TUBERCULOSIS OF THE SPINE IN HIV-NEGATIVE AND -POSITIVE PATIENTS: IMMUNE AND PATHOGEN-RELATED FACTORS IN THE LOCAL DISTRIBUTION OF THE DISEASE

MV Ngcelwane

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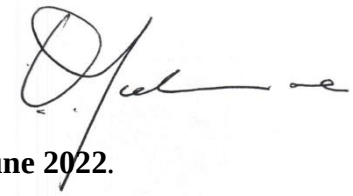
DECLARATION

I, MV Ngcelwane, hereby declare that this work, which I submit for the degree of Doctor of Philosophy in Orthopaedics to the University of Pretoria, is my own original work and has not previously in its entirety or in part been submitted by myself for a degree at this or any other university. Where other people's work has been used, it has been properly acknowledged and referenced. The research was carried out in accordance with the ethical rules prescribed by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria.

Signature: 

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TUBERCULOSIS OF THE SPINE IN HIV-NEGATIVE AND -POSITIVE PATIENTS: IMMUNE AND PATHOGEN-RELATED FACTORS IN THE LOCAL DISTRIBUTION OF THE DISEASE

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SUMMARY

Tuberculosis of the spine is an ancient disease. Evidence of spine involvement has been seen in Egyptian mummies dating back to 900BC. South Africa is the fourth leading country in having a high prevalence of tuberculosis (TB) of the spine, after China, India and Korea. South Africa also has a high incidence of HIV infection. It is not clear in the literature whether the Human Immunodeficiency Virus (HIV) co-infection affects the distribution of the disease in the spine or causes a different radiologic pattern to that found in HIV-negative patients.

The study was therefore undertaken to compare the extent of the distribution of the disease in HIV -negative and –positive patients, to examine the immune factors that might be responsible for any difference in the distribution of the disease and to evaluate any pathogen-related genetic factors that might be responsible for the TB bacilli settling in the spine.

This was a prospective multi-center study on 61 consecutive patients undergoing surgery for tuberculosis of the spine. The HIV status and blood parameters were measured. The angle of kyphosis and the pattern of the disease on plain radiographs were measured. From Magnetic Resonance Imaging, the number of vertebrae involved, the occurrence of skip lesions, the amount of vertebral body loss and the volume of pus formation were measured. The tissue taken at surgery was sent to Anatomic Pathology for histologic examination, to Immunology to examine the expression of cytokines in the granulomas, to Microbiology to identify and culture the bacteria and for drug resistance testing, and for whole genome sequencing to identify any mutations that may be unique to the spine isolates.

The first set of results showed that 70% of the patients with TB of the spine were HIV-positive, which is much higher than that of a similar study done in this country eight years ago in which there

were 40% HIV-positive patients. There was significantly more vertebral bone loss and marginally more pus in HIV-positive patients. This is the direct opposite of what is currently reported in the literature on this subject. These results can be explained based on bone destruction, not only by the bacilli, but mainly by the immune response triggered by the TB and HIV infection. The study also found that non-contiguous lesions occur slightly more frequently in HIV-positive patients.

In the second part of the study, the granulomas were classified according to groups 1-4, where 1&2 were poorly formed granulomas and 3&4 were well formed granulomas. The expression of the cytokine TNF-α in the granulomas was also measured. There were no differences between the HIV status of the patient and the grading of the granulomas. There was also no difference between the HIV status of the patient and the expression of the cytokine TNF-α. Of interest is that the granuloma grades 1&2 had less expression of TNF-α, although this was just marginal and not statistically significant. This finding adds to the number of studies currently investigating host directed therapies in the treatment of TB of the spine.

The last set of data relates to whole genome sequencing (WGS) done at the National Institute for Communicable Diseases in Johannesburg. WGS can identify multidrug resistant strains much more accurately than the phenotypic drug resistance testing methods currently in use. Multidrug-resistant TB occurred in 4.7% of these isolates. The study shows that the *Mycobacterium tuberculosis* lineages that cause disease in the spine are the same as those causing pulmonary TB in South Africa, with the East-Asia (Beijing) strain dominating at 47%. We could not find any mutations specific to these spine isolates, however some genes of unknown function were detected. It is not certain if they encode for any specific functions that may enable the bacilli to survive in the hypoxic environment of the spine. More work needs to be done on these isolates.

