyte counts, NLR and MLR in study participants with microbiologically confirmed TB, by GeneXpert and/or liquid culture, radiologically suggestive TB (group 2) and healthy controls (group 3). Data was separated by symptom presentation **(A)** and HIV status **(B)**.

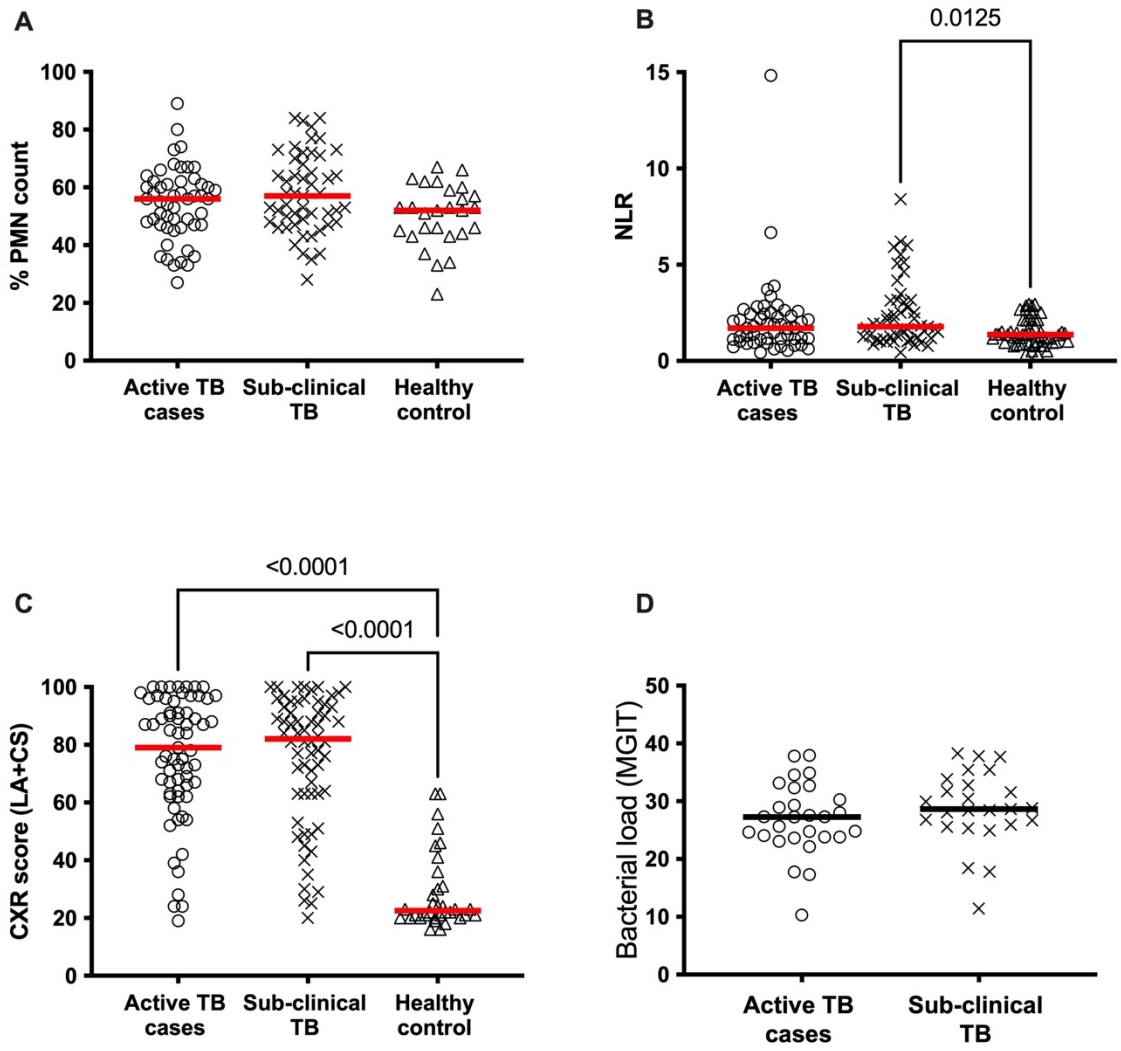


Figure 20: Baseline measures of clinical and inflammatory markers in active and sub-clinical TB. Scatter plots of percent neutrophil counts, NLR, CXR score and TTP of active and subclinical TB cases, and healthy