HOW LATENT IS “LATENT” TUBERCULOSIS?
The radiographic, transcriptional and immunological characterisation of subclinical tuberculosis in HIV infected adults

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For Leila and Seren, may you be inspired to think critically.
ABSTRACT

Background
The central hypothesis of the thesis is that the neat division of tuberculosis (TB) into states of active disease and latent infection is an oversimplification and that the transition between latent and active TB involves passage through a subclinical phase of disease, which may be prolonged, during which pathology evolves. The primary aim of this thesis is to utilise $[^{18}\text{F}]$-fluoro-2-deoxy-D-glucose positron emission tomography combined with computed tomography (FDG-PET/CT) to identify and define intra-thoracic pathology consistent with subclinical TB in a cohort of asymptomatic adults diagnosed with latent TB at high risk of developing active TB (due to HIV co-infection) and then to identify transcriptional and immunological biomarkers that distinguish those with radiographic evidence of subclinical pathology. Such biomarkers may have future translational potential as tests more predictive of active TB compared to the currently available tests (tuberculin skin testing (TST) and interferon gamma release assays (IGRA)) and may also aid our understanding of the biology of TB.

Methodology
Healthy HIV infected, ART naïve, adult outpatients living in an area with very high TB burden (Khayelitsha township, Cape Town, South Africa) were screened to identify 35 participants that were asymptomatic, with CD4 count $\geq 350$ mm$^3$, evidence of latent TB (by QuantiFERON Gold in tube (QFGIT)) and with no history of previous tuberculosis or evidence of current active TB. These participants had FDG-PET/CT performed and were then commenced on isoniazid preventive therapy (IPT) or standard TB therapy if clinically indicated and had repeat FDG-PET/CT following treatment. A number of additional groups of HIV infected and uninfected control participants with and without active TB were also recruited for blood sampling. Microarray, carried out on RNA extracted from whole blood, was used to identify differentially abundant transcripts between those with and without subclinical pathology. A 38-plex assay and ELISA covering a total of 45 analytes were then used to identify serological or QFGIT plasma biomarkers that distinguish those with and without subclinical pathology.
Main Results
Parenchymal abnormalities in the 35 participants were evaluated in detail and interpreted in relation to the historical autopsy data and 28.6% were categorised as having evidence of subclinical TB pathology. Analysis of the whole blood microarray for these 35 participants along with 15 age, sex and CD4 count matched controls with clinical active TB identified 82 transcripts that clustered 80% of those with subclinical TB with active TB. Those with more metabolically active subclinical pathology, as determined by FDG uptake, clustered more effectively with clinical active TB. This signature was confirmed as specific to TB in HIV uninfected controls. Transcripts relating to the classical complement pathway and Fcγ receptor were found to be overabundant in subclinical and active TB in relation to those with latent TB with no evidence of subclinical pathology. Neutrophil related transcripts were over abundant only in clinical active TB, particularly in those that were smear positive. Network analysis of the 82 transcript signature, informed the selection of 45 soluble protein analytes. 10 analytes showed a significant difference in concentration between the 3 groups (active, subclinical and latent TB). IL-1α with a cut-off of 16.9 pg/mL and circulating immune complex (CIC) with a cut-off of 100.9 µg Eq/mL individually classified 50% and together 70% of those with subclinical TB as active TB. In addition when assessed across 5 stages of increasing disease activity by PET findings and smear status all 10 analytes showed a significant increasing trend.

Conclusion
The utility of FDG-PET/CT a novel research tool in the study of latent TB in humans has been systematically evaluated for the first time in this thesis. It has allowed for the identification of pathology within the lungs consistent with subclinical TB not reliably identified on CXR. Microarray analysis of whole blood has contributed of our understanding of which biological process may be pertinent from the early subclinical stages of disease, suggesting that the classical complement pathway and overabundance of Fcγ receptor may be important. Furthermore, the approach has lead to the identification of transcriptional and serological biomarkers that distinguish those with subclinical pathology from those without. These biomarkers may have translational potential as more predictive diagnostic tests for active TB.
ACKNOWLEDGEMENTS

First and foremost, I wish to thank my parents for their lifelong and unquestioning support, love and encouragement. In particular, I am grateful for the sacrifices they have made for me over the years. Their own academic journey has also been a constant source of inspiration and a clear example of the transformative power of education.

This thesis would not have been possible without the help and support of numerous people. I am particularly grateful to my supervisor Professor Robert Wilkinson. From our first meeting, over 6 years ago to discuss research opportunities in Cape Town, he has been a very generous and supportive academic mentor, providing me with numerous opportunities, helpful advice but also the freedom to develop as a clinical researcher. I am also grateful for the mentorship and support of Professor Douglas Young and Dr Clifton Barry 3rd and the exposure they have provided me to a global, collaborative group of TB researchers, which has been both exciting and inspiring.

This research would not have been possible without the group of participants from Khayelitsha, to whom I am greatly indebted to for consenting to take part the study. The study would also not have been feasible without the help, both administratively and clinically, of a very large group of dedicated people, in the Wilkinson Group at the University of Cape Town. I am especially appreciative of all the help and assistance provided by the clinical research workers, nurses and drivers in the recruitment and follow-up of the participants while they were working on a number of other studies. Particular thanks go to Kathryn Wood and Rene Goliath who have always readily provided assistance to ensure the smooth running of the study.

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DECLARATION

Apart from the assistance I have outlined in the acknowledgement section, the work described in this thesis is my own, with everything else being appropriately referenced. The work in this thesis has not been previously submitted for a degree or qualification at this or any other university.

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<td>3TC</td>
<td>Lamivudine</td>
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<tr>
<td>AFB</td>
<td>Acid Fast Bacilli</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>ARI</td>
<td>Annual Rate of Infection</td>
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<td>ART</td>
<td>Anti-Retroviral Therapy</td>
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<td>AUC</td>
<td>Area Under the Curve</td>
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<td>AZT</td>
<td>Zidovudine</td>
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<tr>
<td>BCG</td>
<td>Bacille Calmette-Guerin</td>
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<tr>
<td>BF</td>
<td>Complement factor B</td>
</tr>
<tr>
<td>Bq</td>
<td>Becquerel</td>
</tr>
<tr>
<td>C.I</td>
<td>Confidence Interval</td>
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<tr>
<td>C/EBPβ</td>
<td>CCAAT-enhancer-binding protein β</td>
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<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
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<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
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<td>CEQ</td>
<td>Chromosomal Equivalents</td>
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<td>CFP-10</td>
<td>10 kDa Culture Filtrate Antigen</td>
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<td>Cr</td>
<td>Creatinine</td>
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<td>CRF</td>
<td>Clinical Record Forms</td>
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<td>CRP</td>
<td>C-Reactive Protein</td>
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<td>CT</td>
<td>Computed Tomography</td>
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<td>CXR</td>
<td>Chest radiograph</td>
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<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
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<td>DosR</td>
<td>Dormancy survival Regulon</td>
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<td>EGF</td>
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<td>EHR</td>
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<td>FBC</td>
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<td>FC</td>
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<td>FCGR1A</td>
<td>Fcγ receptor 1A</td>
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<td>FDG</td>
<td>[18F]-fluoro-2-deoxy-D-glucose</td>
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<td>FDR</td>
<td>False Discovery Rate</td>
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<td>FGF-2</td>
<td>Fibroblast Growth Factor</td>
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<tr>
<td>Flt-3L</td>
<td>Fms-related tyrosine kinase 3 ligand</td>
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<td>Fluoromisonidazole</td>
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<td>Granulocyte Colony Stimulating Factor</td>
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<td>Hounsefield Units</td>
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<tr>
<td>IASLC</td>
<td>International Association for Study of Lung Cancer</td>
</tr>
<tr>
<td>ICH-GCP</td>
<td>International Conference on Harmonization of Good Clinical Practice</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGRA</td>
<td>Interferon Gamma Release Assay</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>Interleukin 1 Receptor Antagonist</td>
</tr>
<tr>
<td>INH/H</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>IP</td>
<td>Interferon-gamma inducible Protein</td>
</tr>
<tr>
<td>IPT</td>
<td>Isoniazid Preventive Therapy</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter Quartile Range</td>
</tr>
<tr>
<td>ISGF</td>
<td>Interferon Stimulated Gene Factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>kV</td>
<td>kilovoltage</td>
</tr>
<tr>
<td>LAL</td>
<td>Limus Amebocyte Lysate</td>
</tr>
<tr>
<td>LFT</td>
<td>Liver Function Test</td>
</tr>
<tr>
<td>LLL</td>
<td>Left Lower Lobe</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph Node</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LR-</td>
<td>negative Likelihood Ratio</td>
</tr>
<tr>
<td>LR+</td>
<td>positive Likelihood Ratio</td>
</tr>
<tr>
<td>LTA4H</td>
<td>Leukotriene A₄ hydrolase</td>
</tr>
<tr>
<td>LUL</td>
<td>Left Upper Lobe</td>
</tr>
<tr>
<td>mAs</td>
<td>milliampere-second</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte Chemoattactant Protein</td>
</tr>
<tr>
<td>MDC</td>
<td>Macrophage Derived Chemokine</td>
</tr>
<tr>
<td>med</td>
<td>median</td>
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<tr>
<td>MGIT</td>
<td>Mycobacteria Growth Indicator Tube</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinases</td>
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<tr>
<td>MRC</td>
<td>Medical Research Council</td>
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<tr>
<td>Mtb</td>
<td><em>Mycobacterium tuberculosis</em></td>
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<tr>
<td>NHLS</td>
<td>National Health Laboratory Services</td>
</tr>
<tr>
<td>NIAID</td>
<td>National Institute of Allergy and Infectious Diseases</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-Nucleotide Reverse Transcriptase Inhibitor</td>
</tr>
<tr>
<td>NNT</td>
<td>Number Needed to Treat</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative Predictive Value</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleotide Reverse Transcriptase Inhibitor</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-Nitrophenyl Phosphate</td>
</tr>
<tr>
<td>POC</td>
<td>Point Of Care</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified Protein Derivative</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive Predictive Value</td>
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PZA/Z  Pyrazinamide
QA    Quality Assurance
QC    Quality Control
QFGIT QuantiFERON TB Gold In-Tube
RD    Regions of Difference
RIF/R Rifampicin
RIN   RNA Integrity Number
RLL   Right Lower Lobe
RML   Right Middle Lobe
ROC   Receiver Operating Characteristic
RPE   Rifapentine
rpf   resuscitation-promoting factors
RT-MLPA Reverse Transcriptase Multiplex Ligation dependant Probe Amplification
RUL   Right Upper Lobe
s.d   standard deviation
SLE   Systemic Lupus Erythematosis
Sm    Smear
SOP   Standard Operating Procedure
ss    single-stranded
SUV   Standardized Uptake Value
Sv    Sievert
TB    Tuberculosis
TBNA  Transbronchial Needle Aspirate
TCEP  tris(2-carboxyethyl)phosphine
TDF   Tenofovir Disoproxil Fumarate
TGF   Transforming Growth Factor
TLR   Toll-Like Receptor
TMB   Tetramethylbenzidine
TNF   Tumour Necrosis Factor
TST   Tuberculin Skin Test
TU    Tuberculin Units
tx    treatment
UCT   University of Cape Town
USPHS United States Public Health Service
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>USS</td>
<td>Ultra Sound Scan</td>
</tr>
<tr>
<td>VL</td>
<td>Viral Load</td>
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<tr>
<td>VS</td>
<td>Visual Score</td>
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<tr>
<td>vs.</td>
<td>versus</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
CHAPTER 1: Introduction

1.1 General introduction

Tuberculosis (TB) is a disease of mammals caused by infection with members of the *Mycobacterium tuberculosis*-complex. *Mycobacterium tuberculosis* (Mtb) sensu stricto is the most common cause of tuberculosis in humans and is only occasionally isolated in other animals which are usually domesticated or in captivity and are generally unable to transmit Mtb. The organism, a bacterium characterised by a cell wall rich in mycolic acid, is a slow growing, strict aerobe and an obligate pathogen. Despite its adaptability within the host Mtb is unable to survive for long periods in the environment and hence humans are the main reservoir. Infection is usually transmitted via the respiratory route and the lungs are also the most common sites of disease but disease can occur in any part of the body and most often takes a sub-acute course. Common symptoms of disease are weight loss, night sweats, fever and cough but one of the most characteristic features of tuberculosis is its variable and often prolonged incubation period and the fact that only a small proportion of those infected ever develop symptomatic disease. As a result we conceptualise a non-infectious latent stage of TB during which the host is asymptomatic with low bacillary numbers and a state of symptomatic active disease with significantly increased bacillary numbers when there is potential for infection to be transmitted. The transition between these 2 states can be considered as subclinical TB.

Our ability to predict in whom and when the transition from latent infection to active disease will occur and our understanding of the mechanisms and events that underlie this are poor. However, there are a number of risk factors known to increase the likelihood of progression from latent to active TB. Human Immunodeficiency Virus (HIV) infection is one of the most potent and important of these risk factors and has had a major impact on the global epidemiology of TB. Although the currently available diagnostic tests for latent TB (tuberculin skin test (TST) and interferon gamma release assays (IGRA)) do not distinguish and are poorly predictive of active TB, recently progress has been made in identifying transcriptional and immunological biomarkers that can distinguish latent and active TB. This raises the possibility of developing infection stage specific biomarkers and diagnostic tests more predictive of active TB.
A central aim of this thesis is to contribute to our understanding of latent TB infection and early stages of its transition to active disease, particularly in the context of early HIV infection, with a view that this could facilitate development of diagnostic tests better able to predict active tuberculosis which would be an important step in global TB control. A core hypothesis for this thesis is that this definition of latent infection encompasses a spectrum of infection states.

The concept of a latent phase of tuberculosis even predates the discovery of Mtb by Robert Koch, though understanding developed in the early part of the 20th century with many holding quite a nuanced and sophisticated view of the condition, recognising that the boundary between latent and active TB was often blurred, fluctuated and was influenced by available diagnostics.

“Latent tuberculosis may be defined for convenience as that which is unaccompanied by symptoms and physical signs, causes no obvious disturbance and is not recognised by the physician. There is no sharp distinction between latent and manifest tuberculosis, and in some instances latent tuberculosis is more extensive than that which is recognisable. Ability to distinguish between latent and manifest disease will vary with the means available for diagnosis”

Opie and McPhedran 1926[1]

In the pre-chemotherapy era, when early diagnosis was considered crucial to a favourable outcome, identifying people in this fluctuating transition between latent and active TB was appreciated with mass chest radiograph (CXR) screening for subclinical TB being widely implemented globally. This was perhaps the first example of a mass screening programme in modern medicine. In the post-chemotherapy era a more rigid distinction evolved, with latent and active TB considered as binary outcomes in a diagnostic algorithm that required different management strategies. Latent TB is generally defined as having evidence of immune sensitization by Mtb, in the absence of symptoms or signs of disease and treatment with 1 or 2 anti-tuberculous agents for 3-9 months has been shown to reduce risk of progression to active TB. The diagnosis of active TB is made following identification of Mtb or evidence of active pathology caused by Mtb in a person with suggestive symptoms and treatment with a minimum of 3 effective drugs for a minimum of 6 months is required. However, recently the increasing availability in many parts of the world of advanced imaging more sensitive than CXR for the detection of minimal pathology, such as $^{18}$F-fluoro-2-deoxy-D-glucose (FDG) positron emission tomography combined with computed
tomography (PET/CT), and invasive sampling, such as trans-bronchial needle aspiration (TBNA) of lymph nodes, has again led to the frequent identification of cases that do not neatly fit into this simplistic binary categorisation which usually results in a management dilemma. In this thesis the improved sensitivity of PET/CT was exploited to detect evidence of subclinical pathology in asymptomatic persons otherwise considered to have latent TB. Transcriptional and immunological differences were then evaluated in those with and without subclinical pathology.

This introductory chapter will outline our current and historical understanding of latent and subclinical tuberculosis, the natural history of infection and transition to active TB, the impact of HIV on progression of TB, the pathological and radiographic features of TB, and ability of biomarkers to distinguish active from latent TB.

1.2 Latent tuberculosis

1.2.1 The evolution of latency in tuberculosis

*Mtb* co-evolved with anatomically modern humans over at least 40-70,000 years, originating in Africa and co-migrating with us to cover every corner of the globe. Humans initially lived largely as foragers in small communities of roaming hunter-gatherers. In these small isolated populations infectious pathogens of high virulence and short incubation periods would result in rapidly terminating epidemics due to the lack of susceptible hosts[2, 3]. Persistence of low virulence pathogens capable of initiating a chronic and/or latent infection within the human host would be favoured since transmission to susceptible new birth cohorts years after the initial infection would be possible. Our natural immune response to TB is likely to have been shaped over this period. Starting with the Neolithic Transition Period approximately 10,000 years ago, agricultural development allowed for increases in population density and the development of permanent settlements. More recently the urbanization following the Industrial revolution led to further rapid increases in population density especially in Europe and Asia[4]. Co-incident with this demographic change was a significant expansion in mycobacterial genetic diversity and the development of 3 evolutionary modern lineages (lineage 2 (East Asia), lineage 3 (India/East Africa) and lineage 4 (Europe/America)[5]. It might be expected that through adaptation to higher population densities these modern strains may be more virulent and have shorter incubation periods in comparison to ancient strains (lineage 1(Philippines/ Indian Ocean rim) and lineage 5&6 – *M.africanum* (West Africa)). This would certainly be consistent with the observed success in
terms of global spread of W-Beijing stain (Lineage 2) and is supported by the finding of de Jong et al who showed fewer household contacts of ancient strains developed TB over a 2 year period compared to contacts for evolutionary modern strains[5, 6].

1.2.2 Overview of the lifecycle of tuberculosis

![Diagram of TB lifecycle](image)

Figure 1.1 – Overview of TB lifecycle: Solid lines represent main routes of progression. Dashed lines represent less common or theoretical routes of progression

The general outline of the natural history of TB in humans is widely accepted and forms the basis of the majority of epidemiological models (figure 1.1). However many of the finer details and biological mechanisms underlying this are poorly understood or yet to be established and will be explored in subsequent sections of this chapter.

Although TB can develop in virtually any part of the body, disease involving the lungs (which occurs in 60-75% of cases) is necessary for transmission of infection; in particular pulmonary cavitation facilitates efficient Mtb replication and transmission. There is some evidence that suggests Mtb may specifically exploit the immune response through conservation of immunodominant epitopes[7], which could promote the induction of immunopathology that leads to lung cavitation. Immunocompetent adults contribute most to disease transmission. Individuals who are most effective at transmitting are often sputum smear positive for acid-fast bacilli (AFB) and cough spontaneously, thereby generating infectious particles. Infection is initiated by droplet nuclei (particles formed by evaporation of expectorated droplets, 1-
5μm in size) that can remain suspended in the air for hours (if not disrupted by turbulence) and are inhaled by contacts sharing the same environment[8]. Following an initial exposure to Mtb, infection may fail to establish due to mechanical or cellular element of the innate immune system but around 30-50% of close contacts will develop a primary infection, as evidenced by immune sensitization (a positive TST or IGRA - see below)[9]. In a small proportion (more common in children ≤ 2 years and immunocompromised) primary infection may progress to cause disease, this primary progressive TB is sometimes referred to as childhood disease in the historical literature. In the remainder primary infection is controlled and infection is considered latent. It is possible that in a number of those in whom infection is initially established Mtb is eradicated through the acquired immune response. Disease that subsequently develops is termed post-primary and invariably arises at a site distant to the primary site of infection in the lungs. In immunocompetent adults this is commonly within the apical or sub apical region of the upper lung lobes; in the historical literature this is sometimes referred to as adult disease. Post-primary disease occurs in around 10% of those initially infected (higher in those with immunocompromise) and at least half of which occurs in the 2-3 years following initial reactivation of infection. Although, once active disease is initiated progression of symptoms and pathology is common, spontaneous arrest of disease accompanied by healing of pathology and resolution of symptoms may occur. Data from the pre-chemotherapy era for HIV uninfected persons suggest that if left untreated, case fatality of smear positive TB was approximately 70% and smear negative TB approximately 20%; the average duration of disease from diagnosis to either death or cure was approximately 3 years although relapse after cure was common[10]. Re-infection can occur at any stage but the fate of the bacillus and outcome of reinfection will depend on numerous factors, which are incompletely understood.

1.2.3 Latent tuberculosis in the context of global tuberculosis control

The World Health Organization (WHO) and Stop TB partnership have set the challenge to eliminate TB as a public health problem by 2050[11]. This has been defined as achieving an incidence rate of less than 1 case per million of the global population. For comparison, the 2012 rate is 1220 cases per million[12]. In addition bold interim targets have recently been set for 2025 (to reduce TB mortality by 75% and TB incidence by 50% compared to 2015 levels) and 2035 (to reduce TB mortality by 95% and TB incidence by 90% compared to 2015 levels)[13]. In the 20th century when much of Western Europe, North America and parts of East Asia saw dramatic reductions in TB incidence through major socio-economic progress and implementation of improved TB control and treatment programmes; peak reduction in
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TB cases of 8.8%/year was achieved in these regions after the 2nd world war[14]. In contrast Sub-Saharan Africa and Eastern Europe/Central Asia in particular suffered a steep increase in TB incidence during the 1990s due to the HIV epidemic and the socio-economic disruption following the collapse of the Soviet Union respectively. Latest estimates suggest that in 2012 there were 8.6 million new cases of tuberculosis and 1.3 million deaths, with the global incidence of tuberculosis falling 2%/year over recent years[12]. In this context the 2050 elimination target seems particularly bold requiring a historically unprecedented 20%/year reduction in global TB incidence[14]. Widespread implementation of current TB control measures coupled with continued socio-economic development particularly within the BRICS (Brazil, Russia, India, China and South Africa) countries along with continued Anti Retroviral Therapy (ART) roll out in Sub-Saharan Africa could result in global reductions in TB incidence of 10%/year. However to bring global incidence down towards current levels seen in North America and parts of Western Europe, considered to be in the elimination phase (<100 cases/million/year), development of novel technologies and approaches will be required though research and innovation. Whereas until now TB control has focused on detection and management of active disease, which will continue to be important, a renewed focus on understanding and managing the important reservoir of infected humans with latent infection will be critical to future progress. Modelling suggests that mass treatment of latent TB would be one of the most effective ways to reduce incidence of TB[14, 15]. However, with current treatment and diagnostics this would involve up to 1/3 of the world’s population taking 3-9 months of anti-tuberculous therapy, which is neither desirable nor feasible. The need for novel diagnostic tests for latent TB more predictive of active disease and shorter course preventive treatments are clear, but in order to pursue these a greater understanding of the biology of latency and reactivation is required.

1.2.4 The current diagnostic tests for latent tuberculosis

By the end of the 19th century the notion that a latent period of infection could occur in tuberculosis was widely accepted. Several autopsy case series carried out around the turn of the century demonstrated that Mtb was frequently present in persons who died of causes other than tuberculosis by inoculating material from cadavers into rabbits or guinea pigs and observing secondary infection[16, 17]. Detecting the presence of infection in asymptomatic
living people was more challenging. Koch’s discovery and development of tuberculin, a heat-killed culture filtrate of tuberculosis that was proposed unsuccessfully as a cure in 1890, provided a useful diagnostic test, unmasking occult infection by inducing systemic reaction following subcutaneous injection[18].

Over a period of 65 years tuberculin was refined into an intradermal skin test using a standardised purified protein derivative of tuberculin (PPD) and measuring the induration formed after 48-72 hours (table 1.1). The result, expressed as millimetres (mm) of induration, is a continuous variable where the threshold for a positive result can be varied to modify diagnostic sensitivity and specificity. The dose of PPD was optimized to maximise sensitivity in distinguishing healthy close contacts of TB from healthy non-contacts, and the tuberculin skin test (TST) remains widely used globally today and is generally considered to demonstrate the presence of infection[19]. In general different cut-offs are used for groups depending on their risk of developing TB. Commonly the Centers for Disease Control and Prevention (CDC) recommended cut-offs are used; ≥5mm for those at highest risk, namely those with HIV co-infection, other significant immunosuppression, recent close contact of TB or fibrotic changes on CXR, ≥10mm for those living in or recently migrated from high TB burden countries and ≥15mm for those with no known risk factors for TB[20].
Those with positive TST are clearly at greater risk of developing TB and will derive greater benefit from preventive therapy and hence one can reasonably conclude that they are more likely to be infected with Mtb. However, to what extent can all those with a positive test be presumed to have a viable mycobacterial infection with the absence of studies relating pre-morbid TST result to post-mortem autopsy findings? The guinea pig “natural infection” model, initially developed by Riley to investigate airborne transmission in which air from side rooms or wards where TB infected patients are resident is vented over chambered guinea pigs, can provide some evidence. In these studies the distribution and magnitude of TST reactions found in guinea pigs was similar to humans; at autopsy evidence of infection was found in 0% of guinea pigs with tuberculin reactions 0-5mm (negative reaction), 92% of guinea pigs with TST >=14mm or evidence of necrosis but only in 25% with TST 6-13mm [21]. This finding, that not all “naturally infected” guinea pigs with positive TST have evidence of infection at autopsy, has been confirmed by others [22, 23]. Equally, if positive TST indicates presence of infection and negative TST absence of infection, then treatment of latent infection might be expected to cause a reversion of status. In the United States Public Health Service (USPHS) Trials 13,176 household contacts who were initially TST positive had TST repeated at 12 months after placebo or isoniazid. 6.5% of contacts who received placebo converted to negative and 7.9% of isoniazid treated contacts converted to negative. However, isoniazid reduced the 10-year incidence of TB by 59% in those who remained TST positive at 12 months and 38% in those who converted at 12 months indicating that isoniazid’s efficacy to prevent disease was not associated with a capacity to induce TST reversion[24]. From this we can conclude that TST provides evidence of immune sensitization by Mtb and is a correlate of TB infection but not all those with a positive test will be harbouring viable bacilli.
Because TST can render low-level false positive results due to sensitisation by environmental mycobacteria and Bacillus Calmette-Guérin (BCG) vaccination, IGRA were developed to improve diagnostic specificity. A positive TST represents cell-mediated, delayed hypersensitivity to a collection of secreted mycobacterial antigens (which are not Mtb specific) injected into the skin. Development of IGRA refined this by measuring the cell mediated response to Mtb in circulating blood by detection of interferon gamma (IFNγ) either by enzyme-linked immunosorbent assay (ELISA) or enzyme-linked immunospot assay (ELISPOT) following short term incubation of either whole blood or peripheral blood mononuclear cells (PBMC) with antigens from the region of deletion (RD) 1 locus of the Mtb genome (table 1.1). The RD1 locus of Mtb encodes a number of genes essential to the virulence of the organism including those relating the ESX-1 secretory system. 6 kDa early secretory antigenic target (ESAT-6) and 10 kDa culture filtrate antigen (CFP-10) are highly immunogenic antigens secreted by this system and are the stimulatory antigens used in IGRA (along with TB7.7 in QuantiFERON-TB Gold-In-Tube (QFGiT)). The RD1 locus is absent in the attenuated *M. bovis*-BCG and the majority of environmental mycobacteria (although notably not from *M. kansasii* and *M. marinum*)[25]). While IGRA have been confirmed to be more specific than TST, especially in populations vaccinated with BCG after infancy and, particularly for TSPOT-TB, remain more sensitive than TST in advanced HIV, many other of the test characteristics are similar[26]. In particular although the negative predictive values (NPV) for both tests are very high (IGRA 99.7%, TST 99.4%), they are both poorly predictive of progression to active disease (positive predictive value (PPV): IGRA 2.7%, TST 1.5%)[27, 28]. As a result when these immunodiagnostic tests are used as a guide for administration of preventive therapy (table 1.2) the number needed to treat (NNT) to prevent one case of
active TB is high. In a systematic review of 11 studies involving 73,375 HIV-uninfected participants randomised to isoniazid or placebo the pooled NNT was 100, ranging from 36 in recently infected household contacts to 179 in those remotely infected[29]. This poor predictive value of current diagnostic tests, along with lengthy duration of preventive therapy are the main barriers to implementation of widespread treatment for latent TB (figure 1.2). After the initial need to develop sensitive and then specific tests for latent TB we now need to develop tests that are better able to predict who will develop active TB.

1.2.5 The global burden of latent tuberculosis

One of the most widely quoted statistics is that 1/3 of the world’s population is infected by Mtb emphasising the huge scale of the problem[30]. However, aside from the fact that no test accurately demonstrates the presence of infection, it is useful to consider the data upon which the statement is made. Prevalence of infection in a population is derived from the Annual Rate of Infection (ARI), which can either be directly determined from focused tuberculin surveys (usually in school children) or indirectly estimated from incidence of active TB using the equation \( ARI = Incidence/\text{coefficient} \), as risk of infection is determined by contact with infectious cases[31]. Of note however, the incidence itself is usually estimated from case notification, disease prevalence or mortality data. In addition, in order to accurately determine prevalence of infection the change in ARI over time in the population should ideally be known.

In 1999 WHO convened a consensus group comprising 86 experts and epidemiologists who evaluated the best available data for all countries up to 1997 for a number of TB indicators including prevalence of infection[30]. Recent good quality tuberculin surveys were available for only 24 countries and the rate of change in ARI was only accurately known in a minority. For the majority of countries for which good quality tuberculin surveys were not available (or it wasn’t possible to confidently extrapolate from countries with good data) ARI was derived from the incidence of smear positive disease using the equation above with the coefficient of 50 (for countries where HIV prevalence in TB cases was less than 5%) coming from “Styblo’s rule” [32] (which states that a smear positive pulmonary tuberculosis incidence of 50/100,000/year corresponds to ARI of 1%, potentially an overestimate – see below). The authors estimated that 32% of the world’s population was infected with Mtb but acknowledged the lack of good data and limitations of the models to determine prevalence of infection and did not provide an uncertainty estimate.
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Tuberculin surveys, while widely performed, have several limitations. Interpreting results to determine the proportion that are immune sensitized by Mtb is challenging as the specificity of the test varies between populations, depending upon exposure to environmental mycobacteria and BCG vaccination. Analysing the distribution of TST reactions using mixture models and other techniques to identify bimodal patterns (as the reaction to TST is greater following immune sensitization by Mtb than environmental mycobacteria or BCG) can be performed but not all distributions lend themselves to this form of analysis[33, 34]. Systematic surveys using IGRA have not often been performed and the need for venepuncture, specialist laboratories and cost may limit widespread use. In addition, the dynamics of IGRA conversion and reversion over time and hence sensitivity to detect remote infection are less well understood, although data from IGRA surveys could be used with tuberculin surveys to refine estimates of LTBI prevalence[35]. A recombinant ESAT-6/CFP-10 skin test is currently in development with early clinical studies showing superior specificity to TST and correlation with whole blood Quantiferon results[36], such a test may ultimately prove particularly useful for surveys of this kind.

Modelling the risk of infection from the incidence of smear positive active disease seems appropriate but “Styblo’s rule” makes some key assumptions informed by 6 studies between 1921 and 1971. Namely each smear positive incident case is infectious for 2 years (corresponding to 2 prevalent cases) and each prevalent case results in 10 new infections per year. Hence the rule, an incidence of 50/100,000/year results in infection rate of 1,000/100,000/year. This may be less applicable in the contemporary era as improved diagnosis and treatment may have reduced the average duration of infectiousness significantly and factors such as population density, success of TB control programmes, the prevalence of HIV infection, and the prevalence of drug resistant TB will also have influenced transmission[37]. A recent analysis of data from East Asian countries between 1975 and 1994 by van Leth et al determined that the number of infections per prevalent smear positive prevalent case was 2.6-5.9/ year, so at least in some parts of the world “Styblo’s rule” overestimates infection[37, 38]. Smear positive TB is also not homogenous. Jones-Lopez et al have shown in only 45% of smear positive cases could Mtb be cultured from cough aerosols generated though 10 minutes of strong coughing. In addition the variability in colony forming units (cfu) generated was great (1-378cfu). They also showed that infection of household contacts was significantly greater when the index cases had a high cough aerosol cfu compared to low or no cough aerosol cfu[39]. Confirming that cases of TB
transmit variably, Escombe et al using the Riley guinea pig model of airborne infection in an HIV/TB ward in Peru, showed 8.5% of admissions were responsible for 98% of infections in guinea pigs[40]. Further complicating the situation is the fact that Mtb strains themselves may differ in their transmissibility and nature of immune response they induce. Taken together using a general rule derived from a small sample of cases to establish prevalence of infection in different populations is likely to be extremely inaccurate.

Accurate estimation of the proportion of the world that is infected using the currently available tools is extremely difficult. Better data and a better understanding of how parameters change regionally or in certain situations (such as drug resistance) may allow for the development of more sophisticated models[41]. These may then allow more accurate assessment of the prevalence of infection and provide estimates for important subgroups such as the prevalence of drug resistant latent TB or the proportion of latent TB related to a recent infection. However, ultimately what we want to know is the proportion that is highly likely to develop active disease.

1.2.6 Risk of latent infection progressing to active disease
The lifetime age-weighted risk of TB following infection in settings with low exogenous reinfection is estimated to be 12%[42]. Careful follow-up in placebo-controlled intervention studies has demonstrated that disease is most likely to occur in the first year following infection, with stepwise reduction year on year over the following 5-10 years (see figure 1.3) by which time incidence approaches that of uninfected contacts[24]. The different manifestations of tuberculosis occur at different intervals following infection with pleural TB, TB meningitis and miliary TB occurring after a shorter interval than pulmonary or other extra-pulmonary sites[43].

Reactivation several decades after initial infection occurs[44] but as observational studies with close follow-up rarely continue beyond 10 years it is difficult to assess how common reactivation is outside this timeframe. In addition, conventional observational studies make it difficult to evaluate whether disease relates to the initial infection event or subsequent reinfection. Borgdorff et al applied a molecular epidemiology approach (using Restriction Fragment Length Polymorphism of IS6110 +/- Polymorphic GC Rich Sequence) to 12,222 cases of TB over a 15-year period in the Netherlands and identified 1,095 linked secondary cases from 688 source cases. The median incubation period (time between predicted date
of infection and onset of symptoms in the secondary case) was calculated to be 1.26 years and the serial interval (time between symptom onset in source and secondary case) was found to be 1.44 years with 83% of secondary cases occurring within 5 years of the source case and >95% within 10 years[45]. Studies of immigrants from high burden to low burden countries provide further information. McCarthy showed that of 128 migrants from high burden countries in Asia diagnosed with TB in London in the 1980s (low burden setting) and who had neither returned to Asia since migrating nor had a known UK TB contact, 71.9% had arrived in the UK 0-5 years previously, 23.4% had arrived 6-10 years previously, 3.9% had arrived 10-15 years previously, and 0.8% had arrived 16-20 years previously[46]. This supports the assertion that after more than 10 year of infection reactivation of disease is very rare. However, it is acknowledged that there are several limitations in interpretation of this data in particular relating to stability of migration rate and over this period and ascertainment of all cases. The observation that the elderly in low TB burden settings have a higher incidence of TB is often, possibly incorrectly, interpreted as providing evidence of prolonged latency and reactivation following immunosenescence. However, careful evaluation of birth cohorts shows this apparent increased risk is an artefact of falling transmission, and younger adults are still invariably at greater risk of TB than the elderly[47]. These data show that the common view that reactivation TB disease often occurs decades after initial infection may be overstated: the majority of cases occur within 18 months of infection and disease resulting from reactivation more than 10 years after infection may be
The risk of disease is also not constant over time; following a single infection the risk is approximately 12% over a person’s lifetime, if no disease develops after 5 years the lifetime risk might only be 2% and after 10 years 0.5%.

### 1.2.7 Risk factors for developing active tuberculosis

The risk of developing active TB can be associated with increased risk of exposure to Mtb, increased risk of infection following exposure or increased risk of reactivation (or progression) following infection. Many conventional risk factors can all of these effects and in epidemiological studies it can be difficult to separate what is driving the increased risk [48](table 1.3). The majority of these common risk factors for active TB have complex and often poorly understood effects on the host response to Mtb (HIV will be discussed in detail below) but they are considered to be either generally immunosuppressive or to specifically affect the lung. Anti tumour necrosis factor (TNF) therapy (such as infliximab) by contrast has a very specific effect on host immunity and a particularly striking impact on development of active TB. In patients treated with infliximab the risk of developing active TB in the first 90 days of treatment is almost 20-times that of the background population, with 43% of total TB cases in a 2 years follow up, occurring within 90 days of administration of the drug[49]. Reactivation is thought to be precipitated by disruption of the granuloma by interference with TNFα signalling, however reactivation is by no means universal. In an evaluation of the implementation of latent TB screening prior to anti-TNF therapy in Spain, 56 patients with positive TST (>5mm) were identified who did not receive any isoniazid prophylaxis and in only one case did active TB occur following anti-TNF treatment[50].

Rather than just immunosuppression alone, it is becoming more apparent that the extremes of the immune response may lead to detrimental outcome in tuberculosis with a more balanced response being optimal (the so called “Goldilocks effect”). It has been proposed that a weak host response may lead to unopposed bacillary replication whereas an

<table>
<thead>
<tr>
<th>RISK FACTOR</th>
<th>RELATIVE RISK (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV</td>
<td>8.3 (6.1-10.8)</td>
</tr>
<tr>
<td>Malnutrition</td>
<td>4.0 (2.0-6.0)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3.0 (1.5-7.8)</td>
</tr>
<tr>
<td>Alcohol &gt; 40g/day</td>
<td>2.9 (1.9-4.6)</td>
</tr>
<tr>
<td>Active Smoking</td>
<td>2.6 (1.6-4.3)</td>
</tr>
<tr>
<td>Indoor Pollution</td>
<td>1.5 (1.2-3.2)</td>
</tr>
</tbody>
</table>

Table 1.3 – Relative risk for active TB for some globally common conditions - Adapted from Lönnroth et al [48]
aggressive response may lead to tissue damage and necrosis, which may provide a more favourable environment for the bacillus. An example of one possible mechanism for this “Goldilocks effect” has been recently characterised. Leukotriene A₄ hydrolase (LTA₄H) mediates the balance of pro-inflammatory eicosanoid LTB₄ and anti-inflammatory lipoxin A. Zebrafish larvae in which LTA₄H is over- or under-expressed are made hyper-susceptible to M. marinum infection compared to wildtype either by low levels of LTA₄H resulting in increased lipoxin A and impaired TNFα production, or excessive LTA₄H resulting in increased LTB₄ and increased TNFα production[51]. Humans who are heterozygous for a single nucleotide polymorphism of LTA₄H promoter rs17525495 (C/T) appear to have the best clinical outcome from TB meningitis, those who are homozygotes for the T allele (T/T) have increased LTA₄H expression and increased inflammatory cerebrospinal fluid but derive significantly greater benefit from dexamethasone therapy compared to those with the C/C genotype[51-53].

A further intriguing and poorly understood risk factor is age. It is a consistent and striking feature of tuberculosis that the age at which infection occurs affects the risk of subsequently developing active disease[54, 55]. Infants and young children especially those ≤ 2 years are at considerable risk of developing disease following infection. Older children (5-10 years old) have consistently been shown to be at the lowest risk of active TB following infection, whereas in comparison peri-pubescent adolescents and young adults are at much greater risk of developing cavitary TB[54, 55]. It has been suggested that this may relate to the immunonoendocrine effects mediated by the balance between dehydroepiandrosterone (DHEA – a precursor of sex steroids) and glucocorticoids[56]. DHEA levels start increasing from 7 years old, peak in early adulthood, and reduce in older adults[55]. One of DHEA’s many effects is as a glucocorticoid antagonist and the cortisol:DHEA ratio has important immunological consequences. Recently DHEA has been shown to influence dendritic cell function to promote T₄₅ː responses by increasing interleukin (IL)-12 and diminishing IL-10 production following Mtb stimulation, with increased expression of major histocompatibility complex (MHC) I, MHCII and cluster of differentiation (CD) 86 expression resulting in enhanced T-cell proliferation and IFNγ production[57]. So it seems plausible that pro-inflammatory responses in healthy adolescents and young adults have detrimental effects leading to cavitary disease, anti-inflammatory responses in infants lead to their inability to control replication and there is a more optimal balanced response in older children leading to a beneficial outcome.
Another interesting observation is the seasonality of active TB with increased case notification, which can be 20-25% higher in spring/summer than in autumn/winter[58-61]. This is striking for an infectious disease with relatively prolonged and variable incubation. In common with other respiratory pathogens one explanation would be behavioural, with winter crowding leading to greater transmission, but modelling evidence and analysis of unique and clustered Mtb strains suggests that this cannot fully explain the seasonality of TB[43, 62]. A seasonal precipitating factor such as vitamin D deficiency or a viral respiratory infection (e.g. influenza) is an alternative explanation. Vitamin D, synthesised within the skin requiring UV light, acts as an immunomodulatory and anti-inflammatory agent primarily exerting its effect on macrophages, facilitating enhanced control of mycobacteria through pleiotropic mechanisms[63, 64]. A number of clinical observations provide some support for the role of vitamin D deficiency in inducing reactivation. TB patients are well documented to have significantly lower vitamin D levels than healthy household controls[65, 66], and the spring/summer peak in TB notifications is preceded by a winter trough in vitamin D levels in Cape Town[67]. There is some evidence to suggest that seasonality is more pronounced in foreign born cases (who may be at greater risk of vitamin D deficiency due to skin pigmentation) compared to native cases in Europe[68, 69]. An alternative seasonal precipitant could be a viral infection. It has recently been shown that the type 2 interferon (IFNγ) response critical for mycobacterial control can be impaired by the downstream effects of type 1 interferons (IFNα/β)[70]. It has therefore been hypothesised that viral respiratory infections inducing a type 1 interferon response could lead to reactivation by impairment of type 2 interferon facilitated control of Mtb. In the mouse model, mycobacterial growth is enhanced and survival decreased in mice previously exposed to influenza by a mechanism dependent on Type 1 interferon signalling[71]. In addition, historical observations and modelling of the 1918 influenza pandemic suggest a negative impact of influenza on TB[72].

### 1.3 Subclinical tuberculosis

#### 1.3.1 Mass chest radiograph screening for subclinical tuberculosis

As TB is in general a relatively slowly progressive condition, there is a period of time during the transition from latent to active TB when a person may be asymptomatic despite evolution of the disease. This period can be considered a subclinical phase of TB. Subsequently, there is a period of time following the onset of symptoms during which
healthcare may not be sought and this can be considered a **pre-diagnostic phase of TB**. Screening is an active process that offers a possibility to identify persons in the subclinical or pre-diagnostic phase of TB. The aim of screening is to identify evidence of pathology related to the disease in an acceptable and cost effective way such that treatment or isolation can be initiated earlier than would otherwise have been the case. This could potentially improve prognosis of the condition for the individual and reduce transmission within the community. CXR screening for subclinical tuberculosis was the one of the first examples of a mass screening program in modern medicine that saw millions of people x-rayed globally between the 1930s and 1970s[73].

The primary abnormalities of interest on CXR at screening were those relating to active tuberculosis at a minimal stage of disease (when even in the pre-chemotherapeutic era isolation, sanatoria care or invasive intervention may have been of benefit) as well as evidence of healed, inactive tuberculosis, particularly following the availability of drug treatments to reduce risk of disease progression. Diagnostic standards for these radiographic lesions were established early on[74]. One of the most comprehensive evaluations of minimal lesions identified through a mass screening programme was the Royal College of Physicians Prophit survey. 1,213 adults with minimal TB lesions were identified from 181,391 persons attending 2 mass screening units in London between 1946 and 1948, 99% of whom were tuberculin positive. Approximately 75% of lesions were in the upper zones, mainly unilateral, usually comprising of clusters of small nodules covering on average 10cm². Although almost all participants denied symptoms and reported good health, on direct questioning approximately 50% admitted to at least one TB symptom. 29.5% showed radiographic deterioration over 5 years, 40% of which was within 6 months of
the initial CXR and a further 27% within 6-18 months. In 54% of those with radiographic deterioration Mtb was isolated, but Mtb was also isolated in 21% without radiographic deterioration. Therefore approximately 44% of screened adults with minimal lesions had radiographic or microbiological deterioration over 5 years[75].

Those that had disease identified at a minimal stage on CXR were found to have much better prognosis than those with moderately advanced or far advanced TB, with 1 study showing mortality in these 3 groups with sanatoria care to be 4.1%, 9.2% and 46.8% respectively, however these differences in part will reflect length and lead time bias[76]. In addition, minimal lesions were more likely to improve than moderately advanced lesions. As these lesions improved, radiographic evidence of activity reduced and fibrosis of lesions was observed, however, subsequent deterioration in those in which disease activity had appeared to arrest was common. As a result identifying those with inactive (healed or fibrotic) lesions on CXR became increasingly important, particularly with the availability of drug treatment. Those with inactive lesions on CXR fell into 2 groups those that were known to have had previous active TB, which subsequently healed, and those with no diagnosis of previous active TB in which subclinical TB was presumed to have spontaneously healed. Individuals with fibrotic inactive lesions on CXR with no previous diagnosis of TB were 5-15 times more likely to develop TB than tuberculin reactors with a normal CXR[24, 77]. Isoniazid therapy was also found to significantly reduce the likelihood of those with inactive lesions progressing to active disease[78]. In addition, studies in Europe and America at a time of rapidly falling TB incidence showed that up to 70% of persons developing TB (with no history of TB and usually no clear contact history) had evidence of fibrotic scarring on previous CXR[79, 80].

Taken together the evidence from CXR evaluation of TB suggests, that in a proportion of people, the disease may follow a cyclical waxing and waning course with earlier reactivation initially arrested by the host, delaying disease presentation. In addition, it is clear that the subclinical phase of active disease prior to clinical presentation may be several months.

1.3.2 Limitations of chest radiography

Mass CXR screening probably played a role in the success of TB control in Europe and America over the twentieth century, largely being abandoned due to poor cost effectiveness particularly as prevalence of disease fell. However, CXR used for TB screening has a number of notable limitations as a diagnostic test, summarized by Koppaka and Bock[81]. Several
studies demonstrated that both under-detection and over-detection of abnormalities were common, although of note the gold standards used in such studies were not ideal (usually panel of experts or final clinical diagnosis). Experience of the reader made a small difference with accuracy being improved only in those that read more than 20,000 films a year or had greater than 10 years experience. Inter-observer variability was high (up to 30%) and of greater concern intra-observer variability (when the same reader was asked to interpret the film months apart) there was disagreement with the earlier read in approximately 20% of cases. Fundamentally the more subtle a lesion the greater the disagreement about its presence is as “signal to noise ratio” is high as overlying structures impair ability to identify the lesion of interest, particularly at the apical and subapical regions where tuberculous lesions are most frequent.

CXR screening is still undertaken for groups at high risk of tuberculosis and recently a systematic review evaluating CXR screening compared to a gold standard of sputum culture identified 4 studies in which any CXR changes of TB had a pooled sensitivity of 87% and specificity of 89%. However, a high risk of verification bias potentially overestimating sensitivity was identified in all studies as, in general, sputum culture examination was limited to those that also had symptoms or abnormal CXR[82]. Other imaging modalities such as CT can provide considerably greater detail of the lung parenchyma and would be expected to be significantly more sensitive for smaller parenchymal lesion but its application in this context has been limited (discussed below). Although CT would be generally inappropriate as a screening tool it may have considerable value as a research tool. Dual energy CXR in which a soft tissue image and bone image are produced though a weighted subtraction of a high energy and low energy exposure could be of interest as an improved screening tool for tuberculosis.

1.3.3 Asymptomatic culture positive tuberculosis in HIV
In addition to CXR abnormalities preceding clinical TB, it has also been demonstrated that persons can be culture positive for Mtb in the absence of symptoms. This is more common in the context of HIV co-infection particularly in those with advancing immunosuppression. Oni et al screened 213 asymptomatic HIV-1 infected persons with sputum culture, 17 (8%) were found to be culture positive, of whom 14 had CXR performed of which only 3 were abnormal. Median CD4 count of those with asymptomatic TB was 249/mm³ and just over 50% developed symptoms with 2 months[83]. Whether those with a normal CXR truly had
an absence of pathology within the lungs is unknown, as no studies have performed cross-sectional imaging in such patients.

1.3.4 The spectrum of infection states in tuberculosis

It is becoming clearer that dichotomisation of tuberculosis into a latent and active stages of infection is an oversimplification. Over the past few years many researchers have revisited the notion that asymptomatic people considered to have latent tuberculosis might be better considered as part of a spectrum of infection states where at one end infection may have been eliminated but immune sensitization remains, while at the other end disease may be active but in a subclinical form, and between these two extremes infection is controlled in a quiescent state[84-89]. Considering this in terms of the natural history of infection it seems plausible that soon after initial infection and immune sensitization there are 3 main possible outcomes influenced by predisposing factors, which determine the course of infection during this critical phase and alter the proportions in each group. Some may initially develop primary progressive disease; this may be a very small proportion in adults but would likely be more common in advanced immunosuppression and infants. A second group (a high risk group - the main group from which reactivation disease arises) may enter a more unstable state with infection taking a waxing-waning course during which, periods of disease progression triggered by precipitating factors may be followed by control (which may lead to evidence of immunopathology) or the development of clinical disease. Some precipitating factors may be more potent than others, very potent precipitating factors (such as anti-TNF and HIV) may have the effect of causing rapid progression over a short time interval. It is
also in this group that isoniazid preventive therapy (IPT) may be most effective. A third group may rapidly and effectively control infection and eventually may even sterilize the organism and may be at extremely low risk of progressing to active disease even in the presence of certain precipitating factors (figure 1.5).

1.4 HIV and its impact on Tuberculosis

1.4.1 HIV

HIV is single-stranded ribonucleic acid (ssRNA), enveloped retrovirus transmitted between humans sexually, intravenously or vertically via infected blood, breast milk, seminal or vaginal fluid. The primary cellular target is the CD4 T cell but macrophages and dendritic cells can also be infected. The virus enters the cell via interaction with CD4 receptor and chemokine receptor CXCR4 or CCR5. Once within the cell the ssRNA viral genome is reverse transcribed to complementary deoxyribonucleic acid (cDNA) and integrated into the host genome. Viral replication is then governed by the cell cycle and influenced by cellular transcription factor notably NF-κB.

Two types of HIV virus exist, HIV-1 and HIV-2. HIV-2 has a limited geographic distribution confined primarily to West Africa and is of lower virulence and infectivity. HIV-1 is distributed globally and the predominant virus found in South Africa. HIV is grouped according to variation in the envelope region into groups M, N and O with group M being the most common, this then sub-grouped into 8 clades with clade C being prevalent in South Africa.

The initial phase of HIV-1 infection, termed acute infection, which is non-specifically symptomatic in a proportion, is accompanied by a rapid rise in viral load and a reciprocal fall in CD4 count. This is followed by a period of clinical latency as the viral load stabilizes and CD4 count recovers. Over a period of time, CD4 count slowly falls and the individual becomes more susceptible to characteristic groups of infections and malignancies, which allow HIV to be staged clinically (commonly as WHO stage I-IV). Initially (often while CD4 count is \( \geq 350/\text{mm}^3 \)) a number of minor infections, such as fungal nail infections, shingles, bacterial skin infection are more common, however, as the CD4 count approaches 200/mm3 the individual become susceptible to a number of more unusual and rare opportunistic infections such as toxoplasmosis, cryptococcosis and Pneumocystis jirovecii either following reactivation of a latent source or recent infection. This stage of HIV infection is termed
acquired immune deficiency syndrome (AIDS) and is rapidly followed by death in the absence of antiretroviral therapy (ART). ART suppresses the virus allowing recovery of the CD4 count and reconstitution of the immune system reducing the risk of opportunistic infection.

1.4.2 Interaction of HIV and tuberculosis

The interaction between TB and HIV is complex and differs in some key respects to other opportunistic infections. Most notably risk of TB increases soon after HIV infection and the nature of TB disease characteristically changes with advancing HIV.

The relative risk of active tuberculosis increases within the first year of HIV infection and progressively increases thereafter [90, 91]. In a lower TB transmission setting Selwyn et al showed in a study of intravenous drug users in 1980’s New York, that over a 21 month follow up of 217 HIV infected patients, 7 of 49 (14.4%) that were TST positive at entry developed TB compared to 1 of 168 (0.6%) that were TST negative (rate ratio = 24). In addition none of the 303 HIV uninfected patients, a similar proportion of which were TST positive, developed active TB. This demonstrated the increased pressure to reactivated latent infection in HIV co-infection, although of note a significant number of TST positive people with HIV infection did not develop TB over this period. This is in contrast to the non-human primate model of simian immune deficiency virus (SIV)-TB. In macaques with latent TB infection (defined by absence of disease ≥ 6 months after TB infection), subsequently infected with SIV, reactivation and progression of TB disease occurred in all animals over an 11 month period[92].

In high TB transmission settings like South Africa, molecular epidemiological evidence suggests that in HIV associated TB, 3 times as many infecting strains are unique in early stages (within 2 years of seroconversion) compared with later stages of HIV infection. This suggests that while reactivation of a latent infection is an important factor in developing TB in early HIV infection, in more advanced HIV in regions of high transmission active TB more commonly reflects a rapid progression to disease following recent (re)infection[91].
The impact of advancing immunosuppression on TB incidence in high transmission settings like Cape Town is striking with a marked increase in those with CD4 counts less than 200/mm³ compared to those with CD4 count ≥ 350/mm³ (figure 1.6)[93]. In a recent evaluation of 29,478 cases of TB in Cape Town, 62.1% of the HIV associated cases had CD4 count less than 200/mm³ and 82.7% had CD4 less than 350/mm³ with the proportion of those with extra pulmonary TB increasing with advancing immunosuppression[94]. Commencement of ART has a dramatic effect on reduction of TB incidence but risk of TB is still elevated compared to HIV uninfected persons even in those with CD4 counts greater than 500/mm³.

Supporting this notion that TB in early and advanced HIV have differing pathogenesis, the presentation of TB differs with CD4 count. Chamie et al reviewed the CXR changes in 848 HIV infected and 1,085 HIV-uninfected persons with culture confirmed TB presenting to the National TB centre in Uganda and related CXR and clinical findings to CD4 count. They found that compared to HIV uninfected TB patients there was no difference in the proportion of HIV infected TB patients with cavitation or disease in the upper lung fields on CXR if CD4 ≥ 300/mm³ and no difference in the proportion of smear positivity if CD4 ≥ 400/mm³. By comparison as CD4 count declined, adenopathy, pleural effusion, miliary pattern and lower lung field disease became more common; a pattern that is more closely associated with progressive primary disease[95]. Furthermore numerous studies have shown that median CD4 count at presentation of extra-pulmonary (137-242/mm³) and disseminated TB (40-79/mm³) is markedly lower than in pulmonary TB (250-500/mm³)[96].
Lucas and Nelson carefully reviewing the histopathological appearance of HIV-associated TB identified 3 histologic stages of the cellular immune response that correlated well with stage of HIV infection. They reported that in early HIV infection a typical, organized, granulomatous response was still observed with CD4 T cells clustered closely around abundant epithelioid macrophages and Langhans giant cells. As CD4 count decreased the histological appearance became increasingly hypo-reactive with fewer macrophages and CD4 T cells and increased numbers of AFB present with lesions beginning to coalesce. In the most advanced stages of HIV/AIDS where disseminated disease was often present, the histological picture was anergic. The characteristic granulomatous organization was now absent, with very few CD4 T cells, epithelioid macrophages or Langhans giant cells, and replaced with a suppurative (neutrophil rich) necrosis and large numbers of AFB[96].

The interaction between HIV and Mtb at a cellular level is complex and remains incompletely understood. HIV infection leads to depletion of CD4 cells however it preferentially infects activated and memory CD4 cells which has a profound effect on Mtb specific immune responses. Peripheral Mtb specific CD4 cells in individuals with latent TB have also been shown to be selectively depleted following HIV infection[97] and there is some evidence to suggest that this may be due to selective infection of IL-2 producing cells with a central memory phenotype[98]. In addition HIV affects the functional characteristics of Mtb specific T cells, which may further impair host control[99]. HIV infection may also directly impair granuloma function; activated CD4 cells within the TB granuloma may be targeted by HIV leading to localized increases in HIV replication and further selective killing of T cells. As a result subsequent impairment of macrophage function through loss of T cell help may lead to failure of mycobacterial containment by the granuloma[99]. In addition to the effect on T cell function, direct HIV infection of the macrophage itself results in a dysregulated response to mycobacterial infection for example being shown to attenuate the IL-10 response to Mtb infection resulting in an exaggerated pro-inflammatory response [100].

Taken together we can infer that Mtb which is controlled within granuloma at a time of greater immunocompetence may be prone to reactivation following HIV infection and this may occur soon after acquiring HIV infection. However, if Mtb was sterilised at a time of greater immunocompetence then disease can not develop because of reactivation
secondary to HIV infection but may occur following reinfection and primary progression of infection which would become more likely as immunosuppression progressed.

1.5 The pathology of tuberculosis

The natural history of TB is more complex than most bacterial pathogens. The incubation period is prolonged and the outcome of infection variable depending upon both host and pathogen. However, understanding this natural history of infection is critical to accurate categorisation of TB infected persons, identification of correlates of risk and protection, and development of novel interventions. In addition detailed knowledge of how infection evolves and its pathological correlation in humans will assist interpretation of FDG-PET/CT scans. Much of our current understanding still arises from piecing together results from historical surveys, autopsy studies and evidence from animal models.