Keywords:

Tuberculosis of the spine; HIV infection; Spine Radiographs; Spine MRI scan; Granuloma necrosis; Cytokines in TB granulomas; Whole Genome Sequencing.

PRESENTATIONS AND PUBLICATIONS

Presentations:

1. **Ngcelwane MV**, Suleman F(Dept of Radiology), Maku M, Bomela L, Motsitsi S (UP) Thwala N, Younis A (Wits) Nxiweni L (WSU), Sibanyoni J (SMU), Mabusha S (UKZN)

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   New horizons in the laboratory diagnosis of tuberculosis of the spine - the role of whole genome sequencing.

   67th Congress of the SA Orthopaedic Association

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Manuscripts under review for Publication:

1. Radiological features of TB of the spine in HIV-negative and -positive patients.

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AIDS	Acquired immune deficiency syndrome
AP	Anterior-posterior view
ART	Antiviral treatment
ASIA	American Spinal Injury Association
BCG	Bacillus Calmette Guerin
CI	Confidence Interval
CSF	Cerebrospinal Fluid
CT	Computerised tomography
CTL	Cytotoxic T-cell
DOTS	Direct observation treatment for TB
EC	Ethics Committee
ESR	Erythrocyte Sedimentation Rate
EPTB	Extra-pulmonary tuberculosis
FS	Fat suppression sequence
g/l	grams per litre
HDT	Host Directed therapy
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon gamma
IGRA	Interferon gamma release assay
Il-12	Interleukin-12
IRIS	Immune Reconstitution Inflammatory Syndrome
LPA	Line Probe Assay

MDR-TB	Multi-drug resistance TB
MIGT	Mycobacterial Growth Indicator Tube
ml	millilitre
MRI	Magnetic resonance imaging
M. tuberculosis	*Mycobacterium tuberculosis*
Neg	negative
NICD	National Institute for Communicable Diseases
NK Cells	Natural Killer Cells
NO	Nitric Oxide
NTM	Non-tuberculosis mycobacteria
PCR	Polymerase Chain Reaction
P.E.	Protamine Glutamate
P-PE	Protamine–Protamine Glutamate
PET	Positron Emission Tomography
Pos	Positive
RANK	Receptor activator of nuclear factor-κappaB
RANK-L	Receptor activator of nuclear factor-κappaB ligand
Rif	Rifampicin
STIR	Short Tau Inversion Recovery sequences
TB	Tuberculosis
TNF-α	Tumour necrosis factor alpha
TGF-β	Transforming growth factor beta
TIW	T1 Weighted sequence
T2W	T2 Weighted sequence
UNAIDS	United Nations AIDS

XR	Radiographs
WGS	Whole Genome Sequencing
WHO	World Health Organisation
18-FDG	18-Fluoro Deoxy Glucose

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CHAPTER 1

AN OVERVIEW OF TB SPINE IN HIV-NEGATIVE AND -POSITIVE PATIENTS AND AIMS OF THE STUDY

1.1 Introduction

Tuberculosis (TB) is a chronic infectious disease caused by the *Mycobacterium tuberculosis* bacterium. About a third of the population of the world is infected with *Mycobacterium tuberculosis.*[1] Tuberculosis is still a threat worldwide and kills more than one and a half million people each year with 8-10 million new cases of active TB reported.[2] TB is a disease which is usually associated with poverty and HIV infection. But overcrowding, illiteracy, malnutrition, alcoholism, drug abuse and immunosuppressant therapy are also contributing factors. It is a complex disease with high morbidity and mortality, [3] affecting mostly young adults.