controls. P-values were obtained using the Kruskal-Wallis tests for Dunn's multiple comparisons (A-C) and Mann-Whitney t tests (D).

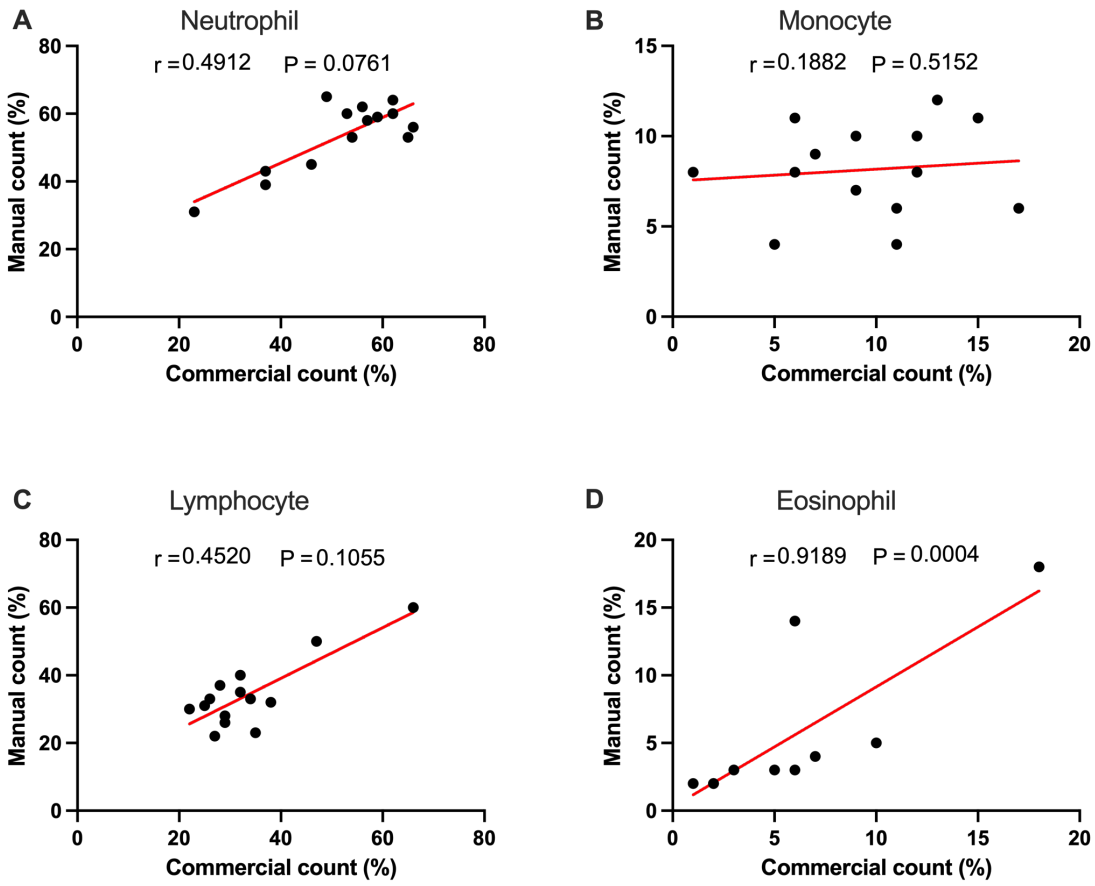


Figure 21: Comparison of baseline blood counts performed commercially and manually. Spearman's rank correlation analysis of neutrophil (A), monocyte (B), lymphocyte (C) and eosinophil (D) percentages measured in a commercial lab and manually in house. R values between 0.7-1 are considered strong; 0,5-0.7 medium; 0.3-0.5 weak and 0-0.3 not correlated.