1.5.1 Primary infection

1.5.1.1 Dissemination following primary infection

Pathological descriptions from autopsies in humans less than 6 weeks following infection are incredibly rare and hence the earliest stages of Mtb infection and dissemination can only be understood from animal models, particularly the guinea pig, rabbit and mouse models. More recently improved understanding has been made possible by cross-sectional imaging (e.g. FDG-PET/CT) in non-human primates, which has allowed for careful follow-up of anatomical changes within animals over-time without need for necropsy. By contrast, events that occur over a period of months and years can only be adequately addressed in studies in humans.

A single droplet nucleus (probably containing 1-10 bacilli) is thought to be able to initiate infection. This long held belief has been supported by recent evidence from animal models. Lin et al infecting macaques with 34 colony forming units (cfu) of Mtb comprised of organisms with 8 different single nucleotide polymorphisms (SNP) (acting as molecular barcodes) determined that 79% of all granuloma identified at 4 weeks grew Mtb of only a single SNP suggesting initiation by a single organism[101]. Animal models then suggest that Mtb deposited at the alveolus is initially taken up by resident alveolar macrophages, which are thought to be maintained at a low level of activation non-specifically through encounter with a wide variety of inhaled particles[102]. If these fail to eliminate the organism, bacillary multiplication within the macrophage resulting in chemokine and cytokine release, leads to
recruitment of inflammatory cells to the region. After an initial lag of approximately 3 days, bacillary numbers increase exponentially over 2-3 weeks before stabilizing. At this stage macrophage and neutrophils are the predominant cell types at the site of infection, though poorly organized. Immature resident dendritic cells transport bacillus via lymphatics to regional lymph nodes and between 14-18 days in the guinea pig model bacilli are culturable within broncho-tracheal lymph nodes. Spread into the blood stream, probably from lymphatics, allows further dissemination to extra-pulmonary sites; this is also the point at which the lung lobes not initially infected via airways and distant to the primary lesion are infected. Secondary lesions then develop at these locations and Mtb is culturable at these sites 18-21 days following infection. Around this time the animal becomes tuberculin positive as a result of developing a mycobacteria specific, cell mediated immune response. Antigen specific lymphocytes accumulate rapidly to the sites of infection while neutrophil numbers drop, with CD4 T cells playing major role in activating macrophages through release of IFNγ and other cytokines. Over this period the granuloma becomes increasingly organized and bacillary numbers begin to fall. At around 4 weeks, caseation is visible at the centre of the granuloma and the characteristic architecture of the tuberculous granuloma is present[103, 104]. During these early stages of development vascularisation of the granuloma increases, mediated by vascular endothelial growth factor (VEGF), which then facilitates cellular recruitment[105]. With developments in intra-vital microscopy it has now become possible to view real-time dynamic changes within granuloma at this stage. Egen et al showed that at 3-4 weeks after intravenous BCG infection, granulomas within the mouse liver were dynamic structures with continuous recruitment of T cells, which displayed rapid motility interacting with a more stable macrophage core. They also showed that efficient T cell recruitment was highly dependent on TNFα[106]. The granuloma continues to mature over the following weeks with macrophages evolving into multi-nucleated giant cells, epithelioid cells and foamy macrophages[105]. While the primary lesion remains macroscopically visible, the majority of secondary lesions regress.

The nature of primary lesions in humans has been carefully studied in numerous autopsy series starting with Parrot (1876), Kuss (1898) and later Ghon (who became the eponym for this lesion) and numerous others[107]. Of particular note the progression and healing of the primary lesion over time in humans both at autopsy and radiographically was carefully documented by Sweany in his 1941 monograph “Age Morphology of Primary Tubercules” in which he systematically documented the microscopic and macroscopic evidence of healing
of the primary lesion in relation to the known ante-mortem TB contact history of the patient at hundreds of autopsies[108]. The earliest changes described are from around 6 weeks after infection at which stage the tubercle is still surrounded by an inflammatory exudate resembling a sub-acute bronchopneumonia, although Cannetti suggests that the extent of this perifocal inflammation is highly variable and is sometimes not present[109]. This infiltration may be visible on CXR and is termed the primary infiltrate. This inflammation begins to recede over a period of months and is followed by the laying down of a fibrous capsule which continues to thicken over a period of years. Calcification is visible after about a year, initially as a ring in the margins and by 7 years after initial infection has filled the core. Bony change is not normally seen until approximately 10 years. At the same time capsule begins to resorb and has all but disappeared by 20 years.

1.5.1.2 Location of primary site of infection from studies in humans

In humans the site of implantation appears to be related to airflow within the lungs and largely follows a chance distribution. Evidence for this comes both from studies documenting location of primary infiltrate on CXR in recent tuberculin converters and from autopsy studies in which location of primary complex or nodules are documented. How commonly primary infiltration is identified on CXR (which is often associated with hilar lymphadenopathy) varies greatly between studies in part related to age of subjects, though the caveats of CXR interpretation are described below. Gedde Dahl and Poulsen conducting separate studies in Scandinavia in the 1930’s/40’s performed CXR on carefully defined, recent TST converters (children and young adults) and characterised the location of the primary infiltrate. They noted that its location was relatively evenly distributed with a slight weighting to middle and lower lung zones and the right lung (however cardiac shadow may have obscured some infiltrates of the left lung) [110, 111].

The autopsy studies in which the location of primary lesions has been carefully documented (2 of which are detailed below) mirror the findings of the CXR studies above. In 1917 Opie evaluated the pulmonary lesions that were present at autopsy in 93 children and in 50 adults. If TB was identified in infants under 2 it was invariably the cause of death, frequently massive, caseous lesions were identified with little tendency to heal. These did not affect the apex of the lung more frequently than other parts and likely represent progression from the primary site infection. From the age of 2 years he observed well-circumscribed lesions between 1mm and 1cm in size, situated in any part of the lung, usually solitary in about two-thirds of cases, and with about 50% of the lesions in contact with the pleural surface. These
focal lesions were often freshly caseous and accompanied initially by tuberculous lymphatic lesions in early childhood and with increasing age the proportion of people exhibiting these lesions increased (as did evidence of healing characterized by encapsulation, fibrosis and then calcification) such that in the elderly they were present in 100%. In adults the lesions were distributed as follows, 26% right upper lobe (RUL), 8% right middle lobe (RML), 23% right lower lobe (RLL), 21% left upper lobe (LUL), 23% left lower lobe (LLL) and found to be calcified in 73%[112]. Medlar performed 1,225 necropsies on adults who died unexpectedly (i.e. presumed not to be as a result of TB) and described localisation of the primary complex (single calcified parenchymal foci ≥ 2mm with and calcified lymph node) and lesions of early disease (described later). 105 cases had evidence of a calcified primary complex and 104 evidence of early disease. Calcified primary foci had a scatter pattern consistent with chance airborne distribution, 85% of foci were within 1cm of pleura with only 12% in a supra-clavicular location, 49% were within right lung, 40% were RUL or LUL, 53% within RLL or LLL and 7% within RML[113] (figure 1.7).

Figure 1.7 – Location of mineralized primary lesions: Lesions from 105 autopsy spatially represented on a chest radiograph from Medlar [113] open circles = lower, closed circles = upper lobe.


The lobar distribution of primary lesions appears to relate to the volume of the lobe and there is some suggestion that the lower portions of the lobes are preferentially infected; these sites are also preferentially ventilated by inspired air in the upright position. It is also a consistent finding that the primary foci of infection are frequently located at the periphery of the lung most commonly close to the pleural surface. Murray has speculated that this may relate to the fact that the peripheral locations of the lung are served by the straightest bronchioles with the largest cross-sectional area (which favours implantation within the alveoli rather than the wall of a bronchiole)[114].
1.5.1.3 Bacillary control and disease progression

The relationship between evidence of tissue healing and presence of viable bacilli was determined early on by researchers. Opie and Aronson in 1927 performed autopsies on 169 adults (dying of causes other than tuberculosis) and examined 304 lung lesions, performing guinea pig inoculation experiments on the majority of these. They determined that viable bacilli were most frequent in lesions that still had evidence of caseation and became increasingly less common if lesions had evidence of fibrosis or calcification[115]. Lin et al have recently provided further insight into the relationship between granuloma and bacillary viability. Infected macaques with 25 cfu of Mtb bronchoscopically the animals were then followed up with FDG-PET/CT and then necropsy was performed at either 4 weeks, 11 weeks or after 6 months when animals were classified either as having a latent infection or active infection on the basis of “clinical” signs (e.g. weight loss, abnormal radiography or positive culture)[101]. Median cfu per granuloma at 4 weeks was $1.8 \times 10^4$, but by 11 weeks (once the acquired immune response is established) median cfu per granuloma had fallen significantly (by 15 fold) and a number of lesions were already sterile. After 6 months median cfu had fallen further still (by 10-100 fold) in both active and latent monkeys and a variety of lesions types (including those that were completely sterile) were present in both groups. However, evidence of TB pneumonia was only present in monkeys with active disease. The authors also measured chromosomal equivalents (CEQ) within the granuloma, which is reflective of the number of viable and non-viable bacilli, and found this to be relatively stable at around $10^5$ CEQ/granuloma, irrespective of cfu or time since infection. Even treatment with isoniazid, which led to significant fall in cfu/granuloma, had little effect on CEQ/granuloma. By creating a ratio of cfu/CEQ they were able to evaluate proportion of viable bacilli (as determined by culturability) within each lesion type. Viability within areas of TB pneumonia was found to be maximal with cfu/CEQ approximately 1, whereas cfu/CEQ ratio was lower within caseous lesions and lowest within fibrocalcific lesions. The authors concluded that granulomas, at least in macaques, had a maximum “carrying capacity” of organism (around $10^7$). They also speculated that if bacillary numbers increased beyond this, secondary granulomas would need to arise to contain the bacilli (as is often seen) but if increase in numbers was too rapid or excessive that this may result in progression to TB pneumonia. They also concluded that this failure of control occurred at a granuloma level, as monkeys with active TB had a distribution of granuloma not dissimilar to that found in the latent monkeys.
1.5.2 Post-primary pulmonary disease in humans

Understanding of the critical events that lead to the failure of Mtb containment following primary infection and the progression of post-primary TB in humans is very limited. Hunter has attempted to describe the progressive pathological changes of post-primary TB seen at autopsy as well as on evaluation of surgical specimens and from historical pathology slides. He then performed extensive literature search to compare and validate these findings to that of earlier investigators. He concluded that one of the earliest manifestations of post-primary disease in humans was the accumulation of foamy macrophages containing numerous AFB within the alveolus. This then progresses endobronchially, with the foamy macrophages, the recruited leukocytes and the fibrin and lipid-rich, cellular debris accumulating within alveoli and bronchioles. The endobronchial spread can be remarkably slow and often halts and heals leaving characteristic fibrotic scaring within the lungs. In addition, Hunter identified endobronchial obstruction as one of the critical points in the pathogenesis of disease leading to development of a lipid-rich pneumonia which can then progress to caseous pneumonia, necrosis and eventually cavitation as the soft friable lung is expectorated. These later stages are accompanied by large increases in bacillary numbers as conditions for growth become more favourable[116]. CT imaging corroborates some of these findings although CT studies are usually opportunistic, related to clinical need rather than systematic. The earliest changes on CT have been described as 2-4 mm centrilobular nodules, which pathological correlation shows relates to caseous necrosis within the bronchioles, as well as linear branching representing cellular infiltration of the airways and endobronchial spread (giving a tree-in-bud appearance). These smaller lesions then
coalesce to form larger 5-8mm hazy, ill-defined nodules and larger areas of consolidation with finally, small areas of cavitation arising at the centre[117].

1.5.2.1 Location of post-primary TB within the lung

In contrast to the site of primary infection it has been understood since at least the 18th century from autopsy studies that tuberculosis disease in adults typically presents in the upper parts of the lung[118]. With the development of CXR, researchers and clinicians were able to identify the early sites of TB disease and a number of terms were used to describe these; Assman focus, Simon focus and Frühinfiltrat (spring infiltrates) being the most common. One of the first attempts to document the evolution of disease radiographically was conducted by Malmros and Hedvall who followed up 3,336 student nurses entering clinical practice into a hospital in Sweden from 1930, a setting with extremely high annual rates of infection and high incidence of disease. All students had tuberculin testing performed at entry and those that were negative were retested annually. In addition, all students had regular CXR performed at short intervals and those with suspected tuberculosis had close follow up over a period of years[119]. From this cohort 133 cases of “indisputably active tuberculosis” were identified, 99 had pulmonary tuberculosis and of these 52 had a previously normal CXR available allowing the authors to give an account of the first localisation of disease within the lungs. In those that were tuberculin convertors initial foci appeared on average 11.5 months post conversion. The initial focus of pulmonary tuberculosis was found to begin in the upper portion of the lung usually within the supra-clavicular region (apical) or infra-clavicularly around the 1st intercostal space (subapical) although the initial focus of disease did not involve these regions in 11% of cases. In those that had an initial normal CXR the subsequent abnormalities were bilateral in only 15%

They went on to describe the initial abnormalities as follows:

“The initial changes most frequently consist of minute and small cloudy spots, occurring singly or in groups (initial foci). These spots have a progressive tendency and in general spread very slowly in an apico-caudal direction, new spots arising lower down the lung or in the other lung. Occasionally, however, the dissemination takes place more rapidly, the spots then becoming fused into infiltrations which may liquefy and give rise to bronchogenic spread”

- Malmros and Hedvall 1940 [119]

The distribution of early post-primary disease has been carefully documented at autopsy. Medlar noted this minimal disease was evenly distributed between right and left lung and
present bilaterally in 19%. 84% of minimal disease was in the upper lobes and most commonly dorsally located in the upper half of the lobe (figure 1.8)[113]. Opie also described post-primary lesions apically, tending to spread diffusely and noted these were not present before the age of 11 but became increasingly common in adolescence and adulthood and often had evidence of partial or complete healing[112].

### 1.5.2.2 Apical localization

The apico-dorsal localization of post-primary tuberculosis is one of the most intriguing and least understood features of the disease. As described above the primary site of infection is no more likely to be in the lung apices than anywhere else within the lung. Dissemination throughout the lungs occurs via haematogenous spread around the 3rd week of infection and unlike primary lesions the secondary lesions that arise often regress and are not apparent macroscopically. Apical localization of disease is undoubtedly reciprocally related to the effect of gravity as while disease in humans commonly initiates at this location, in cows disease is most common at the highest point of the lungs dorsally, while bats have been have been shown to develop disease at the bases. In addition it has been demonstrated that maintaining a rabbit in an upright position for up to 11 hours a day causes TB to develop at the apices rather than dorso-basal region, as usually occurs[120, 121].

The 2 most striking effects of gravity on the highest point of the lung are elevated oxygen tension and very poor perfusion relative to the rest of the lungs (pulmonary artery pressure is only one fifth that of systemic circulation). This has led to competing theories about the explanation for apical localisation, however it is possible that both effects play a role. Peak oxygen tension is a commonly favoured explanation and certainly an oxygen rich environment facilitates growth of Mt. However, bacilli are generally thought to be contained within granuloma, which are known to be profoundly hypoxic and this may not be affected by alveolar oxygen tension[122]. In addition, people that live at high altitude in Peru have been found have similar rates of TB to those that live in lowland regions. Finally apical localization is observed in a number of other non-infectious conditions such as progressive massive fibrosis, ankylosing spondylitis where a role for oxygen tension is harder to understand. A second explanation relates to lymphatic clearance. It has been speculated that areas of the lung where this is poorest will result in ineffective clearance of antigen resulting in more robust and potentially more pro-inflammatory immune response at that site. Lymphatic flow is proportional to microvascular pressure and is greatly reduced at the apex. Lymphatic flow has been shown to be at least 4 fold slower at the apex than the lung.
base. In addition flow is assisted by respiration with the movement of the anterior chest wall, which is more minimal apically. As a result one would expect the dorsal apical region, where TB most commonly arises, to have the most sluggish lymphatic flow. This argument also provides better explanation for the apical localization of pneumoconiosis and is also said to support the anecdotal observation that TB is more common in those with pulmonary stenosis versus mitral stenosis and provides physiological credence to the suggestion that supine bed rest, a common part of sanatoria care, may facilitate recovery from TB[114].

1.5.3 Other patterns of tuberculosis disease that may be present within the lung

1.5.3.1 Miliary tuberculosis
A miliary pattern of disease can occur following massive haematogenous dissemination of Mtb and most likely occurs when a source of infection has persistent access to the vasculature rather than following the transient bacilaemia that occurs to allow initial dissemination (e.g. erosion of a tuberculous focus into the blood stream or focus of infection with the vasculature). Miliary disease may occur as a part of primary progressive disease or later as a part of post primary disease. The characteristic finding on imaging or autopsy are of numerous nodules 2-3 mm in size (similar to millet seeds) evenly distributed throughout the lung fields and other organs, especially those that receive high blood flow. Miliary tuberculosis occurs in approximately 1-2% of TB cases in immunocompetent adults but at higher frequencies in children and the immunocompromised[123].

1.5.3.2 Tuberculoma
Tuberculoma describes a well-circumscribed rounded lesion commonly between 1 and 5 centimetres (cm) diameter. They are often single but can be multiple and are usually found incidentally on CXR in asymptomatic persons where it can resemble a tumour. It probably does not represent a single granuloma increasing in size but more likely a localised area of tuberculous pneumonia that has ceased progression and encapsulated or a small cavitary lesion that has healed following bronchial obstruction[124].

1.6 FDG-PET/CT imaging

1.6.1 FDG-PET/CT
PET/CT is a non-invasive medical imaging technique that not only provides detailed anatomical information but also allows functional assessments through the monitoring of
the distribution and anatomical localisation of positron-emitting radiopharmaceutical tracers. The most commonly used tracer, $^{18}$F-fluoro-2-deoxy-glucose (FDG), is a glucose analogue with the oxygen molecule at the C-2 position of glucose ($C_6H_{12}O_6$) replaced with 18-Fluorine ($^{18}$F). $^{18}$F is a radioisotope of Fluorine and is unstable with a half-life of 109.8 minutes and decays by the emission of positrons (positively charged antielectrons released from protons to form neutrons) to stable 18-oxygen.

FDG is taken up by cells in the same manner as glucose, via glucose transport proteins (GLUT). Once within the cell FDG, like glucose, it is phosphorylated by hexokinases but unlike glucose is unable to enter the glycolysis pathway and is effectively “trapped” within the cell. This occurs preferentially in cells with increased metabolic activity in which GLUT and hexokinases have been up-regulated through greater requirement for glycolysis. The positrons emitted, annihilate with surrounding electrons (typically only having travelled 1-3 mm). A single annihilation event results in the emission of 2 gamma ray photons in opposite directions, approximately 180 degrees to each other. These gamma rays are then localised and quantified by PET camera surrounding the patient circumferentially, typically 1-2 hours follow intravenous administration of FDG. PET imaging is followed in quick succession (usually sequentially) by CT imaging which allow generation of detailed 3-dimensional (3D) X-ray images through reconstruction of multiple 2D slices. Co-registration of these images allows anatomical localisation of sites of metabolic activity. The FDG activity is often just evaluated visually in comparison to physiological uptake of surrounding organs and structures but can then be converted into a standardized uptake value (SUV) for objective comparisons using the following formula;

$$SUV = \frac{\text{Tissue radioactivity concentration}}{\text{(Injected radioactivity(corrected for time)/weight)}}$$

Essentially SUV represents the ratio of, concentration of radioactivity found at a certain time point in a selected region of interest within the body (derived from the image) and the injected radioactivity concentration normalised for body weight and corrected for time since injection. Maximal radioactivity in the region of interest is most commonly used and termed SUVmax, but mean and peak values can also be used. The sensitivity of PET is dependent on the size of the abnormality as well as the intensity of uptake of the tracer. In practice lesions less than 1 cm in size have activity underestimated and may be falsely negative on PET scan
due to a phenomenon known as the partial volume effect, although it is possible to develop algorithms to correct for this.

### 1.6.2 Cellular uptake of FDG in tuberculosis

The primary use of FDG-PET/CT imaging is in oncology as tumour cells demonstrate increase FDG uptake. However inflammatory and immune cells, especially if activated, also have considerable requirement for glucose. As a result FDG uptake is also elevated at sites of infectious and non-infectious inflammatory pathology.

In animal models specific cellular localization of FDG can be determined at necropsy (if FDG is injected into the animal just prior to being euthanized) by a process of autoradiography (as β-particles (like positrons) and gamma rays will develop photographic film similarly to X-Ray). Kaim et al performing this technique in experimental soft tissue abscesses in rats showed that in acute stages of infection considerable FDG uptake was found within the neutrophil layer of the abscess and in chronic infection even higher uptake occurred in the macrophages layer. Of note only limited activity (8-fold less than macrophage layer) was detected within fibroblast enriched granulation tissue [125].

Evidence that FDG might be preferentially taken up primarily by neutrophils and macrophages in humans with TB comes from Mamede et al. In this study patients undergoing thoracotomy and surgical resection of pulmonary lesions in Japan had prior FDG-PET/CT. In 10 patients, lesions were subsequently found to relate to TB with a median SUVmax of 6.6. Immunohistochemical staining for Glucose transport protein -1 (Glut-1) and hexokinase II (HKII) (required for cellular trapping to FDG) was performed on the resected lesions. 9/10 TB lesions stained positive for Glut-1 and 9/10 for HKII with Glut-1 and HKII staining both found to localise to neutrophils and macrophages within necrotizing granuloma[126].

### 1.6.3 FDG-PET/CT in clinical studies of tuberculosis

Increased FDG uptake within active tuberculosis lesions is well described and FDG-PET/CT is often diagnostically useful in localising site of disease [127-129]. As lesions heal (either following treatment of as part of their natural history), their cellular make-up alters as activated immunoregulatory and effector cells are replaced by scar tissue. As a result, we can expect that the metabolic activity will be different between active cellular lesions and
healed lesions that are fibrosed or mineralized. Kim et al have demonstrated differences in histology and FDG uptake in surgically excised culture or polymerase chain reaction (PCR) positive tuberculoma compared with culture/PCR negative tuberculoma. The former group displayed elevated FDG uptake (median SUVmax = 2.3) and histology showed granuloma with numerous cells in the lesion periphery, whereas the latter displayed low FDG uptake (median SUV max = 0.79) and histology showed granuloma surrounded by dense fibrous tissue[130].

Treatment accelerates healing and FDG uptake has been shown to normalize by the end of treatment in a number of case series[131-133]. Martinez et al looked at the early effects of TB treatment on SUV uptake in 20 cases; FDG uptake reduced by a median 31% after 1 month TB treatment the one patient that showed no improvement in FDG uptake remained smear positive at 3 months[134].

Abnormal FDG uptake is seen within anatomically normal mediastinal and hilar lymph nodes in individuals with active tuberculosis. Sathekge et al identified 18 lymph node (LN) sites in 9 TB patients demonstrating abnormal FDG accumulation - mean SUVmax 6.3(range 3.4-9.2) [135]. In 5 of these patients no abnormalities were picked up on CT. Shora et al identified 28 LN sites in 14 TB patients that demonstrated abnormal FDG accumulation (SUVpeak range 2.7-7.7)[136]. Of particular interest Ghesani et al have recently shown that 4 of 5 recent contacts of TB with positive Quantiferon or TST had abnormal FDG uptake within mediastinal nodes, 3 of which improved following IPT and furthermore there was a strong positive correlation with between Quantiferon result and SUV[137].

1.6.4 FDG-PET/CT in animal models

PET/CT has been used as a method to serially evaluate evolving tuberculous pathology both prior to and during treatment in the rabbit, marmoset and macaque models[138-140]. In the macaque model granulomas (as nodules) appeared on PET/CT as early as 2 weeks following bronchoscopic administration of 200-400 cfu of Mtb with 99% of granuloma having visible FDG uptake by 8 weeks. The authors followed a random sample of granulomas over time and demonstrated that granulomas behaved independently with disease outcome dependent on only a subset of lesions. Areas of consolidation (TB pneumonia) could either arise from a single lesion or from the coalescence of multiple lesions, corresponding to histological findings, and appeared to contribute substantially to the development of active TB. Areas of necrosis within TB pneumonia or granuloma were
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evidenced by a characteristic pattern of lack of central FDG uptake. At necropsy cfu within a lesion correlated with its size but not SUV. Following 2 months of treatment with isoniazid (INH), rifampicin (RIF) or a combination of isoniazid, rifampicin, pyrazinamide and ethambutol (HRZE) total SUV reduced influenced largely by reduction in SUV in regions of TB pneumonia. However, at the level of individual granuloma INH treated animals were more variable with some granuloma increasing in SUV while others reduced, this occurred to a lesser extent in animals treated with RIF where the majority of granuloma reduced in SUV with treatment[140].

These findings are consistent with the interpretation that SUV relates to presence of metabolically active cells and may reflect their accumulation within a granuloma or region of consolidation as bacillary numbers increase or reflect the presence of cells such as activated macrophages that are able to adequately control numbers (especially if scans are performed in the early stages of infection). Treatment particularly with INH may result in antigen release, which may result in inflammation and cellular recruitment accounting for increase SUV uptake.

1.6.5 FDG-PET/CT in asymptomatic HIV infected and uninfected individuals

The degree to which abnormal FDG uptake on PET is present in an asymptomatic population both HIV infected and uninfected individuals has also been well established. Over 40,000 healthy (HIV uninfected) individuals have been screened in Japan with whole body FDG-PET [141-144]. Although the rate of abnormal FDG uptake anywhere in the body is approximately 10%[141], abnormalities are most common in organs outside the thorax, with the thyroid and lower gastrointestinal tract being most frequently affected[141]. In a study where 2,911 asymptomatic HIV uninfected individuals 40 years and older were screened by FDG-PET, only 26 (0.9%) had any focal FDG uptake within the lungs [144]. Although FDG uptake within hilar LN is not uncommon, uptake greater than the background mediastinal blood pool is uncommon. In a study assessing FDG uptake in hilar LN of 179 healthy HIV uninfected individuals only 1 (0.6%) had FDG uptake within hilar LN greater than background mediastinal blood pool[145].

Several studies have established patterns of FDG uptake in ART naive and treated HIV infected individuals [146-151]. A distinct pattern of LN activation, felt to represent sites of viral replication, have been identified. In early infection (off ART) multiple, peripheral LN chains within the upper and lower torso (cervical, axillary, inguinal and iliac LN) demonstrate
increased FDG uptake symmetrically. FDG uptake is correlated with viral load and the pattern of LN activation has been shown not to change at repeat scanning after an interval of 4 months[146]. In treated HIV infected individuals with suppressed viral load, similarly to HIV uninfected individuals, very little FDG uptake within lymph nodes is seen. However, if treatment is stopped, resulting in increased viral load, FDG uptake increases [148]. In advanced HIV more extensive LN involvement particularly of mesenteric LN has been demonstrated but this may reflect opportunistic infections rather than as a result if HIV infection in itself.

1.7 Immunological assays to distinguish active and latent tuberculosis

Major limitations of TST and IGRA are that neither are able to distinguish active from latent tuberculosis and, more importantly, they poorly predict who will develop active disease. In order to make progress in this regard, one popular approach has been to identify whether cytokines and chemokines other than IFNγ are released differentially between active and latent tuberculosis following stimulation of whole blood or PBMC with ESAT-6, CFP-10 +/- TB7.7 (usually by taking supernatants from QFGIT or T.SPOT.TB and detecting other cytokines by ELISA or multiplex). Chegou et al have reviewed these studies. Differential release of cytokines between active and latent TB vary between studies and results are often contradictory. The cytokines/chemokines that appear most promising, being more consistently identified across a number of studies and induced to high levels, are IFNγ induced protein 10 (IP-10 or CXCL10), IL-2, monocyte chemotactic protein 1 (MCP-1 or CCL2), MCP-2 (CCL8), macrophage inflammatory protein 1β (MIP1β) and IL-1 receptor antagonist (IL-1RA) although even for these biomarkers there are disagreement between studies and no large confirmatory validation studies have been published[152]. In addition it should also be noted that the approach to selecting cytokines of interest for these studies is determined to a great extent by which cytokines and chemokines are present on commercially available multiplex kits and hence there are numerous potential cytokine/chemokines yet to be fully investigated.

A second approach has been to consider the differential immunogenicity of antigens other than ESAT-6, CFP-10 and TB7.7 between latent and active tuberculosis. In particular there has been interest in the products of genes induced by hypoxia and other conditions that
allow the bacillus to persist in a non-replicating state, especially those that form part of the dormancy survival regulon (DosR) and the extended hypoxic response (EHR). However, on the whole, results have been disappointing in terms of degree of immunogenicity, universality of response and consistency of findings. In addition, it should be noted that in almost all of these studies the read out has been restricted to IFNγ production. Leyten et al initially evaluated immunogenicity of recombinant proteins derived from the 25 most strongly induced genes from the DosR in active TB patients and asymptomatic TST positive controls in Holland. 18 of the 25 antigens were recognised in at least 10% of the participants, with Rv1733c, Rv2029c and RV2627c recognised in greater than 50% of TST positive participants (although none were recognised in greater than 62%). Only Rv2029c was recognised in significantly more TST positive than active TB patients, whereas Rv2031c (heat shock protein - hspX) was recognised by a greater proportion of patients with active TB[153]. Subsequently Black et al, using a 7-day whole blood assay, evaluated IFNγ responses by ELISA to 51 antigens spanning the 48 genes of DosR in 3 African populations with LTBI in South Africa, Uganda and The Gambia. Rv0081, Rv1733c, Rv1735c and Rv2006 were in the top 10 most frequently recognised antigens for all 3 sites and Rv1736c-C, Rv1737c and Rv1997-C were in the top 10 at South African and Ugandan sites. Responses to Rv1735c, Rv1736c-C and Rv1737c appeared to be highly correlated[154]. In a subsequent study this group compared IFNγ response to a total of 118 antigens (including the 51 DosR antigens) between active TB patients and healthy household contacts (HHC) in Cape Town. For 8 DosR antigens (Rv2032, Rv2625c, Rv1996, Rv0081, Rv2624c, Rv2006, Rv2629 and Rv2007c) IFNγ response was greater in HHC than active TB patients with an area under the curve (AUC) > 0.7 in receiver operating characteristic (ROC) analysis. Of the other antigens, all 5 resuscitation promotion factor (rpf) antigens were found to elicit greater IFNγ responses HHC compared to active TB patients all with AUC >0.7[155]. Taking this further for 5 promising DosR antigens and 2 rpf antigens they compared responses for 12 cytokines between active disease and HHC. IL-12(p40), IP-10, IL-10 and TNF-α responses to Rv0081 were most promising being greater in HHC and distinguishing these 2 groups with an accuracy of 100%[156].

Looking beyond DosR, Gideon et al evaluated immugenicity of antigens coded by genes upregulated during more prolonged hypoxia. They specifically focused on genes showing the greatest fold change over a 7 day hypoxic culture that were also likely to demonstrate greater Mtb specificity and identified Rv2658c and Rv2659c (RD11 encoded, absent from
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*M. bovis* and Rv1986c (RD2 encoded, absent from most BCG strains) as antigens of interest. Evaluating IFNγ and IL-2 *ex vivo* ELISPOT responses to these antigens in HIV uninfected participants with active or latent infection they found Rv1986 to have striking immunodominance for an IL-2 response. This was of comparable magnitude to the IFNγ response to ESAT-6/CFP-10, with a greater proportion of those with latent infection recognising this antigen compared to those with active TB[157].

Most of these approaches above have been driven by the hypothesis that the predominant metabolic state of the population of Mtb, and thus the antigens presented to the immune system, may vary between latent and active infection. However recently, Lindestam-Arlehamn *et al* have shown the value of a more unbiased, genome-wide approach to identify the novel immunodominant antigens (in terms of IFNγ response) in latent TB. The authors initially predicted the binding potential of all 15-mer peptides derived from protein sequences of 5 complete Mtb genomes for 22 of the most common human leukocyte antigen (HLA) class II alleles and then synthesised the 20,060 peptides expected to be the most promiscuous epitopes. They then determined the *ex vivo* IFNγ response to these peptides using PBMC from 28 donors with latent TB and identified 82 antigens that accounted for over 80% of the total response in this group, 74% of these antigens were novel[158]. The differential recognition of many of these antigens is yet to be evaluated.

A third common approach to distinguishing active and latent TB immunologically is using flow cytometry to determine differences in the phenotype of Mtb specific cells either with regard to expression of surface markers or functionality in terms of cytokine production. The most common cell types to focus on have been CD4 and CD8 cells, usually characterised according to memory phenotype or activation markers with intracellular cytokine staining most commonly performed for IFNγ, IL-2, TNFα and MIP-1β. Results, particularly with regard to polyfunctionality of Mtb specific CD4 cells, have been inconsistent between studies and methodology, particularly with regard to co-stimulation, varies greatly. Pollock *et al* evaluating differences between active and latent TB in those with and without HIV demonstrated that frequency of PPD specific CD4+ cells secreting IFNγ only, TNFα only and IFNγ/TNFα were greater in those with active TB irrespective of HIV status. PPD and RD1 specific CD4 cells were found to be predominantly of central memory phenotype (CD45RA+/CCR7+) in latent TB and effector memory phenotype (CD45RA-/CCR7-) in active TB, in addition CD127 expression was found to be reduced in active TB[159]. Harari *et al*
similarly demonstrated that there was a substantial increase in proportion of TNFα only producing RD1 specific CD4 cells in active compared to latent TB where as polyfunctional IFNγ/TNFα/IL-2 producing cells showed the opposite pattern[160]. By contrast, Caccamo et al demonstrated that polyfunctional IFNγ/TNFα/IL-2 producing CD4 cells were more common in active compared to latent TB, being detectable in 85-90% of those with active TB and only 10-15% of those with latent TB. This is in keeping with findings by Sutherland et al who also showed that these polyfunctional cells were more common in active TB compared to healthy household contacts[161, 162].

1.8 Host transcriptional signatures in tuberculosis

<table>
<thead>
<tr>
<th>MICROARRAY</th>
<th>RNA SEQUENCING</th>
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<tbody>
<tr>
<td><strong>Overview</strong></td>
<td>mRNA is converted into a library of cDNA fragments. Sequence of each fragment is then determined by next generation high throughput sequencing technology. Sequences are then aligned in relation to a reference genome.</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>1. Established technique that is widely available and been used in numerous publications in the field 2. Data analysis pathways/ strategies well established</td>
</tr>
<tr>
<td><strong>Disadvantages</strong></td>
<td>1. Greater dynamic range of expression level (thousand-fold) 2. Less reliant on predetermined gene sequence</td>
</tr>
<tr>
<td><strong>Overview</strong></td>
<td>1. As only common alleles are represented in probe, coverage is more limited than RNAseq (hundred-fold) 2. Dynamic range of expression level less than RNAseq (hundred-fold)</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>1. Data analysis and bioinformatics more complex 2. Not as widely availability</td>
</tr>
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Table 1.4 – Comparison of microarray and RNA sequencing

Profiling of transcript abundance (transcriptomics), commonly in whole blood, aims to generate a global picture of cellular function by simultaneously measuring the expression of thousands of genes with a broad coverage of the entire genome. However, it should be noted that transcript abundance only partially predicts protein abundance due to a variety of post-transcriptional events[163]. These studies can reveal biological mechanisms relevant to the condition of interest and also provide biomarkers of disease that may have potential utility diagnostically. There are two main techniques in wide use for global transcriptional profiling, microarray and RNA sequencing, each with their own advantages and disadvantages (see table 1.4).

Several studies, all using the microarray approach, have recently been published highlighting differences in transcript abundance between latent and active tuberculosis. In a study of
HIV negative participants, Berry et al identified a 393 transcript signature that distinguished active tuberculosis from latent tuberculosis and healthy controls on a training cohort in London[164]. Applying this signature to test and validation cohorts in London and Cape Town the sensitivity for active TB was 61.7% and 94.1% and specificity 93.8% and 96.7% respectively using k-nearest neighbour class prediction. 5 of 21 (24%) of those latently infected in the test cohort and 3 of 31 (10%) in the validation cohort clustered with active TB. The authors hypothesised that it may be possible that this group of latently infected persons that were misclassified as active TB may have had subclinical tuberculosis or otherwise been at high risk of developing TB. However, it is worth noting that 4 of 21 (19%) in test and 1 of 20 (5%) in validation with active TB clustered with latent TB and healthy controls so misclassification could be related to chance as much as having a biological basis. The authors went on to show that this active TB signature was related to the radiographic extent of disease with the signature being much more apparent in those with moderate and advanced disease. They then went on to demonstrate that an 86 transcript signature distinguished active tuberculosis from other infectious and inflammatory diseases (streptococcal and staphylococcal infection, Still’s disease and systemic lupus erythematosus (SLE)) with a pooled specificity of 83%. Both the 86 and 393 signature diminished with treatment. Analysing the functional components of the host response by modular analysis of transcripts in whole blood and separated cells they demonstrated that the TB signature revealed a decreased abundance of B cell and T cell transcripts and an increased abundance of myeloid transcripts. Although this reduction in T cell transcripts was likely to reflect reduced circulating T cell number in active TB, the increase in myeloid transcripts was less likely to relate to changes in circulating cell types. The most significantly over represented pathway identified in active TB was found to be IFN (type 1 and type 2) signalling, which was particularly over-expressed on neutrophils. In a subsequent study the group recruiting new cohorts from London and Cape Town looked further into the effect of treatment response on transcriptional signature. They derived a 664-transcript signature from differentially expressed genes between pre-treated active and latent TB in Cape Town and showed that this signature significantly and rapidly changed after 2 weeks of TB treatment[165].

Maertzdorf et al performing a study in HIV uninfected participants in South Africa identified 2,048 transcripts that were differentially expressed between TB patients and latently infected household contacts including 918 transcripts that were also differentially expressed with non-infected donors (fold change (FC) cut off $\geq 0.2$ or $\leq 0.2$ (log2scale) with $q=0.01$). $Fc\gamma$
receptor 1B was found to be the most strongly differentially expressed gene. Analysing the functional categorization of differentially expressed genes they determined that decreased apoptotic activity and increased innate host defence response were apparent in TB patients compared to latent TB controls[166]. In The Gambia this group identified 1,661 differentially expressed transcripts between HIV uninfected TB patients and latently infected controls (log2-fold change ≥0.5 or ≤0.5 significance q=0.01). Using signalling pathway impact analysis they demonstrated that genes involved in the complement and coagulation cascades, toll-like receptor (TLR) signalling and SLE were significantly enriched with significant perturbation within Fcγ receptor mediated phagocytosis pathway. Comparing the signatures they identified in The Gambia and South Africa with the signature identified in a UK cohort by Berry et al they determined 74% of genes to be common to all 3 sites in particular genes involved in IFN signalling[167]. In a larger study incorporating 523 HIV infected and uninfected participants from sites in Malawi and Ethiopia as well as The Gambia and South Africa, reverse transcriptase multiplex ligation dependant probe amplification (RT-MLPA) was performed to evaluate expression of a specific set of genes determined to be involved in TB pathogenesis from previous studies. Fcγ receptor 1A (FCGR1A) was identified as the most consistent classifier of active TB regardless of HIV status[168].

Cliff et al taking a different analytical approach, evaluated the dynamics of the host transcriptional response during treatment and were able to determine which genes responded within 1 week of TB treatment, the time of greatest killing of Mtb, as well as which gene responded later in treatment (between 4 and 26 weeks). Specific components of the complement system were found to be rapidly down-regulated after 1 week of treatment. In particular C1q, C2, SerpinG1 and factor B (BF) with C5, CD55 (complement decay accelerating factor) and CD59 (MAC inhibitory protein) down-regulated to a lesser degree. The authors speculated that these changes were as a result of rapid action of isoniazid on actively replicating bacilli and reflected the reduction in bacillary burden[169].

In order to identify common themes in these TB transcriptomic studies, Joosten et al performed an analysis of the biological pathways and process represented by genes identified in 8 recently published studies (the majority of which are outlined above). A total gene set of 409 genes was identified across these studies and then functional analysis was undertaken using 3 platforms (Ingenuity Pathway Analysis (IPA), Gene Set Enrichment
Analysis (GSEA) and modular analysis). Their analysis revealed a less dominant role for interferon related genes and suggested an important role of myeloid cells, in particular signalling through Pattern Recognition Receptors, Fc receptors, fibrosis and TREM1[170].

While helpful to interrogate biological processes that may be involved in a disease process, large transcriptional signatures are less useful as diagnostic signatures. Kaforou et al attempting to define the minimal transcript signature to distinguish TB from latent TB and other diseases carried out a study in HIV infected and uninfected participants in Malawi and South Africa. Using variable selection via elastic net to identify transcripts, they identified 27 transcripts that optimally distinguished active from latent TB and 44 transcripts distinguishing TB from other diseases. In order to evaluate individual risk of TB they developed a disease risk score (DRS) derived by subtracting the total intensity of down regulated transcripts from up-regulated transcripts with the threshold derived from the weighted average risk score for a group of patients. These signatures were found to have sensitivity and specificity of 95% and 90%, respectively, to distinguish active from latent TB and 93% and 86%, respectively, to distinguish active from latent TB in a test cohort of HIV infected and uninfected participants. These signatures performed considerably better in HIV uninfected than HIV infected participants[171].

Though these signatures perform well at distinguishing active from latent TB and from some other diseases, they have been less discriminatory against other diseases most notably sarcoidosis and meliodosis. Several authors have demonstrated that apparently TB specific signatures fail to distinguish active TB from sarcoidosis and that the IFN inducible, neutrophil driven transcriptional signature is also present in active sarcoidosis[172-174]. However, Bloom et al were able to define a signature of 144 differentially abundant transcripts between tuberculosis and sarcoidosis[174]. Koh et al demonstrated that differentially abundant transcripts from healthy controls in melioidosis and tuberculosis followed a similar pattern with IFNγ pathway being most prominent in both diseases[175].

In summary, whole blood transcriptional signatures for active tuberculosis have been demonstrated by several groups at a number of geographically diverse location encompassing participants and Mtb strains of varied genetic background. Interferon signalling is a dominant part of this signature but transcripts involved in other pathways are frequently over-represented, most notably complement pathways and genes relating to Fcγ
mediated phagocytosis. Although these signatures are distinct from certain infectious and inflammatory conditions, they overlap to a large extent with other pulmonary granulomatous diseases and diseases caused by other intracellular bacteria.

Work is now beginning on converting these signatures into assays that may prove more feasible for developing novel diagnostics for active TB. However, it is not clear whether these signatures could be predictive of active TB, which is potentially more significant. It may be that pathways that dominate the active TB signature do not become prominent until later in the disease process while other components of the signature appear earlier in disease. This would have the effect of persons with minimal or subclinical pathology being misclassified because they lack the dominant component of the transcript signature. In addition although a few studies have included HIV infected individuals in their cohorts none have looked in detail at the interaction between TB and HIV on the whole blood transcriptional signature.

1.9 Summary

In order to make progress toward elimination of TB as a public health problem we need to have a better understanding of the reservoir of infection, persons with latent TB. Current diagnostic tests for latent TB poorly predict active disease, development of more predictive tests will allow focusing of resources on those at greatest risk of active disease and improve acceptability of preventive therapy by reducing number needed to treat. From early in the course of HIV infection the risk of reactivating latent infection and progression to active TB is high. Those at highest risk of developing active TB may have evidence of minimal active pathology or fibrotic scarring suggestive of a fluctuating transition from latent to active TB. This subclinical pathology historically was screened for by CXR. FDG-PET/CT is a more advanced imaging modality that provides superior anatomical detail with CT component and assessment of metabolic activity with a PET component and should result in much greater sensitivity for detection of subclinical pathology. Recently transcriptional assessment of whole blood, as well as immunological profiling, has been successful in distinguishing active from latent tuberculosis, not possible with TST or IGRA. This raises the possibility that these modalities may be able to distinguish those with and without subclinical pathology on PET/CT. This may provide an infection stage specific biomarker that may have translational potential as a more predictive diagnostic test. In addition, this would provide greater insight
into the early stages and biological processes that occur in the transition from latent to active TB.

1.10 Thesis scope and aims

1.10.1 Overall Hypotheses and Aims

The overall hypotheses informing this thesis are that:

1. The transition from latent tuberculosis to active pulmonary tuberculosis involves a subclinical phase, which may be apparent on detailed imaging of the lungs with FDG-PET/CT.

2. Those with evidence of subclinical pathology will have similarities in immune response and transcriptional profile to people with active tuberculosis but be distinguishable from those with evidence of latent infection without subclinical pathology.

The overall aims and scope of the thesis is to:

1. Evaluate the utility of FDG-PET/CT to identify intra-thoracic pathology consistent with active tuberculosis in asymptomatic, ART naïve, HIV-1 infected adults with evidence of latent tuberculosis infection by QuantiFERON Gold-in-tube and no history of active tuberculosis; thereby defining a group with evidence of subclinical tuberculosis and a group without evidence of subclinical tuberculosis.

2. Determine the similarities and differences in peripheral blood transcript abundance between those categorized with and without subclinical tuberculosis and age, sex and CD4 matched controls with symptomatic active tuberculosis.

3. Determine the similarities and differences in peripheral blood immune responses between those categorized with and without subclinical tuberculosis and age, sex and CD4 matched controls with symptomatic active tuberculosis.

1.10.2 General Chapter aims

Chapter 1 Aim

• To provide an extensive overview and analysis of the current understanding of latent and subclinical TB and the impact of HIV infection.
Chapter 1 - Introduction

Chapter 2 Aim
• To describe material and methods used in thesis

Chapter 3 Aim
• To describe the recruitment of participants and controls in the study and to explore the performance of the current tools for screening of active and latent TB used in the recruitment of participants.

Chapter 4 Aim
• To evaluate the utility of FDG-PET/CT to identify evidence of subclinical pathology in asymptomatic, ART naïve, HIV infected adults with evidence of latent TB and no previous history of tuberculosis

Chapter 5 Aim
• To determine the differentially expressed transcripts in whole blood in those with and without subclinical TB and in comparison to an active TB control group and to evaluate the effect of HIV on the transcriptional response to TB

Chapter 6 Aim
• Identify serological and QFGIT supernatant markers that distinguish those without evidence of subclinical pathology from those with evidence of subclinical pathology and active TB control group

Chapter 7 Aim
• To draw overall conclusions and define contribution to knowledge by the thesis
CHAPTER 2: Materials and Methods

2.1 Setting

2.1.1 South Africa

South Africa lies at the southern tip of Africa bordering Namibia, Botswana and Zimbabwe to the north, and Mozambique and Swaziland to the east (figure 2.1). It has a population of 53 million of which approximately 80% are of Black African ancestry[176]. South Africa is an upper middle-income country and has the largest and most developed economy in Africa with a Gross National Income (Purchasing Power Parity) per capita of $11010 (2012)[177]. However, it also has one of the highest levels of income inequality in the world (as determined by the Gini index) and as a result has relatively high rates of poverty and unemployment.

![Figure 2.1 – Map of South Africa showing Cape Town, provinces and surrounding countries](image)

2.1.2 HIV/AIDS and tuberculosis in South Africa

Approximately 6.1 million people (11.5% of the population) in South Africa are living with HIV[178] and amongst antenatal women approximately 30% are HIV infected[179]. The national rollout of ART commenced in 2004 and by 2012, 2.2 million people were taking ART, 83% of those eligible[180]. South Africa has both the highest number of people infected with HIV and the largest ART programme in the world. Patients eligible to commence ART under South African guidelines (2013) include, all HIV infected persons with CD4 count less than 350/mm³, HIV infected persons with WHO Stage III or IV disease (including all forms of active tuberculosis) irrespective of CD4 count and pregnant women irrespective of CD4 count[179].
In 2012, 349,582 cases of TB were reported in South Africa, 65% of which were HIV associated. Overall TB case detection was estimated to be 62% and national incidence of TB is estimated to be 1,003/100000 [one of the highest rates in the world][12]. Tuberculosis is the leading cause of death in South Africa and accounts for approximately 12% of all deaths annually[181].

2.1.3 Khayelitsha, Cape Town

![Map of Cape Town showing location of study site.](image)

All participants and controls for this study were resident in Khayelitsha, a peri-urban township approximately 25 kilometres southeast of Cape Town in the Western Cape province. Khayelitsha is one of the largest and fastest growing townships in South Africa with a population of 391,749 living in 118,809 households (2011 census – although this is considered to be an underestimate)[182]. The population is predominantly Black African (99%) and Xhosa speaking (>95%) with the majority of residents being rural to urban migrants from the Eastern Cape province. Levels of socio-economic deprivation are high, 38% of the adult population are unemployed and 74% of households have a monthly income of less than R3200 (£250). Only 36% have completed high school education, although literacy levels are very high at 94%. 55% of household live in informal shack accommodation, 62% have access to piped water, 81% electricity and 72% access to a flush toilet. Use of biomass fuel for cooking and heating is uncommon and reported by less than
1% of households[182]. TB incidence in Khayelitsha is at least 50% higher that the national average at >1,500/100000. Cure rate for new TB patient is approximately 80%. 70% of TB cases are co-infected with HIV and antenatal HIV prevalence approximately 26%. Case detection rate of multi-drug resistant (MDR)-TB is approximately 50/100000[183, 184]. Tuberculosis notifications in Cape Town demonstrates seasonal variation with case notification being lowest April-June and highest October-December and vitamin D deficiency in the winter is common[67]. Approximately 80% of young adults in this environment have evidence of immune sensitization to Mtb by either TST or IGRA and force of TB infection is approximately 4-8%/year during adolescence and early adulthood[185, 186].

Routine healthcare and HIV/TB management in Khayelitsha is becoming increasingly decentralized and nurse-led and is provided through 4 community healthcare centres, 6 general clinics, 2 youth clinics, 1 male clinic and 2 midwife obstetric units. In 2012 a 300-bed district hospital was opened in Khayelitsha to provide secondary care and referral support. Tertiary level care is provided by referral to Tygerberg Hospital in Bellville or Groote Schuur Hospital in Observatory.

2.1.3.1 Khayelitsha Site B Community Health Centre

The site of recruitment and clinical follow up of the participants in this study was the Khayelitsha site B community health centre (CHC). On this site there are several healthcare facilities. A day hospital provides outpatient services for the day-to-day management of stable chronic conditions and includes a wellness clinic for the management and regular follow-up of HIV infected adults that are not eligible for ART. Adjoined to the day hospital is an emergency and trauma unit for the stabilisation of acutely unwell patients that may need onward referral to hospital. In a separate building health services for HIV infected persons requiring ART are provided, adjoined to this is the TB clinic, which has resulted in TB/HIV care being increasingly integrated. Also on the same site is a midwife obstetric unit.

This site has a longstanding relationship with the medical humanitarian, non-governmental organisation, Médecins Sans Frontières (MSF) (who started the first programme to provide ART in South Africa at this site in 2001) and with the University of Cape Town. Several successful and large clinical studies primarily focused on HIV/TB have been conducted at this site since 2004. Over this time research infrastructure and capacity has been expanded to provide additional rooms, buildings and appropriate facilities as well as a core of International Convention on Harmonization of Good Clinical Practice (ICH-GCP) trained
clinical research staff to enable recruitment and follow up of participants in clinical studies and trials.

2.1.3.2 Standard of care for adult HIV/TB patients in Khayelitsha

HIV counselling and testing (HCT) has been widely rolled out in Khayelitsha and uptake and acceptability amongst the population has rapidly increased with 55,000 being tested for HIV in 2010 (20% were positive)[184]. HIV testing is performed by trained healthcare staff using a point-of-care (POC) finger prick test for antibodies to HIV 1/2 with positive results confirmed by a second (different) POC test. Those who are HIV positive have blood taken for CD4 count. Patients with CD4 count ≤ 350/mm³ (or otherwise eligible for ART as mentioned above) are referred to ART clinic and offered treatment. First line treatment is currently a fixed dose combination of 300 mg tenofovir (TDF), 200 mg emtricitabine (FTC), 600 mg efavirenz (EFV) taken once daily. Those not eligible for ART are referred to a wellness clinic where they are reviewed every 1-2 months and provided with healthy lifestyle advice and vitamin tablets (B/C). In addition, specific concerns of the patient are addressed, weight and blood pressure is measured, symptom screens for sexually transmitted infection and TB are carried out and WHO clinical stage is determined. CD4 count is repeated every 6 months to re-evaluate for ART eligibility. Co-trimoxazole prophylaxis is commenced for patients with a WHO stage II/III/IV condition or CD4 count ≤ 200/mm³. In adults no testing for latent TB by TST is performed and currently Isoniazid Preventive Therapy (IPT) is not widely provided within the Western Cape, which is an anomaly nationally[187, 188]. The current national guidelines (2010) recommend 6 months IPT without the requirement for TST in ART naïve patients[189]. The Western Cape province are hoping to implement a more comprehensive approach based on recent evidence and international guidelines suggesting longer duration of IPT, up to 36 months in ART naïve and 12 months in ART established patients may be beneficial[190-192]. Patients in whom TB screen is positive (cough > 2 weeks, weight loss > 1 month, night sweats > 1 month, lymph nodes > 2cm palpable) are referred to TB clinic. At the start of the study in 2011 local guidelines for TB investigation in HIV infected persons included submission of 2 sputum samples for direct smear microscopy and culture. In 2012 this changed to the routine testing of sputum of TB suspects using GeneXpert MTB/RIF (Sunnyvale, CA, USA), a nucleic acid amplification test (NAAT). Culture was then only performed if GeneXpert was inconclusive or suggested rifampicin resistance and smear microscopy only performed if GeneXpert was positive. Anyone diagnosed with TB (sensitive to Rifampicin on GeneXpert) is started on a standard regimen of Isoniazid, Rifampicin, Pyrazinamide and Ethambutol for 2
months (2HRZE) followed by Isoniazid and Rifampicin for 4 months (4HR). ART is usually commenced usually within 2 – 8 weeks of starting of TB treatment.

2.1.4 University of Cape Town

The majority of laboratory work has been carried out within the Institute of Infectious Diseases and Molecular Medicine (IDM) at the University of Cape Town (UCT). The IDM is a world-class, multi-disciplinary, postgraduate research institute comprised of more than 20 research groupings that operates in the fields of infectious diseases and molecular medicine with a strong focus on TB and HIV research. It is housed at the Faculty of Health Sciences campus of the University of Cape Town in close proximity to Groote Schuur Hospital.

Processing of blood samples drawn from study participants in Khayelitsha, was performed within the Wilkinson Group facilities at the IDM, which included a BSL 2 laboratory with 4 x class 2 biosafety cabinets, 2 x 37°C CO2 incubators, 4 x centrifuges, and -20°C, -80°C and liquid nitrogen sample storage facilities. In addition there was a dedicated RNA preparation area. Daily assessment and maintenance of these facilities was carried out by a laboratory team. Freezers were all connected to back up emergency CO2 supply and a text alert system.

2.1.4.1 MVA85A Phase IIB vaccine trial

The recruitment process for the study described in this thesis was initially closely associated with the screening process for the MVA85A Phase IIB vaccine trial. MVA85A is a TB vaccine candidate developed at University of Oxford that has been evaluated in clinical trials in partnership with AERAS. A two centre Phase IIB vaccine trial of MVA85A in healthy HIV infected adult outpatients (ART naïve CD4≥350/mm³ and ART established CD4≥300/mm³) commenced in August 2011; Khayelitsha site B CHC was one of the clinical recruitment sites and Prof R.J. Wilkinson was the local PI. A dedicated building was constructed for the purpose of recruitment and follow-up for the vaccine trial as well as other associated studies and contained 4 consultation rooms, office space, phlebotomy area and pharmacy. A separate container housing a digital CXR machine was erected close by with an adjoining sputum induction booth.

For the MVA85A vaccine trial, prior to randomization to either vaccine or placebo arms, potential trial participants were screened for active TB with symptom screen, sputum culture and CXR and for latent TB with QFGIT first and then TST. Participants with a positive
screen for active TB were referred for on-going investigation and excluded from participation in the vaccine trial. Those with a negative screen for active TB and latent TB were immediately randomised into the vaccine trial. Those that had a negative screen for active TB but a positive QFGIT were considered as potentially eligible for the study described in this thesis and were further evaluated for eligibility as explained below (prior to TST administration). Participants not considered eligible for the study described in this thesis, then had TST performed and were treated with 6 months of IPT prior to randomization into the vaccine trial. From April 2012 there was a sponsor-initiated postponement in recruitment into the vaccine trial followed by a curtailment in scope of the trial following disappointing results from a separate paediatric trial of MVA85A[193]. Therefore from August 2011 to April 2012 participants for the study described in this thesis were recruited within screening process of the vaccine trial but after April 2012 it was necessary for the screening for this study to take place independently from the vaccine trial but using a similar screening approach for active and latent TB.

2.1.5 MRC National Institute for Medical Research, London

The laboratory work for whole blood transcriptomics component of the study and training in analysis of microarray data was carried out at the MRC National Institute for Medical Research in London over a 3 month period between August and November 2013 in the laboratories of Dr Anne O’Garra.