TB is the number one opportunistic infection associated with HIV infection. The risk of TB doubles within a year of infection with HIV, and thereafter it rises exponentially.[4] Patients who are HIV-positive have a 20-37% higher chance of contracting TB infection than patients without HIV.[5] The risk of re-activation of latent TB in patients with advanced HIV is 100 times more than in immune-competent individuals.[6]

In sub-Saharan Africa, we cannot talk about TB of the spine without referring to HIV as the two often co-exist. The increased prevalence of extra-pulmonary tuberculosis (EPTB),

as in TB of the spine, is directly linked to the rising HIV infection rate. HIV patients with TB co-infection have a four times higher risk to develop EPTB.[7]

TB of the spine is an ancient disease. Evidence of spine involvement has been found in Egyptian mummies dating back to 900 BC. Early Babylonian literature and Chinese writers refer to TB infection.[8] Sir Percival Pott, an English surgeon, first described a case of TB of the spine with kyphotic deformity and paraplegia in 1799.[9] A century later, a German physician, Dr Robert Koch, isolated the causative organism in 1905. TB of the spine has since also been known as Pott's disease, and TB known as Koch's disease.

1.2 Mechanism of infection

Mycobacteria enter the body through the lungs. An individual gets infected by inhaling droplets from an infected individual. These droplets are 2-5 micrometers in size. Being small, they can float in air for minutes to hours. They escape the defense mechanisms of the bronchi and land in the terminal alveolar. They are then engulfed by the phagocytic antigen presenting cells, macrophages and dendritic cells.[8] The macrophages secrete cytokines and chemokines to recruit granulocytes and monocytes, as well as T- and B-lymphocytes.[7]

The phagocytosed bacilli replicate intracellularly. The bacterial-laden immune cells cross the alveolar barrier and spread the infection to pulmonary nodes, and by invading the blood vessels, they spread the infection to extra-pulmonary tissue, including the spine. Patients with EPTB, as in TB of the spine, are usually not infectious unless pulmonary TB is present as well. [10]

In immunocompetent patients, an effective cell-mediated immunity develops in 2-8 weeks and stops the multiplication of the bacilli.

1.3 The granuloma

The hallmark of *M. tuberculosis* infection is the ability to invade the host's immune system to form a granuloma, which is a reaction to chronic macrophage stimulation by the mycobacteria. Granulomas are aggregates of innate immune cells that are recruited to the site of infection.

Alveolar macrophages, laden with bacilli, produce immune mediators which are inflammatory cytokines and chemokines that serve as signals for infection. Monocytes, neutrophils and lymphocytes migrate to the site of infection. The bacilli resist the bactericidal mechanisms of the macrophages and cause macrophage necrosis. The released bacilli multiply extra-cellularly. More macrophages try but also fail to kill the bacilli.[8]

Meantime the dendritic cells with engulfed bacteria mature, migrate to the regional lymph nodes and prime T-cells, both CD4 and CD8, against bacterial antigens.

The specific immune response produces primed T-cells which migrate back to the focus of infection, guided by the chemokines produced by the infected cells. The accumulation of macrophages, B- and T-cells, dendritic cells, natural killer cells, endothelial cells, fibroblasts, and cells involved in the formation of extracellular matrix, all lead to formation of a granuloma at the site of infection.

Granulomas are an important protective immune response against mycobacteria and serve to isolate the intruding organisms and to eradicate the infection.[11, 12] Most bacilli are killed in the caseating granuloma.

Histopathology shows that the classic granuloma is composed of epithelial macrophages, neutrophils and other immune cells, surrounded by a cuff of lymphocytes. With time, the granuloma undergoes remodeling, characterized by a central necrotic area, which has dead macrophages and is hypoxic, with bacilli residing inside the macrophages in the hypoxic center.[8] The necrotic center forms caseous material which may liquify, facilitating local spread of the bacilli.

The hypoxic microenvironment of the center of the granuloma has nitric oxide and carbon monoxide, and hence a low pH. It is an environment that increases the expression of several mycobacterial genes involved in dormancy production. The dormant bacilli can inhabit the granuloma for the lifetime of the host but are able to germinate in the event of host immunosuppression. The latent infection in a person without clinical signs of the disease is indicated by the delayed–type hypersensitivity response to Purified Protein Derivative.