Lastly, it is important to note that blood counts here were done using blood smear microscopy, whereas in chapter three, an automated flow based counting system using whole blood was employed. The smear microscopy approach is likely to lead to more technical noise, due to slide preparation, the fields of view examined to generate the count and differences between the manual readers. To test this, I recounted a randomly selected subset of blood slides. **Figure 21** shows a generally poor correlation between counts from the same slides, particularly for monocyte (**Figure 21 A,**C). Distribution between lymphocyte and monocyte counts is potentially a result of morphological similarities making them difficult to distinguish with a 100% accuracy. Regardless, eosinophil show a highly significant and

strong correlation between the two counts (**Figure 21D**). Eosinophil morphology is highly distinct and significantly easier to identify.

Results from this study indicate that blood counts measured from blood smears may not be an accurate means of identifying or distinguishing sub-clinical from active TB in a community setting. Unfortunately, it was not possible to evaluate the performance of absolute blood counts performed by flow cytometry as the appropriate sample was not collected from this cohort. This methodology may be more able to detect the subtle differences and is potentially a more objective approach. In addition, it would be interesting to evaluate the use of plasma cytokine levels and transcriptional profiles to delineate the TB spectrum and as potential biomarkers distinguishing disease states. Pursuing these answers was not possible in this study due to time and budgetary constraints and the intervention of the COVID-19 pandemic.

Final discussion and conclusion

The World Health Organisation ambitiously aims to reduce new TB cases by 90% in the year 2035. A major challenge to meeting this target is poor adherence to the prescribed treatment regimen and the incomplete characterisation of TB disease, which is imperative for TB diagnosis. In this thesis I measured blood neutrophil counts, phenotype and associated plasma cytokine levels at diagnosis and over the course of treatment. I endeavoured to characterise subclinical and overt TB disease and identify novel biomarkers of TB disease severity and treatment response among participants from the clinic and the community.

Briefly, we find that overt TB disease diagnosed in the clinic is associated with elevated neutrophil blood counts and NLR compared to healthy controls. Excessive release of neutrophils during inflammation may contribute to tissue damage and exacerbate disease (Panteleev et al. 2017; Abakay et al. 2015). Similar findings were observed in this study, as neutrophil counts correlated with disease severity as measured by bacterial burden and extent of lung involvement measured by CXR. Following treatment initiation neutrophil count and NLR resolved to heathy control levels, indicating that the neutrophil response follows the path of TB treatment and may serve as potential markers of TB disease and treatment outcome. Monocyte counts and MLR also emerged as a potential markers of TB disease when compared to other WBC subsets. This is not surprising as whole transcriptomic analysis of sorted blood immune subsets showed that, second to neutrophils, monocytes also provide a strong TB associated signature (Bloom et al. 2013) and are able to distinguish ATB from LTBI and healthy controls (La Manna et al. 2017). Monocyte counts were also significantly correlated with disease severity and unlike neutrophil counts distinguished ATB from a distinct group of patients which presented with classic TB symptoms, were GeneXpert positive but culture negative. However, the high turnover of neutrophils during infection makes them prime candidates for monitoring disease states compared to monocytes. None-the-less, monocytes offer an additional source of TB associated biomarkers that warrant further exploration.