2.1.6 PET/CT facilities

FDG-PET/CT scans were performed at 2 different sites during the study both approximately 15 miles from the study site in Khayelitsha with the participants transported too and from these sites for scans to be carried out. Those performed between October 2011 and May 2012 were performed at the Cape PET-CT centre in Panorama Medi-Clinic private hospital using a Siemens Biograph PET/CT machine as this was at the time the only PET/CT scanner in the Western Cape province. From June 2012 to July 2013, FDG-PET/CT scans were performed at the newly opened Western Cape Academic PET-CT centre at Tygerberg provincial hospital which had close links to Stellenbosch University. This facility housed a Phillips Gemini PET/CT machine. The transfer was due to the development of academic collaboration with Stellenbosch Nuclear Medicine department and the cost effectiveness of performing imaging outside of a private hospital. Imaging protocols used on the 2 machines were similar and all repeat imaging was performed on the same machine as the initial scan.
All imaging carried out on the 2 scanners was reported by the same group of radiologists and Nuclear Medicine physicians using the same structured report.

2.1.7 Overview of study timeline

Recruitment for study commenced in August 2011 and was completed in January 2013. Follow-up of patients continued until July 2013. Laboratory work continued until May 2014 with analysis and write up completed by August 2014 (figure 2.3).

2.2 Clinical Recruitment

2.2.1 Approvals for study

Ethical approval for this study was received from University of Cape Town (013/2011) and Stellenbosch University (N12/11/079). Approval to conduct this study at the Khayelitsha Site B Community Health Centre was received from Western Cape Government, Department of Health (RP 23/2012) and the City of Cape Town (10286).

2.2.2 Summary of Clinical Study Design

This was an observational study in which the groups of participants and controls outlined in table 2.1 were recruited, to test the principal hypotheses that:

- The transition from latent tuberculosis to active pulmonary tuberculosis involves a subclinical phase, which may be apparent on detailed imaging of the lungs using FDG-PET/CT.

- Those with radiographic evidence of subclinical pathology will have similarities in immune response and transcriptional profile to people with active tuberculosis but be
distinguishable from those with evidence of latent infection without radiographic evidence of subclinical pathology.

<table>
<thead>
<tr>
<th>No</th>
<th>HIV Status</th>
<th>TB status</th>
<th>PET/CT</th>
<th>RNA</th>
<th>Immune</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>HIV POSITIVE ART naïve CD4 &gt; 350</td>
<td>LATENT TB sub-classified by PET/CT Asymptomatic, No previous TB Screening QFGIT positive Screening TB Culture negative, Screening CKR – No Active TB</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>3,4,5,6</td>
</tr>
<tr>
<td>15</td>
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<td>ACTIVE TB</td>
<td>TOTB Symptoms TB Culture/GeneXpert positive</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>15</td>
<td>HIV POSITIVE ART naïve CD4 matched</td>
<td>NO TB</td>
<td>Asymptomatic, QFGIT negative, TST negative TB Culture negative CKR – No Active or Inactive TB</td>
<td>N</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>8</td>
<td>HIV POSITIVE ART established CD4 matched</td>
<td>LATENT TB Asymptomatic, QFGIT positive, No previous TB Viral Load suppressed on ART TB Culture negative, CKR – No Active TB</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>3,5</td>
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<td>14</td>
<td>HIV NEGATIVE ART naïve CD4 matched</td>
<td>ACTIVE TB</td>
<td>TOTB Symptoms TB Culture/GeneXpert positive</td>
<td>N</td>
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<td>N</td>
</tr>
<tr>
<td>15</td>
<td>HIV NEGATIVE ART established CD4 matched</td>
<td>NO TB</td>
<td>Asymptomatic, QFGIT negative, TST negative TB Culture negative</td>
<td>N</td>
<td>Y</td>
<td>N</td>
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</tbody>
</table>

Table 2.1 – Summary of participants and controls recruited into the study: Table shows clinical characteristics, whether PET/CT was performed and whether participants formed part of transcriptional study (RNA) or immunological study (Immune)

As the primary aim of the study was to establish whether subclinical TB was on a spectrum between latent and active TB with regard transcriptional and immune responses, the principal control group used was HIV infected persons with active TB that were ART naïve but matched for CD4 count, sex and age. The HIV uninfected no TB, HIV uninfected active TB and HIV infected no TB groups were used to establish the independent effects of active TB and HIV on the transcriptional signature. It is acknowledged that these control groups may also have also been of value in exploring the independent effects of HIV and TB on immune responses but these samples were not available. The HIV infected ART established group was used to explore if the effect of HIV on the transcriptional signature was reversible with ART. The important factor therefore was that this group did not have active TB although it is acknowledged that an HIV infected, ART established group with no TB would have been a preferable control group for this purpose. However, as explained recruitment of the ART group was curtailed following postponement of the vaccine trial.
2.2.3 Diagnostic definitions

2.2.3.1 HIV-1 infection
Participants were determined to be HIV-1 infected if they had documented evidence of a positive point of care (POC) test for HIV in their medical notes AND, either a positive HIV-1 viral load and/or a positive HIV-1 ELISA. Participants with inconsistent results had further testing performed if necessary (e.g. HIV PCR, HIV Western Blot).

A participant was determined to be ART naïve if they verbally reported not taking ART AND no evidence of ART prescription was found in the medical notes.

Participants were determined to be established on ART if they verbally reported taking ART for > 6 months AND had evidence of ART prescription in the medical notes AND had a suppressed viral load (<40 copies/mL or Lower than detectable limit (LDL)).

2.2.3.2 Active Tuberculosis
Participants were considered to have active pulmonary tuberculosis if they had any of the following symptoms consistent with tuberculosis:

- Cough ≥ 1 week
- Haemoptysis
- Fever – documented > 38°C or reported history of fever
- Drenching night sweats
- Weight loss

AND sputum either culturing Mtb or positive by GeneXpert for Mtb.

2.2.3.3 Latent Tuberculosis
Participants were determined to have latent tuberculosis if at initial screening they had all of the following features:

- QFGIT positive
- CXR without evidence of active tuberculosis (see below)
- Negative 42-day culture for M. tuberculosis
- No symptoms suggestive of active TB over the screening period
- No previous history of active TB treatment or IPT
Chapter 2 – Material and Methods

TST was not used to confirm latent tuberculosis because of concern over Mtb antigens within PPD boosting mycobacteria specific immune responses and affecting interpretation of any subsequent immunological assays. This has previously been well reported[194].

Asymptomatic HIV infected ART naïve participants with latent TB were then sub-classified into those with and without subclinical pathology on the basis of PET/CT findings (see chapter 4) prior to subsequent transcriptional (chapter 5) and immunological (chapter 6) analyses.

2.2.3.4 No evidence of latent TB
Participant were considered to have no evidence of latent TB if they had the following features:

- QFGIT negative
- TST <5mm if HIV infected or <10mm if HIV uninfected

For HIV infected control in addition they required a CXR with no evidence of active or inactive TB and a negative culture at 42 days.

2.2.4 Consent procedure
All consent documents were provided to potential participants in English or Xhosa and read through with a member of the study team to ensure full understanding and capacity to consent before signing. Participants recruited to undergo PET/CT were recruited in a 2-stage process. At initial screening consent they were provided with an information leaflet in English or Xhosa explaining the study procedures and the risks and benefits of study involvement. At the end of the screening period this leaflet was explained in detail with eligible participants and any queries or concerns addressed prior to final consenting for study entry.
Figure 2.4 Study Flow
2.2.5 Overview of study sampling and procedures

<table>
<thead>
<tr>
<th>Screen Visit</th>
<th>Results</th>
<th>Recruit Visit</th>
<th>W0</th>
<th>W1</th>
<th>M1</th>
<th>M3</th>
<th>M6</th>
<th>HIV+</th>
<th>HIV+ Nil</th>
<th>HIV+ ART</th>
<th>HIV- Active</th>
<th>HIV- Nil</th>
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<tr>
<td>Screen Consent</td>
<td>◆</td>
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<td>Control Consent</td>
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Table 2.2 – Overview of visits and sampling: Red diamond = primary group of interest; Green diamond = controls, Black diamond = samples stored not used in thesis analysis; Green star = CXR not specifically performed for study so not available in all

2.2.6 Recruitment of primary group of interest

Healthy outpatients attending the pre-ART wellness clinic were approached for screening consent. At the initial screening those that provided consent had the following performed:

- CXR
- QFGIT
- CD4 (if very recently available this was not repeated at screening)
- Sputum smear and culture x 2
- Screening history and exam

This screening was either performed as part of the vaccine trial screening or independently as explained above. Participants were reviewed after 1 week with results. Those that were asymptomatic, ART naïve, CD4 ≥350mm³, QFGIT positive, with no previous history of tuberculosis and without CXR evidence of active disease had a detailed history and
examination performed and were asked to return at 6 weeks for results of sputum culture and a repeat CXR (figure 2.4). At 6 weeks those that met inclusion/exclusion criteria were consented for participation in the study and had PET/CT scan arranged.

2.2.6.1 Inclusion criteria

- HIV-1 infected
- ART naïve
- CD4 ≥ 350/mm$^3$ at initial screening visit
- QFGIT positive at initial screening visit
- CXR without evidence of active TB at initial screening visit
- Age ≥ 18 years

2.2.6.2 Exclusion criteria

- Screening sputum culture positive for TB
- Symptoms or signs of active pulmonary or extra-pulmonary TB during screening period
- Evidence of any acute or unexplained chronic illness
- Previously diagnosed or treated TB
- Previous IPT
- Known recent contact of MDR TB
- CXR abnormality within lung parenchyma or lymph nodes known or suspected to related to a condition other than inactive TB
- Age > 50 years
- Smoker of > 30 pack years
- History of working in the mines or evidence of silicosis
- Previously diagnosed malignancy
- Previously diagnosed chronic lung infection (NTM, Fungal)
- Known diagnosis of chronic lung disease (COPD, bronchiectasis)
- Known diagnosis of chronic inflammatory condition associated with pulmonary pathology (Sarcoid, RA, Wegeners granulomatosis)
- Current steroid use
- Uncontrolled diabetes mellitus
- Pregnant or planning pregnancy
- Breast feeding or unable to avoid very close contact with young children/pregnant persons for 12 hours
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- Compelling indication for ART despite CD4>350/mm$^3$
- Inclusion in study to result in annual radiation exposure of > 50mSv
- Investigator assessment of lack of willingness or inability to participate and comply with all requirements of the protocol or identification of any factor felt to significantly increase the participant’s risk of suffering an adverse outcome

2.2.7 Rational for inclusion/exclusion criteria

In the extremely high TB burden setting of Khayelitsha particularly in an asymptomatic HIV infected cohort with evidence of latent tuberculosis and CD4≥350/mm$^3$, the pre-test probability of pulmonary lesions identified on FDG-PET/CT being related to tuberculosis was high. However, inclusion and exclusion criteria were designed to ensure that pulmonary lesions related to alternative causes were minimised and that conditions that may cause false negative or positive FDG uptake were excluded. Malignancy is the main differential diagnosis of concern. To minimise probability of a lesion relating to malignancy, those aged over 50 years or with > 30 pack years of smoking history or with a previous history of malignancy were excluded[195].

Alternative infections similarly could give rise to abnormalities and those with a history of chronic pulmonary infection were excluded. With regard to undiagnosed infection other than TB, those with bacterial pneumonia would be unlikely to remain asymptomatic over 6 weeks. Non-tuberculous mycobacteria (NTM) infection usually presents as disseminated disease in HIV-infected persons with CD4 < 100mm$^3$ (even at this level of immunosuppression disseminated TB is approximately 5 times more common than *Mycobacterium avium and intracellularare infections* (MAI) in South Africa[196]), however, it is also a cause of pulmonary disease in those with pre-existing lung disease. Silicosis, previously treated TB and working > 9 years in a mine were exclusion criteria to minimise this[197, 198]. Other chronic infections considered were pulmonary cryptococcal disease which is uncommon in those with CD4 counts greater than 350 /mm$^3$. Classical histoplasmosis, although present in the Western Cape province of South Africa is relatively rare [199]. Blastomycosis is rarer still with only 81 cases reported in the whole of Africa between 1951 and 1987[200] and only a single case of Paragonimus has been reported in South Africa[201].
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Inflammatory conditions such as rheumatoid arthritis, ankylosing spondylitis or sarcoidosis can cause parenchymal abnormalities and those with history of such chronic inflammatory conditions were excluded. It would be unlikely that parenchymal abnormalities related to these conditions would be present in the absence of other symptoms.

Uncontrolled diabetes mellitus leading to chronic hyperglycaemia can lead to downregulation of GLUT transporters and interfere with FDG uptake into cells hence was an exclusion. Steroid use may also affect cellular recruitment to sites of pathology reducing FDG uptake so was also an exclusion.

2.2.8 Modifications to the study design

In the initial study design it was intended that asymptomatic, ART naïve, HIV infected participants with latent TB recruited to undergo PET/CT would also have evidence of inactive TB lesions on CXR, which were intended to be evaluated in by PET/CT. Screening was therefore incorporated into a much larger study (the MVA85A vaccine trial) as the screen to recruitment ratio was anticipated to be high. Following postponement of the vaccine trial in April 2012 modifications to the study design had to be made and it had already become apparent that CXR was not an effective way to screen for abnormalities on PET/CT (as will be discussed in chapter 4). The inclusion criteria were modified so that participants were required to have no evidence of active TB (i.e. either have evidence of inactive TB lesions or considered to have a normal CXR). This reduced the screen to recruitment ratio and allowed for the study to continue independently of the vaccine trial. In all, 8 participants were recruited into the study via vaccine trial screening and 27 participants independently from it.

2.2.9 Follow-up and treatment

Recruited participants in the primary group of interest were followed up and sampled as outlined in Table 2.2. Participants were seen for study visits at the following time points for assessment and sampling,

- Day of PET/CT scan
- 1 week after PET/CT scan
- 1 month after PET/CT scan
- 3 months after PET/CT scan
- 6 months after PET/CT scan for repeat PET/CT
In terms of clinical management, the majority of participants were commenced on 6 months isoniazid as preventive therapy. Any participant that developed symptoms, radiographic progression or had concerning abnormalities on PET/CT was further investigated as appropriate with participants commenced on full active TB treatment if TB was confirmed microbiologically or suspected as highly probable.

2.2.10 Matching strategy for control groups

Control participants were recruited primarily to provide positive and negative control groups for whole blood transcriptomics experiments and to allow for a degree of validation of any identified transcriptional signatures. The HIV infected active TB control group were also used to provide a positive control for immunological studies.

The HIV infected controls were matched with participants of the primary group of interest by distribution of age, sex and CD4 count as these were potentially confounding factors. Ethnicity in Khayelitsha is almost exclusively Xhosa and therefore did not need to be formally matched. HIV infected participants were not matched for viral load, as active TB infection is known to directly increase HIV viral load and hence matching by this variable would not feasible and would also lead to issues of overmatching. The effect of any difference in viral load between these groups will be considered in analysis.

Matching was performed such that the distributions of the variables (age, sex and CD4 count) were similar between the groups and so that the median values didn’t vary significantly. Recruitment of the primary group and the HIV infected control groups overlapped so distribution was monitored in real time.

HIV uninfected control groups were recruited towards the end of the study over a shorter period of time, after the majority of the other participant groups had been recruited. Therefore precise matching of age and sex was not possible.

2.2.11 Symptomatic active TB controls

HIV infected active TB controls were recruited either within the study screening process, as outlined above in figure 2.4, at the research clinic in Khayelitsha Site B CHC, or independently through the TB clinic at the same site. HIV uninfected active TB controls were all recruited through the TB clinic at this site. The majority of participants recruited through the TB clinic already had a confirmed diagnosis of active TB, either by GeneXpert or culture
and were often co-recruited with other ongoing clinical studies. In these instances sputum sampling was not repeated. In some cases TB treatment had already been commenced, those that had > 24 hours treatment were not eligible. After providing consent eligible active control participants had blood sampling as indicated in table 2.2

2.2.11.1 Inclusion criteria - active TB controls

- Sputum GeneXpert positive or culture positive
- Symptoms consistent with active TB
- Age ≥ 18 years

For HIV infected active controls

- HIV-1 infected
- ART naïve
- CD4 ≥ 350/ mm³ or CD4 count unknown if newly diagnosed with HIV

For HIV uninfected active controls

- Evidence of negative POC HIV test

2.2.11.2 Exclusion criteria - active TB Controls

- On TB treatment for more than 24 hours
- Evidence of acute or unexplained chronic illness other than TB or HIV

2.2.12 Asymptomatic QFGIT negative, TST negative controls

HIV infected, QFGIT negative, TST negative control subjects were identified through the screening process outlined above (figure 2.4). HIV uninfected, QFGIT negative, TST negative control subjects were screened and identified separately at the same site. For all these control participants, blood sampling was carried out prior to TST administration. HIV infected controls also had sputum culture and CXR performed, however, HIV uninfected controls did not. This was in part due to logistics but also because asymptomatic, HIV uninfected persons are less likely to have positive sputum culture even in this setting.

2.2.12.1 Inclusion criteria – QFGIT negative, TST negative controls

- QFGIT negative
- TST negative
- Age ≥ 18 years

For HIV infected controls

- HIV-1 infected
- ART naïve
- CD4 ≥ 350/ mm³
- CXR no evidence of active or inactive TB
- Sputum culture negative for Mtb
For HIV uninfected controls
- Evidence of negative POC HIV test

2.2.12.2 Exclusion criteria - QF/GIT negative, TST negative controls
- Symptoms or signs of active pulmonary or extra-pulmonary TB
- Evidence of acute or unexplained chronic illness

2.2.13 Asymptomatic HIV infected ART controls
HIV infected, QF/GIT positive control subjects established on ART were recruited through the screening process of the vaccine trial and were not recruited following postponement of the vaccine trial and hence numbers were limited to 8.

2.2.13.1 Inclusion criteria - HIV infected ART established, QF/GIT positive controls
- HIV-1 infected
- Established on ART for ≥ 6 months with suppressed viral load
- CD4 ≥ 350/ mm³
- QF/GIT positive
- CXR without evidence of active TB
- Age ≥ 18 years

2.2.13.2 Exclusion Criteria - HIV infected ART established QF/GIT positive controls
- Sputum culture positive for Mtb
- Symptoms or signs of active pulmonary or extra-pulmonary TB
- Evidence of acute or unexplained chronic illness

2.3 Details of Clinical Procedures & Data collection Methods

2.3.1 History and Examination
Medical history and examination was recorded in a structured format directly onto a dedicated clinical research form (CRF) with ability to add free text if desired. A comprehensive medical history and examination was recorded on participants recruited for PET/CT. History and examination were more targeted during screening of participants and for control participants.

2.3.2 Sputum sampling and induction
All HIV infected participants and controls regardless of symptoms were asked to provide sputum samples as part of screening for active TB. If participants could not produce adequate sputum samples spontaneously, they were requested to undergo sputum induction, provided there were no contraindications (primarily asthma). Sputum was
induced in a specially designed cubicle housed outdoors on the study site to minimise the risk of aerosol transmission. This was performed with the assistance of a trained research worker who wore an N95 mask for personal protection. 30ml of 3% hypertonic saline was administered via the mouthpiece of a nebuliser and expectorated sputum was collected over a 1/2hr period.

2.3.3 Tuberculin Skin Testing
Control participants with a negative QFGIT subsequently had a TST administered to confirm lack of evidence of immune sensitisation by Mtb. As explained above participants recruited who underwent PET/CT did not receive TST because of concern about boosting. TST was carried out by ICH-GCP trained research nurses. 2TU (0.1mL) of PPD RT23 (Statens Serum Institut) was injected intradermally with a 27G needle into the volar aspect of the forearm. Participants returned for reading 48-72 hours after injection. The edge of indurated margin was marked using a ballpoint pen and maximal transverse induration was measured. Induration $\geq$ 5mm was considered positive for HIV infected persons and $\geq$ 10mm was considered positive for HIV uninfected persons.

2.3.4 Routine Laboratory Tests

2.3.4.1 Microbiology test
Sputum samples were processed in the accredited laboratories of the National Health Laboratory Services (NHLS) where auramine sputum smear, GeneXpert and mycobacteria growth indicator tube (MGIT) liquid TB culture were performed according to local standard operating procedure (SOP). As part of the quality control (QC) for the vaccine trial, which sent samples to the same laboratory, sterile mock sputa were sent weekly to the laboratory. In all 209 mock sputa were sent to the NHLS laboratory for TB culture between August 2011 and June 2014. None of these were found to be positive for Mtb, demonstrating that cross contamination within this laboratory was very low.

2.3.4.2 Blood Tests
Volume of blood and tubes used for each test is outlined in table 2.3. All blood tests were processed in regional haematology, biochemistry, immunology and virology laboratories with nationally recognised accreditation according to the SOP employed by the individual laboratories.
2.3.4.3 HIV POC tests

All HIV infected persons had a diagnosis of HIV prior to study entry and all HIV uninfected participants had a confirmed negative HIV test using the testing strategy adopted within the Western Cape province. This involved a trained healthcare professional first counselling the patient for an HIV test. A rapid immunochromatographic card test for the detection of antibodies to HIV 1 and 2 was then performed on a capillary blood sample drawn following finger prick (FIRST RESPONSE®, Premier Medical Corporation, Nani Daman, India). Results were interpreted after 5-15 minutes as either Negative, HIV-1 positive, HIV-2 positive, HIV1 & 2 positive or invalid. Those with a positive test had a second rapid immunochromatographic card test performed from a different manufacturer (Determine™ HIV-1/2, Alere Medical Corporation, Chiba, Japan). Results and discussion with patient were recorded on a structured reporting sheet and this was used as evidence of HIV status. As mentioned in 2.2.3.1 additional confirmatory tests for HIV were performed within the study.

2.4 Imaging methods

2.4.1 Chest Radiography

Chest radiographs were all performed using a digital X-Ray machine and captured posterior-anteriorly with the participant standing in full expiration with arms pronated to reduce scapular shadow over the lung fields. High kV (125kV) was used to ensure optimal visualization of the lung fields. Two different machines were used during the study, between August 2011 and February 2012 CXR were performed on a Delft Oldeca DR machine located at UCT, for which the participants were transported to and from Khayelitsha. From February 2012 until July 2013 CXR were performed on a dedicated research Phillips Essenta DR machine located at the study site in Khayelitsha site B. Any
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<th>CXR findings suggestive of ACTIVE TB</th>
<th>CXR findings suggestive of INACTIVE TB</th>
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<td>Infiltrate or consolidation</td>
<td>Discrete fibrotic scar or linear opacity with or without volume loss or retraction</td>
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<td>Opacification of airspaces within the lung parenchyma. Dense or patchy with irregular, ill-defined, or hazy borders</td>
<td>Discrete linear or reticular opacity within the lung with distinct edges and no suggestion of airspace opacification or haziness between or surrounding the linear or reticular lesion. Calcification can be present within the lesion</td>
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<td>Any cavitary lesion</td>
<td>Discrete nodule(s) with or without calcification</td>
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<td>Presence of a significant amount of fluid within the pleural space</td>
<td>One or more nodular opacities with distinct borders and no surrounding airspace consolidation</td>
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<td>Nodule with poorly defined margins</td>
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<td>Round opacity within the lung parenchyma, with margins that are indistinct or poorly defined</td>
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<td>Pleural effusion</td>
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<td>Hilar or mediastinal lymphadenopathy</td>
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<td>Enlargement of lymph nodes in one or both hila and/or within the mediastinum</td>
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<td>Miliary Tuberculosis</td>
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<td>Nodules that are uniform in size, measuring 1 to 2 mm (millet size), distributed throughout the parenchyma</td>
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Table 2.4 Definition of CXR abnormalities: Adapted from [30]

repeat CXR were performed on the same machine as the initial CXR though the imaging protocols used were similar

2.4.1.1 Reporting of Chest Radiographs

The Digital CXR images were viewed on 2 megapixel screens in low ambient light using the OsirIX version 3.8.1 32-bit (Pixemo, Geneva, Switzerland) software package and reported independently by 2 medically qualified researchers (H Esmail and T Oni) blinded to clinical details. Reading of the CXR was performed in a structured way using a slightly modified version of the Chest radiograph Reading and Reporting System (CRRS) report sheet (see chapter supplementary information). The CRRS system has been specifically designed and validated to facilitate reading of CXR performed in research and epidemiological studies for the investigation of TB and provides a structured report form to assess all areas of the CXR for the presence of abnormalities consistent with TB[202]. Both researchers reading the CXR successfully completed the CRRS course prior to commencement of the study. After structured reading, the CXR was determined to be consistent with active or inactive TB according to modified CDC criteria outlined in the Tuberculosis component of Technical Instructions for the medical examination of Aliens in the USA May 2008 [203] (table 2.4). The slight modification for the purpose of this study being that discrete nodules with calcification were kept as part of the definition for inactive TB and the other category for inactive TB (upper lobe bronchiectasis etc.) was not used.
The CXRs were then categorised as:

1. Consistent with Active TB
2. Consistent with Inactive TB
3. Abnormality not consistent with TB
4. Normal

Any disagreement in reporting was resolved by consensus to provide final categorisation of CXR. If consensus could not be achieved CXR were reported by a consultant radiologist (Q Said-Hartley) using a similar structured report and final categorization in these cases was determined by the consultant radiologist.

2.4.2 FDG-PET/CT

Participants undergoing FDG-PET/CT were asked not to eat for 6 hours prior to the scan and were driven to the PET/CT centre escorted by a research worker. At the PET/CT centre a point of care assessment of blood glucose measurement (BM) was performed to ensure BM < 11.1mmol/L (hyperglycaemia can contribute to false negative FDG-PET/CT). A POC pregnancy test was performed prior to scan on all female participants to ensure they were not pregnant. Participants were then given 20mg of propranolol orally to minimize brown fat uptake of FDG (as long as systolic blood pressure was greater than 100mmHg). Thirty minutes later 4MBq/kg of the radiolabelled tracer, FDG, was administrered via a 22G cannula in the forearm. Sixty minutes after FDG administration a PET/CT scan was performed according to the protocol in table 2.5. CT was limited to the chest (although this included neck and upper abdomen to just below the liver) to reduce radiation exposure. Total effective radiation dose per scan was approximately 10mSv (varying with body weight and height). A second PET/CT was performed after approximately 6 months towards the end of treatment with either IPT or 2HRZE/4HR (as indicated). Every effort was made to use a similar dose of FDG and also leave same time between injection and scan as the initial scan.
2.4.2.1 Reporting of PET/CT images

Images were reported using a specially designed, structured report focusing on the Regions Of Interest (ROI); lung parenchyma and mediastinal and hilar lymph nodes (see chapter supplementary information for copy of report). The primary report of the scan was provided by radiologists and nuclear medicine physicians at the Western Cape Academic PET/CT centre. If scans were performed at the Panorama Medi-Clinic they were re-reported by the team at Western Cape Academic PET/CT centre. Detailed reporting instructions were provided to all readers. Parenchymal lesions were categorized as nodules, fibrotic scars, infiltrates or other. The size and location of lesion (both the lobe lesion was in and its relationship to pleura and secondary lobule) were described. The lesions were also evaluated for radiographic signs of active TB as follows:

Consistent with TB with signs of disease activity (within lung parenchyma)

- Poorly defined nodule or infiltrate
- Tree in bud appearance
- Cavitation

Consistent with TB but without signs of disease activity (within lung parenchyma)

- Well defined nodule
- Fibrotic scar
- Mineralization

FDG uptake within the parenchyma lesions was quantified by both a widely used and accepted Visual Score (VS) (see below) and also by SUVmax. SUVmax is the most commonly used measure of PET activity and provides a ratio of the maximum radioactivity (derived from image) within a particular defined ROI to the total injected radioactivity normalised to body weight (taking into consideration radioactive decay over time)

Visual Score

0. No visible uptake
1. Visible uptake within lesion greater that background lung parenchyma but less than mediastinal blood pool
2. Visible uptake within lesion greater than mediastinal blood pool but less that liver
3. Visible uptake within lesion greater than liver

Parenchymal lesions were considered to have signs of activity if VS was ≥ 1 or if VS was 0 but the lesion had radiographic signs of activity.
Mediastinal and hilar lymph nodes were specifically focused upon and were considered abnormal if short axis width was greater than 1 cm, there was evidence of mineralization or there was FDG uptake above that of the mediastinal blood pool (i.e. VS ≥ 2). SUVmax was also recorded for all abnormal lymph nodes. Abnormal lymph nodes were located into 1 of the following lymph node basins following convention of the International Association for Study of Lung Cancer (IASLC) (figure 2.5)

- R or L Superior Mediastinal (2 – 4)
- Aortic (5,6)
- Inferior Mediastinal (Subcarinal) (7-9)
- R or L Hilar (10-14)

In addition to the mediastinal and hilar lymph nodes, any abnormal uptake within cervical LN, axillary LN and thymus were noted and any other pathological abnormalities between mandible and base of liver were commented upon.

The scans were reviewed by a researcher (H Esmail) and any queries or differences of opinion with the primary report were resolved by a 3rd reader (J Warwick).
2.5 Processing of research samples

2.5.1 Quantiferon Gold in tube (QFGIT)

2.5.1.1 Background to Quantiferon Gold in tube
Quantiferon (Qiagen; Linberg, Netherlands) is one of two commercially available IGRA tests used for the in vitro diagnosis of latent tuberculosis (i.e. having evidence of sensitization by Mtb). It is a short term, whole blood assay in which IFNγ release in response to stimulation with Mtb specific antigens is quantified by ELISA. It comprises 3 heparinized tubes; NIL (used as negative control for background subtraction), ANTIGEN (coated with Mtb specific antigens, ESAT-6, CFP-10 and TB7.7) and MITOGEN (phytohemaglutinin- used as a positive control). IFNγ ELISA Optical Density (OD) values are converted into International Units and results are interpreted as in table 2.6.

<table>
<thead>
<tr>
<th>NIL</th>
<th>ANTIGEN-NIL</th>
<th>MITOGEN-NIL</th>
<th>RESULT</th>
<th>INTERPRETATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤8.0</td>
<td>&lt;0.35</td>
<td>≥0.5</td>
<td>NEGATIVE</td>
<td>Mtb infection NOT likely</td>
</tr>
<tr>
<td></td>
<td>≥0.35 and &lt; 25% of NIL value</td>
<td>≥0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥0.35 and &gt; 25% of NIL value</td>
<td>ANY</td>
<td>POSITIVE</td>
<td>Mtb infection likely</td>
</tr>
<tr>
<td></td>
<td>&lt;0.35</td>
<td>&lt;0.5</td>
<td>INDETERMINATE</td>
<td>Results are indeterminate for TB Antigen responsiveness</td>
</tr>
<tr>
<td>&gt;8.0</td>
<td>ANY</td>
<td>ANY</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6 – Quantiferon results interpretation

2.5.1.2 Methodology for QFGIT
One mL of blood was drawn directly into each of the 3 QFGIT tubes (NIL, ANTIGEN and MITOGEN) and immediately inverted 10 times. Blood tubes were transported from the clinical site to the Wilkinson Laboratory where they were incubated in an upright position at 37°C for 24 hours. After incubation the tubes were centrifuged at 2200g for 10 minutes and the plasma supernatant was removed and aliquoted into 2 vials. 100µL was stored at -20°C for the QFGIT assay, which was usually performed within 1 week of collection, and the remainder approximately 300µL was stored at -80°C for use in research multiplex assays and ELISA described below.

The QuantiFERON IFNγ ELISA assay was performed weekly along with vaccine trial samples according to manufacturer instructions by good laboratory practice (GLP) trained research laboratory assistants (F Patel and M Carr). They also had regular evaluation as part of the
quality assurance (QA) of the vaccine trial. The result of the assay was determined as POSITIVE, NEGATIVE or INDETERMINANTE according to manufacturers instructions. INDETERMINANT results were repeated once.

2.5.2 Tempus Blood RNA tubes collection and storage

2.5.2.1 Background to Tempus tubes
Tempus Blood RNA tubes (Applied Biosystems; Foster City, CA, USA) are specifically designed to stabilise and isolate RNA from whole blood for transcriptional analysis. Tempus tubes contain 6 mL of stabilization reagent which when mixed with blood causes immediate cell lysis, inactivation of cellular RNAses and precipitation of RNA thus “freezing” the global gene expression profile.

2.5.2.2 Methodology
Three mL of blood was drawn into 2 x Tempus Blood RNA tubes and shaken vigorously for 10-15 seconds as recommended by the manufacturer. Tubes were then transported to the Wilkinson laboratory and kept for long-term storage between -20°C and -80°C prior to use.

2.5.3 Serum storage
Blood was drawn into 5mL SST™ II Advance tubes (BD Diagnostics; Franklin Lakes, NJ, USA), transported to the Wilkinson laboratory, centrifuged at 1200g for 10 minutes and serum supernatant was aliquot and stored at -80°C for long-term storage prior to use.

2.6 Research laboratory assays and methodology

2.6.1 Transcriptional profiling using microarray (Chapter 5)

2.6.1.1 RNA Extraction
Tempus tubes were in storage between -20°C and -80°C for no longer than 21 months before RNA extraction. RNA was extracted from 1 of the 2 Tempus tubes available using the 5 PRIME PerfectPure™ Blood RNA Kit (5 PRIME; Dusseldorf, Germany) in the Wilkinson laboratories at UCT in a dedicated RNA area by 2 researchers (H Esmail and N Bangani). A modification of the manufacturers protocol developed by C Graham at MRC National Institute for Medical Research London was followed. All equipment and laboratory surfaces were cleaned of RNase with RNaseZAP™ (Sigma-Aldrich; St Louis, MO, USA) prior to extraction. Briefly, tempus tubes were placed on ice for 1 hour to ensure complete slow thaw. The blood/reagent mixture was then transferred into a 50mL universal falcon tube
containing 3mL of cold ethanol and vortexed for 2 minutes of to ensure sample homogenization. The falcon tubes were then centrifuged at 4700 rpm for 60 minutes at 0°C after which the supernatant was poured off and the tubes were inverted onto absorbent paper for 2 minutes. The RNA pellet was then dissolved in 200μL of 1% tris(2-carboxyethyl)phosphine (TCEP) Lysis solution and transferred into a purification column. RNA was then isolated on the purification column filter by centrifugation at 13000 rpm in a microfuge. The RNA was then washed with 400μL of Wash 1 solution and incubated with 50μL of DNase at room temperature for 15 minutes. RNA was then washed twice in 200μL of DNase wash solution followed by washing twice in 200μL of Wash 2 solution. 50μL of RNA Elution Solution was then added to the column and RNA collected into a vial by centrifugation. The collected solution was then pipetted back into the column and centrifuged again to maximise RNA yield. 1.5μL was aliquoted for Nanodrop evaluation, 5μL for RNA integrity number (RIN) evaluation and 20μL was aliquoted into 2 cryovials and stored at -80°C. RNA was shipped on dry ice to the MRC National Institute for Medical Research, London by a specialist clinical, cold-chain courier (World Courier; New Hyde Park, NY, USA) and the remainder of the RNA processing and microarray set-up were performed there by 3 researchers (H Esmail and R Lai with assistance from C Graham).

2.6.1.2 Assessment of RNA purity, concentration and quality

Nanodrop

Nanodrop spectrophotometers allow evaluation of RNA purity by evaluating the spectral absorbance of a sample at 230nm, 260nm and 280nm. Absorbance at 260nm and 280nm is affected by nucleic acids. As thymine and uracil have different spectral absorbance the 260/280 ratio can be used to assess degree of DNA contamination. A 260/280 ratio of approximately 2 reflects “pure” RNA and 1.8-2 was considered acceptable. The 260/230 ratio reflects contamination with other compounds and solvents usually from involved in the processing of RNA. A 260/230 ratio of >1.5 was considered acceptable and 2-2.2 optimal. In addition to purity nanodrop spectrophotometers can evaluate RNA concentration (ng/μL). A total yield of greater than 2000ng was considered acceptable for subsequent processing and microarray.

RNA purity and concentration were determined using a Thermoscientific Nanadrop ND 1000 spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA) in the adjacent Medical Microbiology Research Unit laboratory at UCT. Briefly, the sample pedestal was cleaned with RNase-free water and then 1.2μL of elution solution was then pipetted onto the sample
pedestal to allow assessment of background spectral distribution. 1.2µL of the RNA sample was then pipetted onto the sample pedestal and the spectral measurement taken. The pedestal was wiped with RNase free water between sample measurements. Results were provided in graphical and tabular format on computer connected to the machine.

RNA Integrity Number (RIN)

RIN is generated by a software algorithm which analyses the entire electrophoretic trace of the RNA sample for the detection of degradation products. It provides a more consistent evaluation of RNA integrity than using the 28S to 18S rRNA ratio. A RIN ≥ 7 was considered good quality RNA acceptable for use in the microarray experiments.

RIN evaluation was performed by the Centre for Proteomic and Genomic Research (CPGR) based close to the university using an Agilent Bioanalyser Nano Assay (Agilent; Santa Clara, CA, USA). A written report was provided detailing results.

2.6.1.3 Removal of Globin mRNA

Globin mRNA in whole blood decreases transcript detection sensitivity and increases signal variation on microarray. Samples therefore underwent removal of α and β globin mRNA using Ambion® Human GLOBINclear™ Kit (Thermo Fisher Scientific; Waltham, MA, USA) using a protocol adapted by C Graham. Briefly, first RNA concentration in the samples was normalised to a concentration of 2000ng in 14µL using RNase free water as a diluent. Globin mRNA was then hybridized to biotinylated capture oligonucleotides by addition of 1µL of Capture Oligomix with 15µL of 2 x Hybridisation buffer and incubation at 50°C for 30 minutes. Streptavidin Magnetic Beads were then added to sample and incubated at 50°C for 1 hour to bind the Globin mRNA. Globin mRNA was then immobilized on a magnetic rack and the supernatant containing globin mRNA depleted RNA (grRNA) transferred to a 96 well plate. RNA magnetic Binding Beads were then added to capture grRNA and 400µL of 80% ethanol added for further purification. grRNA was immobilized on a magnetic rack and ethanol aspirated. An elution buffer was then added to recover grRNA from magnetic Binding Beads. grRNA was stored at -80°C. Purity, concentration and integrity of grRNA were assessed by Nanodrop and RIN performed at NIMR.

2.6.1.4 RNA preparation for microarray

grRNA was reverse transcribed to cDNA and then in vitro transcribed into biotinylated cRNA ready for microarray using Illumina® TotalPrep RNA Amplification Kit (Illumina; San Diego,
Assembled onto silica slides (chips). Address sequences are concatenated to each probe to minimise chip related bias.

Table 2.7 Steps in RNA preparation for microarray

<table>
<thead>
<tr>
<th>STEP 1</th>
<th>STEP 2</th>
<th>STEP 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>grRNA → 1st strand cDNA</td>
<td>2nd strand cDNA synthesis</td>
<td>In vitro transcription cRNA</td>
</tr>
<tr>
<td>Master Mix</td>
<td>2 µL 10x 1st Strand Buffer</td>
<td>2.5 µL Biotin-NTP Mix</td>
</tr>
<tr>
<td></td>
<td>4 µL dNTP Mix</td>
<td>2.5 µL 10x T7 Reaction Buffer</td>
</tr>
<tr>
<td></td>
<td>1 µL T7 Oligo (dT) Primer</td>
<td>2.5 µL T7 Enzyme Mix (containing RNA polymerase)</td>
</tr>
<tr>
<td></td>
<td>1 µL RNase Inhibitor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 µL Reverse Transcriptase</td>
<td></td>
</tr>
<tr>
<td>Temp</td>
<td>42°C</td>
<td>16°C</td>
</tr>
<tr>
<td>Incubation time</td>
<td>2 hours</td>
<td>2 hours</td>
</tr>
<tr>
<td>Purification</td>
<td>Nil</td>
<td>Magnetic bead capture and ethanol wash</td>
</tr>
</tbody>
</table>

CA, USA) using a protocol adapted by C Graham. Briefly, the concentration of grRNA was normalised to 200ng in 11 µl using RNase free water as a diluent and samples were then transferred to a 96 well plate. Steps 1, 2 and 3 were then carried out as shown in table 2.7 by the addition of Master Mix to sample and incubation in thermo-cycler at the temperature and duration shown followed by purification. Purity, concentration and integrity of cRNA was confirmed by Nanodrop and RIN performed at NIMR.

2.6.1.5 Illumina HumanHT-12 v4 Expression BeadChips

Samples were then handed over to the MRC National Institute for Medical Research microarray core facility where cRNA was fragmented and hybridized to Illumina HumanHT-12 v 4.0 Expression BeadChips (Illumina; San Diego, CA, USA) and labelled with streptavidin-Cy3. Samples were randomly distributed across chips along with control samples to minimise chip related bias. Microarray chips were then scanned on Illumina HiScan machine (Illumina; San Diego, CA, USA) and florescence emission quantitated to provide raw data.

Illumina HumanHT-12 v 4.0 Expression BeadChips are high density microarrays providing genome wide transcriptional coverage of well characterised genes, gene candidates and splice variants. Each array targets more than 47,000 probes derived from the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq) Release 38 (November 7, 2009).

Array probes are transcript specific 50-mer oligonucleotides, most genes are represented by a single probe but may be represented by more than one if different isoforms of the gene are covered. Hundreds of thousands of copies of each probe (which provides dynamic range for transcript abundance) are bound to 3µm silica beads, which are then randomly assembled onto silica slides (chips). Address sequences are concatenated to each probe to
allow subsequent localisation of probe on the chip. Each bead has approximately 15 replicates, which are randomly distributed across the array to provide technical replicates. In addition some beads are probe free providing assessment of background signal. Up to 12 samples can arrayed on a single chip.

2.6.1.6 Microarray Quality Control

To ensure that technical factors (either chip differences or differences in labelling and staining procedures) didn’t affect downstream analysis and interpretation, 5 QC parameters were evaluated for each sample prior to analysis. Any sample falling greater than 3 standard deviations from the mean was flagged as an outlier and suggested technical concerns. The 5 QC parameters were:

- Intensity value of housekeeping genes
  - Expression level in 12 housekeeping transcripts was recorded. This parameter is influenced by labeling and staining procedures.

- Intensity value of background
  - Expression levels in “empty” probes (beads that do not carry oligonucleotides) was recorded. This parameter is affected by blocking and staining and should be in the range 80-120.

- Intensity value of pre-labeled biotinylated probes
  - Expression level for pre-labeled biotinylated probes is affected by the staining procedure independently of labeling. The biotin signal is commonly highly variable

- Intensity of the 95th percentile
  - This parameter is affected by labeling and staining procedures similarly to housekeeping genes.

- Number of genes detected (p<0.01)
  - This parameter represents the number of transcripts showing expression above background with p value representing confidence with which transcripts are detected above background. Low numbers of detected transcripts usually relate to high background or low signal intensity.

4 samples all on a single chip failed all QC suggesting a technical problem with the chip or its processing. All samples on the chip of concern were repeated randomly distributed across 2 new chips along with a selection of samples that had passed QC. All samples passed QC on repeat array.
2.6.2 Mutiplex Assay (Chapter 6)

Quantiferon plasma supernatants and serum samples from participants that underwent PET/CT (drawn on the day of PET/CT) and HIV infected controls with active TB (drawn on the day of recruitment) and had multiplex assay performed to determine the levels of secreted cytokines and chemokines.

2.6.2.1 Background to Mutiplex assay

Multiplex assays are particularly useful in allowing the simultaneous evaluation of multiple (up to 100) analytes within a single specimen using a small volume of sample. The technology uses 5.6µm polystyrene beads. The beads are internally dyed with a specific combination of red and infrared fluorophores which provide a unique spectral signature for each bead set. Each bead set is coated with thousands of capture antibodies to different analytes. Captured analytes are sandwiched by biotinylated detection antibodies labelled with the green fluorophore streptavidin-PE. The samples are then passed through Green and Red lasers using a fluidic approach similar to flow cytometry. Bead florescence is acquired with bead type characterised by red florescence and concentration of analyte quantitated through green fluorescence. A number of beads (e.g. 50) for each analyte are acquired.

2.6.2.2 Methodology

The assay used was the standard premixed 42-plex MILLIPLEX® Map Kit Human Cytokine/Chemokine Magnetic Bead Panel (Millipore; Billerica, MA, USA) as majority of the analytes of interest as determined by microarray analysis. Specifically it allowed detection of Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF-2), Eotaxin (CCL11/24/26), Transforming Growth Factor (TGF)-α, Granulocyte Colony Stimulating Factor (G-CSF)(CSF-3), Fms-related tyrosine kinase 3 ligand (Flt-3L), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)(CSF-2), Fractalkine (CX3CL1), Interferon (IFN)α2, IFNγ, Growth Regulated Oncogene (GRO)(CXCL1), Interleukin (IL)-10, Monocyte Chemotactic Protein (MCP)-3(CCL7), IL-12p40, Macrophage Derived Chemokine (MDC)(CCL22), IL-12p70, IL-13, IL-15, sCD40L, IL-17A, IL-1ra, IL-1α, IL-9, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IFNγ induced protein (IP)-10(CXCL10), MCP-1(CCL2), Macrophage Inflammatory Protein (MIP)-1α(CCL3), MIP1-β(CCL4), Tumour Necrosis Factor (TNF)α, TNFβ, Vascular Endothelial Growth Factor(VEGF)). In addition RANTES, platelet derived growth factor (PDGF)-AA and PGDF-BB can be quantitated but separately as they require 1:100 dilution which was not performed.

Quantiferon supernatants of the NIL (unstimulated) and ANTIGEN (ESAT-6, CFP-10, TB7.7)
tubes were assayed in singlet. Two plates were required and participant samples were randomly distributed across the plates to minimise bias, however NIL and ANITIGEN from the same participant were plated sequentially and on the same plate. Serum samples were also assayed separately in singlet on a single plate again randomly distributed to minimise bias.

Assays were carried out on the 96 well plate supplied as per manufacturers protocol using the 2 hour incubation method by 2 researchers (H Esmail and K Wilkinson). Briefly, samples and kit were brought to room temperature prior to use. Plates were washed in wash buffer and then 25µL of undiluted sample and 25µL of assay buffer were added to wells along with required standards and controls. 25µL of vortexed, premixed beads were added to each well. Plates were sealed, covered and placed on a plate shaker at room temperature for 2 hours. Plates were washed using a magnetic plate washer to immobilise beads and 25µL of detection antibody was added to each well and then re-sealed, covered and placed on a plate shaker at room temperature for 1 hour. 25µL of Streptavidin-PE was then added to each well and plates were returned to the plate shaker for a further 30 minutes. Plates were then washed on a magnetic plate washer and 150µL of sheath fluid added to all wells. Plates were then read on a Bio-plex 200 reader (Bio-Rad; Hercules, CA, USA) ensuring at least 50 beads for each analyte were read. Standard curves were generated by software for each analyte and concentrations calculated. Quality control samples incorporated into assay were all in the expected range. For GRO, IL-6, IL-8, MIP-1α and IP-10 the majority of values were above the upper limit of detection for QFGIT samples and assay was repeated at a 1:40 dilution.

2.6.3 Enzyme-Linked Immunosorbent Assay (ELISA) (Chapter 6)

ELISA were performed on serum to quantify circulating protein informed by microarray analysis. Assays were carried out by 2 researchers (H Esmail and K Wilkinson) in the University of Cape Town laboratories. All plates were read on a Bio-Rad iMark microplate reader (Bio-Rad; Hercules, CA, USA) with final concentrations calculated from a standard curve using MPM6 software (Bio-Rad; Hercules, CA, USA).

2.6.3.1 CCL23 sandwich ELISA (Abcam)

CCL23 (also known as MIP3 or MPIF-1) ELISA (Abcam; Cambridge, UK) was carried out according to manufacturers instructions. Briefly, samples were diluted 1:2 in supplied assay diluent and standards were prepared by serial dilution 1:2 to obtain a 7-point standard curve.
with the range 8.23-6000 pg/mL. 100µL of standards and diluted samples were added to appropriate wells in a 96-well ELISA plate precoated with CCL23 specific antibody and incubated for 2.5 hours at room temperature with gentle shaking. Plates were washed 4 times with 300µL of supplied wash solution using an autowasher and then inverted onto paper towels to ensure removal of wash solution. 100µL of biotinylated CCL23 detection antibody was added to each well and plate was incubated for 1 hour at room temperature with gentle shaking. The wash step was repeated as previously described and 100µL of Horseradish Peroxidase (HRP)-Streptavidin was added as enzyme conjugate followed by 45 minutes incubation at room temperature with gentle shaking. The wash step was repeated as previously described and 100µL of Tetramethylbenzidine (TMB) substrate was added to each well and incubated at room temperature with gentle shaking for about 30 minutes until optimal colour density developed. Finally 50µL of Stop Solution was added and absorbance was read immediately at 450nm.

2.6.3.2 Haptoglobin competitive ELISA (Abcam)
Haptoglobin competitive ELISA (Abcam; Cambridge, UK) was carried out according to manufacturers instructions. Briefly, samples were diluted 1:2,000 in supplied assay diluent and standards were prepared by serial dilution 1:2 to obtain a 7-point standard curve with the range 0.01-20 µg/mL. 25µL of standards and diluted samples were added to appropriate wells in a 96-well ELISA plate, precoated with Haptoglobin specific antibody. Immediately 25µL of biotinylated Haptoglobin was added to each well and plate was incubated for 1 hour at room temperature with gentle shaking incubated. Plates were washed 5 times with 200µL supplied wash solution using an autowasher and then inverted onto paper towels to ensure removal of wash solution. 50µL of HRP-Streptavidin was added as enzyme conjugate followed by 30 minutes incubation at room temperature with gentle shaking. The wash step was repeated as previously described and 50µL of TMB substrate was added to each well and incubated at room temperature with gentle shaking for about 15 minutes until optimal colour density developed. Finally 50µL of Stop Solution was added and absorbance was read immediately at 450nm in a plate reader.

2.6.3.3 C5 sandwich ELISA (Abcam)
C5 ELISA (Abcam; Cambridge, UK) was carried out according to manufacturers instructions. Briefly samples were diluted 1:20,000 in supplied assay diluent and standards were prepared by serial dilution 1:2 to obtain a 7-point standard curve with the range 0.313-20 ng/mL. 50µL of standards and diluted samples were added to appropriate wells in a 96-well ELISA
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Plate precoated with C5 specific antibody and incubated for 2 hours at room temperature with gentle shaking. Plates were washed 5 times with 200μL of supplied wash solution using an autowasher and then inverted onto paper towels to ensure removal of wash solution. 50μL of biotinylated C5 detection antibody was added to each well and plate was incubated for 1 hour at room temperature with gentle shaking. The wash step was repeated as previously described and 50μL of HRP-Streptavidin was added as enzyme conjugate followed by 30 minutes incubation at room temperature with gentle shaking. The wash step was repeated and 50μL of TMB substrate was added to each well and incubated at room temperature with gentle shaking for about 30 minutes until optimal colour density developed. Finally 50μL of Stop Solution was added and absorbance was read immediately at 450nm in a plate reader.

2.6.3.4 C1q sandwich ELISA (Abcam)
C1q ELISA (Abcam; Cambridge, UK) was carried out according to manufacturers instructions. Briefly, samples were diluted 1:50,000 in supplied assay diluent and standards were prepared by serial dilution 1:4 to obtain a 6-point standard curve with the range 0.039-40 ng/mL. 50μL of standards and diluted samples were added to appropriate wells in a 96-well ELISA plate precoated with C1q specific antibody and incubated for 2 hours at room temperature with gentle shaking. Plates were washed 6 times with 300μL of supplied wash solution using an autowasher and then inverted onto paper towels to ensure removal of wash solution. 50μL of biotinylated C1q detection antibody was added to each well and plate was incubated for 1 hour at room temperature with gentle shaking. The wash step was repeated as previously described and 50μL of HRP-Streptavidin was added as enzyme conjugate followed by 30 minutes incubation at room temperature with gentle shaking. The wash step was repeated and 50μL of TMB substrate was added to each well and incubated at room temperature with gentle shaking for about 15 minutes until optimal colour density developed. Finally 50μL of Stop Solution was added and absorbance was read immediately at 450nm.

2.6.3.5 CXCL17 sandwich ELISA (Cusabio)
CXCL17 ELISA (Cusabio; Wuhan, China) was carried out according to manufacturers instructions. Briefly, samples were used undiluted and standards were prepared by serial dilution 1:2 to obtain a 6-point standard curve with the range 125-8000 pg/mL. 100μL of standards and undiluted samples were added to appropriate wells in a 96-well ELISA plate precoated with CXCL17 specific antibody and incubated for 2 hours at 37°C in the dark. Fluid
was then aspirated from the wells and 100µL of biotinylated CXCL17 detection antibody was added to each well without washing and plate was incubated for 1 hour at 37°C in the dark. Plates were washed 3 times with 200µL of supplied wash solution with 2 minute soak in-between each wash step using an autowasher and then inverted onto paper towels to ensure removal of wash solution. 100µL of HRP-Streptavidin was then added as enzyme conjugate followed by 1 hour incubation at 37°C in the dark. The wash step was repeated as previously described 5 times and 90µL of TMB substrate was added to each well and incubated at 37°C in the dark for about 30 minutes until optimal colour density developed. Finally 50µL of Stop Solution was added and absorbance was read immediately at 450nm.

### 2.6.3.6 SERPING1 competition ELISA (Cusabio)

SERPING1 (also known as C1 inhibitor) competition ELISA (Cusabio; Wuhan, China) was carried out according to manufacturers instructions. Briefly, samples were diluted 1:1000 in supplied assay diluent and standards were prepared by serial dilution 1:2 to obtain a 6-point standard curve with the range 18.75-1200 ng/mL. 50µL of standards and diluted samples were added to appropriate wells in a 96-well ELISA plate precoated with SERPING1 along with SERPING1 specific antibody conjugated to HRP and incubated for 30 minutes at 37°C in the dark. Plates were washed 5 times with 200µL of supplied wash solution with 2 minute soak in-between each wash step using an autowasher and then inverted onto paper towels to ensure removal of wash solution. 90µL of TMB substrate was then added to each well and incubated at 37°C in the dark for about 20 minutes until optimal colour density developed. Finally 50µL of Stop Solution was added and absorbance was read immediately at 450nm.

### 2.6.3.7 Circulating Immune Complex (CIC) ELISA (Bühlmann)

CIC ELISA (Bühlmann; Schönenbuch, Switzerland) was carried out according to manufacturers instructions. Briefly samples were diluted 1:50 in supplied assay diluent. Pre-prepared standards comprising 7 known calibrating concentrations of aggregated IgG were supplied. The 96-well ELISA plate precoated with human C1q (which binds the Fc region of human IgG) was twice using 300µL of supplied wash buffer using an autowasher and then inverted onto paper towels to ensure removal of wash solution. 100µL of standards and diluted samples were added to appropriate wells and the plate was incubated for 1 hour at room temperature with gentle shaking. The wash step was repeated 3 times as previously described and 100µL of Enzyme Label (Protein A conjugated APase) was added
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followed by 30 minutes incubation at room temperature with gentle shaking. The wash step was repeated 3 times and 100µL of p-Nitrophenyl Phosphate (pNPP) substrate was added to each well and incubated at room temperature with gentle shaking for about 30 minutes until optimal colour density developed. Finally 100µL of Stop Solution was added and absorbance was read immediately at 405nm in a plate reader.

2.6.4 Limus Amebocyte Lysate (LAL) assay (Lonza)

The LAL assay (Lonza; Basel, Switzerland) is a quantitative test for Gram-negative bacterial endotoxin (e.g. lipopolysaccharide (LPS)). It was used to evaluate specimens in which there was a concern over LPS contamination of the blood tubes used to collect samples. The assay is based on a protein found in the horseshoe crab, Limus polyhemus, which has been found to react with endotoxin. Assay was carried out according to a slight modification to the manufacturer’s protocol. Briefly, samples were diluted 1:10 in the supplied LAL Reagent Water, and then heat inactivated at 80°C for 15 minutes on a heating block and allowed to cool. Standards were prepared by serial dilution 1:2 to obtain a 7-point standard curve with the range 0.008 EU/mL-0.5 EU/mL (EU = endotoxin units). 50µL of standards and samples were then loaded in triplicate to the manufacturer supplied plate (the first two used as duplicate results and the third for background subtraction). The plate was equilibrated in a 37°C incubator for 5 minutes and then 50µL of LAL was added to the first two wells of each sample and standards. The plate was then returned to the incubator for a further 12 minutes. 100µL of chromogenic substrate was then added to the first two wells for each sample and standard and the plate was then returned to the incubator for 7 minutes. 50µL of Stop solution (25% acetic acid) was then added to the first two wells of each sample and standard and absorbance was read immediately at 405nm in a plate reader. Final concentrations were calculated from a standard curve using Excel software. Samples with > 0.5 EU/mL were considered contaminated with endotoxin.