1.4 Cytokines and immune cells

A wide range of immune components are involved in immunity against mycobacteria.
The most important of these are the CD4 cells, macrophages and the cytokines interferon-gamma (INF-γ) and tumor necrosis factor-α (TNF-α)

Interferon-γ is produced by CD4 T-cells, CD8 T-cells and natural killer (NK) cells.

Early production of INF-γ by CD4 cells and subsequent activation of macrophages determines the outcome of infection. INF-γ is the key cytokine for an effective immune response against mycobacteria. It synergizes with TNF-α in activating macrophages to kill intracellular bacilli. INF-γ does this by augmenting antigen presentation, leading to recruitment of CD4 T-cells and cytotoxic CD8 cells, which participate in killing of mycobacteria. In association with TNF-α, it induces production of nitric oxide and other reactive nitrogen intermediates.[8]

TNF-α is produced by macrophages, dendritic cells and T-cells. In conjunction with IFN-γ, it induces macrophage activation, enhances immune cell recruitment to the site of infection and augments chemokine expression by macrophages through activation of the nuclear-factor-kappa–β signaling pathway. TNF-α mediates cell death by inducing apoptosis.[13, 14]

CD4 T-cells produce INF-γ. They are also responsible for apoptosis of infected macrophages, production of other cytokines (IL-2 & TNF-α), induction of other immune cells (macrophages and dendritic cells) to produce other cytokines, IL-10, IL-12, IL-15, and activation of macrophages through direct contact via CD40 ligand. They also control intracellular growth of Mycobacteria by a nitric oxide-dependent mechanism.

This immune activation is regulated by a group of molecules called Immune checkpoint molecules. Their role is to maintain immune homeostasis and prevent autoimmunity. Uncontrolled immune responses to pathogens can cause inflammatory tissue damage and autoimmune disease.

Some of the soluble, co-inhibitory systemic immune check point molecules are:

- Lymphocyte -activation gene3 (LAG-3)
- Programmed cell death protein 1 (PD-1)
- Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4)
- T-cell immunoglobulin and mucin containing domain 3 (TIM-3)

1.5 Immune response in TB co-infection with HIV

Co-infection with human immunodeficiency virus (HIV) has changed the pathology of the disease. HIV-infected patients develop TB at a rate of 10% per year, while HIV-negative individuals have a 10% chance of contracting the disease in their lifetime.[14]

HIV infection decreases the body's ability to contain mycobacteria in the granulomas. In infection by intracellular organisms, granuloma formation is required to contain the infection. This is a feature of cell-mediated immunity.[15] HIV weakens the host's granulomatous response to TB by inhibiting the macrophage, T-cell and cytokine responses.[16,17-19] CD4+ T-cells secrete interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α), while CD8+ T-cells produce perforin, and granulysin as effector molecules which are essential to the immune response.[11]

Both IFN-γ and TNF-α are important cytokines in the protective reaction against TB infection. IFN-γ is elevated in patients with advanced HIV who are most susceptible to TB infection.[6]

HIV infection causes a progressive loss of CD4 T-cells, while continued virus replication and virus-induced cell death contribute to the depletion of mucosal memory CD4 T-cells during acute HIV infection. Although the numbers of CD4 T-cells are diminished, most opportunistic infections cause complications only after longer periods of progressive HIV disease, while *M. tuberculosis* is one of the few pathogens that cause disease soon after HIV infection. The risk of developing active tuberculosis increases drastically in the first year of HIV infection, regardless of CD4 T-lymphocyte counts.

In developing countries, the presence of pulmonary TB is often the first sign of acquired immune deficiency syndrome (AIDS). This indicates that there is a rapid loss of T cells specific for the *M. tuberculosis* organism during the acute phase of HIV infection.[19]

The HIV virus has an ability to manipulate the TB granuloma. In a study on lymph nodes, Diedrich found that HIV reduces the CD4 T-cell count within the granuloma.