Neutrophil phenotyping using 8 markers involved in neutrophil activation, maturation and transmigration revealed CD15, a carbohydrate adhesion molecule typically expressed by mature neutrophils (Elghetany 2002), as a robust marker of TB disease state. Expression levels were significantly reduced with active disease, inversely correlated with neutrophil counts and bacterial burden and began to resolve within a week of treatment and reached healthy control levels following six months of treatment. CD15 was superior to other neutrophil surface markers as it was unaffected by technical issue I experienced whilst carrying out this longitudinal study. These were of course unintended and may have caused me to miss other potentially useful markers. However, the fact that CD15 still remained highly significant despite these issues may indicate it is indeed a robust and sensitive immune marker of TB disease. Importantly, the strong correlation with neutrophil count and bacterial load indicates potential as a rapid point of care measure of disease severity. Sputum culture is not only the primary means of TB diagnosis; it is also used to determine treatment duration. Patients who remain culture positive at 7 weeks post treatment initiation can continue the regimen extended for up to 24 months (Nahid et al. 2016; Nahid et al. 2019). A unit increase in CD15 expression at baseline was associated with an 83% reduced risk of remaining culture positive at 2 months post-treatment initiation, indicating that the neutrophil response may have value for predicting likelihood of a successful treatment outcome at diagnosis. To put it another way, it may be possible to identify TB patients who are at elevated risk of failing treatment and therefore qualify for enhance follow-up. CD15 could also, therefore, offer an alternative and more attractive approach for monitoring treatment response that is not reliant on sputum, which is not always easy to obtain and is not impacted by HIV co-infection (Zar et al. 2000; Elliott et al. 1993; Maddineni and Panda 2008). CD15 expression remained significantly correlated with bacterial load and neutrophil counts a month post-treatment initiation. Importantly, sputum culture takes up to 42 days to deliver a result, whereas POC measures such as this could allow for on the spot decision making.

As previously mentioned, CD15 expression was significantly reduced among TB cases pre-treatment. A similar observation was made when Zarco et al. treated healthy donors with recombinant G-CSF, a

neutrophil specific growth factor (Zarco et al. 1999), that stimulates immune cell release from the bone marrow (Semerad et al. 2002). I hypothesised the CD15 low neutrophil phenotype could result from an influx of immature neutrophils as a result of chronic inflammation and that neutrophil associated cytokine/chemokines and soluble factors are the key drivers of this response. Exploring the relationship between neutrophil phenotype and cytokine levels also presents an opportunity to identify additional biomarkers of TB disease. I reported that, neutrophil associated analytes (i.e., G-CSF, GM-CSF, IL-8, IL-17A, IL-6, IL-1β, IL-18, IL-6Ra, S100A9, S100A8/9 and TREM-1) were among the cytokines significantly elevated in active disease compared to Xpert-positve/culture-negative and/or healthy controls. These markers have previously been shown to have potential in discriminating active TB from LTBI and HC in other studies (Yao et al. 2017; Luo et al. 2019), however, few studies have directly linked their expression levels as primary contributors to neutrophil associated TB inflammation. Of these analytes S100A9 had strong direct correlations with bacterial burden, CXR score, neutrophil counts and an inverse correlation with CD15 expression. S100A9 is an a potent neutrophil-specific chemotactic factor comprising ~40% of neutrophil proteins (Ryckman et al. 2003) and as a heterodimer (S100A8/9) is linked to neutrophil accumulation in the granuloma (Scott et al. 2020). Therefore, in addition to CD15 expression, S100A9 has potential as a highly specific marker of neutrophil driven TB disease severity. In concordance with the above study, G-CSF was significantly correlated with CD15 expression, supporting the hypothesis that low CD15 could be linked to an increased release of neutrophils from the bone marrow. This molecule, however, showed no correlation with neutrophil counts, bacterial load and CXR score. None-the-less taken together this data suggests a clear relationship between immature neutrophil influx and CD15 expression.