2.7 Challenges faced in undertaking study

This study could only be conducted in a setting of high HIV and TB burden. However, the use of FDG-PET/CT imaging as well as laboratory requirements meant the study needed to be conducted in a setting with adequate clinical and research infrastructure, making Cape Town an ideal study site. As might be expected, there were a number of logistical and technical challenges in conducting a complex clinical and laboratory study in this setting that resulted in the requirement for additional time and modifications to the study design.
As stated previously the study was initially designed to be embedded within the MVA85A vaccine trial which was due to commence in July 2010, however unforeseen problems with the manufacture of the investigational product resulted in the vaccine trial start being delayed until August 2011. This resulted in a 6-month delay to the start of the recruitment for this study. Digital chest radiography was a vital part of TB screening for this study and the vaccine trial, however the machine available on the study site at the start of the study (Sedecal Dragon DR) was not deemed appropriate for this use and thus was not given regulatory approval. This resulted in a re-tendering process to purchase a more appropriate digital X-ray machine (Phillips Essenta DR), which was not installed on site until February 2012. As an interim measure, a suitable digital X-Ray machine (Delft Oldeca DR) was identified at UCT to allow commencement of both the vaccine trial and this study although this required participants to be transported on a 30 mile round trip for CXR screening.

In the initial study design the intention was that CXR screening would be used to identify asymptomatic participants with inactive TB lesions, which would then be evaluated in greater metabolic and anatomical detail by FDG-PET/CT. Diagnostic standards for such inactive TB lesions on CXR have been present since at least 1940 and identification of these lesions are considered an important part of TB screening CXR as they are associated with increase risk of developing active TB. However, it quickly became clear that often the CXR determined lesions were not apparent on CT and that CT frequently identified inactive lesions not obviously visible on CXR.

The vaccine trial had a sponsor-initiated postponement due to staffing and investigational product issues in April 2012, this resulted in a necessary change to study design as recruitment needed to be conducted independently of the vaccine trial. As few changes as possible were made to the recruitment process, however, because of the need to improve the screen to recruitment ratio and the preliminary observations regarding the relationship between inactive TB lesions on CXR and CT, the main modification was to extent recruitment to those with “normal” lung parenchyma on CXR while still excluding participants with active TB on CXR. This also provided an opportunity to compare TB CXR screening to the gold standard of CT for the first time (see chapter 4).
Chapter 2 – Material and Methods

FDG-PET/CT is not widely available in South Africa and at the beginning of the study the only place in the Western Cape with an FDG-PET/CT machine was based in the Panorama Medi-Clinic private hospital. Though well established they had little experience of conducting research studies. Although training and supervision meant protocol adherence was good, the reporting of images was not adequate for the needs of a research study and there was little enthusiasm to address this at the Medi-Clinic. Therefore, a collaboration was developed with the Academic Nuclear Medicine department at Stellenbosch University to develop a structured approach to reporting the images. As a result, for practical and financial reasons, when the Western Cape Academic PET-CT centre at Tygerberg Hospital (the main teaching hospital for Stellenbosch University) opened in 2012 the study imaging was moved to the new facility. As few changes as possible were made to the imaging protocol following the transfer between imaging facilities.

Follow up of the participants was also challenging and only 27 of the 35 participants had follow up PET-CT, which was less that anticipated. The main challenge to follow-up was the regular movement of participants between Khayelitsha and the Eastern Cape province (where most of the Xhosa speaking community living in Khayelitsha originate). This was a particular issue around Christmas and Easter time, as well as throughout the year for funerals and weddings. Also because unemployment was very high in Khayelitsha if job opportunities arose elsewhere during the study, participants rightly felt compelled to prioritize their employment. Pregnancy was also an issue particularly as participants were mainly women aged 20-40, with 3 of 35 participants falling pregnant during the study, meaning repeat PET/CT was not performed. This was despite planning pregnancy being specifically inquired about and being an exclusion criteria for the study.
## 2.8 Chapter supplementary information

### Structured CXR Reporting form

### RADIOLOGY REPORT

**CHEST RADIOGRAPH READING AND RECORDING SYSTEM**

**Vaccine S. N:**

**Date of CXR:**

**Date of Reading:**

**Film Quality:**

**Comments:**

### Parenchymal abnormalities

<table>
<thead>
<tr>
<th>1A Large opacities (&gt;1cm)</th>
<th>Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Round</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irregular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 1.5 mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Round</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 5 cm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Round</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; upper lobe</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1B Small opacities (&lt;1cm)</th>
<th>Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Round</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irregular</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5-3.5 mm</td>
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<td></td>
</tr>
<tr>
<td>Round</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5-10 mm</td>
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<td></td>
</tr>
<tr>
<td>Round</td>
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<td></td>
</tr>
</tbody>
</table>

### Pleural abnormalities

<table>
<thead>
<tr>
<th>2A Calcification/Pleural effusion</th>
<th>Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chest side</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2B Pleural fluid/Fibrosis</th>
<th>Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chest side</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Central abnormalities

<table>
<thead>
<tr>
<th>3A Tracheal deviation</th>
<th>Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chest side</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3B Mediastinal shift</th>
<th>Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chest side</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Other abnormalities

<table>
<thead>
<tr>
<th>Surgical</th>
<th>Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Skeletal</th>
<th>Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lung</th>
<th>Y</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Radiograph completely normal

<table>
<thead>
<tr>
<th>Abnormalities consistent with TB</th>
<th>Y</th>
<th>N</th>
</tr>
</thead>
</table>

- Active TB
- Inactive TB

### Conclusion

- Not Eligible – Stop screening
- To see Dr Hanif Email next visit
# Chapter 2 – Material and Methods

## Structured PET/CT reporting form and instructions

<table>
<thead>
<tr>
<th>PET/CT Structured Report: LTRI Study (UCT)</th>
<th>REPEAT SCAN - YES ☐ NO ☐</th>
<th>Date of Read ___ / ___ / ___</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participant: ____________________________</td>
<td>Date of Scan ___ / ___ / ___</td>
<td>Weight: ___ ___ ___ kg</td>
</tr>
<tr>
<td>________________________________</td>
<td>FDG injected: ___ ___ MBq</td>
<td>Time injection to scan ___ ___ min</td>
</tr>
</tbody>
</table>

### Parenchymal lesions (nodules, fibrotic scar, infiltrate)

<table>
<thead>
<tr>
<th>No</th>
<th>Type</th>
<th>Location</th>
<th>Size (mm)</th>
<th>SUV (max)</th>
<th>CT evidence of activity if TB related (abscess)</th>
<th>SUV/MN</th>
<th>PET</th>
<th>Suitable for TBNA</th>
</tr>
</thead>
</table>

If no lesions present tick ☐

### Mediastinal/Hilar lymph nodes (enlarged, mineralized or avid)

If no avid, mineralized or enlarged mediastinal/hilar LN tick ☐

<table>
<thead>
<tr>
<th>Lymph node lesion</th>
<th>SUVmax</th>
<th>Size (mm)</th>
<th>Mineralized</th>
<th>Score</th>
<th>SUV/MN</th>
<th>PET</th>
<th>Suitable for TBNA</th>
</tr>
</thead>
</table>

If no lesions present tick ☐

### Other abnormalities visible on CT or FDC avid

- Cervical LN
- Submax. ____________
- Axillary LN
- Submax. ____________
- Thymus
- SUVmax - ____________

Notes: (Different diagnosis, follow-up requirement, comments on scan quality etc.)

Reported by: ___________________________ Sign ___________________________ Date ___ / ___ / ___

For urgent results contact Dr Hamid Esmail – c.0422592465, esmailemail@gmail.com

## Reporting instructions

1. Parenchymal lesions
   a. Number of visible lesions to be recorded with details of each lesion. If greater than 6 lesions continue on second reporting sheet.
   b. Describe nature of each lesion as nodule, fibrotic scar, infiltrate or other. In the case of “other” specify nature of lesion in notes section.
   c. Describe location of lesion:
      i. Right or left lung
      ii. Upper, mid or lower zone
      iii. Central or peripheral
      iv. Centrilobular or peribronchial/subpleural (if applicable)
   d. Size should be described as maximal 2 dimensional size in mm.
   e. Consistent with TB? Yes or No. If No alternative pathological process should be described in notes section.
   f. State if radiographic features of activity are present or not and specify which features are present.
   g. Radiographic features consistent with active TB on CT:
      i. Poorly defined nodule
      ii. Tree in bud appearance
      iii. Cavitation
      iv. Well defined nodule or fibrotic band
      v. Mineralized
   h. FDG uptake – view with SUVmax scaled to 10. Comment on qualitative and semi-quantitative uptake within lesions.
      i. Qualitative uptake: - No visible uptake
      ii. Visible uptake above background lung but less than or equal to mediastinal blood pool
      iii. Uptake greater than mediastinal blood pool
      iv. Intense uptake (greater than liver)
   i. SUV max within region of interest

2. Lymph nodes
   a. Specify which lymph node basin is abnormal (abnormal of size > 1cm or mineralized or FDG uptake above background blood pool):
      i. Bilateral superior mediastinal
      ii. Aortic hilar
      iii. Inferior mediastinal (subcarinal, para aortic and infra diahragmatic)
      iv. Hilar
   b. Specify size if >1cm and note presence of mineralization
   c. FDG uptake (qualitative and semi-quantitative):
      i. Qualitative uptake: - No visible uptake
      ii. Visible uptake but less than or equal to that of mediastinal blood pool
      iii. Uptake greater than mediastinal blood pool
      iv. Intense uptake (greater than liver)
   d. SUV max within region of interest
   e. Suitable for TBNA if ≥ 10mm, uptake ≥ 2, location suitable for TBNA (vITAL LN stations 2R, 2L, 3R, 3L, 4R, 4L, 5R, 5L, 7L, 7R, 10L, 10R and 11L)

3. Any abnormal FDG uptake in ROI? If visible uptake ≥ 1 for parenchymal lesion and/or ≥ 2 for mediastinal/hilar LN tick YES

4. For cervical LN, axillary LN and Thymus (control ROI) specify SUV max
CHAPTER 3 – Evaluation of screening tools used in participant recruitment

3.1 Chapter 3 Introduction

In this chapter the recruitment of participants and controls will be detailed and their clinical features presented and compared. Each of the participant and control groups were distinguished by the presence or absence of active and/or latent TB and by HIV status. Active TB in HIV infected participants was excluded by sputum culture, TB symptom screening and CXR screening. These three screening investigations for active TB were performed on almost all potential participants. As a result this provided an opportunity for evaluation and comparison of the performance of screening investigations. Screening was conducted in healthy outpatients so the prevalence of undiagnosed culture positive TB (prevalent TB) could also be assessed. Prevalent TB is either pre-diagnostic (i.e. TB symptoms are present but presentation to healthcare services for diagnosis and treatment has not occurred) or culture positive, subclinical (i.e. no TB symptoms are present). This chapter therefore begins to provide insight into the grey area between latent TB and active TB, which has then been further explored in subsequent chapters. The diagnosis of latent TB was made by QFGIT and although TST was not performed on everyone (as previously explained) comparison of the 2 tests could be made. As healthy HIV uninfected participants were also screened as controls, comparison of the performance of QFGIT in HIV infected and HIV uninfected persons living in the same community could be made.

Evaluation of these screening tools remains important and relevant. Although indiscriminate mass TB screening, widely implemented in the mid-20th century was abandoned from the 1970’s in the face of falling TB prevalence and poor cost effectiveness, in recent years there has been renewed interest in targeted screening of high risk persons especially in high TB burden countries[204]. Symptom screening and CXR remains the mainstay of for active TB screening and TST remains widely used for screening of latent TB although in some contexts IGRA are also implemented. Screening for active and latent TB are both important in the context of IPT administration especially in HIV infected populations.
3.2 Chapter 3 aims

- To describe and compare the clinical features of the main cohort of participants and control participants
- To evaluate the performance of clinical screening tools for the detection of active TB and latent TB.

3.2.1 Specific chapter 3 aims

1. To describe and compare the clinical features of participants of controls
2. To determine the prevalence of active TB and latent TB in a population of healthy, outpatient HIV infected persons that are ART naïve.
3. To determine the utility of symptom screen, CXR and QFGIT screening to detect prevalent TB
4. To compare QFGIT and TST for the diagnosis of latent TB
5. To evaluate the inter-observer variability of screening CXR
6. To evaluate the variability in QFGIT diagnosis of latent TB in HIV infected and uninfected adults

3.3 Chapter 3 methods

Statistical analysis was performed using Stata ver. 12.1. Graphs and figures were created using both Stata ver. 12.1 and GraphPad Prism ver. 5.0a. Area propionate Venn diagrams were derived using the BioVenn application (http://www.cmbi.ru.nl/cdd/biovenn/). CXR images were assessed in and exported from OsiriX 3.8.1 32-bit.

Non paired, non-parametric data was compared using Wilcoxon rank sum (Mann-Whitney U test) or Kruskal Wallis rank test and parametric data compared using t-test or ANOVA. Proportions were compared by χ² test or Fisher’s exact test (if the contingency included a number ≤ 5). Correlation was performed by Pearson and Spearman analysis. Agreement between tests was determined using kappa statistic with degree of agreement defined as follows[205];

- κ < 0 Less that chance agreement
- κ = 0.01-0.20 Slight agreement
- κ = 0.21-0.40 Fair agreement
- κ = 0.41-0.60 Moderate agreement
- κ = 0.61-0.80 Substantial agreement
- κ = 0.81-0.99 Almost perfect agreement
3.4 Results

3.4.1 Screening of HIV-infected ART naïve outpatients for study participation

265 healthy HIV infected, adult outpatients with no history of previous TB, attending pre-ART wellness clinics, were screened for study eligibility by CXR, sputum culture x 2 (induced if necessary), QFGIT, CD4 count and a short structured history (table 3.1). 143 were screened within the vaccine study between August 2011 and May 2012 and 122 independently from the vaccine study between May 2012 and January 2013. A similar strategy was used for both phases of screening. The potential participants that were screened had a median age of 32 years (IQR 28-38 years) and 84.5% were women, the high proportion of women screened reflected attendance at the pre-ART clinic. 89.2% had CD4 ≥350/mm³ with median CD4 520/mm³ (IQR 429-673/mm³). All those with CD4 < 350/mm³ were referred for ART. The prevalence of latent TB as determined by QFGIT in this population was 64.2% (95% C.I 58.3% - 70.0%); the proportion with positive QFGIT did not vary significantly across CD4 quartiles (p=0.77). 5.6% reported previous IPT and had mainly received this through previous clinical studies conducted at the site. Only participants self-reporting as well and free of symptoms were screened, however on direct questioning about TB symptoms (cough > 1 week, haemoptysis, night sweats, weight loss, fever, pleuritic pain, or shortness of breath) 8.4% reported symptoms. Of those with symptoms, weight loss was most commonly reported by 73.9%, followed by cough and fever both reported by 21.7%, haemoptysis 13%, pleuritic pain 8.7% and shortness of breath 4.3%.

The 2 CXR readers were blind to clinical details and laboratory results. Independent structured reports were provided as described in chapter 2 with final report determined by consensus. Where consensus could not be reached a 3rd reader arbitrated. 12.5% of the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
<th>Parameter</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age med – Med (IQR)</td>
<td>32(28-38)</td>
<td>Mtb Culture positive - % (244)</td>
<td>4.1%</td>
</tr>
<tr>
<td>Female - %</td>
<td>84.5%</td>
<td>TB sympt screen +ve - %</td>
<td>8.7%</td>
</tr>
<tr>
<td>CD4 – mm³ Med (IQR) (259)</td>
<td>520 (429-673)</td>
<td>CXR Consensus - Active TB - %</td>
<td>12.5%</td>
</tr>
<tr>
<td>% CD4 &gt; 350/mm³</td>
<td>89.2%</td>
<td>CXR Consensus – Inactive TB - %</td>
<td>31.7%</td>
</tr>
<tr>
<td>QFGIT Positive (%)</td>
<td>64.2%</td>
<td>Previous IPT - % (262)</td>
<td>5.3%</td>
</tr>
</tbody>
</table>

Table 3.1- Clinical characteristics of the 265 screened participants
screened participants were reported to have CXR changes consistent with active TB, 31.7% had CXR changes consistent with inactive TB, 47.6% had a normal CXR and 8.3% had other abnormalities on CXR unrelated to TB. The most common “other abnormality” on CXR was cardiomegaly (cardio thoracic ratio > 50%). Proportion of CXR abnormalities did not vary by CD4 quartile (p=0.69) or with QFGIT status (p=0.54). Significantly more participants with symptoms had CXR changes consistent with active TB than those without symptoms (30.4% vs. 10.7%, p=0.014) but there was no significant association of inactive changes with symptoms (39.1% vs. 31.0% p=0.48).

### 3.4.2 Prevalence of tuberculosis in screened participants

Of the 265 participants consenting for screening, 244 were able to produce sputum either spontaneously or following induction (unfortunately whether sputum was spontaneously produced or induced was not reliably recorded). The 21 participants (7.9%) in whom sputum culture was not available were less likely to have TB symptoms 4.7% vs. 9.0% or CXR changes of active TB 4.8% vs. 13.2% but these differences were not statistically significant. Of those with culture results, 10 participants were culture positive for Mtb (i.e. had prevalent untreated TB) (table 3.2). All were smear negative and GeneXpert was not performed on any samples. Both sputum samples were culture positive in 4 participants, one of two cultures positive for 5 participants and only one sputum was sent for 1 participant (which was positive). Median time to culture positivity was 16.5 days (IQR 14.25-20 days) (for 1st of 2 samples if both positive).

The prevalence of undiagnosed TB in healthy pre-ART, HIV infected, outpatients with median CD4 520/mm³ and no prior history of TB in this setting was therefore to be 4.1% (95% CI 1.6%-6.6%). In the subgroup of participants that were QFGIT positive with no history of IPT,
prevalence was 6.1% (95% C.I. 2.2%-10%). Six participants with prevalent TB were asymptomatic and culture positive (i.e. culture positive subclinical TB) and of these 5 did not have evidence of active TB on CXR. The prevalence of culture positive subclinical TB in healthy pre-ART outpatients in this setting was therefore determined to be 2.5% (95% CI 0.5%-4.4%). TB prevalence was only determined on the basis of screening cultures sent, but likely underestimated the true prevalence as a number of participants with symptoms and/or abnormal CXR that were culture negative at screen were referred to TB clinic. For example one such participant was found to have inguinal LN TB.

There was no significant difference in age, sex, CD4 count or QFGIT positivity between screened participants with or without prevalent TB. Those with prevalent TB, however, were significantly more likely to have TB symptoms on direct questioning 40% vs. 7.7% (p=0.007) and have evidence of active TB on CXR 50% vs. 11.5% (p=0.026) than those that were culture negative. Of the specific symptoms only cough (p=0.016) and haemoptysis (p=0.005) were more common in those with prevalent TB. In participants screened with evidence of active TB on CXR (33 in total) there was a trend to parenchymal opacification without evidence of mineralization being more common in those with prevalent TB (p=0.052). Those with prevalent TB and symptoms were more likely to have CXR suggestive of active TB. All 4 symptomatic participants had CXR evidence of active TB compared to 1 of the asymptomatic participants although this did not reach statistically significance (p=0.07). All of the parenchymal lesions were of minimal extent and 2 participants also had lymphadenopathy (see figure 3.1).
Chapter 3 – Screening for participants

Figure 3.1 – TOP Area proportionate Venn diagram showing symptom screening and CXR screening results in participants with prevalent TB

BOTTOM – CXR of 5 participants with prevalent TB and active TB changes on CXR: Parenchymal abnormalities circled in green, lymphadenopathy circled in red and pleural abnormalities in yellow.
Chapter 3 – Screening for participants

The negative predictive value (NPV) of all screening parameters for prevalent TB was high which would be expected given the prevalence of culture positive TB was 4.1% (table 3.3). The positive predictive values (PPV) for, positive symptom screen, CXR abnormalities and QFGIT were generally poor especially for any TB abnormalities on CXR and QFGIT however these parameters had high sensitivity. Evaluating performance of specific symptoms screen questions, PPV was highest for presence of cough > 1 week and haemoptysis, however, 95% confidence limits around these estimates were very large. Considering the performance of CXR in those with and without symptoms, diagnostic performance was markedly different. Sensitivity and PPV of CXR was good in those with TB symptoms and poor in those without TB symptoms however as numbers were small 95% C.I. overlapped.

3.4.3 Agreement in screening CXR reports between readers

Given that CXR lesions were subtle and usually of minimal extent, agreement between readers was further evaluated. The independent reports of 2 CXR readers blind to clinical details are cross-tabulated in table 3.4 with agreement shown in red. Categorizing CXR into those consistent with active TB, inactive TB or normal/other CXR, observed agreement between readers was 57.5% against an expected agreement of 43.1%, \( \kappa = 0.25 \) (95% C.I. 0.16-0.35). Therefore agreement between CXR readers was fair. Difference in reporting between readers was resolved by consensus. Agreement between individual readers and the consensus read was similar for readers 1 and 2, \( \kappa = 0.61 \) (95% C.I. 0.51-0.70) and \( \kappa = 0.66 \) (95% C.I. 0.56-0.76) suggesting that no one reader influenced the consensus read more than the other. Approximately 25% of images were read independently by a consultant radiologist to validate reads and agreement with consensus read was moderate, observed agreement 65.2%, expected agreement 35.6%, \( \kappa = 0.46 \) (95% C.I.- 0.29-0.63).
3.4.4 Agreement between TST and IGRA

TST was performed after screening for 104 of 143 screened within the vaccine study (weighted slightly towards QFGIT negative), which allowed comparison of TST for a sample of QFGIT results. Taking TST ≥ 5mm as positive, agreement with QFGIT was moderate. Observed agreement was 78.9% compared to expected agreement of 49.2 %, $\kappa=0.58$ (95%C.I. 0.40-0.77) (table 3.5).

<table>
<thead>
<tr>
<th>TST</th>
<th>NEG</th>
<th>POS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEG</td>
<td>43</td>
<td>18</td>
<td>61</td>
</tr>
<tr>
<td>POS</td>
<td>4</td>
<td>39</td>
<td>43</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>57</td>
<td>104</td>
</tr>
</tbody>
</table>

Table 3.5 – Agreement between TST and IGRA in a sample of 104 participants

### 3.4.5 Screen failures

Of the 265 healthy, HIV infected, outpatients screened, 101 participants had no TB symptoms, a CD4 count > 350/mm3, a positive QFGIT, negative TB cultures, no evidence of active TB on CXR and no history of IPT and were considered to have untreated latent TB. As described previously, during screening within vaccine study, an additional inclusion criterion of CXR compatible with inactive TB applied and so only 70 of 101 participants were considered potentially eligible, of these 55 had more extensive medical history and clinical assessment to screen for eligibility. 35 participants met full inclusion and exclusion criteria and provided consent for PET/CT and full participation in the study.
3.4.6 Clinical features of 35 participants undergoing PET/CT

3.4.6.1 Clinical Features at screening

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
<th>Parameter</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age med – Med (IQR)</td>
<td>31 (27-38)</td>
<td>Never smoked - % (n/N)</td>
<td>88.5% (31/35)</td>
</tr>
<tr>
<td>Female - % (n/N)</td>
<td>91.4% (32/35)</td>
<td>Never ETOH - % (n/N)</td>
<td>80% (28/35)</td>
</tr>
<tr>
<td>CD4 screen – mm³ Med (IQR)</td>
<td>564 (505-656)</td>
<td>Current Biomass exposure -</td>
<td>2.9% (1/34)</td>
</tr>
<tr>
<td>QFGIT TAg-Nil – IU/ml Med (IQR)</td>
<td>2.17 (0.96-3.54)</td>
<td>Years since HIV diagnosis - Med (IQR)</td>
<td>2.1 (0.8-5.2)</td>
</tr>
<tr>
<td>2 x Mtib Culture negative - % (n/N)</td>
<td>100% (35/35)</td>
<td>WHO Stage 1 (%); stage 2 (%)</td>
<td>80%; 20%</td>
</tr>
<tr>
<td>Weight – kg Median (IQR)</td>
<td>71.5 (64.5-84.7)</td>
<td>BCG scar present - % (n/N)</td>
<td>37.1% (13/34)</td>
</tr>
<tr>
<td>BMI – kg/m² Median (IQR)</td>
<td>28.7 (24.5-33.3)</td>
<td>Unemployed - % (n/N)</td>
<td>62.9% (22/35)</td>
</tr>
<tr>
<td>Active CXR screen - % (n/N)</td>
<td>0% (0/35)</td>
<td>Hx of TB close contact - % (n/N)</td>
<td>45.7% (16/35)</td>
</tr>
<tr>
<td>Inactive CXR/N CXR</td>
<td>40% / 60%</td>
<td>Hx of TB tx or IPT - % (n/N)</td>
<td>0% (0/35)</td>
</tr>
</tbody>
</table>

Table 3.6 – Clinical Characteristics of the 35 recruited participants at screening

The 35 HIV infected ART naïve participants that underwent FDG-PET/CT were in general representative of the screened population (table 3.6). These participants had a median age of 31 years (IQR 27-38 years), 91.4% were female and the median CD4 count at initial screening was 561/mm³ (IQR 505-651/mm³). The median time since HIV diagnosis was 2.1 years (IQR 0.8-5.2 years) with 80% having WHO stage 1 infection and 20% with WHO stage 2 infection. Of the 7 participants with WHO stage 2 infection, 5 had previous shingles and 2 had a history of fungal nail infection. Two participants were current smokers and 2 ex-smokers, however, none had smoked more for more than 2 pack years. Only 1 participant had current biomass fuel exposure. Biomass fuel use is very uncommon in Khayelitsha but more common in rural Eastern Cape province, year of migration to Khayelitsha was known for 13 participants and median time since migration for these participants was 10 years (IQR 8-15 years). 45.7% of participants reported a history of close contact with someone with TB (almost always a partner or first-degree relative) a median of 7 years previously (IQR 2.5-17 years). 40% had CXR changes consistent with inactive TB and 60% CXR with normal parenchyma, therefore despite the screening strategy that focused on inactive TB initially the final proportions were representative of the 265 screened participants. In those without active TB changes on CXR 36% had CXR consistent with inactive TB and 64% Normal CXR or other non-TB abnormalities, for the overall screened cohort.
3.4.6.2 Stability of clinical features over screening period

FDG-PET/CT was performed a median of 55 days (IQR - 50-73 days) after initial screen usually in the 1-2 weeks following the 42 day culture result. FDG-PET/CT performed outside this time usually resulted from participant unavailability due to travel to the Eastern Cape province, especially over Christmas and Easter periods. The participants were evaluated for symptoms and signs of TB or acute illness 3-4 times over this screening period and no patient developed progressive symptoms consistent with active TB. Weight did not change significantly between screen and scan (median weight change/28 days -0.6% (IQR -1.1% to +0.4%) with no participant losing more than 5% total body weight over the screening period. Any symptoms or conditions developing over this period were appropriately addressed and all participants were asymptomatic at time of PET/CT scan.

On the day of the scan, the participants had a symptom screen, clinical examination, 1 x sputum culture sent (no sputum induction) and clinical blood tests (CD4, VL, FBC, ESR, CRP, Cr, LFT) as well as research blood tests performed (table 3.7). Median ESR was the only parameter to be significantly outside the normal range (defined in HIV uninfected adults). This was not unexpected as ESR is frequently abnormal in “healthy” HIV infected adults[206]. Over the period between screen and scan, not unexpectedly, CD4 count significantly reduced to a median of 517/mm$^3$ (IQR 393-658, p<0.001) and CD4 count was ≤350/mm$^3$ in 4 participants (≥346/mm$^3$ in 3 and 297/mm$^3$ in the fourth). VL was not performed at screening but on day of PET/CT scan, median VL was 10800 (IQR 1982-26052).

<table>
<thead>
<tr>
<th>Observation</th>
<th>Result</th>
<th>N.Range</th>
<th>Observation</th>
<th>Result</th>
<th>N.Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP - mg/L Med(IQR)</td>
<td>1.9(1-4)</td>
<td>&lt;5</td>
<td>RR - bprn Med(IQR)</td>
<td>16(16-19)</td>
<td>12-20</td>
</tr>
<tr>
<td>WCC - x10^5/L Med(IQR)</td>
<td>5.34(4.49-6.53)</td>
<td>4-10</td>
<td>Pulse - bpm Med (IQR)</td>
<td>71(67-75)</td>
<td>60-100</td>
</tr>
<tr>
<td>ESR - mm/hr Med(IQR)*</td>
<td>37.5(27-50)</td>
<td>&lt;15</td>
<td>DBP - mmHg Med(IQR)</td>
<td>76(70-85)</td>
<td>60-90</td>
</tr>
<tr>
<td>CD4 - mm$^3$ Med(IQR)</td>
<td>517(IQR 393-658)</td>
<td></td>
<td>SBP - mmHg Med(IQR)</td>
<td>119(108-129)</td>
<td>100-160</td>
</tr>
<tr>
<td>VL - copies/ml</td>
<td>10800 (IQR 1982-26052)</td>
<td></td>
<td>Temp - °C Med(IQR)</td>
<td>35.9 (35.5-36.4)</td>
<td>&lt;37.5</td>
</tr>
</tbody>
</table>

Table 3.7 – Clinical characteristics of recruited participants on day of PET/CT scan - *ESR n=34
3.4.6.3 Stability of Quantiferon over screening period

Median QFGIT TBAg-NIL on the day of PET/CT scan was 1.76IU/ml (0.59-7.52) and did not differ significantly from screening values (p=0.42). QFGIT TBAg-NIL values at the 2 time-points were also significantly correlated (r= 0.77 – p<0.0001). However, fluctuations in QFGIT resulted in reversion (from TBAg-NIL ≥ 0.35IU/mL → < 0.35IU/mL) in 20% in keeping with what is described in the literature[207-209]. Reversion rate was significantly different across baseline QFGIT TBAg-NIL quintiles (p=0.04) with the majority of reversions occurring in those with screening QFGIT in the 1st and 2nd quintiles as shown in table 3.8. There was no significant difference in CD4 count between reverters and non-reverters (p=0.76; median CD4 - 542/mm³ vs. 565/mm³ respectively).
3.4.6.4 Stability of CXR and sputum culture over screening period

All recruited participants had a single sputum culture repeated on day of scan. Two of 35 participants who had 2x negative Mtb culture at screening had a positive Mtb culture at scan despite remaining asymptomatic (days to culture positivity being 33 and 12). All recruits also had a repeat CXR performed just prior to scan and 2 of 35 showed slight deterioration (1 of whom was also culture positive) though remained asymptomatic (figure 3.4). These 3 participants all developed TB symptoms 7, 30 and 34 days after PET/CT scan while awaiting culture results.
3.4.7 Screening for TST negative / QFGIT negative controls

3.4.7.1 HIV-infected TST negative QFGIT negative controls

From the screen failures, 15 HIV-infected ART naïve participants that were TST and QFGIT negative were recruited to provide age, sex and CD4 matched (to day of scan) controls. For these 15 control participants, median age was 34 years (IQR 28-37), CD4 count 453/mm$^3$ (IQR 398-593), viral load 7042 copies/ml (IQR 4937-71100) and 86.7% were female. These characteristics were not significantly different from the 35 participants undergoing PET/CT scan (p=0.70, p=0.39, p=0.67 and p=1.0) (figure 3.9). None of the controls had TB symptoms or any CXR abnormalities consistent with active or inactive TB. Of the 15 participants, 11 had sputum culture performed and were negative (4 were unable to produce sputum even with induction).

TST was 0 mm in 11/15 participants and 1-4 mm in 4 participants. Median QFGIT TBAg-NIL was 0.17 IU/ml (0.03-0.26). However, the distribution of these values appeared bimodal, raising the possibility that those at the higher end of the distribution may represent a distinct subgroup (see figure 3.5). Those with QFGIT TBAg-NIL ≥ 0.17 IU/ml were not more likely to have TST > 0 mm (p=0.19)

![Figure 3.5 – Distribution of QFGIT TBAg-NIL values in HIV infected participants that were QFGIT and TST negative](image)
3.4.7.2 Evidence of QFGIT conversion following TST boosting in HIV-infected persons
with negative TST and negative QFGIT (in separate cohort)

To further evaluate the hypothesis that there may be different subgroups within HIV infected persons with negative QFGIT results. The effect of administering TST on subsequent QFGIT results was investigated in a separate group (not recruited as controls into this study and of mixed ART status, but recruited into the vaccine trial). The phenomenon of boosting and conversion of QFGIT results following TST has been well described especially in HIV uninfected individuals[194]. This evaluation was possible as part of the vaccine trial protocol, as if screened participants that were TST and QFGIT negative had a > 45 day delay before randomization to placebo or vaccine, repeat TB screening including a QFGIT was performed.

22 HIV-1-infected, TST negative (all 0mm), QFGIT negative adults (median CD4 477/mm$^3$ IQR 439-621) of whom 6 were ART naïve and 16 ART established, had QFGIT repeated after median 62 days (IQR 49-70). 40.9% (95%CI 18.6%-63.2%) converted from negative to positive. In those who converted, median TBAg-NIL increased from 0.21IU/mL (IQR 0.16-0.28) to 0.84IU/mL (IQR 0.6-1.89) compared to 0.02IU/mL (IQR 0-0.07) to 0.06IU/mL (IQR 0-0.1) in non-converters. Both baseline and repeat values were significantly different p=0.005 and p = 0.0001 respectively between converters and non-converters. The groups did not differ by CD4 count or ART status.
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ROC analysis showed a baseline cut off was effective at predicting QFGIT conversion following TST with AUC = 0.87. Baseline cut off of 0.15IU/ml and 0.17IU/ml correctly classified 86% of converters with sensitivity/specificity of 89%/85% and 78%/92% respectively.

3.4.7.3 HIV uninfected TST-ve QFGIT-ve controls

In order to identify HIV uninfected, TST negative/QFGIT negative controls, healthy adults living in the Khayelitsha site B area were screened with rapid POC test for HIV. 90 asymptomatic participants confirmed as HIV uninfected underwent QFGIT and TST of whom five did not return for TST reading. For the 85 participants with complete results, median age was 22 years (IQR 20-31) and 57.7% were female. In line with CDC guidance for HIV infected persons from high TB prevalence regions, TST was considered positive if ≥10mm[20]. The proportion of HIV uninfected adults with a positive QFGIT was 71.8% (95% C.I. 62.0% - 81.5%) and 64.7% (95%C.I. 54.3%-75.1%) had a TST ≥ 10 mm. Latent TB was diagnosed by either test in 82.3% (95% C.I. 74.1%-90.6%). Agreement between QFGIT and TST results was fair with observed agreement being 71.8% compared to expected agreement of 56.4%, κ=0.35(95%C.I. 0.14-0.56). The 15 participants that were TST and QFGIT negative were considered eligible control. Only QFGIT results could be compared with the HIV infected population and there was no significant difference in the proportion that were positive (p=0.23). Median age was 20 years (IQR 19-21) and 66.7% were female, significantly different from the 35 participants undergoing PET/CT scan (p<0.0001 and p=0.04 respectively) although no attempt was made to match these characteristics.
However, in contrast to the HIV infected, TST negative, QFGIT negative controls, distribution of TBAg-NIL results was normally distributed (Shapiro-Wilk test for normality p=0.56) around a mean of 0.03IU/mL (figure 3.7).

The distribution of the TBAg-NIL values in HIV infected and uninfected persons screened with positive QFGIT was then further evaluated. Median TBAg-Nil was significantly higher in HIV uninfected participants (4.66IU/mL vs. 2.38IU/mL, p=0.015). The distribution of values for both HIV infected and uninfected persons screened with positive QFGIT was bimodal though left-shifted in HIV infected participants (Figure 3.8).

### 3.4.8 Active TB controls

#### 3.4.8.1 HIV-infected ART naïve active TB controls

21 HIV infected ART naïve participants with either confirmed or suspected TB were screened at the TB clinic at the Khayelitsha Site B CHC to provide age, sex and CD4 matched (to day of scan) controls (figure 3.9). Two participants did not have culture or GeneXpert confirmed TB and 3 participants had very low CD4 counts (8, 18 and 154/mm³) and were excluded. The remaining 16 participants were considered eligible controls. Of these 16 participants, only 15 had good quality RNA for use in microarray experiment, 14 had available QFGIT plasma supernatants and 13 had available serum samples.
For the 16 participants the median age was 30.5 years (IQR 27-36.5), median CD4 count 447/mm$^3$ (IQR 375-538/mm$^3$) and 81.3% were female. These characteristics were not significantly different from the 35 participants undergoing PET/CT scan ($p=0.97$, $p=0.12$, $p=0.36$ respectively). The median viral load was 69677 copies/ml (IQR 33184-156096) and as expected this was significantly different to those undergoing PET/CT scan ($p=0.0006$).

All participants had positive Mtb sputum culture or positive sputum GeneXpert; 7 were diagnosed by culture and 9 by GeneXpert. Only 1 participant had a history of previous TB and she was diagnosed by Mtb culture. All participants had sputum smear microscopy performed and 9 of 16 were smear positive. Of these 6 were 3+ smear positive and 3 were scanty smear positive. All participants were symptomatic at diagnosis with median duration of symptoms being 3 weeks (IQR 2-8 weeks). Cough and weight loss were reported most commonly, each by 10 of the 16 participants (61.5%) while haemoptysis, fever, night sweats and pleuritic pain were each reported by 5 of 16 participants (31.25%). Nine participants had CXR available; 8 were consistent with active TB and 1 with inactive TB. All participants had less than 24 hours TB treatment at diagnosis with most participants being recruited on the day of diagnosis. QFGIT was positive in 15 out of 16 HIV infected active TB control with median TBAg-NIL 3.46IU/ml (0.81-9.71), this was not significantly different from either screening or day-of-scan values for the 35 participants undergoing PET/CT ($p=0.55$ and $p=0.50$ respectively).

**3.4.8.2 HIV uninfected active TB controls**

20 HIV uninfected participants with either confirmed or suspected TB were screened at the TB clinic at the Khayelitsha Site B CHC. Three TB suspects were not culture or GeneXpert confirmed and 3 were subsequently found to be HIV infected and these 6 participants were excluded. The remaining 14 HIV uninfected, symptomatic, Mtb culture positive participants were considered eligible controls of which 11 were smear positive (4 were smear 3+, 4 were smear 2+, 2 were smear 1+ and 1 scanty).

The median age was 39 years (IQR 35-54) and 35.7% were female, significantly different from the 35 participants undergoing PET/CT scan ($p<0.0001$ and $p=0.0001$ respectively) although no attempt had been made to match these characteristics (figure 3.9).
3.4.9 HIV infected QFGIT+ve, ART established controls

From within the screening process of the vaccine study, 9 asymptomatic participants that were HIV-infected, QFGIT positive but established on ART were consented for participation. Of these 1 participant was sputum culture positive despite being asymptomatic and was excluded. All 8 participants had been on ART for > 24 weeks (median 2.1 years (1.1-2.8)) and all had a suppressed viral load (<40 copies/ml). All participants were prescribed Lamivudine (3TC) with 5 taking Tenofovir (TDF) and 3 taking Zidovudine (AZT) as 2nd nucleotide reverse transcriptase inhibitor (NRTI). All participants were prescribed a non-nucleotide reverse transcriptase inhibitors (NNRTI), with 5 taking Efavirenz and 3 taking Nevirapine. The median age was 34.5 years (32-41), median CD4 count 516/mm$^3$ (IQR 476-569) and 87.5% were female, these characteristics were not significantly different from the 35 participants undergoing PET/CT scan (p=0.20, p=0.70, p=1.0 respectively). All 8 participants were asymptomatic, culture negative with no evidence of active or inactive TB on CXR and QFGIT positive with median TBAg-NIL 7.7 IU/ml (IQR 1.93-10) which was not significantly different from either screening or day-of-scan values for the 35 participants undergoing PET/CT (p=0.08 and p=0.09 respectively). TST was not performed.
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<table>
<thead>
<tr>
<th>Parameter</th>
<th>HIV+ LTBi (PET/CT)</th>
<th>HIV+ Active TB</th>
<th>HIV+ TST/QFGIT+ve</th>
<th>HIV+ ART+ LTBi</th>
<th>HIV- TST/QFGIT-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>35</td>
<td>16</td>
<td>15</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Age med – Med (IQR)</td>
<td>31 (27-38)</td>
<td>30.5 (27-36.5)</td>
<td>34 (28-37)</td>
<td>34.5 (32-41)</td>
<td>39 (35-54)</td>
</tr>
<tr>
<td>Female - % (n/N)</td>
<td>91.4%</td>
<td>81.3%</td>
<td>86.7%</td>
<td>87.5%</td>
<td>35.7%</td>
</tr>
<tr>
<td>CD4 screen – mm³ (IQR)</td>
<td>517 (393-658)</td>
<td>447 (375-538)</td>
<td>453 (398-593)</td>
<td>516 (476-569)</td>
<td>NA</td>
</tr>
<tr>
<td>Viral Load – copies/mL (IQR)</td>
<td>4.2 (3.3-4.4)</td>
<td>4.8 (4.5-5.2)</td>
<td>3.8 (3.7-4.8)</td>
<td>LDL</td>
<td>NA</td>
</tr>
</tbody>
</table>

Figure 3.9 - Comparison of clinical characteristics for participants and controls: TOP – Table of clinical characteristics for participants and controls (shaded region parameters that were matched). BELOW – Graphs showing distribution of age, sex, CD4 and VL for participants and controls.
3.5 Summary of main findings

In a “healthy” outpatient HIV infected population with median CD4 count approximately 500/mm$^3$ in Khayelitsha, the prevalence of untreated culture positive TB was 4,098/100,000 (4.1%). 40% of these cases could be considered pre-diagnostic, i.e. symptoms were present on direct questioning and 60% were subclinical i.e. participant did not have symptoms even on direct questioning. The sensitivity and specificity of positive symptom screen, active CXR findings and any TB CXR abnormality for culture positive TB was found to be 40%/92.3%, 50%/88.5% and 70%/56.8% respectively. Progression from a negative to a positive sputum culture and/or from a CXR without active TB findings to one with active TB findings occurred in 3 asymptomatic participants soon after recruitment. All 3 participants developed symptoms within 5 weeks. CXR lesions were subtle and there was considerable inter-observer variability in CXR reporting with agreement being fair (κ=0.25). The prevalence of QFGIT positivity was 64.1% in HIV infected adults and 71.8% in HIV uninfected adults. Agreement between QFGIT and TST was fair to moderate. Reversion of QFGIT (from positive to negative) occurred in 20% of recruited participants over a median of 55 days and was commonest in those in the lowest 2 quintiles of baseline QFGIT results. In those that were TST and QFGIT negative the distribution of QFGIT results was different for HIV infected (bimodal) and HIV uninfected (normally distributed) adults. In a separate cohort of HIV infected adults, conversion of QFGIT (from negative to positive) following boosting with TST occurred in 40.9%, most commonly in those with a baseline QFGIT TBAg-NIL of 0.15IU/mL.

Compared to the main group of interest, asymptomatic HIV infected adults with positive QFGIT undergoing PET/CT, HIV infected controls were all well matched for CD4 count, age and sex as planned. Viral load was significantly higher in those with active TB and suppressed in those taking ART as expected. In HIV uninfected controls both age and proportion that were female were significantly different as no attempt was made to match these characteristics and is a limitation of the study. All participants and controls were Black African residing in the same community in which > 95% of people are of Xhosa origin.

3.6 Discussion

The point prevalence of sputum culture positive TB identified in this study is in line with previously published studies in this setting. Wood et al reported the prevalence of untreated TB in a similar community, of mixed HIV status, in Cape Town to be 1,575/100,000 (5,140/100,000 in HIV infected adults and 514/100,000 in HIV un-infected adults). However,
little was known about degree of immunosuppression in the HIV infected population. By comparing the untreated point prevalence with the case notification rate, the authors also calculated that the estimated mean duration of untreated TB disease was just over 1 year for both HIV infected and uninfected adults[210]. Oni et al screened HIV infected persons attending the same clinic as used in this study, between 2008 and 2010, and found the prevalence of subclinical TB to be 8.5% though degree of immunosuppression (median CD4 of TB cases 249/mm³) was greater than in this study[83].

Two systematic reviews of screening strategies for TB have recently been published in HIV infected and HIV uninfected persons[82, 211]. The systematic review of HIV infected persons incorporated 12 studies in which symptom screening of 8148 participants was performed (6.1% had TB). A symptom screen of any one of current cough, haemoptysis, weight loss, fever or night sweats was found to have a sensitivity of 78.9% and specificity of 49.6% in contrast to the 40% sensitivity and 92.3% specificity found in this study. Several factors accounted for this difference. The use of any or current cough rather than prolonged cough (cough > 1week was used in this study) would be expected to increase sensitivity and reduce specificity of a symptom screen as a greater proportion of the population will be screen positive. Also the studies contributing to the systematic review were heterogeneous, in particular screening studies from mines were included where symptoms such as cough would be expected to be generally more common. This again would lead to increased sensitivity and reduced specificity of a symptom screen. The high specificity of symptom screen in this study may also relate to recruitment strategy, which was biased toward those self-reporting as healthy and so less likely to have symptoms than all comers. However, of note the sensitivity and specificity of symptom screen for this study were within the 95% confidence limits calculated in the systematic review. They are also similar to 2 community based TB/HIV prevalence surveys conducted in Zimbabwe by Corbett et al which were included in the systematic review[211-213]. No analysis of CXR screening in HIV infected populations was performed in this systematic review owing to limited numbers of studies.

In the systematic review of TB screening in HIV uninfected adults, pooled sensitivity and specificity in for prolonged cough (>2 weeks) was 35.1% and 94.7%, for any cough 62.7% and 77.5% respectively and any symptom 77.0% and 67.7% respectively. Evaluation of CXR screening was based on 5 studies with pooled sensitivity and specificity for any abnormality suggestive of TB being 86.8% and 89.4% respectively and any CXR abnormality being 97.8%
and 75.4% respectively. The pooled sensitivity in particular was higher than reported in this study (50% for active TB, 70% for any TB related abnormality) and this likely relates to methodological differences in the studies included in the systematic review. In 4 of the 5 studies, CXR was not performed in parallel to sputum culture but rather sputum culture was performed after symptoms and CXR screening leading to verification bias that would tend to overestimate sensitivity[82]. As demonstrated in this study the sensitivity of CXR in those with symptoms (100%) was considerably higher than in those without symptoms (16.7%). In addition, even at high CD4 counts, HIV infection may be expected to affect CXR findings in those screened with positive culture. In a recent screening study in which 825 HIV infected adults in South Africa underwent TB screening with symptoms screen, CXR and sputum culture performed in parallel. The sensitivity of any CXR abnormality was found to be 69.4% and specificity 44.9%, similar to findings in this study[214].

In this study 7.9% of participants did not have a sputum culture result and these participants were less likely to have abnormal CXR or TB symptoms. Therefore they may have been less likely to have culture positive TB. This may potentially have lead to a partial verification bias, however, the proportion without sputum compares favourably to other similar studies. In the CXR screening study described by Ahmad Khan et al, 10.9% of participants did not have cultures available[214]. Another potential limitation of this study is that physicians (that had undergone specific training in TB screening CXR reading) reported films rather than experienced radiologists who may be expected to perform better.

Agreement between CXR readers in this study was fair this and is in keeping with historical studies performed in the 20th century which highlighted the significant inter and intra-observer variability in reporting of TB screening CXR[81]. More recently it has been reported that using a structured report, for example the CRRS system, inter-observer variability can be minimised and agreement considerably improved[202]. However, the study setting and aims can have a considerable influence on inter-observer variability. Active screening has been shown to identify persons at earlier stages of disease with more minimal CXR pathology[215] in addition HIV infection may result in more subtle CXR findings. Subtle lesions will affect the signal to noise ratio, as they are harder to distinguish from overlying bony structures, and hence disagreement will be expected to increase. The aim of CXR reporting in a study may also influence observer variability. Structured reporting systems
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such as CRRS train readers to report abnormalities in the same way to minimize variability, however this does necessarily mean that reporting will be more accurate[205].

The finding of a relationship between, presence of TB symptoms and presence of CXR abnormalities suggestive of active TB is of interest. It suggests that the extent of pathology that typically causes symptoms and the extent of pathology that typically is detectable by CXR may be similar. The implication being that, at least in this population, CXR may be better able to identify TB in its pre-diagnostic rather than truly subclinical phase. In culture positive participants that are asymptomatic with no evidence of active TB on CXR it could be questioned as to whether laboratory contamination had occurred (i.e. potentially false positives). As this study was closely linked to a TB vaccine trial in which development of TB was a primary endpoint, anonymous sterile mock sputum samples were sent twice a week to the laboratory over the course of this study as QC. None of the 209 specimens sent over a 34 month period were positive for Mtb suggesting laboratory contamination was highly uncommon. Although subclinical cases were not systematically followed up after referral to TB clinic, in the previously described study by Oni et al, 56% of subclinical cases developed symptoms within 2 months[83].

In this study the difference in QFGIT positivity between HIV infected and uninfected persons was not significant. The CD4 count in this population was relatively well preserved and of note the HIV uninfected participants were significantly younger. However, median TBAg-NIL was significantly lower and the distribution of results was left-shifted in HIV infected participants with positive QFGIT compared to HIV uninfected participants. The proportion of HIV infected adults with a positive TST and IGRA has been found in other studies to reduce as CD4 count falls[216]. Due to the study design TST was not performed on all of the HIV infected participants, however, in line with most studies discordance between TST and QFGIT result was common in both HIV infected and uninfected participants[217]. As previously reported reversions of QFGIT were found to be common, particularly in those with baseline results just above the cut off[218]. Of interest, the distribution of QFGIT TBAg-NIL values differed between HIV infected and uninfected participants that were negative for both TST and QFGIT, with the bimodal distribution suggestive of 2 distinct groups in the HIV infected population. In a separate cohort, administration of TST resulted boosted IFNγ release with conversion of QFGIT in 40.9%. 89% of those that converted had a baseline QFGIT TBAg-Nil ≥0.15IU/mL. The phenomenon of boosting has been well described in HIV
uninfected persons in South Africa although at a lower rate than observed in this study and has been noted to persist for almost 3 months after TST[194, 219]. One explanation for the phenomenon of boosting is that pre-existing memory responses may be stimulated by a cocktail of antigens within PPD (which will include ESAT-6 and CFP-10) when TST is performed. This may then lead to proliferation of antigen specific effector cells which may then be present in greater numbers when peripheral blood is restimulated \textit{ex vivo} in a second QFGit performed weeks later leading to greater IFN\textgreek{\gamma} release. This is also the principle behind the 2-step TST strategy (where an initial negative TST is repeated a few weeks later to see if conversion of response has been induced by PPD administration) which has been recommended to confirm true negative in high risk populations[20].

Taken together this suggests that in a high burden setting in HIV infected participants, it may not be possible to infer that those with a negative QFGit and TST have no evidence of past or current TB infection. It is possible that some of those with negative results are in fact falsely negative as a result of impaired cell mediated immune responses. Given the left shifted distribution of results in HIV infected persons, the bimodal distribution of negative results and the observed high rates of conversion following boosting with TST especially if baseline negative QFGit was above 0.15IU/mL. It therefore seems possible that the current cut off of 0.35IU/mL may not be appropriate for both HIV infected and uninfected individuals. A lower cut off may improve sensitivity in HIV infected persons in a similar fashion to the lower cut off used for TST. This would seem appropriate for an immunodiagnostic test in a population with impaired immune responses. Further assessment and validation would need to be undertaken different HIV infected cohorts with different levels of immunosuppression.

\subsection*{3.7 Conclusions}

All currently available screening tools have limitations for the early detection of active tuberculosis. In particular while symptom screen and CXR perform well in the detection of pre-diagnostic cases of TB they appear less well suited to identify true subclinical cases. Sputum culture is the gold standard investigation but even this has limitation for detection of active TB especially if disease is extra-pulmonary, sputum is scarce or insufficient numbers of bacilli are within the airways. The performance of CXR is significantly impaired as lesions become more subtle and can not be reliably identified from surrounding structures which
leads to high levels of inter observer variability. Novel techniques are therefore required to further interrogate the earliest stages of disease reactivation.
Active pulmonary TB is a comparatively slowly progressive condition that may follow a fluctuating course and has a number of characteristic pathological features. In particular, an apical and sub-apical preponderance, tendency for cavitation and a spontaneous healing phase that is characterised by fibrosis and calcification. Numerous autopsy studies from the early 20th century showed that early stages of disease were present in persons dying of conditions other than TB (i.e. sub-clinically)\[112, 113, 115\]. CXR screening has been and still is widely used identify TB in this subclinical phase but is has considerable limitations in terms of sensitivity and specificity as described in the previous chapter. The more sensitive imaging modality of FDG-PET/CT may be able to identify evidence of subclinical pathology that may not be reliably detected by conventional CXR imaging. This could then allow identification of a group a higher risk of progression to active TB.

While other subacute and chronic conditions can cause similar pathological appearances to TB e.g. histoplasmosis, atypical mycobacteria, sarcoidosis etc. In settings such as Khayelitsha these conditions are orders of magnitude less common than TB. HIV infection considerably increases the risk of developing active TB and while CD4 count is high (≥ 350/mm³) the pattern of disease resembles that in the immunocompetent HIV uninfected persons.

4.2 Chapter 4 hypothesis

FDG-PET/CT can be used to identify evidence of pathology consistent with TB disease activity in asymptomatic, HIV infected persons with CD4 ≥350/mm³ and with evidence of immune sensitization by Mtb (by QFGIT).

4.3 Chapter 4 aim

To evaluate the utility of FDG-PET/CT to identify evidence of subclinical pathology in asymptomatic, ART naïve, HIV infected adults with evidence of immune sensitization by Mtb and no previous history of tuberculosis
4.3.1 Specific chapter 4 aims

1. To describe and categorise baseline parenchymal abnormalities visible on FDG-PET/CT and to evaluate their activity radiographically (by CT) and metabolically (by FDG uptake).
2. To describe baseline FDG-PET/CT findings within the mediastinal LN.
3. To describe baseline FDG-PET/CT findings with cervical, axillary and upper abdominal LN.
4. To evaluate effect of TB treatment (IPT or standard TB treatment) +/- ART on baseline FDG-PET/CT abnormalities.
5. To compare the reporting of CXR to findings on FDG-PET/CT.
6. In asymptomatic persons with evidence of immune sensitization by Mtb to define a group with evidence of subclinical TB and a group without evidence subclinical TB.

4.4 Chapter 4 methods

Statistical analysis was performed using Stata ver. 12.1. Graphs and figures were created using both Stata ver. 12.1 and GraphPad Prism ver. 5.0a. Primary analysis of PET/CT images including lesion size and SUV was performed on Philips Extended Brilliance Workspace version 4.5.3.40140. Additional analysis was carried out using OsiriX 3.8.1 32-bit. All fused PET/CT images are shown with SUV scaled 0-10. Images summarizing lesion location were created to be anatomically accurate as follows. First an outline of the lesion was digitally propagating in the coronal plane and the location of the lesion captured at the level of the carina in the coronal plane using OsiriX. The location of the lesion was then digitally traced onto a representative CXR anchored by anatomical reference points. Non-parametric data was compared using Wilcoxon rank sum (Mann-Whitney U test) or Kruskal Wallis rank test and parametric data compared using t-test or ANOVA. Proportions were compared by $\chi^2$ test or Fisher’s exact test (if the contingency included a number $\leq 5$). Correlation was performed by Pearson and Spearman analysis. Agreement between tests was determined using kappa statistic with degree of agreement as defined in Chapter 3.

4.5 Treatment and follow-up of participants

The 35 recruited asymptomatic participants underwent FDG-PET/CT at baseline. Repeat scans were performed in 27 out of 35 participants after a median of 26 weeks (IQR 25-26.93). The median activity of FDG injected at the 1st scan was 4.03 MBq/kg (IQR 3.71-4.17) and 2nd scan 4.04 MBq/kg (IQR 3.93-4.12); injected FDG at the 2nd scan was between 90-110% of 1st scan for 74% of participants. The time between FDG infusion and scan for
the 1st scan was 60 minutes (IQR 58.5-61) and 2nd scan 60 minutes (IQR 59-65); time from injection to scan for the 2nd scan was between 90-110% of 1st scan for 89% of participants. As calculation of SUVmax factors in the injected FDG dose and time between injection and scan, slight differences in these parameters between scans do not significantly affect interpretation. Repeat FDG-PET/CT was not performed in 8 participants for the following reasons; 3 became pregnant, 2 withdrew consent for study participation, 2 migrated to the Eastern Cape and 1 was lost to follow-up. Participants were prescribed 6 months IPT unless there was a clinical concern regarding the initial FDG-PET/CT. Four participants, who all subsequently developed TB symptoms following the scan, were prescribed (or converted to) standard TB therapy between 7 and 90 days of the baseline scan. Two of these participants also had a positive culture for Mtb having initially been culture negative and 2 remained culture negative but had evidence of evolving pathology on repeat imaging (table 4.1).
4.6 Results

4.6.1 Parenchymal lesions on FDG-PET/CT scan

For the 35 participants undergoing FDG-PET/CT, none of whom had previous TB, 71.4% had CT abnormalities within the lung parenchyma and only 28.6% had normal lung parenchyma. All abnormalities within the lung parenchyma could be categorized into 4 groups

1. Discrete (inactive) nodules
2. Active nodules
3. Infiltrates
4. Fibrotic scars

4.6.1.1 Discrete Nodules

Discrete nodules were defined as small, spherical opacities present within the lung parenchyma where the border was clearly defined with no CT evidence of disease activity (figure 4.3b). Discrete nodules were present in 57.1% of participants. In those with discrete nodules the majority had only 1 present and only 4 participants had > 3 present (figure 4.2). The median size of nodules was 3.8mm (IQR 3.1-4.5) with all nodules being sub-centimetre and 83.3% being less than 5mm. 18.3% of nodules had a CT appearance of complete mineralization and in 35% of those with nodules at least 1 nodule was calcified. 49.2% of nodules were sub-pleural and an additional 30.5% were located within the lobar fissures. Hence 78.3% were in contact with pleura and 20.3% had a centrilobular location. Of the nodules not associated with the pleura, the median distance from pleura was 8.8mm (IQR 5.6-12.4); in total 83.3% of discrete nodules were within 5mm of the pleura. 96.7% of nodules had no visible uptake of FDG above background lung (VS = 0) and for those that did FDG uptake was less than that of mediastinal blood pool (VS = 1). The median SUVmax of discrete nodules was 0.89 (0.68-1.14). The discrete nodules were evenly distributed between the lung lobes with the observed distribution not being significantly different to that expected if equally distributed (p=0.19). In general a slightly higher percentage of nodules were within the right lung (56.6%) and in the lower half of the lung (70%) with very few being located superior to the clavicles (figure 4.1).