The circulating CD4 T-cell count is also decreased. The granulomas thus contain less CD4 and CD8 T-cells, less INF-γ, more neutrophils, more Interleukin-10 and increased mycobacterial numbers.[20] In a study done on autopsy specimens of pulmonary tissue, de Noronha found that granulomas from HIV-negative patients had normal architecture, with typical epitheloid, as well as giant cells, and exhibiting caseous necrosis.[21]

Granulomas from HIV co-infected patients showed extensive necrosis, were poorly formed, and showed a marked presence of polymorphs. TNF-α staining was greatly reduced. This study suggests that the immune response to TB is compromised in HIV-positive granulomas.[14, 21]

In a study on pleural biopsy specimens, Bezuidenhout et al. found that necrotic granulomas were more evident in HIV-positive patients with a clear association between TNF-α and necrosis in the granulomas. [14] This suggests that HIV may affect the ability of the granuloma to contain the infection, hence the wider spread of the disease in these patients. Mathews et al. conducted an analysis of human pericardial tissues in TB pericarditis, to determine the effect of HIV co-infection on the phenotype of the *M. tuberculosis* infection-associated memory cells, and the role of polymorphs using cells from the pericardial fluid.[22] They concluded that HIV infection resulted in an altered phenotype and function of Mycobacterium–specific CD4 T cells at the disease site, which may contribute to increase in developing TB at all stages of HIV infection. IL-8, a cytokine responsible for immune cell recruitment, is released at high levels in focal disease in HIV-positive patients.[23, 24] In a systematic review, Diedrich et al. found that HIV was associated with an increased bacillary load within mycobacteria-infected tissue. The HIV co-infected persons had a reduced circulating CD4 cell count, with poorly formed granulomas and a higher bacterial count.[20]

The cellular response in HIV-positive and -negative patients has been investigated. Bronchoalveolar lavage was used to characterize the response in patients with pulmonary TB. The host response to TB is dependent on activation of cytotoxic and memory CD4

cells, resulting in granuloma formation, and a delayed hypersensitivity reaction.

HIV-positive patients have a decreased proportion of lymphocytes, a marked decrease in CD4 count and a higher proportion of CD8 lymphocytes and lower Interferon-gamma levels. Despite these indices of immunosuppression, HIV-positive patients showed a much higher CD4/CD8 ratio, indicating that TB can increase migration and replication of CD4 lymphocytes in HIV-positive patients.

The most remarkable finding in the study was that the host response to TB was the increase in CD4 cells. In both HIV-positive and -negative patients, the presence of lymphocytes with granules suggested participation of these cells in bacterial killing mediated by apoptosis, cytotoxicity or INF-γ release. IL-8, a cytokine responsible for immune cell recruitment, is released at high levels in focal disease in HIV-positive patients.

Very little is written on immunopathology in the spine. Govender *et al* reported on 42 HIV-positive and HIV-negative patients. They harvested the extradural granuloma from inside the spinal canal during spine decompression operations for TB of the spine with neurologic involvement.[16] In their study they did immunophenotyping of the peripheral blood. Mononuclear cells were isolated from peripheral blood and granulation tissue. They showed that there was decreased CD4 cells in HIV-positive patients, while HIV-negative patients had more interleukin-2 than HIV-positive patients.

Danaviah *et al* investigated the TB granuloma from the epidural granulation tissue in six patients. They examined the granulation tissue for histology, bacterial culture and

molecular assay. [7] They concluded that the HIV granuloma in TB of the spine is a site for independent viral evolution.

1.6 Clinical effect of HIV co-infection

The association between TB and HIV has been studied by several authors.

In a study on mine workers in South Africa, Sonnenberg found that the risk of TB doubles within a year of infection with HIV, and thereafter it rises exponentially.[4]

There were no significant differences in blood levels of haemoglobin and albumin between patients who were HIV-positive or -negative, but lower counts of lymphocytes were found in HIV-positive patients.

Clinical differences in distribution of the disease between HIV-positive and -negative patients have been described. The disease had a wider spread in HIV-positive patients than in HIV-negative patients.[25] TB infection of the spine in HIV-negative patients was confined to the thoracic, thoracolumbar or lumbar regions, while the HIV-positive patients had infection in the cervical, cervical-thoracic and lumbosacral regions as well.[25] There were no differences in the clinical presentation or severity of paralysis or progression of TB of the spine between the HIV-positive and -negative patients. Also, there were no differences in the surgical treatment between the two, although the proponents of the posterior approach would say it is the preferred approach in HIV-positive patients.