Hierarchical clustering revealed four distinct clusters, reflecting a potential spectrum of inflammation present during active TB disease, and suggesting that a group of markers as opposed to individual analytes may be more useful as biomarkers. This was further highlighted by the lack of individual markers who's baseline value was associated with culture conversion at two months post treatment initiation. Indeed, this observation itself support the potential value of measuring neutrophil phenotypes, as measuring CD15 alone appears to provide this discriminatory power. However, several analytes,

such as G-CSF, IL-8, IL-6 and IL-1β did significantly reduce in the first 3/4 weeks post treatment initiation, suggesting cytokine response can change rapidly in response to successful drug treatment. Furthermore, other studies reported associations between IP-10, RANTES, IL-6, IL-1Ra, VEGF with culture conversion and TB cure (Riou et al. 2012; Goletti, Lindestam Arlehamn, et al. 2018; Ronacher et al. 2019; Lee et al. 2015), which may indicate the sample size was too small in this study to detect the effects observed in other studies. Interestingly, IL-8, another potent neutrophil specific marker was not associated with neutrophil counts or disease severity in this instance.

TB prevalence surveys have reported that 60-80% of individuals with prevalent, bacterially or radiologically confirmed ATB report having no clinical symptoms (Brennan et al. 2020; Miller et al. 2000). Characterising the spectrum of TB disease is essential to aid TB diagnosis and thus link the 'missing' TB cases to care. It also provides an opportunity to identify novel biomarkers that can be used to distinguish ATB from incipient and subclinical TB. This is an especially important consideration when one takes into account the fact that TB biomarkers are often evaluated primarily in patients from the clinic, who are often experiencing overt TB disease, and not evaluated in the community where asymptomatic TB disease is prevalent (Hoa et al. 2010; Ayles et al. 2009). Using blood smear obtained from the Vukuzazi TB prevalence survey, differential blood counts were measured. We find no detectable changes in neutrophil blood counts, NLR and bacterial burden among participants with microbiologically confirmed and radiologically suggested TB, and healthy controls. Furthermore, these findings were not impacted by symptom presentation or HIV status. This may indicate that neutrophils are not a sensitive enough measure for characterising the TB spectrum within the community or that blood smears, which are subject to human error, are not a reliable method for detecting the subtle differences in blood counts. This begs the question of how useful other TB associated markers identified in the clinic are when applied in the community.

In conclusion, active TB is associated with detectable neutrophil responses which resolve with treatment. Notably, increased neutrophil counts and down-regulation of CD15 expression correlated with disease severity, i.e bacterial load and CXR scores. Reduced CD15 expression is likely to occur as a result of the influx of immature neutrophils from the bone marrow, due to emergency

granulopoiesis. This coincided with elevated levels of several neutrophil related cytokines including S100A9 and the growth factor G-CSF, which is known to stimulate the release of $CD15^{low}$ neutrophils from the bone marrow. Importantly, CD15 expression was the only baseline marker that correlated with culture conversion at two months, suggesting that monitoring the neutrophil response may potentially be useful in predicting treatment outcomes. Overall, neutrophils play a critical role in TB control and progression and are a likely to represent an important area of biomarkers research. Finally, this work highlights the need to evaluating biomarkers within the clinic and community setting to ensure their viability. Particularly, when the aim is to improve TB diagnosis and case management.

Future work to follow up from this study would include validating the CD15 and S100A9 findings in a separate cohort. Evaluating specificity and sensitivity in discriminating ATB from LTBI and HC and predicting treatment outcomes. Within the community, further evaluation of the use of neutrophil associated plasma cytokine levels to characterise and therefore identify incipient and subclinical TB disease and observe identified cases for disease progression. Monocyte counts showed promise as biomarkers and therefore exploring monocyte specific response to active TB disease may reveal additional biomarkers that can be used in combination with neutrophil associated biomarkers. Ultimately, this work shows the potential for blood neutrophil counts, phenotype and associated plasma cytokines to be developed as a POC test, in addition to the standard microbiological tests, for distinguishing of ATB, non-TB and HC regardless of HIV co-infection.

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