Of the 20 participants with discrete inactive nodules, 14 had a repeat FDG-PET/CT performed. Of the 53 discrete nodules present on these baseline scans, 96.2% were visible on follow up scan, this slight differences most likely resulted from differences in image acquisition between the studies.
**Chapter 4 – FDG-PET/CT**

<table>
<thead>
<tr>
<th>Clinical characteristics participants with and without discrete nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameter</strong></td>
</tr>
<tr>
<td>Age med – Med (IQR)</td>
</tr>
<tr>
<td>Female - %</td>
</tr>
<tr>
<td>CD4 – mm³ Med (IQR)</td>
</tr>
<tr>
<td>Viral load</td>
</tr>
<tr>
<td>WHO clinical stage 2</td>
</tr>
<tr>
<td>Current or Ex smoker</td>
</tr>
<tr>
<td>Qf/GIT</td>
</tr>
<tr>
<td>BMI</td>
</tr>
<tr>
<td>History of household contact</td>
</tr>
<tr>
<td>HIVCs scar (n=34)</td>
</tr>
</tbody>
</table>

**Figure 4.1** – Clinical features of 20 participants with discrete nodules and distribution of these nodules in participants:

- **TOP** - Table showing clinical characteristics of participants with and without discrete nodules;
- **MIDDLE** - CT location of discrete nodules for all 20 participants spatially represented on a single CXR image as filled circles. Numbers signify participant study number, colour represents lobe; BLACK = upper lobe, WHITE = middle lobe, GREEN = lower lobe.
- **BOTTOM** – Tables showing features and distribution of discrete nodules.
The presence of discrete nodules on FDG-PET/CT was not associated with TB household contact history, duration since TB contact, presence of BCG scar, WHO clinical status or any smoking history. In addition there was no significant difference in age, sex, CD4 count, viral load, QFGIT result or BMI between those with discrete nodules and those without.

4.6.1.2 Active nodules

This term was used to describe small, spherical opacities within the lung parenchyma with poorly defined borders (figure 4.4). These were present only in a single participant (an 18 year old female with CD4 count of 802/mm³ and VL of 3948 copies/mL) in whom sputum was culture negative at screening but positive 48 days later on the day of scan (time to sputum culture positivity 33 days). Symptoms of cough and night sweats developed 34 days after the scan. Standard 6 month TB therapy was commenced but ART was not commenced until after TB therapy was complete. 26 nodules were present within the lung parenchyma. The median size was 3.4mm (IQR 3.1-3.9) and SUVmax 0.86 (IQR 0.71-0.95) which was not significantly different from discrete nodules (p=0.23 and p=0.78). All lesions had a VS = 0 and none had evidence of mineralization. Nodules were present in all lung lobes in a random distribution with only 42.3% of nodules in contact with the pleura (either sub-pleural or within pleural fissure) and 57.7% being centrilobular. This distribution was significantly different to discrete nodules (p=0.001) (figure 4.3a/b). Unusually, QFGIT TBAg-NIL for this participant increased from 2.58IU/mL to >10IU/mL between screen and scan date, change of this magnitude was only seen in one other participant (figure 4.3a).

Furthermore in contrast to discrete nodules, following treatment for TB all but 1 of the active nodules disappeared on follow-up FDG-PET/CT.
Figure 4.3a – Distribution of active nodules in 1 participant and change in QFGIT response

LEFT: Graph showing QFGIT TBag-Nil results at screening and on day of scan (participant with active nodules shown in RED);
RIGHT: CT location of nodules in 1 participant with active nodules spatially represented on a CXR image as orange filled circles.

Figure 4.3b – Axial section of CT showing examples of discrete and active nodules

LEFT – Example of sub-pleural, non-calcified, well-circumscribed, discrete nodule (green arrow)
RIGHT – Example of 4 centrilobular, poorly circumscribed, active nodules in left lung (green arrow) and 1 sub-pleural active nodule in right lung (green arrow)
**4.6.1.3 Infiltrates and scars**

In total 9 participants (25.7%) had infiltrates consistent with active TB or fibrotic scars consistent with “inactive” TB present within lung parenchyma, 6 having fibrotic scar and 6 having infiltrates (figure 4.5 and table 4.2). Those with fibrotic scarring within lung were significantly more likely to have infiltrates within the lung than those that did not (p=0.049). Of the 3 participants with fibrotic scars who did not have infiltrates, 2 displayed other evidence of disease activity responding to IPT. One had considerable uptake within a mediastinal LN which resolved with IPT (fig 4.5-G2) and the other had repeat scans showing reduction in size and FDG uptake within mediastinal LN post IPT (and also developed asymptomatic sterile FGD avid nodules – both cases discussed in detail below).

Scars were linear fibrotic or fibro-cystic abnormalities slightly distorting the surrounding lung tissue with no radiographic signs of activity, all had a VS = 0 and median SUVmax 0.9 (0.87-1.0). 50% of scars had evidence of mineralization and the median length was 12.2mm (IQR 7.2-16). All scars were situated within the upper lobes (58.3% within left upper lobe and 41.7% with right upper lobe) with 83.3% being located within the S1+2 (apico-posterior, left lung), S1 (apical, right lung) or S2 (posterior, right lung) broncho-pulmonary segments and 16.7% within the S3 (anterior, left or right lung) broncho-pulmonary segments (figure 4.4).

Infiltrates represented irregular, poorly defined, airway consolidation with radiographic signs of activity. In all 6 participants with evidence of infiltrates, only a single infiltrate was present. Median SUVmax was 2.4 (IQR 0.84-5.1) and in 5, the lesion had VS ≥ 1. Mineralization was not present in any lesion and median length was 16.3mm (IQR 5-19.8). 2 participants with largest areas of infiltration had evidence of tree-in-bud opacification and a small area of cavitation was present in the centre of the largest infiltrate measuring approximately 3mm (fig 4.6). Lesions were within the upper lobe in 5 of 6. However, the participant with evidence of infiltration within the left lower lobe had a fibrotic scar within
Figure 4.4 – Distribution of infiltrates and scars in 9 participants.
TOP – CT location of infiltrates and scars from 9 participants represented on a single CXR as triangles. Length of triangle proportional to length of lesion. Colour represents activity; RED = FDG avid Infiltrate, ORANGE = Infiltrate without FDG avidity but CT evidence of activity, BLUE = fibrocystic scar, BLUE/BLACK = mineralized fibrocystic scar. Numbers represent participant.
BOTTOM – CT location of infiltrates represented on a single axial section. Colour represents broncho-pulmonary segment; WHITE – S1, R lung, GREEN – S2, R lung and S1/2, L lung, YELLOW – S3 R/L lung, BLACK – lower lobe. Number represents participant (blue number = scar, red number = infiltrate)
Figure 4.5 - Coronal sections through fused FDG-PET/CT showing infiltrates and scars in all 9 participants. Letter signifies individual participant where another image relates to same participant letter is followed by “2”, post-treatment image represented by ‘’. Pre-treatment lesion circled in green and post-treatment in red. Green arrow in “B2” identifies LN within porta hepatitis (confirmed on USS) and red arrow increased uptake within spleen with spherical lesions (confirmed as splenic micro-abscesses on USS). All images scaled to SUVmax =10 (scale shown)
the right upper lobe. Of those with upper lobe lesions 4 of 5 (80%) had lesions within the S1 or S2 broncho-pulmonary segment and 1 (20%) within the S3 segment. Of those with infiltrates 1 participant (with small cavity) was culture positive on day of scan (time to positivity 12 days) having been culture negative at screening but had shown progression of CXR changes between day of screening and day of scan (see chapter 3 section 5.3.2). She developed symptoms of fever and night sweats approximately 7 days after scan and was commenced on quadruple therapy. ART was commenced after 66 days of TB therapy. Repeat FDG-PET/CT was performed 22 days after completing TB therapy and lesion showed substantial improvement in FDG uptake (SUVmax 6.31 → 1.59) (Fig 4.5 – F/F’).

Two additional participants with infiltrates developed TB symptoms after 90 and 30 days, and were commenced on TB therapy though TB culture was negative. The former had a small lung infiltrate which was not FDG avid but had enlarged FDG avid lymph nodes within the porta-hepatis and increase patchy uptake within the spleen (splenic FDG uptake being greater than liver uptake). Splenic micro-abscesses and LN were confirmed by abdominal ultrasound scan (USS) and were suggestive of abdominal TB. Symptoms of malaise and left upper quadrant pain resolved after TB therapy but FDG-PET/CT was not repeated. The other had a clinically indicated contrast CT scan of the chest performed approximately 4 weeks after initial scan while awaiting results of TB culture which showed evidence of bronchogenic progression of infiltrate down an adjacent lobule, which may represent how TB progresses in the early stages (figure 4.6). She developed symptoms of night sweats 30 days after scan, which responded to TB therapy. ART in this participant was commenced after 18 days of TB treatment. FDG-PET/CT was repeated 71 days into TB treatment showed improvement in FDG uptake (fig 4.5 – E/E’). The remaining participants with infiltrates continued on IPT.
HIV, being a median of 4.5 years from diagnosis, those with evidence of disease activity there was a suggestion of slightly more advanced participants that had either no parenchymal abnormalities or previous disease activity. Their clinical characteristics were compared with the 25 participants that had either no parenchymal abnormalities or only discrete nodules (table 4.3). There were no statistically significant differences between the groups. However, in those with evidence of disease activity there was a suggestion of slightly more advanced HIV, being a median of 4.5 years from diagnosis with median CD4 count of 406/mm³ and

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Infiltrate/ scar/ active nodules (10)</th>
<th>Normal parenchyma or discrete nodule (25)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age med – Med (IQR)</td>
<td>29.5(27-35)</td>
<td>32(27-39)</td>
<td>0.17</td>
</tr>
<tr>
<td>Female - %</td>
<td>90%</td>
<td>92%</td>
<td>0.85</td>
</tr>
<tr>
<td>CD4 – mm³ Med (IQR)</td>
<td>406(384-548)</td>
<td>517 [435-724]</td>
<td>0.14</td>
</tr>
<tr>
<td>Viral load</td>
<td>18949(6878-51166)</td>
<td>9958(625-20702)</td>
<td>0.07</td>
</tr>
<tr>
<td>WHO clinical stage 2</td>
<td>30%</td>
<td>16%</td>
<td>0.35</td>
</tr>
<tr>
<td>Days positive HIV</td>
<td>4.5(0.8-7.5)</td>
<td>1.7(0.9-3.5)</td>
<td>0.32</td>
</tr>
<tr>
<td>CRP</td>
<td>2.1 (1-4)</td>
<td>1.9 [1-3.9]</td>
<td>0.95</td>
</tr>
<tr>
<td>WCC</td>
<td>5.59(4.24-7.38)</td>
<td>5.34 (4.92-6.42)</td>
<td>0.97</td>
</tr>
<tr>
<td>ESR</td>
<td>41(28-83)</td>
<td>36 (26-46.5)</td>
<td>0.39</td>
</tr>
<tr>
<td>QFGIT</td>
<td>4.57(0.69-9.1)</td>
<td>1.76(0.39-4.11)</td>
<td>0.53</td>
</tr>
<tr>
<td>Inactive CXR</td>
<td>60%</td>
<td>32%</td>
<td>0.23</td>
</tr>
<tr>
<td>Discrete nodules on CT</td>
<td>30%</td>
<td>60%</td>
<td>0.11</td>
</tr>
<tr>
<td>BMI</td>
<td>24.8 (23.3-29.5)</td>
<td>28.8 (26.8-34.1)</td>
<td>0.09</td>
</tr>
<tr>
<td>% weight change/28 days</td>
<td>-0.84% (-1.38—0.50)</td>
<td>-0.24% (-1.03-0.93)</td>
<td>0.10</td>
</tr>
<tr>
<td>History of household contact</td>
<td>40%</td>
<td>48%</td>
<td>0.67</td>
</tr>
<tr>
<td>%BCG scar (n=34)</td>
<td>33.3%</td>
<td>40%</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Table 4.3 – Comparison of clinical characteristics of 10 participants with infiltrates/scars and active nodules and 25 participants with normal parenchyma of discrete nodules only

Of the 3 participants with infiltrates who also had scars, the infiltrates did not develop directly from or adjacent to scars in any case. Furthermore, in 2 participants the scars and infiltrates were in different broncho-pulmonary segments. One participant had multiple distinct fibrocystic scars within both lungs (figure 4.5 – G only 1 cluster of scars shown).

Three scars were closely clustered peripherally within the S1/2 broncho-pulmonary segment in the left lung and showed significant calcification with a 4th non-calcified scar more apically located within the same broncho-pulmonary segment. Two further non-calcified scars were clustered within the S2 broncho-pulmonary right lung. If scars represented prior spontaneous healing of sites of active infection. This could suggest episodes of reactivation in this participant on 2 or 3 separate occasions with the clustered scars relating to bronchogenic spread down adjacent lobules followed by healing.

The 10 participants with evidence of upper lobe infiltration and scarring and the participant with the active nodular pattern were grouped together as having evidence of current or previous disease activity. Their clinical characteristics were compared with the 25 participants that had either no parenchymal abnormalities or only discrete nodules (table 4.3). There were no statistically significant differences between the groups. However, in those with evidence of disease activity there was a suggestion of slightly more advanced HIV, being a median of 4.5 years from diagnosis with median CD4 count of 406/mm³ and
viral load of 18949 and 30% being WHO stage 2. In comparison, the 25 participants without evidence of activity were a median of 1.7 years from HIV diagnosis with a median CD4 count of 517/mm$^3$ and viral load of 9958 with 16% being WHO stage 2. In addition, those with evidence of disease activity had a non-significant trend towards a lower median BMI (24.8 vs. 28.8 – p=0.09) and a greater change in weight over the screening period (-0.84%/28 days vs. -0.24% /28 days – p=0.10) compared to participants without these changes. Otherwise there were no differences between the groups particularly with regard to inflammatory markers, history of household TB contact or presence of BCG scar.

There was no apparent relationship between discrete nodules and presence of infiltrates, scars and active nodules and no evidence that such lesions arose from or in relation to discrete nodules.

4.6.2 Lymph node abnormalities on FDG-PET/CT scan

Mediastinal lymph nodes (LN) were categorized into the following LN basins. R and L superior mediastinal, aortic, R and L hilar and subcarinal/inferior mediastinal and were considered abnormal if any of the following were present:

1. Mineralization
2. Enlargement $\geq$ 10mm in short axis
3. Increased FDG uptake greater than mediastinal blood pool

At repeat scan any changes in specific LN identified on initial scan were evaluated. In addition maximal FDG uptake was defined in visible cervical and axillary peripheral LN chains and within LN in the upper abdomen abnormal FDG uptake within lymph nodes was recorded.

4.6.2.1 Lymph node mineralization

Mineralized LN were identified in 11 participants (31.4%) with 3 participants having greater than 1 mineralized LN basin resulting in a total of 15 mineralized LN basins being identified (table 4.4). Hilar and subcarinal LN were most commonly mineralized. Discrete parenchymal nodules were present in 7 participants with mineralized LN (63.6%), of which in only 1 was the parenchymal nodule also mineralized. This distribution was not significantly different from participants without mineralized lymph nodes (p=0.40). A consistent relationship between location of parenchymal nodules and location of mineralized lymph nodes was not easily discerned, however, lymphatic drainage of lungs though well
characterised is complex and variable. In total 17 participants (48.6%) had evidence of mineralized parenchymal nodules or LN. Any mineralization within lung parenchyma or LN was not associated with history of TB contact, BCG scar, QFGIT result, employment history or HIV indices.

4.6.2.2 Lymph node enlargement and FDG uptake

Both HIV and TB infection are known to lead to enlargement and increased metabolic activity of LN. Abnormal FDG uptake was present in the cervical LN in 70.6%, axillary LN in 97.1% and mediastinal LN in 45.7%. One participant had significant FDG uptake within upper abdominal lymph nodes (2.9%). The presence of FDG avid cervical, axillary or mediastinal lymph nodes was not associated with significant differences in viral load, CD4 count or number of days since HIV diagnosis and there was no significant correlation of SUVmax with VL, CD4 or baseline QFGIT for any of the 3 groups of LN. However, abnormal axillary lymph nodes had significantly greater SUVmax than either cervical or mediastinal nodes (see figure 4.7).

The 10 participants with infiltrates, scars or active nodules were no more likely to have abnormal uptake within cervical or axillary nodes compared to those without these parenchymal features of activity (p=0.68 and p=1.0 respectively) and the median SUVmax for cervical and axillary nodes was also not significantly different. However, they were significantly more likely to have abnormal uptake within mediastinal lymph nodes (p=0.022), although for those with abnormal LN median SUV was not significantly different (p=0.75). In addition the only participant with abnormal FDG uptake within abdominal LN had an infiltrate within the lung parenchyma. The distribution of SUVmax within abnormal central LN (mediastinal LN and abdominal LN) appeared bimodal (figure 4.8). A significantly greater proportion of those with infiltrates, scars or active nodules had a central lymph nodes with SUVmax ≥ 5 compared to those without parenchymal evidence of activity (40% vs. 4%; p=0.017). The single participant without parenchymal evidence of activity had extensive

Table 4.4 - Location of mineralized lymph nodes

<table>
<thead>
<tr>
<th>Lymph Node Basin</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>R/L Hilar</td>
<td>53.3%</td>
</tr>
<tr>
<td>Subcarinal/ Inf Mediastinal</td>
<td>26.7%</td>
</tr>
<tr>
<td>R/L Sup Mediastinal</td>
<td>13.3%</td>
</tr>
<tr>
<td>Aortic</td>
<td>6.7%</td>
</tr>
</tbody>
</table>
abnormal FDG uptake within all visible LN basins. For this participant there was initial concern regarding ground glass opacification of lung parenchyma but this was dismissed at review of follow up scan. Bronchoscopy and transbronchial needle aspirate (TBNA) were performed with samples sent for cytology and culture not revealing a cause for the abnormalities. The participant did complain of intermittent cough post bronchoscopy but these symptoms resolved without treatment there was no weight loss by the time of second scan she remained asymptomatic and all cultures were negative for TB. LN abnormalities remained present at the second scan. However, it is possible that there was an undiagnosed pathology other than HIV responsible for these abnormalities.

Enlarged central lymph nodes (>10mm) were present in 14 (40%) of participants. There was no significant correlation between size of enlarged lymph node and its SUVmax (r = 0.2, p=0.24). Median VL and CD4 were not significantly different to those without enlarged lymph nodes (p=0.17 and 0.62 respectively). Enlarged central lymph nodes were present in 6 of 10 participants (60%) with infiltrates, scars or active nodules compared to 8 of 25 (32%) with no evidence of TB disease activity (p=0.151).

Of the 27 participants who had a repeat scan, the majority of were treated with IPT and not commenced on ART prior to repeat scan at 6 months. For these participants there was a significant correlation of SUVmax, between initial and repeat scan for cervical, axillary and mediastinal LN. This relationship was particularly strong for axillary LN (r = 0.89, p<0.0001) and mediastinal LN (r = 0.87, p<0.0001) compared to cervical LN (r = 0.51, p=0.02). One
participant was commenced on standard TB therapy but not ART, 1 participant commenced on ART and remained on IPT and 2 participants were commenced on standard TB therapy and ART. This therefore allowed some assessment of the effect of TB therapy and ART on FDG uptake particularly within axillary and mediastinal LN. For axillary LN, neither IPT nor TB treatment alone had a significant impact on FDG uptake suggesting that TB infection or disease may not contribute significantly to metabolic activity of axillary LN (closed symbols figure 4.9 (TOP)). ART initiation, however did appear to result in a reduction in FDG uptake irrespective of whether IPT or standard TB therapy was prescribed (open symbols figure 4.9 (TOP)) with reduction in FDG uptake being greatest in participants that had a longer duration of ART. All participants that commenced ART also had marked improvement in FDG uptake within the cervical LN.

For FDG avid mediastinal LN, the impact of ART initiation on FDG uptake for the participant on IPT was less dramatic. Abnormal mediastinal LN showed a median reduction in SUVmax of 12% after 126 days of ART in comparison to a median 7% reduction for all those on IPT alone. Four participants were reported to have a significant reduction in SUVmax of mediastinal LN on repeat scan, 3 of whom were treated with IPT alone (2 of whom also had fibrotic scarring) and 1 that was treated with standard TB therapy and ART (Fig 4.5 E/E’, Fig 4.9 (BOTTOM) labelled “B”). For all these cases there were no technical differences between the 1st and 2nd scans; administered FDG and time between injection and scan for pre and post scans were within 90-110% in all cases. One of the participants with fibrotic scarring treated with IPT alone had an especially dramatic improvement with a 59% reduction in SUVmax (SUVmax 6.1 → 2.5) and almost total improvement in visual score within a very
Figure 4.9 – Correlation of SUVmax in axillary and mediastinal LN between pre and post scan.
TOP – Scatter-plot showing correlation of SUVmax in axillary LN on pre and post FDG-PET/CT scan
BOTTOM – Scatter-plot showing correlation of SUVmax in mediastinal LN on pre and post FDG-PET/CT scan.
Effect of different IPT, standard TB therapy and ART is shown (see text for reference to "A" and "B")
enlarged (19.4mm), R superior mediastinal LN which also reduced in size. There was very little change within other mediastinal LN (6% reduction) or axillary LN (11% reduction).

Hence, this was consistent with tuberculous lymphadenitis responding to isoniazid (fig 4.5 G2/G2’, fig 4.9 BOTTOM labelled “A”). The other participant with fibrotic scarring had a median 20% reduction in SUV following IPT within a significantly enlarged mediastinal LN (up to 20mm) accompanied by reduction in size of LN and development of mineralization. This was accompanied by a 1% increase in axillary SUV and therefore suggested a response to IPT (figure 4.10 – TOP). In the final participant on IPT who was reported as showing a visual improvement within mediastinal LN, had no fibrotic scarring. The median SUVmax with mediastinal LN (which were not enlarged) reduced 38.5% but there was also a 23% reduction within axillary LN. The interpretation therefore was less clear but may have represented a response to IPT. Of note in only 2 of the 4 participants with improvement in FDG uptake within mediastinal LN was the baseline SUVmax > 5 (median SUVmax 4.4 range 2.4-6.47) suggesting that there may be considerable overlap in SUVmax for resulting directly from HIV infection alone and that related to TB infection. Of interest for these 4 participants in whom there was a response within the mediastinal LN to TB therapy there was a strong correlation between QFGIT (performed on day of scan) and baseline SUVmax of mediastinal LN.
LN (r=0.84, p=0.16), however this was not significant due to small sample size, but reflects findings by Ghesani et al[137].

Of note all 6 participants with infiltrates, scars or active nodules that had a repeat scan had a significant improvement in baseline abnormalities reported, either within lung parenchyma or mediastinal LN, compared to only 1 of 21 participants without evidence of TB disease activity who had a repeat scan (p<0.0001).

4.6.3 New abnormalities on follow-up FDG-PET/CT scan

Of the 27 participants that had a follow-up scan only 3 showed significant new findings on repeat scan. One participant had a R lower lobe infiltrate and developed a productive cough and pleuritic pain within 1 week, which resolved following a short course of amoxicillin. This deterioration was attributed to a bacterial bronchopneumonia. The other 2 participants developed new FDG avid, poorly defined nodules. One of these participants had fibrotic scar visible on the initial scan, which had not changed, but an improvement was reported within the mediastinal LN (reduction in size and FDG uptake, as well as increased mineralization (as described above)) (figure 4.10). In addition to this, 3 distinct, large (>1cm), FDG avid, nodules (SUVmax 2.86-2.96) appeared within the apex of the RLL. Compliance with IPT appeared good as judged by pill count. These lesions did not appear similar to type of lesions described on the initial scans, appearing as distinct lesions with little evidence of endobronchial spread (unlike infiltrates) and larger and less numerous than the active nodules described. The second participant, similarly, had a single large FDG avid nodule appear within the R lower lobe. Compliance with IPT in this case was difficult to assess as pills were rarely returned for counting. Neither of these 2 participants developed any TB symptoms and sputum cultures were negative. Lesions were not visible on CXR and repeat CXR did not show any further deterioration. Both participants underwent bronchoalveolar lavage (BAL) and BAL cultures were also negative for Mtb and bacteria with unremarkable cytology.

4.6.4 Comparison of CXR and FDG-PET/CT

Consensus reading of screening CXR was used to exclude active TB and categorize participants as having either normal CXR or evidence of inactive TB. As described 2 participants had evidence of progressive abnormalities at repeat CXR performed just prior to the initial scan consistent with active TB and 33 had no evidence of progression on CXR.
Comparison between the CT findings and the consensus CXR diagnosis for the CXR performed just prior to the initial scan is shown in figure 4.11.

To allow for broad comparison between FDG-PET/CT and CXR, FDG-PET/CT parenchymal lesions were classified as follow

- Normal
  - No parenchymal abnormalities
  - Discrete non-calcified nodules (as these were commonly <5mm and when present usually <3 per patient these would not be expected to be detectable on CXR)
- Inactive
  - Fibrotic scar
  - Discrete calcified nodules (these were included in CXR definition of inactive TB hence to allow for fair comparison)
- Active
  - Infiltrate
  - Active nodules

Using these criteria, agreement between the consensus report and the FDG-PET/CT scan was moderate. The observed agreement was 68.6% against an expected agreement of 42.3%, $\kappa=0.46$ (95%CI 0.24-0.70 – p=0.0001). Of note only 2 out of 7 (28.5%) of those with active changes on FDG-PET/CT were identified by CXR and there was tendency to over estimate inactive TB abnormalities. The sensitivity and specificity of the consensus CXR report compared to the FDG-PET/CT scan (for active TB changes (active/inactive) vs. normal) was 73.3% and 80% respectively.

These CXR diagnoses were as reported by consensus of medical officers following viewing of 2 CXR for each participant (screening and pre-scan CXR). In order to get a more realistic
comparison of CXR screening and FDG-PET/CT, 7 consultant radiologists with median experience of 8 years (IQR 5.5-11) were shown the single CXR for each participant taken closest to FDG-PET/CT (median 9 days apart (IQR 7-14 days). The consultant readers were given minimal clinical information (asymptomatic, HIV infected from Khayelitsha, screening CXR for TB). However, they were given detailed written and verbal reading instructions including CXR definitions of active TB and inactive TB to be used. All readers had experience in the reporting of TB screening CXR for migration purposes and were familiar with the definitions provided, they all reported between 100-5000 CXR/year. The CXR were reported by these readers independently, on medical diagnostic monitors, in a hospital reporting room with low ambient lighting. A structured reporting sheet was used to ensure all sections of the CXR were focused on. All readers reported images in a different random order to minimize reading bias.

Individual reader agreement with FDG-PET/CT (for the 3 categories active TB, inactive TB and normal/no TB) was slight to fair for all readers as measured by kappa statistic (figure
4.12 - C). There was no significant correlation of accuracy of CXR reporting with years of experience as a radiologist ($r = 0.22, p=0.63$) and no significant effect of number of CXR reported/year on accuracy ($p=0.86$). None of the readers reported the culture positive participant with active nodules on FDG-PET/CT as active. 6/7 reported the second culture positive participant with CXR infiltrate as active. Reader 6 was an outlier with regard to distribution of reports. Excluding reader 6, the overall agreement between the remaining 6 readers with each other was still only slight $\kappa=0.15$, with agreement being better for active TB ($\kappa=0.28$) and worse for inactive TB ($\kappa=0.10$). In general there was a tendency to over report inactive TB changes. Evaluating the CXR report as a binary outcome of TB related changes or no TB related changes, sensitivity of CXR readers varied considerably from 33.3%-100% (median 86.7%) as did specificity 25%-94.7% (median 45%). A reciprocal relationship for these parameters was apparent for each reader (figure 4.13), with 5 readers (1,2,4,6,7) having high sensitivity and low specificity (over-reading) and 2 readers (3 and 5) having low sensitivity and high specificity (under-reading). The positive predictive value of CXR readers varied from 50%-83.3% and negative predictive value from 66.7%-100%. In contrast readers in general had poor sensitivity for changes consistent with active TB (median 28.6%) but specificity was generally good (median 92.6%) again suggesting the main tendency was to over call inactive TB changes.
4.7 Summary of main findings

Amongst a cohort of 35 asymptomatic, ART naïve, HIV infected adults with a median CD4 count of 517/mm$^3$, recent evidence of a positive QFGIT and no history of previous TB treatment, 71.4% had anatomical abnormalities within the lung parenchyma and 45.7% had abnormal FDG uptake with central LN (mediastinal and upper abdominal). Four patterns of parenchymal abnormality were observed, potentially representing different stages of TB infection; discrete nodules, active nodules, infiltrates and scars. Those with fibrotic scars were significantly more likely to have infiltrates or other signs of disease activity and hence were grouped with those with infiltrates and active nodules as having some evidence of current or prior TB disease activity. There was no significant difference in clinical characteristics between these 10 participants and the 25 without evidence of disease activity, although there was a tendency towards more advanced HIV infection. The 10 participants with parenchymal evidence of disease activity were more likely to have abnormal uptake within central LN (mediastinal and abdominal), in particular SUVmax $>$ 5, but not within peripheral LN (cervical and axillary). These 10 participants were also significantly more likely to have improvement in baseline abnormalities following IPT or TB treatment. Two participants developed unexplained new FDG avid nodules though remained asymptomatic and had negative culture at bronchoscopy.

Agreement between FDG-PET/CT and CXR for each of the 7 consultant radiologists was slight to fair and agreement between the radiologists was again only slight. Radiologists identified abnormalities consistent with TB with either high sensitivity/low specificity or high specificity/low sensitivity suggesting that some had a tendency to over-read and others under-read screening CXR.

4.8 Discussion

Discrete, well-circumscribed, nodules on CT scan were identified in 57.1 % of participants. These nodules were less than 10mm in size, solid in appearance, generally spherical with no evidence of spiculation or characteristics suspicious of malignancy. When present, nodules were most commonly single, sub-pleurally located with no lobar preference (though more commonly found in the lower half of the lung field) and 18.3% were calcified. Smoking, biomass fuel exposure and traditional risk factors for cancer in this population were very low and all participants were under 50 years with only 14% (5 of 35) being over 40 years old. No
CT screening studies have been performed in this specific population to compare this result, although numerous CT screening studies for lung cancer have been performed. The majority of these studies focus on groups that are at high risk of lung cancer, usually age > 50 years with at least a 15 pack year smoking history, living in high income countries[222]. In these high-risk populations, lung nodules are common being present in up to 51% of smokers over the age of 50[223, 224]. The vast majority of nodules in smokers are benign and are likely represent reactions to inhaled irritants and particles. In smokers that have nodules, the median number is 5-7[225] in comparison to the median of 2 in those with nodules in this study. The prevalence of lung nodules is likely to be considerably lower in non-smokers. Only a single large screening study has included non-smokers and these participants were all > 40 years old. Sone et al conducted a population lung cancer screening trial using low dose spiral CT in Japan involving 5,483 participants with a median age of 64 years, of whom approximately 50% had never smoked. The results were not reported by smoking status but in the study as a whole only 7.2% had benign lesions and 5.1% suspicious lung lesions, substantially fewer abnormalities than in screening studies of higher risk participants[226]. Hence it appears that lung nodules are much more common in our population than would be expected in non-smokers from high-income countries and also follow a different pattern to that found in smokers.

Pathogens that cause granulomas are a common cause of pulmonary nodules with M. tuberculosis, Cryptococcus neoformans and Histoplasma capsulatum being amongst the most common causes globally. Histoplasmosis is a relatively uncommon condition in South Africa although outbreaks have historically been associated with a history of cave exploration[227]. In recent times disseminated histoplasmosis has been shown to occur in advanced HIV but in a retrospective, 8 year review of histological samples of deep fungal infections at a teaching hospital in Cape Town, histoplasmosis was only confirmed in only a single case[228]. Cryptococciosis is a well-known cause of incidental pulmonary nodules but the literature is generally limited to case reports and series. Two case series have reported CT findings of pulmonary cryptococciosis, suggesting that nodules are frequently 6-20mm in size and more often multiple than single which does not correspond to the pattern found in this study[229, 230]. In addition Cryptococcus is environmentally ubiquitous globally, not limited to low-middle income countries, with positive antibodies detectable in the majority of children by the age of 5. A study from New York has shown that 70% of children > 5 years had detectable cryptococcal antibodies[231]. Disseminated cryptococcal disease is a
common presentation in advanced HIV (CD4<100/mm³) in South Africa but increased identification of pulmonary lesions has not been reported in HIV infected persons with higher CD4 counts. Environmental outbreaks of Cryptococcus sp. may lead in increases in pulmonary nodules relating to the organism but even in the well characterised outbreak of Cryptococcus gatti on Vancouver Island overall numbers of pulmonary cryptococcosis were low[232].

It is more likely that these nodules relate to mycobacterial infection with Mtb infection being the most likely. While non-tuberculous mycobacteria can also cause lung granulomas, Mtb is the most commonly sensitizing mycobacteria in this setting with >75% having a positive Mtb IGRA. Furthermore in this study 100% of recruited participants had recent evidence of immune sensitization by Mtb (positive QFGIT). Those with advanced HIV are highly susceptible to both Mtb and Mycobacterium avium intracellulare (MAI) (one of the commonest non-tuberculous mycobacterial infecting causing disease). However, prevalence surveys in these populations in South Africa have shown Mtb to be at least 5 times more common than MAI[196].

The distribution of discrete nodules found on CT in this study is remarkably similar to descriptions of the primary site of initial TB infection (Ghon focus) reported in numerous autopsy studies. Although most autopsy studies were carried out in America and Europe in the early 20th century. This was at a period when prevalence of TB would have been similar to what is seen in Cape Town currently and hence they provide a valid source for comparison. In Ghon’s original monograph “The primary lung focus of tuberculosis in children” he reported on the presumed primary foci identified in 26% of children undergoing autopsy in a Vienna hospital between 1902 and 1910. Of these the majority had a single focus and only 6.4% had 4 or more foci visible. These lesions were most commonly “pea sized” (approx. 5mm), calcified in 37% and located peripherally in in 94%; specifically sub-pleural in 40.5% and within the lobar fissures in 21.5%. Lesions were identified within the right lobe more commonly than the left (55:45) with lobar distribution being as follows: RUL-28.5%, RML-7%, RLL 19.5%, LUL 25.0%, LLL 20.0%[107]. In a survey of 1225 necropsies, Medlar described to location of calcified primary foci as being within 1 cm of pleura in 85% and in the lower half of lungs in two-thirds of cases with only 12% being supra-clavicular (as described in greater detail in chapter 1)[113]. This distribution of presumed primary TB foci; peripheral location, slight right lung dominance with no lobar preference but greater in the
lower half of the lung is repeatedly described in the literature. This likely reflects preferential airflow within the lungs related to airway size suggesting that implantation of the TB bacillus is a random event[114]. In this study a greater proportion of lesions were within the R middle lobe than previously described. One possible explanation for this is that a number of lesions that were identified within the inter-lobar fissures between R upper and middle lobes and R middle and lower lobes could have been misallocated. In addition although 17 nodules were situated within the RML these related to 8 participants which may have slightly skewed results. In autopsy studies of adults a greater proportion have been found to have primary lesions as reported in this study and this will also relate to force of infection[112]. In addition a greater proportion of these lesions are described as mineralized. The finding in this study that 18.3% of nodules are mineralized is likely to be an underestimate in comparison to autopsy studies. Entirely mineralised nodules are easily detectable on CT as such lesions typically will have a density of > 100 Hounsfield unit (HU). However, Sweany in his monograph “The age morphology of primary tubercule” has described that calcification of tubercles occurs in layers and that full calcification takes place over a period of years[108]. Partial calcification of this nature in small nodules would be difficult to detect by CT but would be easily detectable at autopsy as it is readily palpated. It is also likely that nodules smaller than 3mm would not be identified with the CT imaging protocol used and that micronodules (<3mm) may have been present in some of those labelled as having normal scans. It is also very difficult to accurately evaluate the metabolic activity of sub-centimetre nodules; only 3.3% of discrete nodules were described as having visible FDG uptake above background lung parenchyma. However, this could be an underestimate due to the partial volume effect which results in loss of apparent activity in objects less than 1cm due to the poor spatial resolution of the FDG-PET scan and spill-over of activity into the surrounding background regions. Equally, activity is sometimes overestimated when activity form surrounding structures overlay the region of interest.

In comparison to the discrete nodules described above, active nodules were identified in a single participant. Of note this participant was Mtb culture negative at screening and Mtb culture positive (although remained asymptomatic) on day of scan 48 days later. The time to culture positivity was prolonged at 33 days, suggesting a low number of bacilli were transmitted from airways into the sputum. The nodules were far more numerous than discrete nodules described above. Twenty-six nodules were easily visible on the scan although it is likely that total number was in excess of this. These nodules, which were
present in all the lung lobes, were poorly defined in contrast to discrete nodules and were significantly more likely to have a centrilobular location (57.7% vs. 20.3%). In addition, all but 1 active nodule disappeared following TB treatment in contrast to discrete nodules. The fact that these lesions did not demonstrate increased FDG uptake is again not surprising due to the factors described above. No lesions were easily visible within liver or spleen in this participant, which may be due to the limitations of a non-contrast CT scan. This anatomic distribution is suggestive of a haematogenous dissemination of TB and supported by the observation that IFNγ release in QFGIT increased from 2.58 to >10IU/mL between screen and scan. It is therefore possible that this participant was in an early stage of miliary TB. Pathology would have been likely to be considerably more advanced by the time symptoms appeared which occurred 34 days after the culture was taken. It is not immediately apparent from the FDG-PET/CT scan if such a miliary presentation was as a result of recent (re)infection or following focal reactivation and haematogenous dissemination. Of note there were no sites of more extensive disease to suggest an initial focal site of reactivation. Furthermore there was no significant mediastinal LN enlargement/FDG avidity, which might be expected during primary dissemination of infection. However, the participant did report potential close contact with TB within the year prior to recruitment. It is possible there may have been a tuberculous focus with the vascular intima as the source of haematogenous spread which may not have been easily visible on FDG-PET/CT scan which has been suggested in the pathogenesis of miliary TB[123].

Two large autopsy studies, the first involving 8800 consecutive autopsies conducted at Cook County Hospital, USA between 1917 and 1928 and a second involving 4066 consecutive autopsies conducted at Boston City Hospital between 1937 and 1941, reported acute miliary tuberculosis in 1.09% and 1.55% autopsies respectively and would be in keeping with the single case (2.86%) found in this study[123, 233, 234]. In advanced HIV (especially if CD4 count <50/mm³), in high burden TB settings disseminated miliary TB is far more common, however, this participant’s CD4 count was 802/mm³ when such a presentation would be no more common than in HIV uninfected persons.

Fibrotic scarring was present in 6 of 35 (20%) participants, exclusively in the upper lobes and lung infiltration in 6 of 35 participants, of which 5 of 6 were in the upper lobes. The presence of scarring was significantly associated with a concurrent active disease process, which was present in 4 of 6 participants (3 with lung infiltration and 1 with mediastinal
lymphadenitis responding to isoniazid). In all 9 of 35 participants had evidence of upper lobe fibrosis or infiltration which was mainly apico-dorsally located. A number of conditions can cause upper lobe predominant infiltrates and fibrosis globally, the most common being tuberculosis, sarcoidosis and histoplasmosis. As stated histoplasmosis is rare in South Africa. Sarcoidosis commonly presents with bilateral disease and fibrosis is associated with late (stage IV) disease by which time symptoms are invariably present. In addition, in this setting sarcoidosis is very much less common than TB; although good data is scarce incidence may be 50x less than tuberculosis[235]. This pattern of disease arising predominantly in the apico-dorsal sections of the lobes (upper > lower) is characteristic for tuberculosis described in both autopsy and imaging studies. Medlar described findings of minimal tuberculosis suggesting that they were apico-dorsal located in 75%[113]. Sweany reported in detail the location of primary cavities in 204 patients with pulmonary tuberculosis using stereoscopic X-ray imaging. He concluded that initial cavities were most commonly in the posterior, outer parts of the upper lobes usually subapical or within the apical part of the lower lobe[236]. More recently Yeh et al performing high resolution (HR) CT in 279 TB patients at presentation to hospital identified that consolidation was most common in the s1, s2, s1/s2 and s6 bronchopulmonary segments and cavitation in the s1, s2, and s1/s2 bronchopulmonary segments. Furthermore they showed that pathology located in these regions was highly predictive of pulmonary TB[237].

Infiltrates are likely to represent endobronchial spread of tuberculosis. The most advanced infiltrates in this study showed evidence of tree-in-bud opacification, which results from bronchioles filling with inflammatory exudate. In one participant a clinically indicated repeat scan performed 1 month after the initial scan showed retrograde endobronchial spread down what was likely to be an adjacent pulmonary lobule. This may be how denser regions infiltration eventually arise.

Fibrocystic scarring is a characteristic pathological finding within lung tissue as infiltrates and cavities related to active tuberculosis heal. This occurs following treatment but is also thought to occur spontaneously and is presumed to result from reactivation being arrested and reversed following a shift towards a more beneficial host response to mycobacterial infection. Such upper lobe scaring in persons with no history of TB is a common finding in both autopsy and imaging studies. In addition, in the pre-chemotherapy era improvement in infiltrates was commonly described (as outlined in detail in chapter 1). The presence of
fibrotic scarring, however, is well known to be a risk factor for developing TB, increasing risk up to 15 fold[77]. Stead and other have also described that those with sporadic TB (i.e. not a part of a cluster or outbreak) frequently had evidence of fibrotic apical scarring within the lungs on previous CXR, although these authors did not systematically report the spatial relationship of old scars to disease at presentation[79, 80, 238]. Results in this study support these findings as those with fibrotic scars were significantly more likely to have infiltrates and more likely to have evidence of active disease. However, the results also show that disease reactivation does not necessarily occur at the site of old fibrotic scarring as has previously be thought to be most likely. This idea that multiple independent reactivation events can occur is also supported by findings in 1 participant who had 3 separate clusters of fibrotic scars within both the right and left upper lobe showing different degrees of calcification. This is also supported by autopsy studies most notably in Opie and Aronson’s series of persons dying of causes other than TB where fibrotic apical scarring was not uncommonly bilateral[115]. These finding suggest that although recrudescence at the site of fibrotic scarring may occur, a proportion of those that are latently infected may undergo sporadic reactivation and healing initiated at different sites and may be at higher risk of disease occurring when reactivation can not be controlled.

Those with upper lobe infiltration were significantly more likely to have abnormal FDG uptake with the mediastinal nodes. In some instances this may have been a manifestation of disease but this relationship was not consistent as neither of the 2 culture positive participants had abnormal mediastinal lymph nodes present and the largest mediastinal lymph nodes were identified in 2 participants with scars and no infiltrates. It is possible that in latently infected participants at risk of reactivation Mtb is more regularly trafficked between the site of reactivation and regional lymph nodes resulting in pathological abnormalities at these sites.

Almost all participants had abnormal FDG uptake within peripheral (axillary and cervical) lymph node chains and 45.7% had abnormal uptake within mediastinal lymph nodes and a single participant had abnormal uptake within abdominal lymph nodes. Uptake, at least within axillary lymph nodes appeared to reduce rapidly following ART, however response did appear to lag behind suppression of viral load. In this study no significant relationship between lymph node SUVmax and viral load or CD4 count was identified in contrast to previous reports[146], but in keeping with findings by Brust et al[148]. Lelievre et al have
recently shown that SUVmax in lymph nodes of HIV infected persons is highly correlated with percentage of CD4 and CD8 cells expressing the T cell activation markers HLA-DR and CD38[239]. Although abnormal uptake within central lymph nodes was not uncommon, uptake was rarely intense in participants without scars and infiltrates with only a single participant with SUVmax > 5. In contrast, in the 10 participants with evidence of reactivation, 40% had a central lymph node with SUVmax > 5. However, it is unlikely that a cut off of this nature would entirely accurately distinguish HIV related uptake from uptake relating to TB or other opportunistic infections as 2 of the 4 participants that has improvement in uptake within the mediastinal LN on IPT had SUV max < 5. This suggest that although TB related LN abnormalities may be more likely than HIV related LN abnormalities to result in high FDG avidity there may be significant overlap in SUVmax.

Only one other study has systematically performed FDG-PET/CT in persons with latent TB, though the scale and setting of the study differed (5 HIV uninfected participants that were recent contacts of TB in USA)[137]. In their study 4/5 participants were shown to have FDG uptake within the lymph node of which 3 responded to IPT. Lung parenchyma was not evaluated in detail as a low dose CT protocol was used[137]. They also showed a very strong positive correlation between QFGIT TBAg-NIL and SUVmax. In this study despite HIV infection as a considerable confounder, the proportion of those with abnormal uptake within mediastinal LN was less than 50% and only 4/27 (14.8%) showed response to TB treatment or IPT (and of interest there was a strong but non-significant correlation of QFGIT with SUVmax in LN for these participants). This difference between the studies is likely to be due to the recency of (likely primary) infection in the study by Ghesani et al compared to the remote infection of participants in this study. In the early stages of infection, trafficking to regional LN is clearly important and lymphadenopathy visible on CXR is common in children. Furthermore in non-human primate studies there is often considerable FDG uptake within mediastinal LN following infection[240]. As infection stabilizes, activity within LN may reduce.

Two participants had deterioration following IPT not easily attributable to alternative pathology. Large FDG avid nodules that developed were similar in both participant but different to the pattern of lesions seen in the initial scans. In one participant this was accompanied by evidence of a treatment response within the mediastinal lymph nodes. Neither of the participants developed symptoms or evidence of progression of these lesions.
One possible explanation for these lesions would be that they represent expansion and increased metabolic activity in micronodules not visible on the initial FDG-PET/CT following isoniazid monotherapy. Lin et al have used FDG-PET/CT to monitor lesion dynamics in response to INH, RIF or HRZE in non-human primates and showed that treatment with INH in contrast to other regimens frequently lead to increases in FDG uptake within lesions after 2 months treatment even within lesions that were sterile[140]. This may be due to an INH specific effect relating to its mechanism of action. Disruption of the cell wall with isoniazid has been shown to result in the release of antigenic material and isoniazid in contrast to rifampicin containing regimen leads to a boosting of mycobacteria specific immune responses during treatment, which may result in increased inflammation within a lesion[241, 242].

Evaluation of radiologist reads compared to CT as a gold standard were revealing. Agreement between the radiologists categorizing images into active TB, inactive TB and not consistent with TB was slight (κ=0.15) and this was particularly poor for inactive TB changes. There appeared to be 2 patterns of reading; over-reading of TB related abnormalities resulting in a high sensitivity/low specificity and under-reading of TB related abnormalities resulting in low sensitivity/high specificity. With regard to active TB abnormalities, sensitivity was generally poor while specificity was generally very good suggesting that readers in general under-read for active TB and over-read for inactive TB. Poor inter and intra observer variability had been described before, however, this is the first study in which reading of screening CXR have been compared to a gold standard of CT. Previous studies have show high levels of over reading and under reading when compared to other gold standards such as expert consensus or final clinical diagnosis[81].

One of the limitations of the approach taken in this study is that lesions considered to demonstrate evidence of activity relating to TB could not be confirmed as such. Observing progression to disease in participants with evidence of latent TB infection, known to be at high risk due to HIV infection would be ethically inappropriate especially as IPT is a recommended intervention. In this study repeat FDG-PET/CT at the end of treatment was used to demonstrate that lesions considered to relate to TB responded to treatment. However, loss to follow-up meant not all participants received a repeat scan (although treatment response was demonstrated in the 6/10 that had a repeat scan). In addition, as decisions on clinical management were often made independent of the study and in the
participant’s best interests, the administered interventions differed with regard to commencement of ART and standard TB therapy. Furthermore compliance with medication especially IPT could not be confirmed beyond questioning and pill count. An alternative approach would have been to focus on participants that were contacts of DR-TB where national and international recommendations are to observe rather than provide preventive treatment, however recruitment of such a cohort in the available timeframe would have presented challenges in our setting where case notification of DR-TB is approximately 50/100,000. Further microbiological proof of TB could have been sought by routine BAL of lesions but benefit to participants, when IPT was available, would have been minimal. Confirming pathological cause of discrete nodules is essentially not possible as invasive biopsy in this context is not appropriate or ethically justifiable hence aetiology can only be speculative.

It is clear that HIV itself can cause FDG uptake within the mediastinal LN, which has made interpretation activity relating to TB within the mediastinal LN more challenging. Performing such a study in HIV uninfected persons may have simplified interpretation however it is likely that sample size to identify adequate numbers with evidence of TB activity would have been considerably larger.

4.9 Conclusion

In this study for the first time FDG-PET/CT has been used to characterise pathological changes within the lungs in asymptomatic persons with evidence of sensitization by Mtb living in a setting of extremely high burden and with the additional risk factor of HIV. A group of 10 participants (28.6%) were identified that had evidence of reactivation and either bronchogenic spread (in the form of infiltrates and scars) or haematogenous spread in the form of active nodules. From within this group of 10 participants, 2 participants converted to culture positive, a further 2 developed TB symptoms and a further 3 had a pathological response to IPT on FDG-PET/CT. It seems reasonable to classify this group of participants as a high-risk group with subclinical disease in contrast to those without evidence of activity on FDG-PET/CT who may be considered at lower risk of progression. In subsequent chapters this categorisation of subclinical TB and low risk latent TB will be used to evaluate transcriptional and immunological biomarkers.
In addition this study has directly compared a screening CXR for TB to an imaging gold standard and demonstrated the extent of reader variability and the natural tendency of experienced readers either to over-read or under-read images.
CHAPTER 5 – Transcriptional profile

5.1 Chapter 5 introduction

The host response to active pulmonary tuberculosis has local as well as systemic manifestations. This response is influenced by cytokine and chemokine signals generated at the site of disease, which amongst other things lead to recruitment of cells from circulating blood into the lungs. Several studies have shown that the host response to active pulmonary tuberculosis results in a distinct pattern of transcript abundance within whole blood[243]. This is driven by altered gene expression within particular cell types but also in part reflects altered cellular proportions in circulating blood[164]. Components of this active pulmonary TB transcriptional signature overlap with other diseases while other components may be more disease specific[174]. The host response, and thus the transcriptional profile, might be expected to evolve as disease progresses and different pathological processes become prominent. For instance tissue destruction and repair may be a more prominent feature later in the disease process. As a result elements of the TB transcriptional profile maybe absent during the early, subclinical stages of disease. In addition the more minimal extent of pathology in subclinical disease might be expected to register a “weaker” blood transcriptional signature than advanced disease. In Southern Africa, the majority of TB disease is HIV associated and HIV co-infection might also be expected to affect the blood transcriptional profile. However, the effect of HIV on the TB transcriptional signature has not been closely investigated.

5.2 Chapter 5 hypothesis

HIV infected persons with evidence of subclinical tuberculosis on FDG-PET/CT will have components of an active TB transcriptional signature not present in those without evidence of subclinical tuberculosis on FDG-PET/CT.

5.3 Chapter 5 aims

To determine the differentially expressed transcripts in whole blood in those with and without subclinical TB and in comparison to an active TB control group and to evaluate the effects of HIV on the transcriptional response to TB.
5.3.1 Specific chapter 5 aims
1. To characterise a whole blood transcriptional signature for HIV associated active TB.
2. To explore the similarities and differences in the transcriptional signature of subclinical and active TB and to identify a list of transcripts that can distinguish those with and without subclinical TB (a subclinical TB signature).
3. To validate this subclinical TB signature in other groups of patients.
4. To determine the biological significance of the transcriptional signatures using pathway and modular analysis.
5. To determine the similarities and differences in transcriptional response between HIV infection, active TB and HIV associated active TB.

5.4 Chapter 5 methods
5.4.1 Quality control and raw data processing
The quality of extracted RNA was high both in terms of purity, as determined by 260/280 ratio and 260/230 ratio, and integrity as determined by RIN (see chapter 2 methods). Sample yield was suboptimal for only one sample (an active TB control), which was not included on the microarray (see figure 5.1). Primary analysis to evaluate the transcriptional response in subclinical TB was carried out on 50 samples, the 35 participants that underwent PET/CT and 15 age, sex and CD4 matched participants with active pulmonary tuberculosis. Additional analysis to evaluate the interaction of TB and HIV on the whole blood transcriptional response was carried out on 92 samples (using the additional participants outlined in section 5.5).

All samples fell within 3 s.d. of the mean for QC parameters (see chapter 2 methods and figure 5.2). Raw background subtracted expression data was pre-processed using GeneSpring GX version 12.6 (Agilent). For each sample, probes were assigned a signal intensity detection p-value in comparison to background hybridization and probes were assigned a “present” flag using a 0.99 cut off. Raw signal value <1 were then set to a threshold of 1 and all values were Log2 transformed. To minimise technical variation, each sample was then normalised using a 75th percentile shift algorithm in which the Log2 transformed intensity value corresponding to the 75th percentile was subtracted from Log2 transformed intensity value for each probe within a sample. Baseline transformation was then carried out to rescale intensity values to the median of all samples. Samples were visualised using a principal component analysis (PCA) plot to confirm no outliers. Five
samples fell between 2 s.d. and 3 s.d. from the mean for at least 1 QC parameter and these were also visualised on PCA plot to ensure they were not clustered. Following this probes that were not “present” in at least 10% of samples were then filtered out before further analysis. Of 47231 transcripts, 18919 transcripts remained after filtering for the 50 samples in the primary analysis and 18751 transcripts for the 92 samples in the additional analysis.

5.4.2 Microarray analysis

Statistical analysis of microarray and data visualisation were carried out in GeneSpring GX ver. 12.6 (Agilent). For supervised analysis statistical filtering was carried out by moderated t-test (for pairwise comparisons) and ANOVA with a posthoc Tukey honest significant difference (HSD) test (for group comparisons). Benjamini Hochberg false discovery rate (FDR) multiple testing correction was applied with a p(corr) cut-off=0.05. Where hierarchical clustering was used to visualise data, Pearson Uncentered (cosine) distance metric and average linkage rule was used. Pathway analysis was performed using Ingenuity Pathway Analysis software (Qiagen).
Figure 5.2 – Graphs of QC parameters for microarray.

Showing Intensity of biotinylated probes (top), Intensity of housekeeping genes (2nd graph), Intensity of the 95th percentile (3rd graph), Intensity of background “empty” probes (bottom) for all processed samples.
Module analysis and visualisation was performed using a Module Analysis Tools (for Illumina V2, V3 and V4) program (version 1/6/2011) provided by Damien Chaussabel at the Baylor Institute for Immunological Research. Modules, which are groups of co-regulated transcripts derived from multiple datasets whose function is inferred from unbiased literature search, were an updated version of those that have been used in a number of recent publications[164, 174, 244]. This program was used to generate module maps which allowed for visualization of the proportion of transcripts within a module that were significantly under or over abundant in comparison to a control group. The program was also used generate average transcript abundance values for modules which were then further analysed using Stata ver. 12.1. From the 260 modules included in the program, analysis was conducted on the 39 modules with an assigned function (which could be grouped into 18 functional categories). Data was log transformed where this facilitated the use of parametric tests or regression analysis and Bonferroni multiple testing correction was used to adjust p-value cut off to take into consideration the 18 functional categories in the analysis.

5.4.3 Clinical features of participants used in chapter 5

Of the 35 asymptomatic, ART naïve, HIV infected participants that underwent FDG-PET/CT the 10 participants considered to have evidence of disease activity on FDG-PET/CT (subclinical TB – see chapter 4) were the primary group of interest. The group of 25 participants with no evidence of disease activity on FDG-PET/CT (classified as “low-risk” latent TB – in this chapter they will be termed latent TB group) were considered as TB exposed, but disease free controls. The categorization of participants into a group with subclinical and latent TB was finalised prior to the analysis of microarray data. As a positive control, 15 HIV infected, ART naïve patients with culture or GeneXpert confirmed active pulmonary TB who were age, sex and CD4 count matched were included. Of the active TB controls 9/15 were sputum smear positive (3+; n= 6, scanty positive; n=3).

In order to interrogate the transcriptional differences between HIV infection and active TB more generally, samples from further control participants were included.

- 15 HIV uninfected participants with no symptoms of TB who were TST and QFGIT-ve
- 14 HIV uninfected participants with TB symptoms who were sputum culture or GeneXpert positive (10/14 smear positive (3+; n=4, 2+; n=3, 1+; n=2, scanty positive; n=1)
• 15 HIV infected, ART naïve participants with no symptoms of TB, who had no evidence of active TB on CXR and were Mtb culture negative if able to produce sputum sample and were TST and QFGIT-ve.

• 8 HIV infected participants, established on ART with a suppressed viral load and no TB symptoms, who had no evidence of active TB on CXR and were Mtb sputum culture negative but were QFGIT +ve.

It is acknowledged that the asymptomatic control groups differ by QFGIT status but for this analysis the comparison of active TB and HIV was of primary interest. In addition HIV uninfected participants were not matched for sex and age (although of similar ethnicity), this is acknowledged as a limitation (Chapter 3 - figure 3.9).

5.5 Results

5.5.1 Deriving a transcriptional signature for subclinical TB

Initially unbiased hierarchical clustering without statistical filtering was carried out on the 18919 transcripts to identify any strong patterns in the data but clustering of the participants by TB status was limited. Volcano plots of the 18919 transcripts were then used to visualise the differential abundant transcripts (in terms of significance and fold change (FC)) using a moderated t-test to make pairwise comparisons of active, subclinical and latent TB groups (figure 5.3). This demonstrated that a larger number of transcripts were differentially abundant between active and latent TB than active and subclinical TB and that a smaller number of transcripts were differentially abundant between subclinical and latent TB. Using Benjamini Hochberg FDR correction to control for potential false discoveries this pattern was maintained. However, this resulted in no transcripts being identified that were significantly differentially abundant (p(corr) < 0.05) between subclinical and latent TB, although 2469 transcripts had ≥1.25 fold and 675 transcripts had ≥1.5 fold difference in expression values between subclinical and latent TB.

In order to identify biologically relevant transcripts that were unlikely to be false discoveries that differentiated subclinical and latent TB, and to identify the biological processes that were similar between active and subclinical tuberculosis the 3 groups were analysed together. The aim of the analysis was to identify transcripts that clustered participants with active and subclinical TB but differentiated these 2 groups from participants with latent TB. First, transcripts that were differentially abundant between the different stages of TB were defined by statistically filtering of the 18919 transcripts by ANOVA using Benjamini Hochberg
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FDR multiple testing correction with \( p(\text{corr})=0.05 \) as a cut off. This generated 2274 transcripts if 3 groups (active, subclinical and latent) were defined and 2573 transcripts if 4 groups were defined (smear positive active, smear negative active, subclinical and latent). The larger group of transcripts was favoured, as it may have contained biologically important transcripts distinguishing different stages of disease. The 2573 transcripts were then filtered for those with at least a 1.5 fold difference in expression between active and latent TB to define a list of 893 transcripts considered biologically relevant to active TB. Performing hierarchical clustering on this group of 893 transcripts demonstrated complete clustering of active TB away from latent TB and 2/10 with subclinical TB clustering with active TB (figure 5.4).

Clustering of subclinical TB with active TB may have been prevented by transcripts related to later stages of disease and that expression of genes relevant to both active and subclinical TB may be expressed in blood at a lower level in the early stages of disease. Therefore, the 893 transcripts were then filtered to identify which transcripts were also concordantly differentially abundant between subclinical and latent TB using increasingly stringent fold change cut offs. This was achieved by first filtering the 2573 transcripts to define transcripts

![Volcano plots for 18919 transcripts demonstrating differentially abundant transcripts:](image-url)

Figure 5.3 – Volcano plots for 18919 transcripts demonstrating differentially abundant transcripts: Differentially abundant transcripts are shown in red as having \( p \text{-value} \leq 0.05 \) and fold change \( \geq 1.5 \) between active TB vs. “low risk” latent TB (left column), active TB vs. subclinical TB (middle column) and subclinical TB vs. “low risk” latent TB. In the top row, \( p \)-value is uncorrected and in the bottom row \( p \)-value is corrected by Benjamini Hochberg (FDR).
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Figure 5.4 – Heatmaps showing hierarchical clustering of individual participants for the 893, 639, 203 and 82 transcript lists. Active TB (Red), Latent TB (Blue), Subclinical TB (Orange – Upper lobe reactivation, Pink – miliary pattern)
that were at least 1 fold, 1.25 fold or 1.5 fold over and under abundant between subclinical and latent TB and then identifying the common over and under abundant transcripts in the 893 transcripts list. Filtering the 893 transcripts, which differentiated active and latent TB, in this sequential manner, resulted in stepwise improvement in clustering of subclinical with active TB while maintaining good differentiation from latent TB (figures 5.4 and 5.5).

Filtering at ≥1 FC reduced transcripts to 639 and increased clustering of subclinical TB with active TB to 4 out of 10. Further filtering at ≥1.25 FC reduced transcripts to 203 and improved clustering of subclinical with active TB to 6 of 10 and 3 of 25 with latent clustered with active TB. Finally filtering at ≥1.5 FC differential abundance between subclinical and latent TB reduced transcripts to 82 and increased clustering of subclinical with active TB to 8/10 and latent with active TB to 4/25 (figure 5.4). Of note the participant with the miliary pattern of disease did not cluster with active TB and the only participant with an upper lobe reactivation pattern not to cluster with active TB had the smallest infiltrate with only slight
FDG uptake (Chapter 4- figure 4.5D). The 4 participants categorized as latent but clustering with active, were not distinguished by in any way with regard to HIV VL, CD4 count, QFGIT or lung or mediastinal LN abnormalities. One of the 4 had an FDG avid breast lesion, which at biopsy was determined to be benign, xanthalamatous, granulomatous inflammation.

Next, in order to confirm that these transcripts effectively distinguished subclinical and latent tuberculosis in the absence of active TB influencing hierarchical clustering, PCA was performed on the subclinical and latent group alone using the 893, 639, 203 and 82-transcripts. Even with the 893 transcripts, derived as a signature of active TB, the tendency for subclinical and latent TB to cluster separately was apparent, albeit with considerable overlap. Clustering improved as transcript numbers reduced and separation of subclinical and latent was very clear using the 82-transcripts (figure 5.5).

To further investigate the effect of FDG uptake on this signature, participants with subclinical TB were classified into 5 participants showing intense FDG uptake (VS >3) within lung parenchyma or lymph nodes considered to be related to TB and 5 participants who did not show intense FDG uptake on PET/CT (Visual score 0-2) (chapter 4 - table 4.2).

Performing group-wise hierarchical clustering using the 82-transcripts for these 4 groups of participants (latent, active, subclinical (low FDG uptake) and subclinical (high FDG uptake)) demonstrated that the group of subclinical participants with high uptake clustered more closely with active TB participants than those with low FDG uptake. PCA analysis also confirmed that those with greater metabolic activity within regions of interest were more distinct from those with latent TB (figure 5.6)
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Figure 5.7 – Hierarchical clustering of HIV uninfected controls with and without active TB using 82 transcripts (left) and 203 transcripts (right):
Active TB (red), No TB (black)

Figure 5.8 – Hierarchical clustering using the 82 transcripts and 203 transcripts on a dataset participants with TB and other diseases from Bloom et al [1].
Active TB (red), Active sarcoid (orange), Inactive sarcoid (yellow), Pneumonia (green), Lung cancer (blue), Healthy controls (black)
5.5.1.1 Validation of signature in other participants

The 82-transcripts, and to a lesser extent 203-transcripts, derived from the 893 transcript signature for active TB were effective at distinguishing subclinical TB particularly in those with evidence of upper lobe reactivation. To confirm that these transcripts were able to identify TB independently of HIV they were applied to the 14 HIV uninfected controls with active pulmonary TB and the 15 that were HIV uninfected and TST/IGRA negative. Hierarchical clustering demonstrated that both the 82 and 203 transcript signatures were effective at distinguishing HIV uninfected persons with and without TB. Only 1 participant with active TB and 1 with no TB clustering incorrectly with the 82 transcripts and 2 participants with active TB and 1 with no TB clustering incorrectly with the 203 transcripts (figure 5.7). To establish if these transcripts were specific to TB in comparison with other diseases. A dataset of normalised expression values from HIV uninfected participants with TB and a number of control diseases from the Bloom et al study (2013) was downloaded from Gene Expression Omnibus (GEO - http://www.ncbi.nlm.nih.gov/geo/) [174]. The participant diagnoses in this dataset were active TB (n=11), pneumonia (n=6), lung cancer (n=8), inactive sarcoid (n=9), active sarcoid (n=16) and healthy controls (n=52). Performing hierarchical clustering with the 82 and 203 transcripts, both were effective at clustering TB from other diseases, although active sarcoidosis had a tendency to cluster with active TB particularly with the 82 transcripts (figure 5.8). The 203 transcripts performed particularly well with 10/11 participants with active TB clustering together with only 3/16 with active sarcoid and 1/8 with lung cancer.

5.5.1.2 Comparison to other signatures

The 203 and 82 transcript signatures that were found to be more specific to subclinical TB were compared to 3 other available active TB transcript signatures to assess the proportion of transcripts that were in common (393 transcript signature from Berry et al 2010[164], 664 transcript signature from Bloom et al 2012[165], 1446 transcript signature from Bloom et al 2013[174]). 34% of transcripts from the 203 transcript list and 27% of transcripts from the 82 transcript list were present in at least one of the other published TB signature (figure 5.9). 14% of transcripts in the 203 transcript list and 10% of transcripts in the 82 transcript list were present in all of the TB signatures. Kaforou et al have recently derived a considerably smaller list of 27 transcripts sensitive for the detection of TB in a diverse population of mixed HIV status[171]. 37% of these transcripts were present in the 203 transcript list and 7% in the 82 transcript list.
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Fig 5.9 – Common transcripts between the 203 and 82 transcript signatures with 3 other published active TB signatures. Berry et al (Nature 2010) 393 transcript signature (only 387 transcripts compared due to microarray platform differences), Bloom et al (PLOS ONE 2012) 664 transcript signature, Bloom et al (PLOS ONE 2013) 1446 transcript. Common transcripts outlined in yellow.
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#### Figure 5.10

Module map showing significantly over and underabundant transcripts in active and subclinical TB compared to latent TB.

Red dots represent significant overexpression of module and blue dots represent significant underexpression of module compared to control group (latent TB). Intensity corresponds to percentage of transcripts significantly under or over expressed within a module compared to control group.

<table>
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<th>Percentage of transcripts in modules significantly underexpressed (p&lt;0.05)</th>
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<th>Smear -ve</th>
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<td>M4.11 Plasma Cells</td>
<td>M4.12 Monocytes</td>
<td>M1.1 Platelets</td>
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<td>M3.1 Erythrocytes</td>
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<td>M6.2 Mitochondrial Respiration</td>
<td>M6.12 Mitochondrial Stress</td>
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5.5.1.3 Biological processes represented in the subclinical TB signature

First, in order to gain a visual overview of how the functional components of the transcriptional response varied at the different stages of HIV associated tuberculosis, a modular analysis of the 18919 transcripts (significantly detected compared to background hybridization in at least 10% of the 50 samples) was undertaken using the “low risk” latent TB as the control group. Those with 3+ smear positive active TB had the most significant differences in modular expression when compared to latent TB. A significant decrease in abundance of B cell and T cell transcripts was apparent as well as increased abundance of transcripts in interferon, inflammation, cytotoxic, cell death, dendritic cells/apoptosis and neutrophil modules. This pattern of modular response is very similar to that previously described in tuberculosis[174]. This modular signature was much less obvious in participants with scanty smear positive and smear negative tuberculosis and in subclinical TB, few modules had significant differences in modular expression when compared to the latent TB controls (figure 5.10).

IPA Ingenuity software was then used to further explore which biological processes were common to and which distinguished between subclinical TB and later stages of tuberculosis. Analysis was performed on the 4 transcript signatures (893, 639, 203 and 82), which were progressively more specific to subclinical TB, to identify pathways which were significantly over represented in these transcripts. The complement system was the most significantly over represent pathway for over abundant transcripts all 4 signature (figure 5.11a). The components of the complement system showing overabundance related to the classical pathway, which is most commonly initiated by C1q binding of the Fc portion of antibodies in immune complexes. Complement component 1, q subcomponent, B chain (C1QB), complement component 2 (C2), complement component 5 (C5) and serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (SERPING1) all showed increased expression in those with subclinical TB and active TB compared with latent TB (figure 5.12). Acute phase response signalling also remained prominent but represented a number of pathways with complement components also forming part of this. Of note transcripts relating to the Fc fragment of IgG, high affinity Ia/b/c receptor (CD64) (FCGR1A/B/C) were also over abundant in subclinical and later stages of disease compared with latent TB, being present on all 4 transcript lists. FCGR1A, FCGR1B and FCGR1C were all over abundant in subclinical compared to latent TB (+1.36FC, +1.38FC and +1.58FC respectively) and in active compared
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893 Transcripts

- Complement System
- Interferon Signaling
- Salvage Pathways of Pyrimidine Deoxynucleotides
- Role of Jak family kinases in IL-6-type Cytokine Signaling
- Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses
- Role of Jak1 and Jak3 in IL-1 Cytokine Signaling

639 Transcripts

- Complement System
- Interferon Signaling
- Salvage Pathways of Pyrimidine Deoxynucleotides
- Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses
- Role of Jak family kinases in IL-6-type Cytokine Signaling
- Role of Jak1 and Jak3 in IL-1 Cytokine Signaling

203 Transcripts

- Complement System
- Acute Phase Response Signaling
- Salvage Pathways of Pyrimidine Ribonucleotides
- Systemic Lupus Erythematosus Signaling
- Role of CHK Proteins in Cell Cycle Checkpoint Control
- ATM Signaling

82 Transcripts

- Complement System
- Granulocyte Adhesion and Diapedesis
- Acute Phase Response Signaling
- Agranulocyte Adhesion and Diapedesis

Figure 5.11a – IPA ingenuity pathway analysis for transcripts overabundant in subclinical TB compared to latent TB. Maximum of top 6 pathways showing significance (p≤0.05 (log(p-value)>1.30), Fisher’s exact) with at least 2 transcripts in pathway. Blue bar = –log(p-value), yellow squares = ratio (proportion of pathway overabundant)
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893 Transcripts

639 Transcripts

203 Transcripts

82 Transcripts

Figure 5.11b – IPA ingenuity pathway analysis for transcripts underabundant in subclinical TB compared to latent TB. Maximum of top 6 pathways showing significance (p<0.05 (log(p-value)>1.30), Fisher’s exact) with at least 2 transcripts in pathway. Blue bar = –log(p-value), yellow squares = ratio (proportion of pathway underabundant).
Figure 5.12 – Complement pathway showing components overabundant in subclinical and active compared to latent TB.
Overabundant components (pink), FC active vs. latent (red text) and FC subclinical vs. latent (brown text)
to latent TB (+2.66FC, +2.32FC and +2.67FC respectively). As Fcγ receptor 1 is found on the surface of cells and binds the Fc portion of antibodies in immune complexes this again suggested the potential significance of immune complexes in subclinical and active TB. The only cytokines and chemokines that were overabundant in subclinical and active TB were CXCL17 and CCL23, which are both known to relate to pulmonary pathology. Interferon signalling pathways were prominent in the 893 and 639 transcript lists but not in the 203 list of transcripts which showed at least 1.25 fold change between latent and subclinical TB.

To evaluate the biological processes that may have been more specific to later stages of disease and less relevant in subclinical TB, the 254 transcripts that were initially filtered from the 893 TB signature were examined in greater detail. The 6 filtered transcripts with the greatest overabundance in active TB all related to neutrophils; human neutrophil peptide 4 (α-defensin 4(DEFA4)), DEFA1, neutrophil expressed elastase (ELANE), cathepsin G (CSTG), carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) and CEACAM8. To further evaluate this, the modular composition of the 893 and 639 transcripts was assessed. Although the latter list of transcripts represented 71.5% of the former, the reduction was not equal across the modules (figure 5.13). A significantly smaller proportion of transcripts in the 639 list were neutrophil and platelet related compared to the 893 list (p=0.011 and p=0.0001 respectively). 54% of transcripts in the neutrophil related module (M5.15) and 24% in the platelet related module were present in the 893 transcript list whereas in the 639 transcript list only 4% and 1% respectively were present.

To evaluate whether the difference in neutrophil and platelet modules could have related to differences in cell counts between the groups, the relationship between cell counts and modular expression values was explored for participants and controls where these results were available (platelet count n=38, neutrophil count n=26). There was a highly significant positive correlation between platelet count and expression of the platelet module (M1.1), r = 0.51 (p=0.0009) suggesting that in part differences in platelet module expression between active and subclinical TB might be explained by differences in platelet count. However, there was no significant correlation between percentage of white blood cells that were neutrophils (nor for absolute neutrophil count) and expression of the neutrophil module, r = 0.11 (p=0.6), suggesting that differences in neutrophil module expression between active and subclinical TB were unlikely to be relate to simple differences in neutrophil count but rather genes being expressed by these cells. In order to further evaluate if the abundance of neutrophil related transcripts related to the stage of disease, the expression of the 21
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Fig 5.13 – Expression of neutrophil and platelet modules and correlation with cell counts.

(Top) Graph showing the difference in modular distribution for the 839 and 639 transcript lists.

(Middle) Showing correlation of proportion of neutrophils in white blood cells (WBC) with expression in neutrophil module (n=26).

(Bottom) Showing correlation of platelets count with expression in platelet module (n=38).
transcripts of the neutrophil module (M5.15) that were present in the 18919 transcript list was compared for different stages of TB disease. Expression in the neutrophil module was significantly higher in smear positive compared to smear negative TB (p≤0.001) (with expression of all 21 transcripts being greater in smear positive disease) and also smear negative compared to subclinical TB (p≤0.001). However, there was no significant difference in expression of the neutrophil module between subclinical TB and latent TB.

IPA Pathway analysis of the 436 transcripts filtered from the 639 transcripts to generate the 203 transcript list demonstrated that the most prominent pathway lost related to Interferon signalling. However, expression within the interferon modules was still present in the 203 list of transcripts with 8.3% of transcripts forming part of one of the 3 interferon modules. This suggested that while there was differential expression in interferon induced transcripts between subclinical and latent TB it may not have been a may contribute to discriminating these conditions.

5.5.2 HIV TB interaction in whole blood gene expression

Genes related to interferon signalling have previously been demonstrated to be prominently overabundant in active TB. However, being an intracellular viral infection, HIV also induces an interferon response and so interaction between TB and HIV on the whole blood transcriptional signature was further assessed. A modular analysis was performed on 92 HIV infected and uninfected participants with and without active TB. Participants with subclinical TB were excluded from the analysis so the independent effects of HIV and TB on transcriptional response could be assessed. HIV uninfected participants with no evidence of active TB were used as the control group. The 18751 transcripts that were significantly detected compared to background hybridization in at least 10% of the 92 samples were used for the modular analysis (table 5.1, fig 5.14, fig 5.15). In comparison to healthy HIV uninfected controls (n=15) those with HIV but no evidence of TB disease activity (n=40), exhibited significantly lower abundance in the T cell related module after Bonferroni correction for multiple comparisons (p=0.0019). This may have in part related to the reduced circulating CD4 cells in the HIV infected participants. However they had significantly higher abundance in transcripts of interferon related modules (p≤0.0002) as well as modules relating to cell cycle (p=0.0021), cytotoxic/NK cells (p=0.0022) and immune responses (p=0.0001). Transcript abundance in all these modules normalised in HIV infected participants taking ART (n=8) with suppressed viral load but comparable CD4 count. HIV infected participants on ART only had reduced abundance in a mitochondrial respiration
	  

	  

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Chapter	  5	  –	  Transcriptional	  Profile	  

	  

Table	  5.1.	  Median	  modular	  abundance	  values	  by	  clinical	  group:	  	  Values	  significantly	  different	  from	  
controls	  after	  multiple	  testing	  correction	  (p≤0.0027)	  stared	  and	  marked	  in	  red	  	  	  	  	  

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Figure 5.14. Module map showing significantly over and underabundant transcripts in HIV, TB and HIV/TB participants compared to health HIV uninfected controls.
module in comparison to HIV uninfected controls (p=0.0019), although sample size was small for this group. Those that were HIV uninfected with active TB (n=14) also had significantly lower transcript abundance in the T cell related module in comparison to healthy HIV uninfected controls (p=0.0009) and also had reduced abundance of transcripts in B cell related modules (p≤0.0002) and lymphocyte activation (p=0.0026). Transcript abundance in the interferon related modules (M3.4 and M5.12) were also significantly increased (p≤0.0009), as were transcripts in modules relating to inflammation (M4.2, M5.1 and M7.22) (p≤0.002), neutrophils (p=0.0019), dendritic cells/apoptosis (p=0.0009) and cell death (p=0.0012). In HIV infected participants with TB, the modular pattern had features of both HIV infection and TB infection alone. Notably transcript abundance in the interferon modules was greater than in TB or HIV alone but lower in inflammation and neutrophil modules in comparison to TB alone and not significantly different to abundance HIV uninfected controls without active TB. However, transcript abundance in cell cycle related modules was greater in HIV associated TB than HIV alone. These differences in patterns of modular transcript abundance are summarised for individual participants in a module map (figure 5.14) and for the key modules in the main participant groups in a radar plot (figure 5.15)
Expression in interferon, inflammation and cell cycle modules was more closely evaluated to gain greater insight into of TB/HIV interaction. To explore the effect of TB and HIV on inflammation modules, 2-way ANOVA was performed on the average expression of inflammation modules (log transformed to normalise). Inflammation was significantly increased by active TB (p<0.0001) but significantly reduced by HIV infection (p=0.03), there was no significant interaction between HIV and TB. The distribution of smear status differed between HIV infected and uninfected participants with a greater degree of smear positivity in HIV uninfected participants (see section 5.5). Average transcript abundance in the inflammation modules was significantly affected by smear status (p=0.01), however, stratifying by smear status showed that the average expression in the inflammation modules was consistently lower in HIV-associated TB irrespective of smear status (figure 5.16 - A). Therefore, inflammation in HIV associated TB was affected by the distribution of smear status and by HIV infection itself.

Transcript abundance in cell cycle modules was higher in HIV associated TB than HIV alone, however, viral load was also elevated in active TB (as expected despite CD4 being well-matched). Analysing the correlation between viral load and cell cycle expression, a single point was > 3 s.d. from the mean and considered a potential outlier, correlation (log transformed average cell cycle expression) with outlier was r=0.35 (p=0.009) and without r=0.53 (p<0.0001) (fig. 5.16 - B). Linear regression gave an adjusted $r^2=0.27$ suggesting 27% of variance in expression being explained by viral load. Adding TB to this regression model showed there was no independent effect of TB (p=0.20) and this had little impact on the adjusted $r^2$ ($r^2=0.28$) confirming that the increased cell cycle modular abundance in HIV associated TB appear to relate to increased viral load.
Fig 5.17. Expression In 3 interferon modules in TB and HIV: (left column) box plot showing expression in those with active TB and those without active TB by HIV status. (right column) scatter plot of expression in module against Log10 VL.
Transcript abundance in the 3 interferon modules (M1.2, M3.4 and M5.12) was more complex in HIV associated TB. Both HIV and TB were independently associated with increased interferon expression and HIV associated TB had the highest expression in all modules (table 5.1, figure 5.15). To establish if active TB itself or increased viral load as a result of active TB was responsible for elevated transcript abundance, multiple regression was carried out with Log10 HIV VL and the presence of active TB as the explanatory variable for Log10 expression in the 3 interferon modules. For module M1.2, HIV VL alone accounted for 32% of variance in expression (adjusted $r^2=0.317$) and addition of TB into the multiple regression model had minimal effect, adjusted $r^2$ to 0.314, the regression model showed the effect of viral load on expression was significant (p<0.0001) whereas the effect of TB was not (p=0.393). By contrast for the M5.12 interferon module, HIV VL alone accounted for 18% of variance in expression (adjusted $r^2=0.178$) whereas the addition of TB to the multiple regression model had a large effect, increasing the adjusted $r^2$ to 0.392, the regression model showed the effect of viral load on M5.12 expression was not significant (p=0.054) while effect of TB was highly significant (p<0.0001). For module M3.4, HIV VL alone accounted for 26% of variance in expression in M3.4 (adjusted $r^2=0.262$) whereas the addition of TB to the multiple regression model had a large effect, increasing adjusted $r^2$ to 0.453. In the multiple regression model the effect of both viral load and TB on M3.4 modular expression was significant (p=0.005 and p<0.0001, respectively). This analysis

Fig 5.18. IPA interferon signalling pathway. Transcripts in the different interferon modules highlighted
suggested that in HIV associated TB, HIV may have a stronger effect on transcript abundance in interferon module M1.2 where as TB may have a stronger effect on transcripts in modules M3.4 and M5.12. Although, there is known to be considerable overlap of genes induced by type 1 and type 2 interferon, IPA Pathway analysis of the transcripts in the 3 interferon modules revealed that while genes represented in M3.4 and M5.12 modules formed a part of both type 1 (interferon α/β) and type 2 interferon (interferon γ) signalling genes, transcripts in the M1.2 module appeared more associated with type 1 interferon signalling (figure 5.18). To further evaluate if the M1.2 module was more comprised of transcripts related to type 1 interferon stimulation, transcripts from the 3 interferon modules were compared a published list of transcripts found to be more specifically induced by IFNα than IFNγ (genes that were induced by IFNα at least 3-fold over IFNγ after 2.5 hour stimulation of bone marrow derived macrophages in Liu et al [245]). This also demonstrated that significantly more transcripts in the M1.2 list were more specifically induced by IFNα in comparison to M3.4 (p<0.0005) and M5.12 (p<0.0005). This suggests that in the context of HIV associated TB disease, HIV replication contributes more specifically to the type 1 interferon response where as active TB may induce both type 2 and type 1 interferon responses.

5.6 Summary of main findings

This chapter demonstrates that a proportion of transcripts that form part of an active TB whole blood signature can effectively distinguish HIV infected persons with and without subclinical TB pathology determined by FDG-PET/CT. Using 82 transcripts, 8 of 9 persons with an evidence of an upper lobe reactivation pattern of subclinical disease cluster with active TB. Those with regions of presumed tuberculous pathology showing greater metabolic activity by FDG uptake cluster more strongly with active TB. The transcript signature for subclinical TB remains discriminatory for active TB, even in HIV uninfected participants and is relatively specific for TB in comparison to pneumonia, lung cancer, inactive sarcoidosis and to a lesser extent active sarcoidosis.

Overabundance of components of the classical complement pathway compared to latent TB remains prominent in subclinical disease however an overabundance of neutrophil related transcripts appears to relate more to later stages of active TB. Interferon induced transcripts are overabundant in TB alone and HIV alone with further increased expression in
HIV associated TB although the effect of HIV may be more prominent on type 1 interferon induce transcripts.

5.7 Discussion

Analysis in this chapter has shown that a proportion of transcripts within a whole blood gene expression signature for active TB can be used to distinguish groups of asymptomatic HIV infected individuals with and without parenchymal pathology consistent with subclinical TB. If generalizable and validated such a signature might identify those with at higher risk of developing TB. In addition the data presented has shown that there are common features of the independent effects of HIV infection and TB disease on whole blood transcript abundance and that in co-disease and these pathogens have an interactive effect on whole blood transcript abundance.

The 639, 203 and 82 signatures identified transcripts from the 893-transcript active TB signature that were also concordantly differently abundant in those with subclinical pathology compared to those without subclinical pathology, with stepwise increasing fold change. As they were derived from an active TB signature and those with subclinical pathology clustered with active TB they were unlikely to be “falsely discovered”. The 82 transcripts most effectively distinguished those with latent TB with and without subclinical pathology. Of the 2 participants with subclinical pathology that did not cluster with active TB, 1 participant had a miliary pattern of haematogenous disease spread and the other had the smallest of the infiltrates, with minimal FDG uptake. All 8 participants that clustered with active TB had evidence of upper lobe reactivation. Of note all 3 participants with fibrotic scarring without pulmonary infiltrates clustered with active TB. It is possible therefore that these 82 transcripts relate to a particular disease presentation that reflects risk of bronchogenic reactivation and may be insensitive to haematogenous spread of disease although a much larger study would be needed to investigate this hypothesis. No previous study has investigated differences in the transcriptional response between pulmonary and miliary TB. Of note those with greater FDG uptake at sites of pathology consistent with TB clustered more strongly with active TB than those with lower FDG uptake, demonstrating a positive association between transcript abundance and the extent of disease activity. The 4 of 25 participants with latent TB who clustered with active disease did not retrospectively have any unusual features and may reflect either participants at risk of progression (and since IPT was administered this would be less likely to develop in follow-
up) or could just reflect a reduced specificity of the signature. However, in the context of a diagnostic signature that might categorise a high risk group of latent TB for prioritized treatment, such a degree of misclassification would still substantially improve the numbers needed to treat to prevent a case of symptomatic active TB.

Applying these transcripts to existing cohorts of participants with a number of other diseases, the 82-transcripts performed well at clustering active TB from other diseases except active sarcoidosis. This is unsurprising given the known biological similarities between these granulomatous diseases and has been noted in a number of previous studies[172, 174]. The implication of this in terms any diagnostic potential for such a signature will relate to the pre-test probability of TB and sarcoidosis in the population of interest. If used specifically in those at high risk of TB as would be likely, the impact of a rare disease such as sarcoidosis on positive predictive value would be minimal.

In terms of biological processes represented by a whole blood transcriptional signature, it must be acknowledged that the impact of a localised disease process on whole blood transcript abundance is complex. The collection of cells at the site of disease release cytokines and chemokines, which can alter gene expression in circulating cells within the pulmonary vasculature. These cells may then be return to the systemic circulation following recirculation via the left atrium and alter the whole blood transcriptional profile. However, these soluble factors may also lead to the recruitment of leukocytes and their depletion from the circulation might reflect as an under abundance of certain transcripts in a whole blood transcript signature. In addition some cells may be able to recirculate from the site of disease back into the systemic circulation either via the lymphatic system or reverse transmigration.

The complement pathway was the dominant pathway for overabundant transcripts in the 893, 693, 203 and 82 transcript list suggesting activation of this pathway may occur from the earliest subclinical stages of TB. In particular, complement factors relating to the classical complement pathway were differentially overabundant abundant in those with subclinical pathology in comparison to those without (SERPING1 (+1.89FC), C1q(+1.54FC), C2(+1.30FC), C5(+1.28FC)). Transcripts relating to classical complement pathways have consistently been identified as overabundant in active compared to latent TB in a number of studies[164, 165, 171, 174, 246]. In addition recently Cliff et al have showed that a number of transcripts
related to complement pathway are substantially down regulated within the first week of treatment for active TB\cite{169}. This suggests that the abundance of transcripts relating complement pathway may be a marker of bacillary load rising early in disease and this falling rapidly with treatment. The classical complement pathway is initiated most commonly by binding of C1q to the Fc\gamma component of immunoglobulin on immune complexes (although direct binding of C1q by mycobacteria has been reported\cite{247}). This results in activation of the complement cascade, which then promotes opsonisation (through C3b binding to microbial surface), acute inflammatory response and neutrophil recruitment (through C3a and C5a production) and cytolysis (via formation of the membrane attack complex). Ex vivo studies have shown that while Mtb predominantly activates complement via the alternative pathway in serum (binding C3b following spontaneously hydrolysis of C3), in lung BAL fluid, where the concentration of complement components is lower, activation via the classical pathway predominates\cite{248}.

Although the majority of complement factors are produced by hepatocytes within the liver, myeloid cells, such as, tissue-resident macrophages and dendritic cells are major extra-hepatic producers of complement, in particular C1q\cite{249}. It has been demonstrated that while monocytes express C1q mRNA, the protein is not present until differentiation into a macrophage\cite{250}. This extra-hepatic production is thought to allow rapid, localised complement synthesis and activation following myeloid cells recruitment to infected tissue where concentration of complement proteins might otherwise be low. Expression of C1q has been shown to be induced by IFN\gamma\cite{250}. In addition to secreting C1q, macrophages have been shown to have membrane bound C1q (mC1q), which may directly facilitate phagocytosis\cite{250}.

The complement system is a central pillar of the innate immune response. However, recently its role in modulating the adaptive immune response has been increasingly recognised. Recently C1q has been shown to be deposited by cells in the surrounding extracellular matrix\cite{251}. The implications of this are still being established but in in vitro studies, immobilized C1q has been demonstrated promote maturation of dendritic cells enhancing their production of IL-12 and TNF\alpha and their capacity to stimulate T cells\cite{252}. Other studies have shown that immobilized C1q can have an immunomodulatory effect on T cells by reducing their IL-4 production and enhancing IL-10 production\cite{251}. Further more
C1q plays a role in the removal of apoptotic cells by direct binding to cells and promotion of C3 binding to cells both of which facilitate opsonisation[251]. The pro-inflammatory effects of complement activation can lead to significant tissue damage and hence it is regulated by a number of inhibitors. SERPING1 is one such protease inhibitor that prevents activation of complement by irreversibly binding to and inactivating the C1r and C1s proteases of the C1 complex. SERPING1 expression is also induced by IFNγ and has frequently been shown to be overabundant in active TB[164, 165, 174]. Recently it has been speculated that dysregulation of complement activation and altered balance of SERPING1 and C1q expression plays a part in pathogenesis of TB-IRIS[253].

Consistent with the significance of the classical complement pathway, stimulatory FcγR1 was also overabundant in those with subclinical pathology compared to those without (FCGR1C (+1.58FC), FCGR1B(+1.38FC), FCGR1A(+1.36FC)) again suggesting that immune complex formation may occur in the earliest subclinical stages of tuberculosis. Transcripts relating to FcγR1 have been shown to be overabundant in TB in a number of studies[164, 165, 168, 171, 174]. In addition numerous studies report increased circulating immune complexes in active TB[254].

Whereas complement activation appeared to occur from the early stages of disease reactivation, neutrophil activation appears to be specific to later stages of disease with expression increasing as disease advances. While there was no significant difference in abundance of transcripts in the neutrophil module between latent TB and subclinical TB, abundance was significantly greater between subclinical TB and smear negative active TB, and between those with smear negative and smear positive disease. The overabundance of neutrophil related transcripts seemed unlikely to relate to differences in neutrophil count as there was poor correlation between proportion of WBC that were neutrophils and expression of neutrophil module. Although characteristically tuberculous granuloma are composed primarily of macrophages and lymphocytes, neutrophils appear to increase in abundance locally as disease progresses. Eum et al have shown that neutrophils were more abundant than macrophages within the sputum, BAL fluid and cavities (removed at surgery) of predominantly smear positive patients[255]. While Condos et al and Dhand et al have shown that the proportion of neutrophils in BAL is greater in more advanced TB[256, 257]. A critical stage in reactivation and progression of tuberculosis appears to be failure of the granuloma to contain Mtb, which leads to the development of TB pneumonia[101]. It has
been suggested that the failure of T cell immunity or loss of responsiveness to IFNγ may lead to increased neutrophil recruitment to the site of disease[258]. Once at the site of disease, activated neutrophils are thought to be relatively short lived and may contribute to further inflammation, tissue damage and ongoing neutrophil recruitment especially if apoptotic neutrophils are not removed by macrophages and their secondary necrosis leads to release of cytotoxic contents. Neutrophils within the blood may be particularly susceptible to having their gene expression altered by locally secreted cytokines from the sites of disease within the lung. The lung vasculature has been shown to be enriched for mature neutrophils even in the absence of disease and intravascular transit time for neutrophils within the lung is much greater than for other organs. Direct observation of neutrophil trafficking has shown that neutrophils appear to be “crawling” round the pulmonary vasculature even in the normal state[259]. In addition it has been shown that it is possible for recruited neutrophils to reverse transmigrate back to the blood further contributing to the whole blood signature[259].

Transcripts induced by interferon were shown to be overabundant independently in active TB and HIV infection. Abundance in HIV-associated active TB was greater than in either disease individually. This was in part contributed to by the increased HIV viral load seen in active TB. Of interest, the analysis showed that the dominant effect of HIV and TB appears to be on different modules of co-regulated interferon related transcripts with HIV having a more prominent effect on type 1 interferon specific transcripts. While the prominence of interferon induced genes in the transcriptional response in blood to TB and HIV independently has been demonstrated previously, this is the first study to look at the effect of co-infection on expression of interferon stimulated genes[101, 260].

Although type 2 interferon (IFNγ) is critical to the host response to Mtb the role and induction of type 1 interferon in Mtb infection is less well understood. Mtb clearly induce a type 1 interferon response, this is thought to be via the secretory system ESX-1, a key virulence factor for the phagolysosome bound Mtb, which allows bacterial products into the cytosol[261]. There is some evidence that mycobacterial peptidoglycan secreted into the cytosol interact with NOD2 (an intracellular pattern recognition receptor) to induce a type 1 interferon response[262]. However, the type 1 interferon response following TB infection, rather than being protective, has been shown to result in a deleterious outcome in mice[263]. It has been demonstrated that this negative impact of type 1 IFN on control of
Mtb may in part relate to suppression of the pro-inflammatory cytokines IL-1α and IL-1β which may be in part mediated by the induction of IL-10 by type 1 interferon[264]. Recently this group has also shown that IL-1 induction of eicosanoids may limit excessive type I IFN production, promoting containment of Mtb by the host[265]. In addition, it has been shown that the observed detrimental effect of prior infection with influenza A on control of Mtb in mice, is dependent on a type 1 interferon response[71].

The type 1 interferon response is a key part of the host antiviral response and acute and chronic HIV infection have been demonstrated to induce a type 1 interferon response[266]. Plasmacytoid dendritic cells have been shown to be the major source of interferon α/β production in HIV, despite forming a small proportion of circulating blood cells[266]. In addition overabundance of interferon stimulated genes has been shown to be positively correlated with plasma viral load, as has been confirmed in this study[260]. While type 1 interferon has been shown to restrict viral replication it is also thought to promote CD4 cell depletion and chronic immune activation both of which may contribute to progressive immune suppression[267]. In addition HIV has developed ways to both evade the anti-viral effects of interferon in some cell types and developed mechanisms to suppress the induction of interferon in other cell types notably macrophages[266]. Recently HIV-1 has been show to evade induction of type 1 interferon in primary macrophages by minimising exposure of viral DNA following reverse transcription to cytosolic pattern recognition receptors, but overcoming this leads to increase interferon production and promote restriction of viral replication[268]. Of note it has also been demonstrated that Mtb can suppress HIV replication within macrophages but not monocytes through triggering an interferon response through production of interferon-stimulated gene factor 3 (ISGF-3) and induction of inhibitory CCAAT-enhancer-binding protein β (C/EBPβ) transcription factor[269]. Although this observation is of interest, it is likely that this suppressive effect of Mtb on HIV replication is not of major importance given a number of other mechanisms by which TB increases HIV replication.

Interaction of HIV and TB is complex; HIV infection increases risk of active TB primarily through depletion of CD4 T cells but active TB increases HIV replication primarily through immune activation and co-localization of HIV infected and susceptible cells at the site of TB disease. However, it is possible that induction of type 1 interferon by HIV may further affect complexity of HIV TB interaction. Similarly to influenza, HIV induction of type 1 interferon

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may impair host control of Mtb by suppression of inflammatory cytokines. In keeping with this the analysis has demonstrated that while TB is associated with increase in expression of inflammation modules, HIV is associated with reduced expression with these modules. There are several limitations in study design. In particular the transcriptional signatures have not been validated in a separate cohort either to demonstrate specificity to subclinical TB or prediction of active TB, which would increase confidence in their potential. Further more confirming that these transcripts are differentially expressed on a different platform e.g. multiplex RT-PCR, would be important.

As stated making inferences about biology from a whole blood signature is not without caveats. Separating whole blood into cell type and individually interrogating transcript abundance in different groups of white blood cells would have minimised uncertainty about whether over/under abundance of transcripts related to alteration in circulating numbers or true differences in gene expression. Ultimately cells from the site of disease may be more useful in informing about biology. Direct sampling of cells is only possible in surgically or biopsy obtained samples which due to there invasive nature are only opportunistically available. However, BAL samples can provide cells from closer to the site of disease.

5.8 Conclusion

There is a clear need to develop diagnostic tests that are more predictive of active TB. In this chapter proof of concept has been demonstrated that a transcriptional signature could be used to identify a group of people with pathology in lung that may place them at higher risk of developing TB. However, even if validated there are numerous technical hurdles in translating a transcriptional signature into a low cost diagnostic test. If expressed proteins could be detected in serum or plasma that could similarly identify a high-risk group this may be more easily translated into a diagnostic test using available technology.
5.9 Chapter supplementary figures

Complete list of 82 transcripts

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<th>FC SUB vs LAT</th>
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Supplementary table 1 – Complete list of 82 transcripts. Left section showing transcripts over-abundant with active and subclinical TB compared to latent TB. Right section showing transcripts under-abundant in active and subclinical TB compared to latent TB.
6.1 Chapter 6 Introduction

In the previous chapter transcripts that related to different stages of TB infection and disease were identified. Transcriptomics can inform about the biology and be useful for the generation hypothesis regarding biological mechanisms in a condition of interest. A key biological purpose of transcribed mRNA is to serve as a template for translation of proteins, which are in general the primary mediators of biological function condition. Therefore measuring levels of protein predicted by transcriptional data may help to validate and provide support for hypotheses generated by this data. Transcriptional signatures also have a potential role as diagnostic biomarkers. However, translating these signatures into low cost diagnostics remains a significant challenge. Identification of serum and plasma biomarkers that can discriminate those with subclinical and active TB from those with latent TB would provide an alternative and simple route for diagnostic development. As individual serum biomarkers are often not specific to a single disease, the use of mycobacterial antigens to first stimulate the blood is one approach to improving specificity of diagnostic tests and is the principle behind QFGIT. As a result screening the supernatant of this commercially available assay is an attractive approach to TB biomarker discovery.

6.2 Chapter 6 hypothesis

HIV infected persons with subclinical TB (as defined on PET/CT) and active TB will have differences in concentration of cytokines, chemokines and soluble proteins (informed by transcriptional analysis) within serum and/or QFGIT plasma compared to those with latent TB.

6.3 Chapter 6 aim

To identify differences in cytokines, chemokines and soluble proteins between different stages of HIV associated TB infection and disease, both in serum and in QFGIT supernatants.

6.3.1 Specific chapter aims

1. To determine differences in median serum concentration of cytokines, chemokines and soluble proteins determined by multiplex assay (MILLIPLEX) or ELISA method for different stages of TB infection and TB disease.
2. To explore the relationship between HIV viral load on concentration of cytokines, chemokines and soluble proteins.

3. To determine optimal cut off for cytokines, chemokines or soluble proteins to distinguish active, subclinical and latent TB.

4. To compare concentration of cytokines and chemokines in serum with plasma from QFGIT NIL tube by MILLIPLEX assay.

5. To evaluate differences in plasma cytokines and chemokines, detected by the Luminex method, following stimulation of whole blood by Mtb specific antigens (in QFGIT assay) for different stages of TB infection and disease.

### 6.4 Chapter 6 methods

#### 6.4.1 Choice of cytokines, chemokines and soluble proteins for evaluation

The choice of cytokines, chemokines and soluble proteins was informed by the transcriptional analysis. Unbiased network analysis was performed on the 82 transcripts in Ingenuity Pathway Analysis (IPA) software. The networks around transcripts of interest are determined by algorithm using the Ingenuity Knowledgebase, which is a curated database of experimentally demonstrated relationships between molecules based on extensive review of literature conducted by developers of IPA. Of the 82 transcripts, 30 were identified to form part of a network of 140 molecules involved in the inflammatory response, cell death and survival and cellular movement, with a network score of 49 ($p=1 \times 10^{-49}$ that these transcripts would be grouped together by chance). A number of cytokines and chemokines formed part of this network suggesting that their abundance may differ in subclinical and latent TB (see figure 6.1). It was not possible to create a customised multiplex assay to include all these predicted cytokines and chemokines and so a commercially available 38-plex MILLIPLEX assay covering the majority of these cytokines and chemokines was used (Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF-2), Eotaxin(CCL11/24/26), Transforming Growth Factor (TGF)-α, Granulocyte Colony Stimulating Factor (G-CSF)(CSF-3), Fms-related tyrosine kinase 3 ligand (Flt-3L), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)(CSF-2), Fractalkine(CX3CL1), Interferon (IFN)α2, IFNγ, Growth Regulated Oncogene (GRO)(CXCL1), Interleukin (IL)-10, Monocyte Chemotactic Protein (MCP)-3(CCL7), IL-12p40, Macrophage Derived Chemokine (MDC)(CCL22), IL-12p70, IL-13, IL-15, sCD40L, IL-17A, IL-1ra, IL-1α, IL-9, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IFNγ induced protein (IP)-10(CXCL10), MCP-1(CCL2), Macrophage Inflammatory Protein (MIP)-1α(CCL3),
MIP1-β (CCL4), Tumour Necrosis Factor (TNF)α, TNFβ, Vascular Endothelial Growth Factor (VEGF)). This 38-plex assay was performed on both serum and QFGIT NIL and Ag plasma. Specific ELISA for CCL23 and CXCL17 were also performed on serum and QFGIT plasma as transcripts for these cytokines formed part of the differentially expressed 82 transcripts. In addition to these cytokines, ELISA were performed on serum for the acute phase and complement proteins Haptoglobin, SERPING1 and C1q, as these formed part of the 82 transcript signature, and additionally C5, which formed part of the 203 transcript signature. Due to the prominence of C1q and Fcγ receptor in the 82 transcript signature both of which bind immune complex, Circulating Immune Complexes (CIC) in serum were also quantified by ELISA using immobilised C1q to bind CIC.

Fig 6.1 – The most significant network of related transcripts derived from 82 transcripts identified by IPA network analysis: Of the 140 transcripts shown, the 30 shown in colour form part of the 82 transcripts. Green signifies underabundant subclinical TB vs. latent TB, red signifies overabundant subclinical TB vs. latent TB. Soluble protein in this network as shown in the “extracellular space”
6.4.2 Handling of out of range values

Standard curves for MILLIPLEX assay were derived using Bio-Plex Manager software 6.1. Points in the standard curve for which calculated/actual concentrations were <70% or >130%, were invalidated and curve fit was made using a 5-parameter logistic method as recommended by the manufacturer. For the MILLIPLEX assay, QC samples were run on every plate and for each cytokine and chemokine, calculated concentrations were within the acceptable limits provided by the manufacturer. The software interpolated values that were within the minimum and maximum standard concentrations and extrapolated values that were above the highest standard concentration but within the maximum asymptote or below the minimum standard concentration but within the minimum asymptote. Extrapolated values were accepted unless the value was less than the manufacturer defined minimal detectable limit in which case values were assigned 0. Values that were below the minimum asymptote were also assigned 0. Values that were above the maximum asymptote, if this applied to a small number of values for an analyte, were assigned either the observed concentration of the highest standard or the highest extrapolated value (if extrapolated values were present). Large numbers of values were out of maximum range in QFGIT plasma for GRO, MCP-3, IL-6, IL-8, IP-10, MCP-1, MIP-1α and MIP-1β. The specimens were diluted 40-fold and repeated, and for these analytes the diluted results were used. Serum sCD40L was completely out of upper range and was not able to be repeated, this analyte was omitted from analysis. ELISA standards failed for the CXCL17 assay twice and therefore this analyte was also not included in analysis. Although as no colour change was visible in samples for this assay it is likely that this was undetectable in all samples. When data required Log10 transformation or evaluation of fold change, 0.1 was added to all values to ensure no 0 values were present.

6.4.3 Samples used in analysis

All 35 participants undergoing FDG-PET/CT had both serum and QFGIT plasma samples available. Participants were classified into 10 with evidence of subclinical pathology on FDG-PET/CT (referred to as “subclinical” in this chapter) and 25 without subclinical pathology (referred to as “latent” in this chapter) as described in chapter 4. Of the 16 participants with active TB, 13 had serum samples available and of those 11 had QFGIT plasma available (Table 6.1). Samples had all been handled in the same manner prior to storage at -80°C for a
minimum of 1 year and a maximum of 2.5 years and were then aliquoted for the required number of tests on a single occasion to keep freeze thaw cycles to a minimum.

### 6.4.4 Statistical methods

Statistical analysis was performed using Stata ver. 12.1. Graphs and figures were created using both Stata ver. 12.1 and GraphPad Prism ver. 5.0a. A number of analytes were non-normally distributed even following log transformation so non-parametric statistics were used for main analysis. Comparisons between non-paired data were compared using Wilcoxon rank sum (Mann-Whitney U test) or Kruskal Wallis rank test. Dunns post-hoc test was used following Kruskal Wallis test to determine significance of individual comparisons. Paired data was compared using Wilcoxon matched pairs sign rank test. Correlation was performed by Spearman analysis if the majority of analytes were non-normally distributed, Pearson’s correlation was also used if the analytes were normally distributed. Bonferroni correction was used to account for number of analytes in analysis. Trend analysis was performed using Cuzick non-parametric test for trend. Non-parametric Receiver Operating Characteristic (ROC) analysis was also performed in Stata ver. 12.1

### 6.5 Results

#### 6.5.1 Concentration of analytes in serum

Of the 43 analytes analysed (not including CXCL17 and sCD40L), 9 were undetectable in serum in at least 75% of participants in each of the 3 groups (Fractalkine, IL-12p40, IL-13, IL-9, IL-1β, IL-2, IL-3, IL-4 and TNFβ). Twenty-four analytes had significantly different concentrations across the 3 groups (p≤0.05) of which 10 remained significant after Bonferroni multiple testing correction (p≤0.0012) (IFNγ, IL-15, IL-1α, IL-5, IL-6, IL-7, IP-10,
TNFα, CIC and CCL23. Dunns post hoc test demonstrated that IL-5 was significantly elevated in serum of those with subclinical pathology compared to latent TB and that all 10 analytes were significantly elevated in serum of active TB participants compared to latent TB (table 6.1 & figure. 6.2). Of note MCP-3 was also significantly elevated in subclinical TB and active TB compared to latent TB although not after correcting for multiple comparisons. As participants with active TB also had significantly greater HIV VL, which may have contributed to differences in serum concentration of analytes, this was further explored. Linear regression analysis was not appropriate for the majority of analytes due to non-normality despite log transformation. Unsurprisingly, 6 of the 10 analytes (IFNγ, IL-1α, IP-10, TNF-α, circulating immune complex and CCL23) that were significantly different between active and latent TB also had a significant positive correlation (Spearman) with HIV VL when all participants were considered. Only IP-10 remained significant after correction for multiple comparisons. In order to evaluate the effect of VL independently of active TB, correlation of VL with analytes for those with latent TB only was performed. EGF, MDC and CS were found to have a significant negative correlation with VL, and IL-17A, IP-10 and SERPING1 a
Figure 6.2 – Graphs showing difference in serum concentration for 10 analytes, significant after multiple testing correction ($p < 0.0012$) 
$\pm$ Dunns posthoc test; significant for individual comparison.
significant positive correlation with VL although none of these remained significant after multiple testing corrections. Interaction between VL and TB was further visualised on scatter plots (figure 6.3) with the independent effect of TB on serum concentration being apparent for the analytes. As IP-10 appeared to be independently affected by VL this was further explored by linear regression (using Log10 values) acknowledging that data was slightly heteroscedastic and that distribution of residuals were slightly skewed (although data were better suited to linear regression than other analytes). This analysis confirmed that VL had a significant effect on IP-10 accounting for 14% of variance in serum concentration (adjusted $r^2=0.136, p=0.005$). However, when TB status was added to the model the 2 factors accounted for 32% of variance in serum IP-10 concentration (adjusted
r²=0.329, p<0.001) with TB having a stronger independent effect on IP-10 concentration than VL (p<0.001 and p=0.01 respectively).

To further evaluate the relationship between these 10 variables and CRP, a correlation matrix was created. This demonstrated a significant positive correlation between the 11 analytes with correlation of IP-10 and IL-1α being particularly strong (Spearman rho = 0.90). In addition all analytes were positively correlated with serum CRP.

### 6.5.1.1 ROC analysis to identify optimal cut off values

ROC analysis was then undertaken to establish which of the 10 analytes had the greatest potential to distinguish active TB from latent TB with CRP and VL as comparators. All analytes had AUC ≥0.731 with 3 analytes being more discriminatory than CRP (AUC=0.928), IP-10 (AUC 0.982), IL-1α (AUC=0.942) and IFNγ (AUC=0.934). Two optimal cut offs were then derived to establish what proportion of those with subclinical disease would be classified as active. The first cut off value was the one which best classified those with active and latent TB (tending to have a higher specificity). The second cut off value considered was the one with the lowest negative likelihood ratio (i.e. a cut off below which active TB was best ruled out),
### Table defining optimal cut-offs for optimal correct classification of active and latent TB (correct class) and optimal negative likelihood ratio (LR-). % of subclinical TB categorized as active TB in final column. (CENTRE) ROC curves for 4 most discriminatory analytes by AUC (IP-10, IL-1α, IFNy & CIC). (BOTTOM) Graph showing discriminatory ability of CIC (≥100.9 μg Eq/mL) and IL1α (≥16.9 pg/mL).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>AUC (95% CI)</th>
<th>Cut off</th>
<th>units</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Correct Class</th>
<th>LR+</th>
<th>LR-</th>
<th>% Subclinical class active</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP-10</td>
<td>0.982 (0.943-1)</td>
<td>2331.8</td>
<td>pg/mL</td>
<td>92.3</td>
<td>100.0</td>
<td>97.4</td>
<td>.</td>
<td>0.08</td>
<td>20.0</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0.942 (0.838-1)</td>
<td>37.6</td>
<td>pg/mL</td>
<td>84.6</td>
<td>100.0</td>
<td>94.7</td>
<td>.</td>
<td>0.15</td>
<td>20.0</td>
</tr>
<tr>
<td>IFNy</td>
<td>0.934 (0.854-1)</td>
<td>9.1</td>
<td>pg/mL</td>
<td>92.3</td>
<td>93.0</td>
<td>93.1</td>
<td>11.54</td>
<td>0.08</td>
<td>50.0</td>
</tr>
<tr>
<td>CIC</td>
<td>0.886 (0.777-0.995)</td>
<td>100.9</td>
<td>μg Eq/mL</td>
<td>84.6</td>
<td>88.0</td>
<td>86.8</td>
<td>7.05</td>
<td>0.17</td>
<td>50.0</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.885 (0.770-0.995)</td>
<td>1.4</td>
<td>pg/mL</td>
<td>92.3</td>
<td>84.0</td>
<td>86.8</td>
<td>5.77</td>
<td>0.09</td>
<td>30.0</td>
</tr>
<tr>
<td>TNFa</td>
<td>0.849 (0.724-0.974)</td>
<td>20.4</td>
<td>pg/mL</td>
<td>69.2</td>
<td>88.0</td>
<td>81.6</td>
<td>5.77</td>
<td>0.35</td>
<td>10.0</td>
</tr>
<tr>
<td>CCL23</td>
<td>0.837 (0.696-0.976)</td>
<td>176.1</td>
<td>pg/mL</td>
<td>69.2</td>
<td>96.0</td>
<td>86.8</td>
<td>17.30</td>
<td>0.32</td>
<td>0.0</td>
</tr>
<tr>
<td>IL-7</td>
<td>0.831 (0.686-0.975)</td>
<td>3.4</td>
<td>pg/mL</td>
<td>84.2</td>
<td>76.9</td>
<td>83.0</td>
<td>2.10</td>
<td>0.14</td>
<td>40.0</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.709 (0.628-0.91)</td>
<td>0.5</td>
<td>pg/mL</td>
<td>53.9</td>
<td>100.0</td>
<td>84.2</td>
<td>.</td>
<td>0.46</td>
<td>40.0</td>
</tr>
<tr>
<td>IL-33</td>
<td>0.731 (0.590-0.872)</td>
<td>2.0</td>
<td>pg/mL</td>
<td>46.1</td>
<td>100.0</td>
<td>81.6</td>
<td>.</td>
<td>0.54</td>
<td>0.0</td>
</tr>
<tr>
<td>CRP</td>
<td>0.928 (0.803-1)</td>
<td>18.4</td>
<td>mg/L</td>
<td>84.6</td>
<td>100.0</td>
<td>94.7</td>
<td>.</td>
<td>0.15</td>
<td>0.0</td>
</tr>
<tr>
<td>VL</td>
<td>0.840 (0.686-0.993)</td>
<td>19425</td>
<td>copies/mL</td>
<td>76.9</td>
<td>92.0</td>
<td>86.8</td>
<td>9.61</td>
<td>0.25</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Figure 6.5 – ROC analysis for 10 analytes, CRP and VL: (TOP) Table defining optimal cut-offs for optimal correct classification of active and latent TB (correct class) and optimal negative likelihood ratio (LR-). % of subclinical TB categorized as active TB in final column. (CENTRE) ROC curves for 4 most discriminatory analytes by AUC (IP-10, IL-1α, IFNy & CIC). (BOTTOM) Graph showing discriminatory ability of CIC (≥100.9 μg Eq/mL) and IL1α (≥16.9 pg/mL).
which tended to have a higher sensitivity and lower specificity (the 2 cut offs were the same for 5 analytes). Neither of the cut off values for CRP classified any of those with subclinical TB as active TB. The best performing analytes in this regard were IL-1α with cut off of 16.9 pg/mL (which had a sensitivity of 92.3% for active TB, classified 50% of subclinical TB as active and only 7.9% of latent TB as false positive) and CIC with cut off 100.9 µg Eq/mL (which had a sensitivity of 84.6% for active TB, classified 50% of subclinical TB as active and only 12% of latent TB as false positive). If either CIC or IL-1α were above these cut offs, sensitivity for active TB was 92.3%, 70% of subclinical TB was classified as active and 20% of latent TB were false positive (figure 6.5).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Latent n=25</th>
<th>Subclinical (PET low) n=5</th>
<th>Subclinical (PET high) n=5</th>
<th>Active (Smi neg) n=6</th>
<th>Active (Smi pos) n=7</th>
<th>Test for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>FNa2</td>
<td>0.55±0.03</td>
<td>18.77±20.3</td>
<td>0.0±5.53</td>
<td>10.73±4.11</td>
<td>20.91±11.00</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>L-15</td>
<td>0.0±0.00</td>
<td>0.0±0.00</td>
<td>0.0±0.00</td>
<td>0.0±0.26</td>
<td>1.85±0.46</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>L-Sa</td>
<td>0.0±12.77</td>
<td>15.72±20.83</td>
<td>47.13±6.82</td>
<td>53.35±31.11</td>
<td>55.35±31.11</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>L-S</td>
<td>0.0±0.00</td>
<td>0.0±0.00</td>
<td>2.55±2.43</td>
<td>1.39±0.12</td>
<td>0.68±0.46</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>L-6</td>
<td>0.0±0.00</td>
<td>0.0±0.00</td>
<td>0.0±2.67</td>
<td>33.18±12.52</td>
<td>43.25±11.98</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>CIC*</td>
<td>0.0±3.33</td>
<td>0.0±10.47</td>
<td>4.2±11.38</td>
<td>11.38±3.31</td>
<td>11.38±3.31</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>IP-10</td>
<td>924.45±2104.46</td>
<td>1278.72±1518.33</td>
<td>1895.73±885.55</td>
<td>3589.28±2331.65</td>
<td>5087.97±4017.07</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>TNFα</td>
<td>6.24±4.32</td>
<td>7.01±3.57</td>
<td>15.97±4.32</td>
<td>57.36±12.35</td>
<td>47.2±12.95</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>CIC</td>
<td>47.15±28.05</td>
<td>54.8±24.58</td>
<td>102.6±177.3</td>
<td>299.82±100.75</td>
<td>261.55±112.25</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>CCL23</td>
<td>170.75±66.72</td>
<td>25.11±25.11</td>
<td>259.18±82.33</td>
<td>711.71±253.98</td>
<td>1008.19±326.81</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>CRP</td>
<td>0.9±0.30</td>
<td>1.0±0.40</td>
<td>2.7±3.55</td>
<td>62.4±312.7</td>
<td>70.5±184.31</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>VL</td>
<td>9.9±8.25 (2.0-2070)</td>
<td>21399±(10800-51166)</td>
<td>16498±(6878-50854)</td>
<td>95542±(59791-99786)</td>
<td>79998±(1233-200589)</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 6.6 – Effect of disease activity on serum concentration of analytes: (TOP) – Table showing serum concentration of 10 analytes, CRP and VL in participants stratified into 5 groups by increasing activity of disease (Latent, Subclinical VS 0-2, Subclinical VS 3, Active Sm neg, Active Sm pos) with analysis for trend in far column. BELOW – Box plots showing serum concentration for 4 most discriminatory analytes (IP-10, IL-1α, IFNγ and CIC) by disease activity.
6.5.1.2 Relationship between disease activity and serum concentration of analytes

To further investigate whether levels of these analytes increased as TB progressed, participants with active TB were divided into, 7 participants that were smear positive and 6 participants that were smear negative and those with subclinical TB were divided into, 5 participants with evidence of increased metabolic activity considered to be TB related on PET (VS=3 within lung parenchyma or central LN) and 5 within lower activity (VS=0-2) (The same groupings as used in for Chapter 5 – Fig 5.6). Non-parametric trend analysis was then performed to establish if concentration of analytes rose with increasing disease activity. Significant trends were determined for all 10 analytes as well as CRP and VL. These trends were also apparent graphically (figure 6.6).

6.5.1.3 Correlation of transcript abundance with serum concentration of analytes

Transcripts for Haptoglobin, C1q, SERPING1, C5 and CCL23 formed part of the 82 transcript signature and for these analytes expression values were compared with serum concentrations of protein measured by ELISA. There was no significant correlation of expression data with serum protein concentration for any of the analytes apart from Haptoglobin, which showed significant positive correlation (r=0.39, p=0.006). As C1q binds Fcγ on immune complexes, the correlation of C1q expression with CIC was assessed and found to be significant (r=0.38, p=0.007) suggesting that a high proportion of serum C1q may be bound in CIC (fig 6.7). CIC was then correlated with other complement components and also found to be significantly correlated with SERPING1 expression (C1 esterase inhibitor – which irreversibly binds C1r and C1s) (r=0.40, p=0.005) but not with C5 levels (r=0.19, p=0.20) suggesting a high proportion of SERPING1 may also be bound to CIC.

6.5.2 Concentration of analytes in QFGIT Plasma

First, concentration of IFNγ as detected by MILLIPLEX assay in plasma from QFGIT NIL and Ag tubes was compared with initial values obtained from the commercial QFGIT assay, which had been performed within 1-2 weeks of initial sample collection. Values were highly correlated in both NIL and Ag tube (Spearman rho 0.71 and 0.84 respectively (p<0.0001 for both)). This provided reassurance that despite prolonged storage cytokine concentration remained stable and that errors in plate set up had been avoided. Concentrations of cytokine and chemokines were then compared between QFGIT NIL plasma and serum. Correlation of serum and plasma values was very poor for the 37 analytes. Only 11 analytes showed a significant correlation, of which only 4 analytes remained significantly correlated.
Figure 6.7– Scatter plots showing correlation of transcript abundance against serum concentration of protein product.
after multiple testing correction (EGF, Eotaxin, MDC and IL-2) and of these only EGF, Eotaxin and MDC had QFGIT NIL plasma values similar to serum concentration. For most analytes there was a highly significant difference between concentration in QFGIT NIL plasma and serum. Only IL-3 and IL-4 had undetectable values for > 75% in each group in comparison to the 9 analytes in serum. For several of the analytes, notably MIP1α, IL-6, IL-8, IL-1β, MCP-3 and GCSF concentration was often 3 or 4 orders of magnitude higher in QFGIT NIL plasma in comparison to serum. Increase in analyte concentration was not proportionate across the 3 groups with ratio of plasma to serum concentration being significantly higher (after multiple testing correction) in latent TB for TNFα, IP-10, IL-1α, IL-6, IFNγ and IFNα2. As a result a similar pattern of cytokine concentration was not seen in QFGIT NIL plasma and serum.

Given the significant differences in serum and QFGIT NIL plasma concentrations of cytokines, the possibility of contamination of QFGIT tubes was further evaluated. QFGIT tubes are heparin coated which raised the possibility of endotoxin contamination. Twenty-four QFGIT NIL tubes were assessed for endotoxin level by Limulus Amebocyte Lysate Assay. None of the tubes had endotoxin level > 0.2 EU/mL making endotoxin contamination unlikely. QFGIT samples are incubated for 24 hours at 37°C and another possibility was that HIV infection itself might have led to further cytokine release. Correlation of VL with level of cytokines and chemokines was therefore evaluated. EGF had a significant negative correlation (p<0.05) similarly to serum EGF and no analytes showed significant positive correlation suggesting that HIV infection itself was unlikely to be responsible for difference between serum and QFGIT plasma values. Concentration of cytokine and chemokines was then compared to Ag stimulated tube. For the majority of analytes levels were higher in the background than in the antigen stimulated tube and hence Ag-NIL concentration were often negative. Only IFNγ, IP-10 and IL-2 had higher levels in Ag tube for almost all participant.
Chapter 6 – Serum and QFGIT biomarkers

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Latent med (IQR) pg/mL</th>
<th>Subclinical med (IQR) pg/mL</th>
<th>Active med (IQR) pg/mL</th>
<th>Kwalls p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>-21.4 (-44.72–14.4)</td>
<td>-33.6 (-36.37–18.28)</td>
<td>-32.7 (-46.16–16.95)</td>
<td>0.570</td>
</tr>
<tr>
<td>FGF-2</td>
<td>-26.1 (-63.16–16.9)</td>
<td>-68 (-137.79–13.47)</td>
<td>-11.24 (-62.18–16.2)</td>
<td>0.449</td>
</tr>
<tr>
<td>EOTAXIN</td>
<td>-5.23 (-11.2–0.7)</td>
<td>-9.03 (-15.18–3.32)</td>
<td>-9.11 (-37.43–0.5)</td>
<td>0.710</td>
</tr>
<tr>
<td>TGFα</td>
<td>5.75 (-10.22–1.91)</td>
<td>-1.54 (-6.21–2.83)</td>
<td>1.26 (6.23–6.9)</td>
<td>0.077</td>
</tr>
<tr>
<td>GCSF</td>
<td>-1793.17 (-3471.26–520.59)</td>
<td>-818.94 (-2238.06–138.46)</td>
<td>-641.62 (-1172.59–236.34)</td>
<td>0.238</td>
</tr>
<tr>
<td>FLT-3L</td>
<td>-7.2 (-14.76–5.35)</td>
<td>0.59 (-14.4–8.74)</td>
<td>-8.78 (15.5–10.4)</td>
<td>0.825</td>
</tr>
<tr>
<td>GMCSF</td>
<td>-48.74 (-183.37–20.49)</td>
<td>-13.09 (-48.21–78.65)</td>
<td>-9.02 (19.04–42.89)</td>
<td>0.014</td>
</tr>
<tr>
<td>FRACRTALKINE</td>
<td>-30.72 (-77.08–3)</td>
<td>-51.52 (60.53–22.61)</td>
<td>-38.2 (95.12–11.7)</td>
<td>0.804</td>
</tr>
<tr>
<td>IFNa2</td>
<td>-40.34 (-57.71–13.55)</td>
<td>-21.84 (59.24–6.22)</td>
<td>-16.59 (34.4–18.15)</td>
<td>0.153</td>
</tr>
<tr>
<td>IFNγ</td>
<td>131.36 (31.84–221.18)</td>
<td>398.31 (67.62–1095.57)</td>
<td>400.55 (73.72–1419.27)</td>
<td>0.148</td>
</tr>
<tr>
<td>GRO</td>
<td>-1500 (-2900–9365.8)</td>
<td>-2300 (-3300–3911.44)</td>
<td>-4900.88 (19000–13762.22)</td>
<td>0.201</td>
</tr>
<tr>
<td>IL-10</td>
<td>-152.05 (-381.33–84.79)</td>
<td>-115.28 (-261.72–11.25)</td>
<td>-26.86 (46.06–5.52)</td>
<td>0.006</td>
</tr>
<tr>
<td>MCP-3</td>
<td>385.93 (2337.56–329.86)</td>
<td>299.19 (1803.39–1060.19)</td>
<td>1893.48 (1768.78–2967.84)</td>
<td>0.080</td>
</tr>
<tr>
<td>IL-12P40</td>
<td>-16.68 (-27.67–1.48)</td>
<td>5.48 (21.03–2.68)</td>
<td>0.18 (29.85–5.4)</td>
<td>0.325</td>
</tr>
<tr>
<td>MDC</td>
<td>-139.07 (-306.56–137.01)</td>
<td>-182.92 (-342.5–28.13)</td>
<td>-351.91 (476.13–156.09)</td>
<td>0.124</td>
</tr>
<tr>
<td>IL-12P70</td>
<td>-31.01 (-47.8–19.99)</td>
<td>25.92 (51.53–13.02)</td>
<td>-17.8 (21.8–0.6)</td>
<td>0.097</td>
</tr>
<tr>
<td>IL-13</td>
<td>2.35 (-1.48–12.93)</td>
<td>23.28 (3.05–89.36)</td>
<td>22.96 (3.13–87.8)</td>
<td>0.128</td>
</tr>
<tr>
<td>IL-15</td>
<td>-1.01 (-3.67–0)</td>
<td>0.12 (3.16–0.97)</td>
<td>-2.33 (5.42–0.27)</td>
<td>0.511</td>
</tr>
<tr>
<td>IL-17A</td>
<td>-2.19 (-5.57–0.26)</td>
<td>3.46 (5.22–0.57)</td>
<td>-1.51 (3.83–1.61)</td>
<td>0.628</td>
</tr>
<tr>
<td>IL-3RA</td>
<td>-153.67 (-226.79–68.13)</td>
<td>27.84 (190.47–166.16)</td>
<td>86.57 (364.32–291.28)</td>
<td>0.006</td>
</tr>
<tr>
<td>IL-1α</td>
<td>-328.68 (-528.25–74.93)</td>
<td>-377.7 (-1126.72–76.74)</td>
<td>4.36 (60.98–36.73)</td>
<td>0.033</td>
</tr>
<tr>
<td>IL-9</td>
<td>0 (2.16–2.92)</td>
<td>0 (0.24–3.08)</td>
<td>0 (1.38–2.74)</td>
<td>0.830</td>
</tr>
<tr>
<td>IL-1β</td>
<td>-1442.68 (-5806.3–519.65)</td>
<td>-846.08 (4123.73–209.72)</td>
<td>-171.92 (547.27–697.51)</td>
<td>0.007</td>
</tr>
<tr>
<td>IL-2</td>
<td>122.01 (49.237.68)</td>
<td>448.17 (63.05–867.7)</td>
<td>130.66 (17.05–868.3)</td>
<td>0.308</td>
</tr>
<tr>
<td>IL-3</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.972</td>
</tr>
<tr>
<td>IL-4</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.337</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.96 (1.68–2.95)</td>
<td>1.82 (4.35–12.07)</td>
<td>0.41 (2.18–9.45)</td>
<td>0.486</td>
</tr>
<tr>
<td>IL-6</td>
<td>-7345.05 (-12000–2254.13)</td>
<td>-6624.59 (-18000–1184.73)</td>
<td>-1134.39 (4548.87–228.71)</td>
<td>0.025</td>
</tr>
<tr>
<td>IL-7</td>
<td>-9.44 (-17.91–3.32)</td>
<td>-6.53 (-10.7–0.88)</td>
<td>-7.34 (10.53–1.51)</td>
<td>0.350</td>
</tr>
<tr>
<td>IL-8</td>
<td>-3600.0 (-6500–14000)</td>
<td>-3800.0 (-9800–9668.63)</td>
<td>-10000 (-57000–783.23)</td>
<td>0.229</td>
</tr>
<tr>
<td>IP-10</td>
<td>26605.6 (11357.31–46555.74)</td>
<td>25796.35 (13614.60–64855.02)</td>
<td>29161.37 (14624.61–68926.16)</td>
<td>0.658</td>
</tr>
<tr>
<td>MCP-1</td>
<td>-13000 (12000–9999.5)</td>
<td>-5703.63 (30000–1470.29)</td>
<td>12965.78 (16000–2062.42)</td>
<td>0.045</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>-13000 (21000–5568.89)</td>
<td>-12000 (36000–2218.02)</td>
<td>-5763.06 (12000–2254.81)</td>
<td>0.284</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>-5305.97 (-8593.32–1206.22)</td>
<td>-4041.58 (-9867.97–449.12)</td>
<td>-934.13 (2057.32–51.82)</td>
<td>0.034</td>
</tr>
<tr>
<td>TNFα</td>
<td>-1109.71 (-2312.56–543.69)</td>
<td>-870.25 (-1420.68–201.96)</td>
<td>-880.99 (1089.62–187.83)</td>
<td>0.287</td>
</tr>
<tr>
<td>TNFβ</td>
<td>-4.9 (-13.39)</td>
<td>-2.79 (3.04–10.49)</td>
<td>-5.1 (13.61–3.9)</td>
<td>0.081</td>
</tr>
<tr>
<td>VEGF</td>
<td>-80.43 (-162.67–33.44)</td>
<td>10.87 (156.86–88.92)</td>
<td>-72.85 (152.96–26.9)</td>
<td>0.855</td>
</tr>
</tbody>
</table>

Figure 6.10 – QFGIT Ag-NIL concentration of analytes: (TOP) - Table comparing Ag-NIL values by disease status for 37 analytes. (BOTTOM) - Graphs showing distribution of values for IP-10, IFNγ, IL-2, IL-1RA, IL-10.
samples as would be expected for those considered to have evidence of immune sensitization by TB and provided further reassurance that no errors in tube labelling or mixing up of NIL and Ag tubes had occurred. Although 8 analytes showed significant differences (p≤0.05) across the latent, subclinical and active TB none of these remained significant after Bonferroni correction for multiple comparisons. IL-10 and IL-1RA showed the most significant differences across the groups, however this largely related to Ag-NIL values being more negative in the latent TB group (Fig 6.10).

### 6.6 Summary of main findings

Analysis in this chapter has shown that from a group of 43 analytes derived from analysis of the 82 transcript signature, serum concentration of 10 (IFNγ, IP-10, TNFα, IL-6, IL-1α, IL-7, IL-15, IL-5, CCL23 and CIC) was significantly greater in active TB compared to latent TB and concentration of IL-5 was also significantly greater in subclinical compared to latent TB (after multiple testing corrections). Of the 10 analytes only IP-10 was significantly correlated with VL independently of active TB. However, the effect of active TB on IP-10 serum concentration appeared stronger than VL. All 10 analytes were positively correlated with each other and CRP. ROC analysis determined that serum IP-10, IL-1α and IFNγ were all more discriminatory than CRP in distinguishing active and latent TB and that IL-1α (≥16.9 pg/mL) and CIC (≥100.9 µg Eq/mL) individually classified 50% and in combination 70% of subclinical TB as active TB. In addition when assessed across 5 stages of increasing disease activity by PET findings and smear status, all 10 analytes showed a significant increasing trend in serum concentration. Correlation of transcript abundance with serum concentration of the protein product was very poor for all transcripts other than haptoglobin. However C1q and SERPING1 levels were highly correlated with CIC, suggesting that a high proportion of these proteins may be bound to CIC. The majority of cytokines and chemokines were present in significantly greater concentrations in QFGIT NIL plasma compared to serum. This did not appear to represent endotoxin contamination of tubes or be related to viral load. For the majority of cytokines, with the notable exception of IP-10, IFNγ and IL-2 cytokine and chemokine concentrations were lower in the Ag stimulated tube than in the NIL tube with this being more prominent in latent than active TB.
6.7 Discussion

A number of studies have explored differences in the serum concentration of cytokines, chemokines and other soluble factors between active and latent tuberculosis using multiplex assays and ELISA[270]. These studies vary considerably in methodology, in particular the analytes measured. Several features are notable about this study; firstly the selection of the analytes was informed by analysis of a whole blood transcriptional signature and therefore included analytes not included in commercial multiplex assays. Secondly imaging was used to characterise evidence of subclinical pathology in a population that would ordinarily be considered as “healthy latent infection” allowing for comparison of a potentially lower risk group of latent TB with subclinical and active TB. Furthermore, not only were the groups in this study matched for age and sex, almost all participants were of Xhosa ancestry. This is particularly important as ethnic variations in the inflammatory response to Mtb are becoming increasingly apparent[271, 272]. In addition this is one of the few studies to have focused on HIV associated TB.

Some common themes have emerged from this and previous studies (although often findings are study specific and occasionally contradictory)[270]. As part of a more extensive metabolomic profiling of active and latent TB, Weiner 3rd et al performed a similar MILLIPLEX assay as used in this study, in HIV uninfected South African adults. Of the 35 analytes evaluated, they identified IFNγ, IP-10, IL-6, G-CSF, VEGF and sIL2ra as being elevated in active TB compared to latent TB or healthy controls[273]. Several previous studies have also found Tn1 cytokines, in particular, IFNγ, IP-10 and TNFα to be elevated in the serum and plasma of active TB patient compared to healthy controls with latent TB[274, 275]. This is unsurprising given their role in the immune response to Mtb. Similarly IL-6, a pro-inflammatory cytokine, has been previously found to be elevated in the serum in active tuberculosis and is associated with presence of systemic symptoms and fever[276]. Data for IL-1, another pro-inflammatory cytokine and an important component in host control of Mtb, are more contradictory. Although previous studies have reported that IL-1 levels are elevated in the serum of HIV uninfected persons with active TB[277]. Recently, Mayer-Baber et al have demonstrated in 2 separate cohorts, in India and China, that IL-1 levels were reduced in HIV uninfected patients with active TB compared to latent TB[265]. In addition, IL-10, an immunoregulatory cytokine that modulates the Tn1 response is frequently reported to be elevated in the serum of active TB patients[274, 278]. This is in contrast to findings in this study where IL-10 was not found to be elevated in serum of HIV infected active TB
patients whereas IL-1α was. It is possible that this relates to the HIV status of participants in this study. Recently, Tomlinson et al showed that in vitro, HIV co-infection of macrophages resulted in a diminished IL-10 response and an enhanced pro-inflammatory response to Mtb and that this was mirrored in the airway samples of HIV infected TB patients who had low levels of IL-10 and high levels of IL-1β[100].

In this study we were not able to compare HIV infected and uninfected participants, although, recently Mihret et al have investigated the effect of HIV on circulating cytokine levels in TB. Using a 17-plex assay (EGF, Fractalkine, IFNγ, GM-CSF, IL-1, IL-10, IL-12, IL-17, IL-4, IL-7, IL-9, IP-10, MCP-1, MCP-3, MIP-1β, TNFα and VEGF) to compare plasma cytokine levels in HIV infected ART naïve persons with active TB (mean CD4 310/mm³) with HIV uninfected adults they found that the cytokines and chemokines measured were not affected by HIV status[279]. Few studies have investigated the effect of HIV infection alone on circulating cytokines, Keating et al using a similar MILLIPLEX assay to this study, measured 32 analytes in serum of predominantly African American women that were either HIV infected ART naïve, HIV infected established on ART, or HIV uninfected. Serum levels of TNFα and IP-10 were found to be significantly higher and IL-12(p40), IL-15 and FGF-2 levels significantly lower in HIV infected ART naïve women compared to HIV uninfected women. Of these 5 analytes only IP-10 was significantly affected by viral load in keeping with the findings in this study[280]. In other studies elevated levels of IL-7 (involved in lymphocyte maturation and differentiation) have been reported in HIV infected persons (as well as other lymphopenic states) showing inverse correlation with CD4 count and reducing with ART initiation. It is thought this may represent a compensatory homeostatic mechanism to lymphopenia[281, 282]. It is likely therefore that the elevated IL-7 found in this study relates more to HIV infection than active TB.

In addition to the Th1 and pro-inflammatory cytokines, in this study IL-15, IL-5, CCL23 and CIC were also found to be elevated in active TB. IL-15 has not previously been reported to be elevated in the serum of active TB patients, however, in a study by Frahm et al in which 25 analytes were evaluated by multiplex assay in QFGIT supernatants and Ag-NIL values compared between active and latent TB, IL-15 was found to be the most discriminatory cytokine[283]. IL-5 has also not been reported to be elevated in serum of HIV uninfected active TB patients, however, recently a role for IL-5 has been suggested in the pathogenesis of HIV associated TB in an SIV/TB macaque model. This has shown that SIV induces
production of IL-5 in monocytes which in turn inhibits T-cell production of Th1 cytokine which would be expected to impair host control of Mtb leading to progression of disease[284] (of note IL-5 levels were not measured in the study by Mihret et al discussed above).

CCL23 (also known as MIP-3 and MPIF-1) was differentially expressed between active/subclinical and latent TB and was part of the 82 transcript list. The serum concentration of CCL23 was also significantly elevated in active TB despite being poorly correlated with transcript abundance. CCL23 is expressed in lung tissue, liver as well as cells of myeloid origin and has been shown to display chemotactic activity for T cells and monocytes[285]. It is has rarely been studied in TB and does not form part of most commercially available multiplex assay. It has been shown to be elevated in serum of patients with systemic sclerosis, particularly in those with pulmonary hypertension[286]. Of note however in a microarray study of Mtb and M.bovis infection of bovine alveolar macrophages (b.a.mϕ). CCL23 was found to be the most overabundant transcript in M.bovis (more virulent in cows) compared to Mtb infected bovine alveolar macrophages[287]. This suggests the possibility that in humans, infection of alveolar macrophages with virulent Mtb may result in CCL23 release and this cytokine merits further investigation in TB.

Circulating immune complexes were investigated because of the findings from the microarray demonstrating an over abundance of transcripts for Fcγ receptor and C1q, which typically bind immune complexes. The ELISA assay used, measured binding of immune complex to immobilised C1q. Circulating immune complex have frequently been reported to be elevated in active tuberculosis and are increased in smear positive compared to smear negative disease and reduce with treatment[288, 289]. They have been found to be composed of mycobacterial antigens, IgG and IgM antibodies and complement components such as C1q, C3 and C4 [289, 290]. In particular these antibodies have been shown to be specific for mycobacterial glycolipids such as LAM although it is likely that antibodies to soluble proteins and other mycobacterial cell surface components are also present[291, 292]. However, circulating immune complexes are not unique to TB and are reported in a number of chronic inflammatory, autoimmune and neoplastic conditions and also in HIV infection. However, at least in acute HIV infection it has been show that HIV VL and levels of circulating immune complex are not correlated[293]. In this study although there was a positive correlation of VL with CIC in those with latent TB (Spearman rho = 0.32) it was not
significant even before correction for multiple comparisons. Using linear regression to distinguish effect of VL and TB was not considered appropriate, as residuals were not normally distributed. However, there was a significant trend for increasing CIC levels with disease activity. Of note CIC levels were significantly elevated in those with subclinical TB with greater evidence of activity on PET compared paired to those with latent TB despite no significant difference in viral load. It is therefore likely that the increased levels of CIC observed in subclinical and active TB relate more to progression of TB rather than increasing HIV VL that accompanies active TB. However, further characterisation of circulating immune complexes in HIV associated TB would be required to determine this.

The ability to distinguish active from latent TB for the 10 analytes particularly IP-10, IL-1α and IFNγ was extremely good and superior to that reported previously in a number of studies[294, 295]. The reason for this is likely two-fold. The use of FDG-PET/CT allowed the identification of those with evidence of subclinical TB and thus allowed comparison of active TB directly to those with latent TB and no evidence of active pathology. Comparison of active TB with those clinically determined to have latent TB (i.e. combining the latent and subclinical groups) would result in inferior performance characteristics of the analytes. Additionally as discussed above, and notably for IP-10 but possibly CIC and other analytes, the increased serum levels in active TB would result from both TB disease and increased HIV viral load accompanying it. This would be expected to enhance the discriminatory ability of these analytes. However, this is not of major concern, the fact that active TB results in increasing HIV VL for a given CD4 count means that HIV VL itself has a reasonable ability to discriminate active from latent TB. Increases in viral load as well as the identified serum markers are likely to be part of the natural progression of HIV associated TB.

Of major significance is the ability of the analytes, in particular CIC and IL1α, to classify those with subclinical TB as active TB (70% if CIC ≥100.9µg Eq/mL and/or IL1α ≥ 16.9pg/mL) and the clear trend of increasing serum concentration of analytes with increasing disease activity. The implications of this are that these serum markers may be to an extent predictive of active TB and could be used a screening tool for those at high risk of active TB in HIV co-infected populations. It is clear that these markers need validation not only to confirm findings in this population (HIV infected, ART naive, CD4≥350/mm³) but also in other HIV infected population (low CD4 count, established on ART and other ethic groups) and HIV uninfected populations to determine generalizability. Although as discussed above it is likely
that these markers may be better suited to HIV associated TB. In addition, the specificity of these analytes needs to be further explored especially in diseases also common in the Southern African setting.

Of note transcriptional abundance in whole blood was poorly correlated with serum levels of protein. Although haptoglobin expression level was significantly correlated with serum levels, transcript abundance only accounted for 15% of the variance in serum levels. There are several explanations for this. The central dogma of molecular biology (DNA→RNA→protein) is now considered an over-simplification and there are several reasons why transcript abundance may not accurately reflect protein abundance, as has often been observed. In particular post-transcriptional processes, regulation of translation and protein degradation may have a significant impact on protein concentration[163]. However, if a high proportion of a protein is bound, assays measuring free protein levels may not accurately represent total protein concentration. Both SERPING1 and C1q expression were highly correlated with CIC levels rather than the free serum levels detected by ELISA, suggesting that a high proportion may be bound to CIC. Furthermore whole blood transcriptomics only reflects transcript abundance of circulating cells, however, other cells not present in blood may contribute significantly to serum abundance of a protein. Notably complement factors such as C5 are produced in significant quantities by hepatocytes and CCL23 is also produced by lung and liver tissue. However of note despite being poorly correlated with transcript abundance, serum levels of CCL23 remained significantly elevated in those with active TB.

As levels of individual proteins are rarely specific to a single disease process, various strategies can be used to improve specificity of protein biomarkers. The identification of multiple factors in serum or plasma using proteomics to give a disease signature is one approach but translation into low cost diagnostic tools is a challenge. Hence, using Mtb specific antigens to stimulate blood prior to detecting cytokines and chemokines is an attractive strategy, particularly, as QFGIT provides a commercially available, validated platform for this. As a result numerous studies have utilised this methodology.

The difference in levels of cytokines in serum and plasma from the QFGIT NIL tube was striking, with several of the cytokines 3 or 4 orders of magnitude higher in QFGIT plasma compared to serum. Not all cytokines were affected with the effect being greatest on pro-
inflammatory cytokines. The main consequence of this was that the cytokine patterns observed in the serum were not observed in the QFGIT NIL plasma. Of note, the levels of IFNγ were well correlated with the commercial assay performed within 1-2 weeks of initial sampling. In addition, stimulation with Mtb specific antigens resulted in increases in IFNγ (again well correlated with commercial assay), IP10 and IL2 as expected (but reduction in may other cytokines). This suggests that technical or set-up errors in the multiplex assay were not responsible for this finding. Other studies have shown QFGIT NIL levels to be higher than would be expected in the serum and have observed the similar pattern of reduction in cytokine levels with stimulation[296]. Little explanation has been offered for these findings, although, the effects were less marked than for this study. The tubes were confirmed not to be LPS contaminated and the manufacturer states tubes are tested for endotoxin before dispatch.

De Jager et al using a multiplex assay have shown that there is very little difference between cytokine and chemokine levels in plasma and serum, when isolated soon after blood draw[297]. However a key difference between the serum samples and plasma samples from QFGIT is that the latter have been incubated at 37°C for 24 hours. It is clear from the literature on blood transfusion that storage of whole blood products leads to increases in pro-inflammatory cytokines and that this can be mitigated by the depletion of white blood cells[298]. L. Beer (in an unpublished thesis) has recently systematically explored the effect of time and temperature on plasma and serum cytokine concentrations in whole blood assays[299]. He confirmed little difference in cytokine or chemokine concentration in serum and plasma with no incubation. However, incubating the whole blood of healthy volunteers at 37°C for 24 hours (without stimulation) resulted in massive increases in cytokines and chemokines (IL6, IL8, GRO and MCP-1) in the serum tube but also large increases in levels in plasma particularly in heparinised tube compared to EDTA tube. He then demonstrated that 24 hour, 37°C incubation of PBMC with the addition of serum or fibrin resulted in similar large increases cytokine compared to medium alone and concluded that coagulation products were likely to be responsible for these observed increases. Furthermore, fibrinogen has been shown result in a similar pattern of cytokine release to LPS in monocytes, mediated by TLR-4 stimulation leading to up-regulation of pro-inflammatory cytokines such as MIP1α, MIP1β, MIP2 and MCP1[300]. Heparin should prevent clotting of blood however it is likely that a degree of clotting does occurs after 24 hours of incubation. HIV infected individuals have been shown to have higher circulating levels of both fibrinogen
and fibrin degradation products, such as d-dimer, and this may be an explanation to why certain cytokine and chemokines were elevated in this study[301]. An alternative hypothesis is that cells such as neutrophils may undergo necrosis during the 24 hour incubation leading to the release of contents that may be pro-inflammatory. Again this may be more likely to occur in the context of HIV infection. The fall in cytokine levels following Ag stimulation that was observed for many cytokines is harder to explain. To speculate Ag stimulation may either promote the consumption of certain cytokines, reduce the tendency of blood to coagulate or stabilise cells preventing necrosis.

QFGIT tubes have been specifically optimized for use as an interferon gamma release assay. If a similar strategy is to be used for other cytokines, a more systematic evaluation of the optimal tubes to be used, incubation times and the effect of HIV on assays is warranted. It is clear from this study that using the existing QFGIT platform may not be the best way to approach novel biomarker discovery.

There are several limitations to the study outlined in this chapter. Firstly, the commercial multiplex assay used did not cover all the cytokines and chemokines predicted by analysis of the transcriptomic data. Hence, there may be additional biomarkers that perform well and distinguish different stages of TB infection. Secondly, distinguishing the effect of HIV from TB was challenging. The inclusion of controls that were HIV infected on ART and HIV uninfected (with and without active TB) may have helped to better differentiate the effects of TB and HIV. However, in high burden settings such as South Africa the exclusion of subclinical TB in these control groups would still be required. Control groups without TB but with other diseases such as pneumonia may have also allowed evaluation of the specificity of these biomarkers. Furthermore validation of the promising biomarkers identified in a additional cohorts would have helped to confirm findings and improve generalizability.

### 6.8 Conclusions

The use of FDG-PET/CT to distinguish those with evidence of subclinical pathology within the lungs has facilitated identification of biomarkers that distinguish active from latent TB. Furthermore, some of these serological biomarkers show promise in the identification of those with subclinical TB especially those at the more active end of the spectrum. In addition, the value of using transcriptional data to inform the selection of analytes has been shown and in particular, further study of CCL23 and circulating immune complexes seems
warranted. Finally the data has highlighted some key concerns regarding the use of QFGIT tubes for biomarker discovery particularly in an HIV infected population.


**7.1 Scope of Thesis**

*Mycobacterium tuberculosis* is primarily a disease of humans and as such those that are infected by Mtb form the principal reservoir of the disease. Transmission, via the respiratory route occurs when infection progresses accompanied by bacterial replication and lung damage allowing aerosolisation of the organism. The later stages of disease are usually accompanied by symptoms and at this stage an individual is considered as having active clinical disease and may self-present to the health care system for treatment, it is also when the individual is most infectious. The core principles of TB control strategies are (outlined in figure 7.1):

1. Preventing those with active disease transmitting to susceptible individuals by early case detection through active case finding and promotion of good infection control practices.
2. Preventing progression to active disease in those with latent infection by administration of preventive therapy (usually with isoniazid).

Although this appears a conceptually attractive approach to TB control, there are practical challenges. Current diagnostic tests for latent TB do not stratify risk of disease progression, classify a very large proportion of the population as having evidence of latent infection and are poorly predictive of active TB. As a result when used as a guide to preventive treatment the number needed to treat to prevent a case of active TB is very high, even when incorporating additional epidemiological risk factors such as recent contact and immunocompromise. The tests also have the effect of simultaneously exaggerating the scale of the problem and reducing the perception of risk of latent TB (1/3 of the world population are infected but have only a 5-10% life time risk of active disease), leading to inaction on effectively tackling the reservoir of disease, which is a necessary step to TB elimination.

On the other hand active case finding using symptom screening, although a simple and effective way to identify TB cases with symptoms yet to present to the healthcare system (pre-diagnostic TB), may miss a number of truly asymptomatic cases (subclinical TB) who may be culture positive or culture negative but in the early stages of reactivation. This is a particular issue in high HIV burden, high transmission settings such as Khayelitsha and presents a further logistical challenge for the implementation of IPT, due to the concern of inappropriate treatment leading to increased drug resistance. CXR has been widely used for decades as a screening tool to identify people with subclinical or pre-diagnostic TB but it has a number of limitations as has been discussed in previous chapters. There is a clear need for a diagnostic test to fill this gap, that can identify asymptomatic persons at high risk of developing active TB which would facilitate TB control. However, a greater understanding of latent TB and its transition to active TB is required which is the area in which this thesis aimed to further knowledge.

The population of interest were healthy HIV infected outpatients, CD4 count ≥ 350/mm$^3$, ART naïve living in Khayelitsha and the scope of this thesis was to:

1. Explore the performance of the current tools for screening of active and latent TB used in the recruitment of participants for the study (Chapter 3).
2. Evaluate the utility of FDG-PET/CT to identify evidence of subclinical pathology in asymptomatic, ART naïve, HIV infected adults with evidence of latent TB and no previous history of tuberculosis (Chapter 4).

3. Determine the differentially expressed transcripts in whole blood in those with and without subclinical TB and in comparison to an active TB control group and to evaluate the effect of HIV on the transcriptional response to TB (Chapter 5).

4. Identify serological and QFGIT supernatant markers that distinguish those without evidence of subclinical pathology from those with evidence of subclinical pathology and active TB control group (Chapter 6).

7.2 General discussion of findings

The data outlined in Chapter 3 demonstrate the scale of the problem of latent TB in this community but also highlighted the limitations of the current diagnostic tests. Of 235 healthy, outpatient, HIV-infected, ART naïve persons with median CD4 count of 520/mm$^3$, 64.2% of the population had a positive QFGIT. This was less than the 71.8% of (younger) HIV uninfected persons from the same community that were QFGIT positive. Although not a significant difference, this highlights that current immunodiagnostisic tests for latent TB may perform less well in HIV infected persons. In addition, the median value of a positive QFGIT was significantly less in HIV infected persons (2.38IU/mL vs. 4.66IU/mL) and the distribution of positive results was clearly left-shifted. This is unsurprising given the depletion of CD4 cells in HIV infection, which are the main contributors to IFN$\gamma$ release. The distribution of TB Ag-NIL values in those with a negative QFGIT and TST was similarly different between HIV infected and uninfected persons. While in HIV uninfected persons the negative results were normally distributed around a mean TbAg-NIL of 0.03 IU/mL, the HIV infected participants by contrast had a bimodal distribution of TbAg-NIL with half clustered around 0 IU/mL and half clustered around the cut-off value of 0.35 IU/mL. During the screening period for the study, participants were observed for a 6 week period and during this time 20% of participants with a positive QFGIT reverted to negative particularly those with initial results in the bottom 2 quintiles, in keeping with what has been described previously[209]. In a similar population of 22 HIV infected persons screened for the vaccine study (of mixed ART status) with an initially negative QFGIT and TST, 40.9% converted from QFGIT negative to positive following administration of TST. A QFGIT cut-off ≥0.15 IU/mL correctly classifying 86% of those converting following TST boosting. Taken together this suggests that the universal cut off
used for QFGIT of 0.35IU/mL may be less appropriate for HIV infected individuals to detect all those immune sensitized by Mtb and in a similar manor to TST, a lower cut off possibly around 0.15-0.17 IU/mL may improve sensitivity of the test. This will need validation in other cohorts with differing CD4 counts and on and off ART and will need to be evaluated against a clinically relevant endpoint. Clearly the effect of this may be to further reduce the already poor positive predictive value of QFGIT, however the main value of the current test for latent tuberculosis is as a marker of exposure and immune sensitization by Mtb. Ensuring that the test has maximal sensitivity for this will in fact improve its utility epidemiologically when evaluating transmission and long-term impact of TB control programs.

The high burden of undiagnosed, untreated culture positive TB (or prevalent TB) in this population was also demonstrated in Chapter 3. Prevalence was estimated at 4,098/100,000 highlighting the importance of active case finding. However, only 40% were identified by symptom screening (although the screen used did differ from the WHO symptom screen with cough for 1 week rather than any cough being used) and 50% by CXR screening. Of note there was considerable overlap between those with CXR abnormalities and those with symptoms. The sensitivity of CXR screening for culture positive TB in those without symptoms was particularly poor (16.7%). In addition, the CXR abnormalities in persons at the earliest stages of clinical TB were minimal in extent and in general all CXR abnormalities were subtle. In keeping with previous studies, there was poor intra-observer agreement (κ=0.25) in reporting[81]. Recent evaluations of CXR as a screening tool for TB that report better sensitivity have been noted to have study designs resulting in verification bias in a systematic review[82]. The data presented in this thesis suggest that CXR may be best suited to identifying pre-diagnostic TB rather than true subclinical TB, especially in HIV infected persons in high burden settings. This warrants further evaluation in larger studies but also confirms the need for novel screening tools.

Although CXR appears useful to detect pathology around the stage at which symptoms commence, it was hypothesised that those in the early stages of reactivation might have evidence of pathology within lung and lymph nodes that might be visible using a more sensitive imaging technique such as FDG-PET/CT. In Chapter 4 asymptomatic participants with a clinical diagnosis of latent TB (evidence of immune sensitization by Mtb (positive QFGIT) but no evidence of active TB following screening) and no previous history of TB, had
FDG-PET/CT performed. The anatomical and metabolic abnormalities within the lung and lymph nodes in this population, at high risk of active TB, were then defined. This was a novel approach, which has not previously been performed systematically on this scale. Acknowledging that FDG-PET/CT changes (like CXR abnormalities and macroscopic abnormalities defined at autopsy) are not specific for but consistent with TB, 4 broad categories of parenchymal abnormality were defined. Only 28.6% of participants had normal lung parenchyma on PET/CT. Discrete nodules were found in 57%, a rate far higher than would be expected in a relatively young population with little smoking history, although few suitable studies are available for comparison. The anatomical nature and distribution of these lesions was highly suggestive of the Ghon focus of primary infection. One participant had active nodules accompanied by positive culture for MTB in a pattern suggestive of haematogenous rather than bronchogenic spread of TB. 28.6% of persons had infiltrates and scars with only a single lesion not located within the upper lobe (although this participant did have a second lesion within the upper lobe). Infiltrates were suggestive of active bronchogenic spread of TB and scarring suggestive of spontaneous healing. Of significant interest was that those with scarring of the lungs were significantly more likely to have infiltrates of the lungs (and also other evidence of disease activity) than those without scars. This supports the epidemiological observations that those with inactive lesions on CXR (notwithstanding the limitations of CXR) are more likely to develop disease. However, the active lesions on FDG-PET/CT were not within scars but often in unrelated areas of the lungs. The implication of this, although speculative given the limited numbers, is that those with inactive lesions are at high risk not necessarily because residual organisms within these lesions recrudesce (although this may still happen as certainly viable bacilli can be present within fibrotic scars) but because they are more likely to have repeated episodes of reactivation. This also explains the findings of at least 3 distinct clusters of fibrotic scaring at different stages of healing in 1 participant.

Lymph node abnormalities were more difficult to evaluate because of the presence of HIV co-infection. Supporting previous findings in the ART naïve population, FDG uptake within peripheral lymph nodes was common[146]. Almost all participants had abnormal axillary lymph nodes and over 70% had abnormal uptake in the cervical lymph nodes. Activity in the axillary lymph nodes in particular remained stable over time, however, initiating ART resulted in a reduction in FDG uptake, as previously reported[148]. Activity within central nodes (mediastinal and upper abdominal) was more complex and appeared to have a
bimodal distribution. Those with infiltrates/scars and active nodules within the lungs were significantly more likely to have highly avid central LN (SUVmax > 5). Furthermore the reduction in FDG activity in central LN appeared to relate more to treatment of TB than to HIV. This suggests that during reactivation involvement of regional lymph nodes occurs, this may simply reflect increased trafficking of cells or may reflect active infection of the regional lymph nodes (in which case granulomatous inflammation would be expected histologically). However, not all those with such parenchymal abnormalities had central lymph node uptake to this degree, notably the 2 participants that that were asymptomatic and culture positive had SUVmax < 5 within the central lymph nodes. Consideration of the observed abnormalities on FDG-PET/CT, in relation to literature from historical autopsy and imaging studies, allowed categorization of this group of 35 asymptomatic persons with evidence of immune sensitization but with negative symptom screen into a group of 10 with evidence of reactivation of latent infection potentially at higher risk of progression (termed subclinical TB) and 25 without evidence of reactivation considered to be a lower risk of reactivation. Of note, programmatically they would all have been eligible for IPT, but in practise just received 2 month follow up at the pre-ART clinic.

The design of this study allowed, for the first time, comparison of abnormalities reported on screening CXR to a gold standard test of FDG-PET/CT; reported in Chapter 4. The data highlighted that for 7 consultant radiologists, individual agreement with FDG-PET/CT, for the diagnosis of active, inactive and no TB was fair at best and agreement between readers was slight (based on \( \kappa \)-statistic). Of greater interest was the observation that when classifying screening CXR as having evidence of TB, readers either had a tendency to over-read (high sensitivity/low specificity) or under-read (low sensitivity/high specificity) which may reflect an inherent bias in individual readers. In general the data suggested that the utility of screening CXR for the identification of inactive TB changes was particularly poor, with high variability between readers. It is worth noting however that the task presented to the readers was particularly challenging. The participants had all been pre-screened approximately 6 weeks prior to FDG-PET/CT and repeat CXR, to ensure that they were symptom free, culture negative, had no history of previous TB and no evidence of active TB on an initial CXR (as determined by 2 medical officers). The careful anatomical description of parenchymal abnormalities on FDG-PET/CT and their comparison to CXR, may help in the training of readers, as improved understanding of the expected location and nature of early
TB related changes on CXR could lead to greater consistency and accuracy in the reporting of screening CXR.

If those with abnormalities consistent with subclinical TB represented a group in the early stages of reactivation, at higher risk of progressing to clinical active TB, then it was hypothesised that they may have similarities in transcriptional response with active TB that distinguished them from those with latent TB and no evidence of subclinical pathology. In Chapter 5, 893 transcripts were identified that were differentially abundant between an age, sex and CD4 matched group with active TB and those with “low risk” latent TB (no subclinical pathology) using statistical and fold change filtering. These transcripts were then analysed by sequential, stepwise fold change filtering to explore which were differentially expressed between subclinical and latent TB and which were more specific to the later stages of clinical TB. A transcriptional signature of 82 transcripts was defined which clustered 8 of 10 participants with subclinical TB with active TB. Of the 2 participants that did not cluster, 1 had active nodules suggesting haematogenous spread of disease and the other the most minimal of the bronchogenic infiltrates. Although speculative given the small numbers, it is possible that the biological differences between disease that spreads bronchogenically and that which spreads haematogenously also results in differences in transcriptional response. Furthermore, those with subclinical pathology showing greater metabolic activity within lung parenchyma or central lymph nodes clustered more strongly with active TB in comparison to those with less metabolic activity, raising the possibility that metabolic activity in part reflects disease activity. 22 of 82 transcripts were common to at least 1 of the 3 published active TB transcriptional signatures defined in HIV uninfected persons and when applied to a cohort HIV uninfected persons, these 82 transcripts effectively identified those with active TB from healthy controls with no evidence of TB. In addition, when applied to an existing dataset, which included participants with lung cancer, pneumonia, inactive sarcoidosis, active sarcoidosis and healthy controls, active tuberculosis effectively clustered from other diseases, although to a lesser extent from active sarcoidosis. This suggests that the signature relates to tuberculosis (and not HIV) and is specific for granulomatous inflammation. However, what sets it apart as novel is the ability to identify subclinical TB and hence the potential that it is more predictive for active TB, although it is clear that further validation is required to confirm this.
As well as providing a biomarker, the transcriptional signature allowed evaluation of the biological processes (albeit those represented in whole blood) that are associated with the subclinical stages of disease and those that are more specific to later stages of clinical disease. The complement system was consistently the most overabundant pathway at each stage of signature characterization and in particular transcripts relating to the classical complement pathway were overabundant in both subclinical and active TB in comparison to latent TB. Of note, SERPING1, C1q and C5 were common to all 3 of the other published active TB transcriptional signatures assessed[164, 165, 174]. Classical complement pathway is initiated by C1q binding to Fcγ component of immune complexes (most commonly) and so the over abundance of Fcγ receptor 1 also suggested that increased immune complex formation maybe playing a role early in disease. Similarly to the complement system, transcripts for Fcγ receptor 1 are commonly identified as over abundant in active TB[243]. By contrast over abundance of neutrophil related transcripts appeared to occur in the later stages of clinical disease. In keeping with this, abundance of neutrophil related transcripts was greater in smear positive compared to smear negative TB and supported findings that proportion of neutrophils in BAL is greater in more advanced TB[256, 257].

As no previous study has addressed in depth the impact of HIV on the TB transcriptional signature this was further evaluated in Chapter 5. The data showed that both HIV and TB individually resulted in a significant overabundance of transcripts within interferon related modules and in HIV associated TB this overabundance was further increased in part by increasing viral load. However, it was notable that the effect of TB and HIV was greatest on different interferon modules, which may reflect a greater impact on type 1 interferon for HIV and type 2 interferon for TB. Of practical importance this prominent overabundance of interferon modules in HIV may be why interferon signalling transcripts were less prominent in the subclinical signature as they would be expected to be less discriminatory.

Following on from this in Chapter 6, it was reasoned that if transcriptional differences were present between these different stages of TB infection and disease then differences in the concentration of proteins in either serum or QFGIT supernatant may also be present. The selection of 45 analytes was informed by network analysis of the 82 transcriptional signature and in addition, concentration of circulating immune complexes were determined to further investigate the biological hypothesis posed above. Ten analytes were found to have a significantly increased concentration in serum in those with active compared to latent TB
after multiple testing corrections and 1 analyte, IL-5, was also significantly higher in subclinical compared to latent TB. The ability of these 10 analytes to distinguish active from latent TB as determined by ROC analysis was generally very good and 3 analytes were more discriminatory than CRP (which has been suggested as a screening tool in HIV associated TB[302, 303]); IP-10, IL-1α and IFNγ. IL-1α with a cut-off of 16.9 pg/mL and CIC with a cut-off of 100.9 µg Eq/mL individually classified 50% and together 70% of those with subclinical TB as active TB. In addition when assessed across 5 stages of increasing disease activity by PET findings and smear status all 10 analytes showed a significant increasing trend. Serum analytes are attractive biomarkers as unlike transcriptional biomarkers they can easily be developed into low cost diagnostic tests using existing technologies. These biomarkers require validation in other cohorts. However these analytes could be particularly well suited to HIV associated TB as levels of these analytes, in particular IP-10, may be increased both by TB disease itself and the accompanying increase in HIV VL.

Of particular note the data highlighted CCL23 as having a potential role in HIV associated TB as both transcript abundance and serum levels were both elevated in active TB. However, this chemokine has received little attention previously and warrants further study. In general transcript abundance did not appear to correlate well with free serum levels of the corresponding protein other than for haptoglobin. However, both C1QB expression and SERPING1 expression correlated well with CIC suggesting that in serum most of these proteins may be bound to CIC. Both the transcriptomics and serological studies suggest that increases in immune complex formation may occur from the early stages of reactivation but little is known of the antibody specificity of these complexes particularly in the context of HIV associated TB or to what extent they also occur at the disease site, which would be an interesting area for further study.

The use of QFGIT supernatant in biomarker discovery is attractive as it provides a standardized commercial method of stimulating whole blood with mycobacteria specific antigens. However, the data presented in Chapter 6 highlight that levels of pro-inflammatory cytokines are orders of magnitude higher in plasma from the QFGIT NIL tube compared to serum in the population studied. This may result in masking of potentially important findings. This effect may be exaggerated for HIV infected samples and potential explanations may be, high circulating levels of fibrinogen or fibrin degradation products that can activate TLR-4 or neutrophil necrosis leading to release of pro-inflammatory contents.
The results highlight the need to systematically investigate the utility of the QFGIT assay in broader biomarker discovery especially in HIV infected individuals and consideration of ways to optimize a whole blood assay so that potentially valuable cytokine and chemokines are not overlooked.

7.3 Impact of thesis on the field of tuberculosis research

Latent tuberculosis is a challenging field of study. Asymptomatic infection is defined only through demonstration of immune sensitization by Mtb, the vast majority of those immune-sensitized do not go on to develop disease and in those that do, duration between infection and disease presentation is variable and sometimes prolonged. Despite the need to have a greater understanding of how and why persons with latent infection develop active TB to facilitate both novel diagnostic and vaccine development, studying the natural history of latent TB has clear ethical tensions given the availability of effective, though sub-optimal, preventive treatments. Another significant challenge is that no animal model of tuberculosis accurately and completely represents human disease (especially not disease seen in adults) and therefore much of our understanding of the natural history of latent tuberculosis infection has come from historical autopsy studies and observational studies from the pre-chemotherapeutic era.

The use of FDG-PET/CT described in this thesis, therefore provides a novel research tool to facilitate greater understanding of latent TB and is the first study to systematically utilise it in this way. Its utility has already been demonstrated in non-human primate models of TB were it has been used to track evolution of granuloma over time in vivo without the need for necropsy and it is being used in a number of studies of active TB to evaluate the response to treatment[139, 240]. It has a number of strengths and weaknesses as a research tool in this field. Firstly, it is comprised of 2 separate imaging modalities that independently and when combined provide valuable information on underlying pathology. The CT provides anatomically detailed information of normal and abnormal structures and pathology to a resolution of 1-3mm (depending on protocols used), which in this study was utilised as the primary means to define pathology to categorise participants. The FDG-PET component provides a tool to evaluate metabolic activity by providing a semi-quantitative marker of glucose uptake within structures that can be anatomically defined on CT. In the thesis this was valuable in categorising participants with subclinical TB into those with high and low
metabolic activity in order to relate to transcript abundance and concentration of serological markers. The FDG-PET component is particularly useful in characterising lymph node abnormalities especially when the CT is non-contrasted. However, PET tends to underestimate activity of lesions smaller than 1 cm although partial volume correction can mitigate this to a certain degree. This underestimation was apparent in this study as active nodules, which were likely to contain numerous metabolically active cells, being <5mm in size did not display increased FDG activity. Furthermore, FDG is a non specific marker of activity as was clear in this study as lymph node activity, likely relating to HIV infection, displayed considerable FDG uptake especially within axillary lymph nodes. A setting where FDG-PET/CT may be particularly well suited is in the evaluation of recent HIV uninfected household contacts of TB, in a recent case series of 5 household contacts reported by Ghesani et al. 4 had evidence of increased metabolic activity within mediastinal lymph nodes, 3 of which showed improvement following IPT[137]. FDG is the most commonly used tracer used both in clinical practice and research and in the context of TB is primary taken up by neutrophils and macrophages. However, there are numerous other markers that can be used for the in vivo study of inflammatory processes as recently outlined in a review by Wu et al.[304]. Of particular interest to TB research is the prospect of using radiolabeled matrix metalloproteinases (MMP), IL-2 and TNFα which are currently being evaluated in pilot studies[304]. \(^{18}\text{F}\)-fluoromisonidazole (FMISO) is another marker that has been utilised in TB research to evaluate hypoxia within TB lesions[305]. TB specific tracers are also in development with a radiolabelled analogue of trehalose (two 1-1 α, α-linked glucose monomers that are incorporated into the mycobacterial cell wall by antigen 85A enzymes) being developed by the Barry laboratory at the National Institute of Allergy and Infectious Diseases (NIAID). Although a promising tool in TB research, one of the ethical considerations around its use is the ionizing radiation accompanying the scan (in this study effective radiation dose per scan was approximately 10mSv), which is a particular factor when performing follow-up imaging.

A major contribution of this thesis has also been to demonstrate that the neat division of tuberculosis into active and latent infection states is an oversimplification and supports the idea of latent tuberculosis as a spectrum of infection states as has been proposed in a number of recent studies[84-86, 89]. The careful phenotyping of latent TB into potentially lower and higher risk groups may facilitate biomarker discovery and genetic markers of risk previously impeded by unrecognized heterogeneity in latent TB. Risk stratification of latent
TB is currently most commonly performed on epidemiological grounds e.g. HIV infection, recent migration from high burden setting etc. and is used to help make decisions on the appropriateness of investigation for active TB and the administration of preventive therapy. This strategy has limitations in that the majority of those with epidemiological risk factors are still unlikely to develop disease and those considered at low risk that eventually develop disease may have benefitted from receiving preventive therapy. The approach taken in this thesis may lead to the development of biological markers of risk, to give an individual risk of disease development rather than population risk of disease development. One consideration is over what timeframe would such as biomarker be predictive; as it is likely that these makers may perform better the in more advanced subclinical TB (as has been suggested by data presented in this thesis). As a result they may be predictive over a relatively short period of time – e.g. risk of TB over 6 - 12 months rather than predictive of life time risk.

The implications of the data presented in this thesis could have a significant effect on our approach to managing latent TB, particularly in the setting of HIV infection. A proportion of HIV infected persons considered to have a latent tuberculosis infection but no evidence of active disease by the current diagnostic definitions (and hence eligible for IPT), have been shown to have evidence of subclinical disease activity by FDG-PET/CT. This subclinical group have transcriptional and serological similarities with active TB and are likely to be at greater risk of progressing to clinical disease. Four out of 10 with subclinical TB developed symptoms and were commenced on standard TB treatment over the follow-up period in this study. This suggests HIV infected persons with latent TB infection do not represent a homogenous group but contain a biologically distinct and identifiable group at greater risk of disease. An immediate concern would be that 6 months IPT, the most common intervention and recommended in the current South African guidelines, might be an inappropriate therapy in this subclinical group. This is suggested by a recent study conducted by Samandari et al in Botswana. HIV infected persons were randomised to 6 or 36 months IPT irrespective of TST status, tuberculosis incidence began to increase in the group randomised to 6 months IPT within 6-12 months of stopping therapy[191]. However, this increased TB incidence was only seen in the TST positive group making reinfection after cure of latent TB an unlikely explanation. A more plausible explanation of these findings would be recrudescence of sub-optimally treated subclinical disease. As a result of this and other trials, 36 months IPT in HIV infected persons with latent TB infection has become a
Chapter 7 – General discussion and conclusions

conditional recommendation of the WHO. 36 months IPT may effectively treat subclinical TB, however, optimal treatment is unknown and may fall between the current 3-6 month preventive therapy and 6 month standard therapy regimens (if drug sensitive). Furthermore, similarly to active TB, co-administration of ART may be appropriate to effectively manage subclinical TB. An obvious concern of sub-optimally treated subclinical TB by isoniazid monotherapy is the potential for acquired drug resistance.

The concept of subclinical TB also has implications for screening for active TB. Currently in HIV clinics in South Africa, patients undergo a symptom screen for active TB at each appointment. If the screen is positive, patients are referred to the TB clinic for sputum investigation (currently with GeneXpert) and commenced on TB therapy if TB is confirmed. The aim of this approach is to identify cases of pre-diagnostic TB (symptomatic but yet to present clinically). If a low cost diagnostic test could be developed for subclinical TB which was found to be predicative of risk of tuberculosis over the subsequent 6-12 months this could transform our approach to TB screening. This might allow for all patients to be screened annually in the clinic irrespective of symptoms and then treated appropriately before symptoms develop. The ability to detect subclinical TB also has implications for mass screening and treatment strategies more generally. Recently, the theoretical benefits of 5-year cycles of mass treatment of an entire population with standard TB therapy in order to achieve TB elimination have been explored[306]. Although modelling shows such an approach could be highly effective at rapidly reducing TB incidence there are numerous ethical and practical barriers to implementation. The approach of regular mass screening for subclinical disease and targeting treatment at this population may prove a similarly effective strategy that may be more easily accepted and implemented.

A fundamental question is whether those with evidence of reactivation on PET/CT will inevitably go on to develop disease given that spontaneous healing can occur. However, even if spontaneous healing was to occur, data presented in this thesis and previous epidemiological studies suggest that subsequent reactivation would be more likely. It may be that such a group have a relatively unstable form of latent TB that follows a waxing and waning course during which periods of progression triggered by precipitating factors may be followed by control or the development of clinical disease.
Several studies over the past few years have utilized whole blood transcriptomics both to develop signatures that distinguish active from latent TB but also to gain insight into the biology of the disease. A number of common biological themes have been identified including interferon signalling, complement system, Fcγ receptor mediated phagocytosis and the role of neutrophils [243]. This thesis has contributed to the understanding of which of these biological pathways are pertinent in early disease and which relate to more advanced disease and what the effect of HIV infection is. The data show that abundance of neutrophil related transcripts may be a feature of more advanced disease and increased complement system and Fcγ receptor related transcripts and immune complex formation may occur from early on in disease. Overabundance of interferon related transcripts are a feature of both HIV and TB. This suggests that renewed focus on the role of B-cells and immune complex formation in TB may be required and that further investigation into the interaction of HIV and TB through their mutual induction of type 1 interferon would be of great interest. Some of the hypotheses generated by the transcriptomics data were then validated in serum in particular the observation that CIC were elevated in serum in both active and advanced subclinical TB in comparison to latent TB.

Serum biomarkers that reliably identify subclinical TB would clearly have significant translational potential diagnostically. Furthermore the possibility of a low cost POC test would be feasible, as the lateral flow immunochomatographic platform has been widely used for this purpose. The analysis described in this thesis was limited to the evaluation of individual analytes to discriminate active and subclinical TB from latent TB using ROC analysis. Although several individual analytes discriminated active and latent TB effectively, the performance of individual analytes to distinguish latent and subclinical TB was relatively poor. This analysis could be taken forward to attempt to identify a panel of biomarkers that could be used distinguish these 2 groups more effectively. Classification and regression tree (CART) analysis would be one approach to create a decision tree of the smallest number of analyte cut-offs that effectively separated those with subclinical and active TB from those with latent TB. A panel of 3-6 serum analytes could be easily developed initially into a multiplex assay and taken forward for further validation in a new cohort to determine the sensitivity and specificity of such a decision tree. These potential biomarkers could either be evaluated in a similar cohort where PET/CT imaging is used to define subclinical pathology or ideally in a natural history cohort or the placebo arm of a vaccine or preventive therapy trial.
where the predictive potential of the biomarkers for clinically active TB could be better determined.

While CXR screening may be reasonably effective at identifying pre-diagnostic cases of TB, the data presented in this thesis show that CXR performs poorly at detecting truly subclinical cases of TB whether culture positive or culture negative. In these cases where pathology is subtle even highly experienced radiologists disagree on findings and perform poorly against a gold standard imaging modality. Furthermore, the data highlight that the performance of CXR as a screening “test” in terms of sensitivity and specificity can vary considerably from radiologist to radiologist. Unlike laboratory tests, there is no “limit of detection” for CXR reporting, however, there comes a point at which the “noise” from overlying bony structures prevents reliable detection of more subtle lesions. Advances in digital CXR technology may help improve reliability of TB CXR screening. Dual energy subtraction radiography (GE Healthcare) and ClearRead bone suppression software (Riverain technologies) are 2 techniques that can digitally remove overlying bony structure. With dual energy subtraction radiography 2 exposures of different energy are acquired and then bony structures are digitally removed to create soft tissue only and bone only images in addition to the standard image. ClearRead bone suppression is a software package that digitally suppresses bony structure on the image viewer i.e. not requiring special imaging equipment and not providing increased radiation exposure. These technologies have been shown to improve detection of pulmonary nodules[307]. In addition computer aided detection has also been shown to further enhance detection of nodules alone and when combined with these technologies[308].

Finally, two potential limitations of the QFGIT assay have been highlighted, firstly the data have questioned whether it is appropriate to have the same cut off for HIV infected and HIV uninfected populations. Secondly, although the QFGIT platform is well suited to detect interferon gamma release in whole blood following stimulation with mycobacterial antigens, the platform may not be the ideal assay for biomarker discovery for other potentially discriminatory biomarkers.
7.4 Future direction for the field and research priorities

The challenge set to eliminate TB as a public health problem by 2050 is undeniably ambitious but one that if successful will have a profound impact on human health[11]. TB is estimated to have been responsible for 1 billion deaths globally between 1700 and 1900, is currently responsible for 1.4 million deaths annually and is still the leading cause of death in South Africa. The WHO has recently approved a road map, which sets out a strategy and interim targets to achieve this goal[13]. A core part of this strategy is a commitment to TB research and development as it is recognized that without novel vaccines, drugs and diagnostic tests that the 2050 target is not feasible.

How then should we move forward in the field of latent TB, recognizing that the effective management of this reservoir of TB will become increasingly important as progress in TB control is made? This thesis has highlighted that a proportion of those labelled as having latent TB have evidence of reactivation and subclinical disease which may put them at higher risk of disease progression. However, little is known about the precipitants for reactivation and the critical events that lead to loss of host control or indeed the subsequent regaining of control that leads to spontaneous healing. Furthermore, it is not clear whether
all those that show evidence of immune sensitization, hence labelled as having latent TB, are all infected with viable bacilli. It may be that some individuals have succeeded in eliminating the organism, while maintaining immune sensitization, and are thus at no risk at all of reactivation. In addition, and perhaps most intriguingly, the epidemiological data suggest that those that are considered latently infected, may be at reduced risk of disease following reinfection\([42, 309-311]\). This suggests that for some, there maybe a protective effect from natural infection supported by animal studies\([312]\). Embracing and trying to understand this heterogeneity within the spectrum of latent TB may give important insight into desirable and undesirable immune responses, markers of protection and risk, that will be of value in the future development of both vaccines and diagnostics (figure 7.2).

In addition to defining markers of risk and protection, developing an optimal marker of exposure may also be of value. Although TST and IGRA are recognised to be poorly predictive, they are also suboptimal markers of exposure as they both undergo spontaneous reversion and sensitivity in those with active tuberculosis (hence undeniably exposed) is at best 85-90%. A more sensitive marker of Mtb exposure would give a better understanding of TB transmission dynamics. Ultimately, it is worth recognising that one test can’t do it all, as a single test cannot be both highly predictive for active TB and a sensitive marker of exposure, as these test characteristics are to a degree a mutually exclusive.

Finally, diagnostics are only one side of the coin to latent TB. If preventive treatment is prolonged or has unacceptable side effects, widespread implementation in an asymptomatic population is challenging. Isoniazid, in many ways, is an unusual drug of choice for treatment of latent TB given its main effect on actively replicating organisms and poor sterilizing ability. Several novel agents are coming through the TB drug discovery pipeline which may be promising candidates for preventive therapy either alone or in combination (not with standing toxicity issues), such as bedaquiline, delamanid, sutezolid and moxifloxacin. However, a critical issue is also how best to evaluate these new drug regimens. Phase 3 studies for novel preventive treatments in latent TB will require thousands of participants to be followed up for several years in multisite studies as the outcome of active TB is comparatively rare. Validated biomarkers of risk would greatly facilitate these studies by potentially reducing the sample sizes required by concentrating the study on a population at greater risk of disease. In addition validated surrogate end
points that related to successful treatment of latent TB could help in selection of promising candidates in Phase 2 studies, this could take the form of blood, urine or imaging biomarker.

Our approach to latent tuberculosis and TB control would be transformed by, an inexpensive point of care diagnostic that provided an accurate assessment of likelihood of progression, coupled with a single dose preventive treatment. This would enable roll out of mass testing and treatment programs with a realistic chance of achieving TB eradication.
APPENDIX A – Brief summary of PBMC experiments not included in thesis

Blood was collected at each time point in NaHep tubes for the isolation of PBMC (see Table 2.2). PBMC were then cryopreserved and experiments performed after the completion of follow-up. The PBMC experiments were not included in this thesis for the reasons explained below. The primary purpose of PBMC isolation was to perform a longitudinal ELISPOT experiment to address the hypothesis that isoniazid therapy would result in a greater increase in T cell responses to TB antigens during treatment in those with subclinical TB compared to those with latent TB and no evidence of subclinical pathology. The rationale for this was that, isoniazid is rapidly bactericidal against actively replicating bacilli (a greater number of which would be expected in subclinical TB) by means of cell wall damage. A 20-fold increase in secretion of mycobacterial proteins has been demonstrated to occur shortly after isoniazid administration in vitro and in clinical studies in HIV uninfected persons, preventive treatment of latent TB resulted in a 1.8 fold increase in IFN-γ production by T cells to TB antigens [241, 242].

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Description</th>
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<tbody>
<tr>
<td>ESAT-6/CFP-10</td>
<td>Antigens used in commercial IGRA – immunodominant for IFN-γ Not present in BCG</td>
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<tr>
<td>Rv1737c</td>
<td>Up-regulated as part of the Dormancy survival Regulon (DosR) in hypoxic stress Preliminary data from Mexico shows IFN-γ response increases with IPT Not present in BCG</td>
</tr>
<tr>
<td>Rv0849</td>
<td>Up-regulated as part of the Extended Hypoxic Response (EHR) Preliminary data from Mexico shows IFN-γ response increases with IPT</td>
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<tr>
<td>Rv1986</td>
<td>Up-regulated as part of the Extended Hypoxic Response (EHR) Immunodominant for IL-2 Not present in BCG</td>
</tr>
<tr>
<td>Ag85A</td>
<td>Immunodominant for IFN-γ Antigen used in a number of vaccine candidates</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified Protein Derivative used in TST</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr Virus antigen Unrelated control antigen</td>
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</tbody>
</table>

Table A1 – TB antigens used in ELISPOT experiment

T cell responses were measured by IFN-γ and IL-2 ELISPOT at screening (~6 weeks), baseline (day of PET/CT), 1 week, 1 month, 3 months and 6 month to the antigens outlined in Table A1. Samples were collected over a period of 24 months, during this period 2 batches of NaHep tubes were subsequently found to have been contaminated by LPS. This had the effect of increasing the background number of spot forming cells in the ELISPOT assay resulting in indeterminate/invalid results for a number of the follow-up samples. Most
participants were affected for at least 1 follow-up time point. In addition, because participants were managed as clinically indicated during follow-up, 4 participants (all in the subclinical group) were commenced on standard TB therapy with 2 of these participants and an additional participant commenced on ART. This created confounding factors not initially anticipated. Furthermore, the difficulties in follow-up described previously (section 2.7) resulted in only 89.4% of samples required being available. Taken together this meant that meaningful analysis to address the hypothesis posed was not possible and hence data was not included as part of the thesis.

PBMC samples from the day of PET/CT scan were also used for a smaller flow cytometry experiment to address the hypothesis that functionality (in terms of IFN-γ, IL-2 and TNF-α production) and memory/senescence phenotype (in terms of CD45RA/CD27/CD57 expression) of ESAT-6/CFP-10 specific CD4 T cells would differ between those with subclinical TB and those with latent TB and no evidence of subclinical pathology. The rationale being that chronic antigen exposure in subclinical TB may lead to terminal differentiation and senescence of antigen specific T cells with accompanying change in functionality as has been described in chronic viral infection. Though the samples taken on the day of PET/CT scan were less affected by LPS contamination the results were not considered substantial enough to form a chapter of the thesis and were not related to work carried out in any of the other chapters so this data has not been included in the thesis.
APPENDIX B – Approvals for Study

University of Cape Town – Approval 013/2011

Amendment Form

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<tr>
<td>HREC REF Number</td>
<td>013/2011</td>
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<tr>
<td>Protocol number (if applicable) &amp; Protocol Title</td>
<td>How inactive is “inactive” tuberculosis? PET/CT benchmarking of latent tuberculosis infection</td>
</tr>
<tr>
<td>Principal Investigator</td>
<td>Prof R. J. Wilkinson</td>
</tr>
<tr>
<td>Department/Office, Internal Mail Address</td>
<td>Room 3.03 &amp; 5, Wolfson Pavilion, Institute of Infectious Diseases and Molecular Medicine, Wernher and Beit Building South, Faculty of Health Sciences, Observatory, 7925</td>
</tr>
</tbody>
</table>

List of Proposed Amendments with Revised Version Numbers and Dates

All changes relate to Protocol Version 5, 3rd November 2011

1. Low-dose Propranolol administration prior to FDG-PET/CT: To reduce FDG uptake in brown adipose tissue to minimize false positive scans
2. Addition of control group: 20 HIV positive participants established on ARVs treated with IPT for 6 months with blood sampling at 0, 1 week, 1 month, 3 months and 6 months.
3. Alteration of definition of active TB: from smear and culture positive to culture positive (smear positive or negative).
4. Recruitment of active TB controls from local TB clinic: Recruitment of active TB controls (smear and/or culture positive) from local TB clinic as well as vaccine study of targets not being met
5. Sampling of active controls: Sputum samples and blood for CD4 and VL to be taken if required (ie not recently done or available for storage)

RESEARCH ETHICS COMMITTEE

- 6 DEC 2011

HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN
Appendix B

Stellenbosch University – Approval N12/11/079

Approval Notice
New Application

26 Nov 2012
Wilkinson, Robert R
Stellenbosch, WC

Ethics Reference #: N12/11/079
Title: How inactive is "inactive" tuberculosis: PET-CT benchmarking of latent tuberculosis infection.

Dear Prof Robert Wilkinson,

The New Application received on 19-Nov-2012, was reviewed by members of Health Research Ethics Committee 2 via Expedited review procedures on 23-Nov-2012 and was approved. Please note the following information about your approved research protocol:


Please remember to use your protocol number (N12/11/079) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:

Please note a template of the progress report is obtainable on www.sun.ac.za/ids and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year, if necessary. Annually a number of projects may be selected randomly for an external audit.

Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB00005239

The Health Research Ethics Committee complies with the SA National Health Act No 61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research, Principles Structures and Processes 2003 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Mr Claudette Abrahams at Western Cape Department of Health (claudette.abrahams@wcd.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and documents please visit www.sun.ac.za/ids

If you have any questions or need further assistance, please contact the HREC office at 0219480307.

Included Documents:
  Checklist
  Synopsis
  Protocol
  Investigators declaration
  Application Forms
  Cover Page
  Other Documentation

Sincerely,
City of Cape Town Approval - 10286

2012-03-09

re: Research Request: How inactive is “Inactive” TB (ID NO: 10286)

Dear Dr Esmail

Permission has been granted to do your research as per your protocol.

Khayelitsha Sub District: Site B Clinic
Contact People: Dr V de Azvedo (Sub District Manager)
Tel: (021) 390-1289/ 083 629 3344

Please note the following:
1. All individual patient information obtained must be kept confidential.
2. Access to the clinics and its patients must be arranged with the relevant Managers such that normal activities are not disrupted.
3. A copy of the final report must be sent to the City Health Head Office, P O Box 2815 Cape Town 8001, within 3 months of its completion and feedback must also be given to the clinics involved.
4. Your project has been given an ID Number (10286). Please use this in any future correspondence with us.

Thank you for your co-operation and please contact me if you require any further information or assistance.

Yours sincerely

Dr G H Visser
MANAGER: SPECIALISED HEALTH

cc: Dr Azvedo
Dr K Jennings
Ms Caldwell
Appendix B

Western Cape Province Approval

REFERENCE: RP 23/2012
ENQUIRIES: Dr Sikhumbiso Mabunda

Room 3.03
IIDMM
Wolmarans Pavilion
UCT: Faculty of Health Sciences
Observatory
7925

For attention: Dr Hanif Esmail, Prof Robert J Wilkinson, Prof Douglas Young, Dr Clifton Barry

Re: How inactive is “inactive” TB

Thank you for submitting your proposal to undertake the above-mentioned study. We are pleased
to inform you that the department has granted you approval for your research. Please contact the
following people to assist you with any further enquiries.

Khayelitsha Site 8 Ubuntu clinic  M Notsha  (021) 361 4835
Khayelitsha Site 8 Ubuntu TB clinic  M Notsha  (021) 361 4835

Kindly ensure that the following are adhered to:
1. Arrangements can be made with managers, providing that normal activities at requested
facilities are not interrupted.
2. Researchers, in accessing provincial health facilities, are expressing consent to provide the
department with an electronic copy of the final report within six months of completion of
research. This can be submitted to the provincial Research Co-ordinator (research@pprc.gov.za).
3. The reference number above should be quoted in all future correspondence.

We look forward to hearing from you.

Yours sincerely,

[Signature]

DR N T Naledi
DIRECTOR, HEALTH IMPACT ASSESSMENT
DATE: [Date]

CC: DR G Perez
DIRECTOR, EASTERN/KHAYELITSHA

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APPENDIX C – Consent forms

Screening Consent Form

UNIVERSITY OF CAPE TOWN

My name is ______________________

We would like to SCREEN you to see if you can participate in our study.

Tuberculosis infection is very common in Khayelitsha where 60-70% of are infected with tuberculosis but are well and can’t pass the infection on, this condition is known as latent tuberculosis infection. Some people with latent tuberculosis especially if they have HIV as well will become sick from tuberculosis. The purpose of our study is to see if everyone living with HIV that is infected with TB is at the same risk of becoming sick from tuberculosis. This will help us to develop tests to identify those who most and least need treatment for TB and will help us make progress toward getting rid of TB as a major health problem in the long run. This study is being run by the Institute of Infectious Diseases and Molecular Medicine at the University of Cape Town and has been funded by the Wellcome Trust. Prof Robert J Wilkinson is the Principal Investigator. This study has also been approved by the Human Research Ethics Committees of the University of Cape Town and Stellenbosch University and will be conducted according to international standards of Good Clinical Practice, applicable South African government regulations, and Institutional research policies and procedures.

You will receive a Subject Information Sheet which explains the study and the risks and benefits of taking part in more detail.

Before you can participate fully in the study we need to make sure that there are no reasons why you wouldn’t be able to take part. IT IS ENTIRELY UP TO YOU TO DECIDE WHETHER OR NOT YOU WHAT TO ALLOW US TO SCREEN YOU FOR THIS STUDY. If you agree we would like to ask you permission to allow a doctor working with the study to ask you some questions and examine you. Also we would like to take 8 -11 tea-spoons of blood to check that you have TB infection and to check your CD4 count, a urine sample to make sure that women are not pregnant, we would like you to produce a cough sample for us which might involve breathing in a fine mist of salty water (called a nebuliser) to see if we can grow any TB, this will cause you to cough but is generally well tolerated, please inform study staff if you have asthma in which case you should not have sputum induced. In addition we would like to take a CXR. Some of these tests may have already been done as part of your screening.
for other studies in which case they will not be repeated. We would then like to see you in 6 weeks to repeat your chest X-Ray and go through your test results with you. You will not be liable for any expense relating to these investigations and will be reimbursed R30 as compensation for time and travel.

Please note that with your consent some of your blood and urine that you provide will be stored safely at the IIDMM for use in this research study or future studies related to this project.

Although these tests very rarely causes problems, if anything goes wrong the University provides insurance to cover this possibility. If you have any concerns regarding following any procedure performed as part of the study you may contact the study doctor.

Please take time to read through the Subject information sheet before your next appointment and make a note of any questions that you might like to discuss further. We will answer these for you when we go over your results with you.

Today you are only being asked to give consent to be screened to see if you have the right features for this study, you are not agreeing at this stage to take part formally in the study. We will ask for you consent again at the next visit if you wish to take part, but the decision to participate is entirely your own. IF YOU DECIDE NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DIASDVFANTAGED IN ANY WAY. In addition, at any point during the trial you are free to withdraw without telling us why. We are aiming to recruit 56 participants from Cape Town.

Since this is a research test, the results will not be made available to the participants however you will be informed of any new relevant information that arises during the course of the trial. Throughout the trial your privacy will be maintained and nobody other than the doctors and nurses looking after you will know that you are participating. Samples will be labelled with code numbers and hence the laboratory staff will not know your identity. When the results of the study become available, names of the participating patients will not be included. The Research Ethics Committees may inspect the research records if required. You may inform your general practitioner of involvement in the study.

Do you have any questions? You may contact either the UCT Research Ethics Committee (021 406 6492) Prof Robert Wilkinson (021 406 6084) or Dr Hanif Esmail (021 406 6389) if you have further questions. Please remember that Prof Wilkinson and Dr Esmail will not be directly responsible for your medical care which will be conducted by your regular doctors and nurses.

Consent to be SCREENED to participate in the study:

I have read the above / the above has been read to me and I have had the opportunity to discuss the study with Dr __________________________ and ask any questions. I consent to take part in this study:

Signature __________________________

Name __________________________

Date __________________________

Name of person consenting __________________________

Signature __________________________

Date __________________________
How inactive is “inactive” Tuberculosis?
PET/CT Benchmarking of Latent Tuberculosis Infection

INFORMED CONSENT FORM FOR STUDY ENTRY

My name is ________________________

We are asking you to take part in our study. You were found to have latent tuberculosis infection as part of screening for the vaccine study. We have seen you on 2 occasions over the last few weeks and have made sure that your X-Ray has not got any worse, that you remain free of symptoms and that you meet all the criteria for our study.

You should have received a Study Information Sheet, which explains in detail the risks and benefits of the study as well as what to expect once you join, and had plenty of time to read it and go through any questions you have with a study staff member. If there are any outstanding questions feel free to ask now.

The purpose of our study is to see if everyone that is infected with TB is at the same risk of becoming unwell. This will help us to develop tests identify those who most and least need treatment for TB and will help us make progress toward getting rid of tuberculosis as a major health problem in the long run. This study is being run by the Institute of Infectious Diseases and Molecular Medicine at the University of Cape Town and has been funded by the Wellcome Trust. Prof Robert J Wilkinson is the Principal Investigator. This study has also been approved by the Human Research Ethics Committees of the University of Cape Town and Stellenbosch University and will be conducted according to international standards of Good Clinical Practice, applicable South African government regulations, and Institutional research policies and procedures. 56 participants from Cape Town are planned to be recruited into this study.

If you agree we will start you on 9 months of standard treatment for inactive TB after you have had a PET/CT scan. This will occur at some point in the next week. After starting treatment you will be seen after a week and then monthly until you complete treatment. You will receive a repeat PET/CT after 6 months of treatment. At 5 of the visits we will take between 8 and 11 teaspoons of blood for the study and a urine sample. You will receive a total of R400 spread over the course of the study to compensate you for time and loss of earnings. Please note that once you start your treatment it is extremely important that you continue to take it until the end of the study. If there are any problems with taking the medicine you must inform the study staff. Prior to the PET/CT scan we may give you a single oral dose of propranolol (up to 40mg) which is commonly used to reduce activity of your fat deposits to improve the quality of the PET/CT scan, we do not expect you experience any side effects from this tablet but if you suffer from asthma you will not receive it. You will not be liable for any expense relating to these investigations.
One of the blood samples will be stored for genetic tests. Genes are codes in you body that determine your individual characteristics, including how your immune system (your body’s defence system) functions. The only genetic tests that would be done on your sample would be to test for genes involved in you body’s response to tuberculosis. No tests will be done for genetic diseases or any other conditions not related to tuberculosis.

Although the tests in the study very rarely causes problems, if anything goes wrong the University provides insurance to cover this possibility. If you have any concerns regarding following any procedure performed as part of the study you may contact the study doctor.

The decision to participate is entirely your own. **IF YOU Decide NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DIASDVANTAGED IN ANY WAY.** In addition, at any point during the trial you are free to withdraw without telling us why.

Since these are research tests, the results will not be made available to the participants, however you will be informed of any new relevant information that arises during the course of the trial. Throughout the trial your privacy will be maintained and nobody other than the doctors and nurses looking after you will know that you are participating. Samples will be labelled with code numbers and hence the laboratory staff will not know your identity. When the results of the study become available, names of the participating patients will not be included. The Research Ethics Committees may inspect the research records if required. You may inform your general practitioner of involvement in the study.

Please note that with your consent some of your blood and urine that you provide will be stored safely at the IIDMM for use in this research study or future studies related to this project.

You may contact either the **UCT Research Ethics Committee** (021 406 6492) **Prof Robert Wilkinson** (021 406 6084) or **Dr Hanif Esmail** (021 406 6389) if you have further questions at any point during the study. Please remember that Prof Wilkinson and Dr Esmail will not be directly responsible for your medical care which will be conducted by your regular doctors and nurses.

**Consent to participate in the study:**

I have read the above / the above has been read to me and I have had the opportunity to discuss the study with Dr _______________________ and ask any questions. I consent to take part in this study:

Signature ______________________

Name ______________________

Date ______________________

Name of person consenting ______________________

Signature ______________________

Date ______________________
Normal CXR, LTBI Control Consent Form

UNIVERSITY OF CAPE TOWN

Study Doctor
Dr Hanif Esmail MA, MRCP, FRCPath
Wellcome Trust Clinical Research Training Fellow
Honorary Research Associate (IIDMM)
h.esmail@imperial.ac.uk

Principal Investigator
Prof. Robert J. Wilkinson MA, PhD, FRCP
Wellcome Trust Senior Research Fellow
Honorary Professor (IIDMM)
r.j.wilkinson@imperial.ac.uk

How inactive is “inactive” Tuberculosis?
PET/CT Benchmarking of Latent Tuberculosis Infection

LTBI CONTROL CONSENT FORM

My name is ______________________

The purpose of our study is to see if in some people with “silent” or latent TB, we can find differences that mean they might be more likely to become unwell. This will help us to develop tests to identify those who most and least need treatment for latent TB and will help us make progress toward getting rid of TB as a major health problem in the long run. The main part of the study involves using a PET/CT scan to assess the activity of scars caused by tuberculosis that can be seen on a chest x-ray. This study is being run by the Institute of Infectious Diseases and Molecular Medicine at the University of Cape Town and has been funded by the Wellcome Trust. Prof Robert J Wilkinson is the Principal Investigator. This study has also been approved by the Human Research Ethics Committees of the University of Cape Town and Stellenbosch University and will be conducted according to international standards of Good Clinical Practice, applicable South African government regulations, and Institutional research policies and procedures.

Your chest x-ray is normal. however, we have found that you have latent tuberculosis infection that requires treatment with isoniazid (INH). We do not need to perform a PET/CT scan.

We would like to ask you to be a control subject for our study, to see if you respond to INH treatment in the same way as people with scars on there chest x-ray. 20 control participants with latent tuberculosis from Cape Town are planned to be recruited into this study. This will involve taking 8-11 teaspoons of blood and a urine sample, 5 times during the course of your INH treatment (at the start of treatment, then after 1 week, 1 month, 3 months and finally at the end of treatment). You will not be liable for any expense relating to these investigations and will be reimbursed up to R30 per visit as compensation for time and travel.

One of the blood samples will be stored for genetic tests. Genes are codes in you body that determine your individual characteristics, including how your immune system (your body’s defence system) functions. The only genetic tests that would be done on your sample would be to test for genes involved in you body’s response to tuberculosis. No tests will be done for genetic diseases or any other conditions not related to tuberculosis.

Although the tests in the study very rarely causes problems, if anything goes wrong the University provides insurance to cover this possibility. If you have any concerns regarding following any procedure performed as part of the study you may contact the study doctor.
The decision to participate is entirely your own. IF YOU DECIDE NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DISADVANTAGED IN ANY WAY. In addition, at any point during the trial you are free to withdraw without telling us why.

Since these are research tests, the results will not be made available to the participants, however you will be informed of any new relevant information that arises during the course of the trial. Throughout the trial your privacy will be maintained and nobody other than the doctors and nurses looking after you will know that you are participating. Samples will be labelled with code numbers and hence the laboratory staff will not know your identity. When the results of the study become available, names of the participating patients will not be included. The Research Ethics Committees may inspect the research records if required. You may inform your general practitioner of involvement in the study.

Please note that with your consent some of your blood and urine that you provide will be stored safely at the IIDMM for use in this research study or future studies related to this project.

You may contact either the UCT Research Ethics Committee (021 406 6492) Prof Robert Wilkinson (021 406 6084) or Dr Hanif Esmail (021 406 6389) if you have further questions at any point during the study. Please remember that Prof Wilkinson and Dr Esmail will not be directly responsible for your medical care which will be conducted by your regular doctors and nurses.

Consent to participate in the study:

I have read the above / the above has been read to me and I have had the opportunity to discuss the study with Dr _________________________ and ask any questions. I consent to take part in this study:

Signature _______________________

Name _______________________

Date _______________________

Name of person consenting _______________________

Signature _______________________

Date _______________________

Name of person consenting _______________________

Signature _______________________

Date _______________________
Active TB Control Consent Form

UNIVERSITY OF CAPE TOWN

Study Doctor
Dr Hanif Esmail MA, MRCP, FRCPA
Wellcome Trust Clinical Research Training Fellow
Honorary Research Associate (IIDMM)

h.esmail@imperial.ac.uk

Principal Investigator
Prof. Robert J. Wilkinson MA, PhD, FRCP
Wellcome Trust Senior Research Fellow
Honorary Professor (IIDMM)

r.j.wilkinson@imperial.ac.uk

How inactive is “inactive” Tuberculosis?
PET/CT Benchmarking of Latent Tuberculosis Infection

ACTIVE TB CONTROL CONSENT FORM

My name is ______________________

The purpose of our study is to see if in some people with “silent” or latent TB, we can find differences that mean they might be more likely to become unwell. This will help us to develop tests to identify those who most and least need treatment for latent TB and will help us make progress toward getting rid of TB as a major health problem in the long run. The main part of the study involves using a PET/CT scan to assess the activity of scars caused by tuberculosis that can be seen on a chest x-ray. This study is being run by the Institute of Infectious Diseases and Molecular Medicine at the University of Cape Town and has been funded by the Wellcome Trust. Prof Robert J Wilkinson is the Principal Investigator. This study has also been approved by the Human Research Ethics Committees of the University of Cape Town and Stellenbosch University and will be conducted according to international standards of Good Clinical Practice, applicable South African government regulations, and Institutional research policies and procedures.

You have been found to have active TB and will be started on TB treatment.

We would like to ask you to be a control subject for our study, to see what differences we can find between you and people with latent TB. We plan to recruit 15 control participants with active TB. This will involve taking just up to 9 teaspoon of blood before and at the end of TB treatment, most of this will be for research tests but we will also check your CD4 count and viral load as needed. In addition we may request 2 sputum samples from you and a Chest X-Ray if not available. We would also like to check your laboratory results and medical notes during treatment. You will not undergo PET/CT scan. You will not be liable for any expense relating to these investigations and will be reimbursed R30 as compensation for time and travel.

Although the tests in the study very rarely causes problems, if anything goes wrong the University provides insurance to cover this possibility. If you have any concerns regarding following any procedure performed as part of the study you may contact the study doctor.

The decision to participate is entirely your own. IF YOU DECIDE NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DISADVANTAGED IN ANY WAY. In addition, at any point during the trial you are free to withdraw without telling us why.

Since these are research tests, the results will not be made available to the participants, however you will be informed of any new relevant information that arises during the course of the trial. Throughout the trial your privacy will be maintained and nobody other than the doctors and nurses
looking after you will know that you are participating. Samples will be labelled with code numbers and hence the laboratory staff will not know your identity. When the results of the study become available, names of the participating patients will not be included. The Research Ethics Committees may inspect the research records if required

Please note that with your consent some of your blood and sputum will be stored safely at the IIDMM for use in this research study or future studies related to this project.

You may contact either the UCT Research Ethics Committee (021 406 6492) Prof Robert Wilkinson (021 406 6084) or Dr Hanif Esmail (021 406 6389) if you have further questions at any point during the study. Please remember that Prof Wilkinson and Dr Esmail will not be directly responsible for your medical care which will be conducted by your regular doctors and nurses.

Consent to participate in the study:

I have read the above / the above has been read to me and I have had the opportunity to discuss the study with Dr ______________________ and ask any questions. I consent to take part in this study:

Signature ______________________
Name ______________________
Date ______________________

Name of person consenting ______________________
Signature ______________________
Date ______________________
How inactive is “inactive” Tuberculosis?
PET/CT Benchmarking of Latent Tuberculosis Infection

TST- IGRA- CONTROL CONSENT FORM

My name is ________________

The purpose of our study is to see if in some people with “silent” or latent TB, we can find differences that mean they might be more likely to become unwell. This will help us to develop tests to identify those who most and least need treatment for latent TB and will help us make progress toward getting rid of TB as a major health problem in the long run. The main part of the study involves using a PET/CT scan to assess the activity of scars caused by tuberculosis that can be seen on a chest x-ray. This study is being run by the Institute of Infectious Diseases and Molecular Medicine at the University of Cape Town and has been funded by the Wellcome Trust. Prof Robert J Wilkinson is the Principal Investigator. This study has also been approved by the Human Research Ethics Committees of the University of Cape Town and Stellenbosch University and will be conducted according to international standards of Good Clinical Practice, applicable South African government regulations, and Institutional research policies and procedures.

You have NOT been found to have latent TB. However, we would like to ask you to be a control subject for our study, to see what differences we can find between you and people with latent TB. We plan to recruit 15 control participants with no evidence of latent TB. This will involve taking just over 7 teaspoon of blood on a single occasion. You will NOT undergo PET/CT scan. You will not be liable for any expense relating to these investigations and will be reimbursed R30 as compensation for time and travel.

Although the tests in the study very rarely causes problems, if anything goes wrong the University provides insurance to cover this possibility. If you have any concerns regarding following any procedure performed as part of the study you may contact the study doctor.

The decision to participate is entirely your own. IF YOU DECIDE NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DISADVANTAGED IN ANY WAY. In addition, at any point during the trial you are free to withdraw without telling us why.

Since these are research tests, the results will not be made available to the participants, however, you will be informed of any new relevant information that arises during the course of the trial. Throughout the trial your privacy will be maintained and nobody other than the doctors and nurses looking after you will know that you are participating. Samples will be labelled with code numbers and hence the laboratory staff will not know your identity. When the results of the study become
available, names of the participating patients will not be included. The Research Ethics Committees may inspect the research records if required.

**Please note that with your consent some of your blood will be stored safely at the IIDMM for use in this research study or future studies related to this project.**

You may contact either the UCT Research Ethics Committee (021 406 6492) Prof Robert Wilkinson (021 406 6084) or Dr Hanif Esmail (021 406 6389) if you have further questions at any point during the study. Please remember that Prof Wilkinson and Dr Esmail will not be directly responsible for your medical care which will be conducted by your regular doctors and nurses.

**Consent to participate in the study:**

I have read the above / the above has been read to me and I have had the opportunity to discuss the study with Dr _____________________ and ask any questions. I consent to take part in this study:

Signature _____________________

Name _____________________

Date _____________________

Name of person consenting _____________________

Signature _____________________

Date _____________________
How inactive is “inactive” Tuberculosis?
PET/CT Benchmarking of Latent Tuberculosis Infection

BRONCHOSCOPY INFORMED CONSENT FORM AND INFORMATION SHEET

My name is ______________________

Thank you for agreeing to take part in the study. This consent form relates to an additional procedure that we would like to perform. The decision to participate in this part of the study is entirely your own. IF YOU DECIDE NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DIASDVANTAGED IN ANY WAY. In addition, at any point you can change your mind.

This part of the study is also being run by the Institute of Infectious Diseases and Molecular Medicine at the University of Cape Town and has been funded by the Wellcome Trust. Prof Robert J Wilkinson is the Principal Investigator. This study has also been approved by the Human Research Ethics Committees of the University of Cape Town and Stellenbosch University and will be conducted according to international standards of Good Clinical Practice, applicable South African government regulations, and Institutional research policies and procedures.

You will have an FDG-PET/CT scan in the next few days. We have found these scans to be frequently abnormal in persons with HIV and latent TB. This is likely to relate to both the HIV infection and the latent TB infection. By repeating the scan after treating your latent TB we will be able to see which abnormalities relate to TB. However there may be other reasons for these abnormalities (active TB, other infections or cancer), the scan will not be able to tell these apart. The only way to be sure about what is causing the abnormality is to take a sample. **If your scan is abnormal and the abnormality is easy to get to we would like to perform a routine medical procedure called a bronchoscopy and transbronchial biopsy** to sample the abnormality to find out what it is. This is a very common procedure that is performed when people have abnormal FDG-PET/CT scans.

If you agree, after the scan you will not be started on the treatment for latent TB straight away but this may be delayed by up to 2 weeks, we do not expect this to have any negative impact for you. We will get your scan reviewed as quickly as possible by a specialist. If he thinks your scan is abnormal and the abnormality can be reached easily we will arrange a bronchoscopy for you. You will have an additional blood test to make sure your blood clots normally. The bronchoscopy will be performed at the Pulmonology unit at Tygerberg Hospital and will be performed by an experienced senior doctor.

This is what will happen during the procedure, which will take around 30-45 minutes. Firstly the nose and back of your throat will be sprayed with anaesthetic fluid, which will numb any discomfort that might be caused. A sedative drug (midazolam and/or propofol) may be given through a drip to make you sleepy before the procedure, you will also be given fluids through a drip, given oxygen to breathe
and be monitored during the procedure. The bronchoscope is a thin flexible tube about as wide as a little finger with a camera on the end. It will be inserted via the nose or mouth, pass the vocal cords and into the windpipe and air tubes of the lung. The abnormality on the scan will be located just the other side of these air tubes. Using the scan to guide him the doctor will place a thin needle into the abnormality after first making it numb with anaesthetic. The sample will be checked by another doctor to make sure it is in the right place and then up to 5 samples will be taken. After the procedure you will sleep for a period at the clinic. The doctor will see you to check everything is fine and tell you when you can eat or drink again.

Usually the procedures do not cause any problems. The bronchoscope may be uncomfortable and can cause coughing during and immediately after the procedure. Rarely (in less than 1%) a small hole in the lung might cause air to get trapped outside the lungs, very rarely this might require you to stay in hospital for a chest tube to be inserted. Slight coughing of blood can arise in 3% of cases but the risk of severe bleeding is very low (<1/1000). If you take blood-thinning tablets (warfarin or aspirin) or have a personal or family history of abnormal bleeding you should tell the doctor and nurse.

There is no cost to you for having this procedure done and because of the inconvenience we will offer you an additional R350 compensation. The acceptance of this payment in no way affects your rights should anything go wrong. In the event that anything does go wrong we will arrange full treatment through the public health service. If you do not feel well after the bronchoscopy, you can call Dr Hanif Esmail on (0820592465)

The samples will be sent to a laboratory to try and find out what the reason for the abnormality is and you will be informed of any significant findings that impact your health. However, as some of the tests are research tests the results will not all be made available to you. Throughout the trial your privacy will be maintained and nobody other than the doctors and nurses looking after you will know that you are participating. Samples will be labelled with code numbers and hence the laboratory staff will not know your identity. When the results of the study become available, names of the participating patients will not be included.

Please note that with your consent some of your blood will be stored safely at the IIDMM for use in this research study or future studies related to this project.

You may contact either the Research Ethics Committee (021 406 6492) Prof Robert Wilkinson (021 406 6084) or Dr Hanif Esmail (021 406 6389) if you have further questions at any point during the study. Please remember that Prof Wilkinson and Dr Esmail will not be directly responsible for your medical care that will be provided by your regular doctors and nurses.

Consent to participate in the study:

I have read the above / the above has been read to me and I have had the opportunity to discuss the study with Dr ___________________________ and ask any questions. I consent to take part in this study:

Signature ___________________________

Name ___________________________

Date ___________________________

Name of person consenting ___________________________

Signature ___________________________

Date ___________________________
HIV-ve Active TB Consent Form

UNIVERSITY OF CAPE TOWN

Study Doctor  
Dr Hanif Esmail MA, MRCP, FRCPath  
Wellcome Trust Clinical Research Training  
Fellow  
Honorary Research Associate (IIDMM)  
h.esmail@imperial.ac.uk

Principal Investigator  
Prof. Robert J. Wilkinson MA, PhD, FRCP  
Wellcome Trust Senior Research Fellow  
Honorary Professor (IIDMM)  
r.j.wilkinson@imperial.ac.uk

How inactive is “inactive” Tuberculosis?  
PET/CT Benchmarking of Latent Tuberculosis Infection

HIV –VE ACTIVE TB CONSENT FORM

Thank you for agreeing to take part in the study. This consent form relates to a blood test that we would like to perform. The decision to participate in this part of the study is entirely your own. IF YOU DECIDE NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DIASADVANTAGED IN ANY WAY.

The main purpose of our study is to see if some people with “silent” or latent TB have similar features to those with active TB in the context of HIV infection. This will help us to develop tests to identify those who most and least need treatment for latent TB and will help us make progress towards getting rid of TB as a major health problem in the long run. This study is being run by the Institute of Infectious Diseases and Molecular Medicine at the University of Cape Town and has been funded by the Wellcome Trust. Prof Robert J Wilkinson is the Principal Investigator. This study has also been approved by the Human Research Ethics Committees of the University of Cape Town and will be conducted according to international standards of Good Clinical Practice, applicable South African government regulations, and Institutional research policies and procedures.

You have been found to have active tuberculosis and we would like to ask you to be a control subject for our study so that we can compare how your blood is responding to those with HIV and latent TB. We plan to recruit 15 control participants with active TB but no evidence of HIV infection. In addition to tests and investigations that you are already receiving we would like to take an additional 3mL of blood. You may have been recruited into other research studies if so we would like to use the results of additional investigations or other assessments you are having. We may also use results that have been performed as part of routine medical care so that they are not repeated unnecessarily. If you have not had these additional test we would like to perform them, these might include Chest X-Ray, HIV test, sputum culture, full blood count, medical history and examination. You will not be liable for any expense relating to these investigations.

Although the tests in the study very rarely causes problems, if anything goes wrong the University provides insurance to cover this possibility. If you have any concerns regarding following any procedure performed as part of the study you may contact the study doctor

The decision to participate is entirely your own. IF YOU DECIDE NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DIASADVANTAGED IN ANY WAY. In addition, at any point during the trial you are free to withdraw without telling us why.

Since these are research tests, the results will not be made available to the participants, however you will be informed of any new relevant information that arises during the course of the trial. Throughout the trial your privacy will be maintained and nobody other than the doctors and nurses...
Appendix C

looking after you will know that you are participating. Samples will be labelled with code numbers and hence the laboratory staff will not know your identity. When the results of the study become available, names of the participating patients will not be included. The Research Ethics Committees may inspect the research records if required

Please note that with your consent some of your blood will be stored safely at the IIDMM for use in this research study or future studies related to this project.

You may contact either the UCT Research Ethics Committee (021 406 6492) Prof Robert Wilkinson (021 406 6084) or Dr Hanif Esmail (021 406 6389) if you have further questions at any point during the study. Please remember that Prof Wilkinson and Dr Esmail will not be directly responsible for your medical care which will be conducted by your regular doctors and nurses.

Consent to participate in the study:

I have read the above / the above has been read to me and I have had the opportunity to discuss the study with Dr ______________________ and ask any questions. I consent to take part in this study:

Signature ______________________
Name ______________________
Date ______________________

Name of person consenting ______________________
Signature ______________________
Date ______________________
Thank you for agreeing to take part in the study. This consent form relates to an additional blood and skin test that we would like to perform. The decision to participate in this part of the study is entirely your own. IF YOU DECIDE NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DIASADVANTAGED IN ANY WAY.

The main purpose of our study is to see if some people with “silent” or latent TB have similar features to those with active TB. This will help us to develop tests to identify those who most and least need treatment for latent TB and will help us make progress toward getting rid of TB as a major health problem in the long run. This study is being run by the Institute of Infectious Diseases and Molecular Medicine at the University of Cape Town and has been funded by the Wellcome Trust. Prof Robert J Wilkinson is the Principal Investigator. This study has also been approved by the Human Research Ethics Committees of the University of Cape Town and will be conducted according to international standards of Good Clinical Practice, applicable South African government regulations, and Institutional research policies and procedures.

You do not have any symptoms and are being recruited for participation in the “Tissue destruction in TB” study we would also like to ask you to be a control subject for our study so that we can compare how your blood is responding to those with HIV and latent TB. We plan to recruit 30 control participants that are asymptomatic and have no evidence of HIV infection. In addition to the blood tests and investigation that you are already receiving (one of which will confirm your HIV status) we would like to take an additional 6mL of blood and perform a skin test. Some of the blood and the skin test will be used to establish if you have evidence of latent TB. We would like to use the results of the other investigation you are having for the “Tissue destruction in TB” study. You will not be liable for any expense relating to these investigations.

Although the tests in the study very rarely causes problems, if anything goes wrong the University provides insurance to cover this possibility. If you have any concerns regarding following any procedure performed as part of the study you may contact the study doctor

The decision to participate is entirely your own. IF YOU DECIDE NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DIASADVANTAGED IN ANY WAY. In addition, at any point during the trial you are free to withdraw without telling us why.

Since these are research tests, the results will not be made available to the participants, however you will be informed of any new relevant information that arises during the course of the trial. Even if you
are found to have latent TB no treatment is recommended for this if you are HIV-ve. Throughout the trial your privacy will be maintained and nobody other than the doctors and nurses looking after you will know that you are participating. Samples will be labelled with code numbers and hence the laboratory staff will not know your identity. When the results of the study become available, names of the participating patients will not be included. The Research Ethics Committees may inspect the research records if required

Please note that with your consent some of your blood will be stored safely at the IIDMM for use in this research study or future studies related to this project.

You may contact either the UCT Research Ethics Committee (021 406 6492) Prof Robert Wilkinson (021 406 6084) or Dr Hanif Esmail (021 406 6389) if you have further questions at any point during the study. Please remember that Prof Wilkinson and Dr Esmail will not be directly responsible for your medical care which will be conducted by your regular doctors and nurses.

Consent to participate in the study:

I have read the above / the above has been read to me and I have had the opportunity to discuss the study with Dr __________________ and ask any questions. I consent to take part in this study:

Signature __________________

Name __________________

Date __________________

Name of person consenting __________________

Signature __________________

Date __________________
HIV-ve Asymptomatic Consent Form – repeat blood

UNIVERSITY OF CAPE TOWN

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How inactive is “inactive” Tuberculosis?
PET/CT Benchmarking of Latent Tuberculosis Infection

ASYMPTOMATIC HIV –VE CONSENT FORM - Repeat Blood test

Thank you for agreeing to have an additional blood test for our study. This consent form relates to an additional blood test that we would like to perform. The decision to participate in this part is entirely your own. IF YOU DECIDE NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DIASDVANTAGED IN ANY WAY.

You were seen a few weeks ago and had tests for latent tuberculosis (a tuberculin skin test and a quantiferon blood test) and an HIV test. The quantiferon blood test for latent tuberculosis was negative and the HIV test was negative. We have discovered that in HIV infected persons that the quantiferon blood test for latent tuberculosis can change from negative to positive when performed 6 weeks following the tuberculin skin test in as many as 40%. We would like to compare this with what occur in HIV uninfected persons. This will help us to understand what the best way to diagnose latent tuberculosis is.

This study is being run by the Institute of Infectious Diseases and Molecular Medicine at the University of Cape Town and has been funded by the Wellcome Trust. Prof Robert J Wilkinson is the Principal Investigator. This study has also been approved by the Human Research Ethics Committees of the University of Cape Town and will be conducted according to international standards of Good Clinical Practice, applicable South African government regulations, and Institutional research policies and procedures.

We would like to perform the quantiferon blood test again which will involve taking 3 mL (less than one teaspoon) of blood and ask you a few further questions. You will not need to return for the results and will not require any treatment even if the result has changed.

Although the tests in the study very rarely causes problems, if anything goes wrong the University provides insurance to cover this possibility. If you have any concerns regarding following any procedure performed as part of the study you may contact the study doctor.

The decision to participate is entirely your own. IF YOU DECIDE NOT TO PARTICIPATE, YOUR TREATMENT WILL NOT BE DIASDVANTAGED IN ANY WAY. In addition, at any point during the trial you are free to withdraw without telling us why.

Since these are research tests, the results will not be made available to the participants, however you will be informed of any new relevant information that arises during the course of the trial. Even if you are found to have latent TB no treatment is recommended for this if you are HIV-ve. Throughout the trial your privacy will be maintained and nobody other than the doctors and nurses looking after you...
will know that you are participating. Samples will be labelled with code numbers and hence the laboratory staff will not know your identity. When the results of the study become available, names of the participating patients will not be included. The Research Ethics Committees may inspect the research records if required

**Please note that with your consent some of your blood will be stored safely at the IIDMM for use in this research study or future studies related to this project.**

You may contact either the **UCT Research Ethics Committee** (021 406 6492) **Prof Robert Wilkinson** (021 406 6084) or **Dr Hanif Esmail** (021 406 6389) if you have further questions at any point during the study. Please remember that Prof Wilkinson and Dr Esmail will not be directly responsible for your medical care which will be conducted by your regular doctors and nurses.

**Consent to participate in the study:**

I have read the above / the above has been read to me and I have had the opportunity to discuss the study with Dr __________________________ and ask any questions. I consent to take part in this study:

Signature __________________________

Name __________________________

Date __________________________

Name of person consenting __________________________

Signature __________________________

Date __________________________
How inactive is “inactive” Tuberculosis?
PET/CT Benchmarking of Latent Tuberculosis Infection.

How did I get Latent TB?
Tuberculosis (TB) is a disease that is seen very commonly in Cape Town and, indeed, in most of South Africa. It is caused by bacteria that people breathe in and the infection results in cough, fevers and weight loss. However, in many people disease does not develop because they can stop the bacteria growing and the TB falls asleep, this is called latent TB infection. When the body is trying to stop the bacteria from growing, the lung can get damaged and cause scarring. Sometimes this scarring is visible on a chest X-ray or a CT scan it is called inactive TB.

Why are we asking you to take part in our study?
As you are in the early stages of HIV infection there is a chance that the TB bacteria might wake up and multiply, and you may become unwell.
The purpose of our study is to see if everyone that has latent TB infection has the same chance of becoming unwell. This will help us to develop tests to find the people that really need treatment for TB and the people that don’t. This will help us make progress toward getting rid of TB as a major health problem in South Africa and across the world in the long run.

This study is being run by the Institute of Infectious Diseases and Molecular Medicine at the University of Cape Town. Professor Robert Wilkinson is the Principal Investigator.
Why Does it matter?

Latent TB infection can restart growing after many years, and people with HIV infection are more likely to become unwell because they are less well able to stop the bacteria from growing. One way to prevent this would be to treat them before they became sick and passed on the infection to other people.

Millions of people in South Africa and over the world are thought to have latent TB, treating everyone therefore would be very difficult. However, we believe that not everyone with latent TB has the same chance of becoming unwell, if we could understand the condition better we might be able to identify and treat those most at risk of becoming unwell with TB.

latent TB is a very difficult condition to study because the infection is so silent. In the past the only way to study what happens to people with latent TB infection was to monitor them for extremely long periods of time, but even after 20 years of monitoring we cannot tell if the infection is still there or not.

What is a PET/CT scan? How will it help to understand Inactive TB?

PET/CT stands for Positron Emission Tomography and Computed Tomography. These are 2 ways of taking pictures of the inside of the body that are done at the same time and merged together. This provides doctors with detailed information about where the abnormal tissue inside your body is and how much energy it is using.

PET/CT scan is a new way to look how the body is fighting infection in latent tuberculosis. Scars in the lung where the body has been fighting infection can be looked at in much more detail with the PET/CT. PET/CT can tell whether the body is still fighting an infection by indirectly measuring the number of active cells in the area. We think that if there is TB infection present the body will send active cells to the area especially if the person has HIV infection as well. This will make the scar look a bright orange colour on the scan (example on left).

After treatment when the infection is cured the body will no longer have to send any more active blood cells to the area to fight infection. If we repeat the PET/CT scan at this stage we think the scar will turn from bright orange on the scan to black (normal).
If I enter the study what will happen to me?

If you enter the study we will provide you with treatment for latent TB which is 9 months of a very commonly used drug called isoniazid. This medicine is recommended for all persons living with HIV who have latent TB infection in South Africa and has been shown to reduce the chances of you becoming unwell from TB. A very small number of patients (about 1 in every 100) can get side effects which would mean the medicine would stop but the benefits of taking this medication far outweigh the risks in your case.

You will come to the clinic on the day you start treatment and then every 1 week, 1 month then every month until 9 months to collect your medication. At each visit a doctor or nurse will give you a check up to make sure that you are still well and you are taking your medicine. On 6 occasions over the year the study staff will also take between 8 and 11 tea-spoons of blood and a urine sample for the research study.

In addition before you start the treatment and after 6 months of treatment you will go with one of the study staff members to a medical centre in Panorama or Tygerberg Hospital to have a PET/CT scan.

What happens in a PET/CT Scan

When you get to the PET/CT centre, you will be met by the reception staff. A small blood sample will be tested to check your sugar levels. You will then be taken into a room to change and where a drip will be set up and a small amount of radioactive sugar will be injected into the drip. You will then rest quietly for 60-90 minutes while the radioactive sugar distributes around your body. You will then be taken to the PET/CT scanner where you will be positioned lying down on a table as comfortably as possible. The scanner is like a donut with a large, thin hole in the middle that your body will pass through twice. The whole process will take about 3 hours and you will be accompanied by a study staff member to and from Khayelitsha.

As the scan will take up most of your day we will compensate you after the scan to make up for any lost earnings.

Almost all the radioactive sugar will have left your body by the end of the day. However, we advise that you wait a few hours before you have very close contact with pregnant women or a baby.
Are there any risks involved?
Both the PET and CT scan involve the use of radiation to generate pictures but the doses are too small to produce any immediate harmful effects and you will not notice anything.

We are all exposed to natural radiation every day from the ground, sky and air we breathe and food we eat. You will have already been exposed to at least 2 times the radiation in the PET/CT scans just naturally during your lifetime.

Radiation is one of the things that can increase the chance of developing cancer, like smoking cigarettes. We all naturally have around a 20% chance of developing a cancer over our lifetimes. Scientists are not clear if the small amount of radiation in the PET/CT scan will increase the chance of developing cancer. If it does the increase risk would be about 0.2% over a lifetime, so the additional risks are very small.

What are the Benefits?
The main reason to join our study is to because you believe that being a part of medical research will help us to understand tuberculosis infection better and provide better treatments to future generation so that in years to come tuberculosis becomes less of a problem in South Africa.

You will get a very careful check up at the start of the study to see if you have actively growing TB so that we can treat you properly. If you don’t have actively growing TB we will treat you with the recommended drug, isoniazid but for 9 rather than 6 months. This will protect you from becoming ill with TB for longer. We will also see you more often than you would be if you weren’t in the study so we can keep a closer eye on things and you will be able to contact us if you start becoming unwell.

The PET/CT scan is the most advanced way to look at abnormal lung. If we are worried that you have any abnormal lesions because of something like a cancer we will refer you to the hospital so that you can get the correct tests and treatment if necessary as quickly as possible.

Thank you for taking the time to read this information sheet. Please write down any questions you may have to discuss with study staff at your next clinic visit.

If you have any urgent questions please contact:
Dr Hanif Esmail on 021 406 6389
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