Regarding medical management, the Immune Reconstitution Inflammatory Syndrome (IRIS) is an important clinical entity in altering the clinical presentation and course of TB

infection. It occurs in 10.6% of patients on ART.[26] IRIS results from dysregulation of the recovering immune response, driving exaggerated inflammation directed at the antigens of opportunistic pathogens. It is most frequently described in association with mycobacterial, fungal and herpes virus infections.

In the case of TB, two forms of IRIS are recognized:

i) Paradoxical TB IRIS occurs in patients with established TB on TB treatment, before anti-retroviral treatment (ART) is initiated, who then manifest with recurrence or new TB signs and symptoms.

ii) Unmasking TB IRIS occurs in patients who are not on TB treatment when they start ART, who then have an unusual inflammatory presentation of TB in the first three months of ART.[26, 27]

1.7 Pathology in the spine

The basic lesion in TB of the spine is a combination of osteomyelitis and arthritis, where more than one vertebra is affected. The rich blood supply of the vertebra contributes to the spread of the disease to the spine[28] The disease causes progressive bone destruction, vertebral collapse and a kyphosis of the vertebral column. The anterior aspect of the vertebral body, which is next to the subchondral plate, is generally involved first.

Paralysis is caused by compression of the spinal cord by pus from the cold abscess, extruded intervertebral disc, fragments of necrotic bone, granulation tissue into the canal

and by a vasculitis causing direct ischemia to the spinal cord. The incidence of neurologic involvement varies and is dependent on the time of diagnosis.[29]

The granuloma is at the center of this tissue destruction. The necrotic center can liquefy and drain, causing cavitating disease.

In the lung, cavitation breaks down into the airway, promoting aerosol formation which increases the spread of infection. In the spine, the granulomas can mature and grow larger, but when the necrotic center liquefies it cannot drain away and this causes formation of para-spinal, psoas and epidural abscesses.[7]

It is generally recognized that the fate of the granuloma (like the liquefaction of the granuloma in this case), is responsible for most of the clinical morbidity.[30] Thus, the clinical effects of TB in the spine are caused more by the immune response rather than by the bacilli. It is the reason why host directed therapies, in the form of immunomodulators like cytokines, that act via a host-mediated response to pathogens rather than directly on the pathogen, are becoming more attractive in the treatment of TB.

1.8 Spread of TB of the spine in HIV co-infection

The classic lesion of Tuberculosis of the spine on X-ray is a two-body disease affecting contiguous vertebral bodies, with destruction of the intervening disc space.[31, 32, 33]

Over the last two decades we have been seeing a different radiological pattern, with involvement of the posterior elements of the vertebrae, skip lesions and more extensive pus formation. Pande described this as atypical TB.[34]

HIV-positive patients with TB have a higher incidence of skeletal lesions compared to HIV

-negative patients.[33] In our clinical experience, presented to the South African Orthopaedic Association Congress in 2010 (unpublished) we presented a series of patients with TB of the spine with and without HIV. Those with HIV showed more of the atypical type of TB described by Pande.

Polley reported that non-contiguous TB lesions as described by Pande, were not an obvious manifestation of HIV-positive TB of the spine,[35] while Emmel concluded that non-contiguous TB was an expression of a more fulminant type of TB.[36] Anley reported on a series of patients on whom MRI scans of the spine were performed, and found the HIV-positive patients had a tendency to form bigger epidural abscesses, but could not confirm if there was more bone destruction in these patients.[25] HIV-positive patients with TB have a higher incidence of skeletal lesions compared to HIV-negative patients.[37]

We have no knowledge of a study that shows either a specific radiologic pattern for TB of the spine in HIV-positive patients, or if there are differences in the distribution of the disease in the two types of patients. A prospective and well controlled study will be able to shed more light on the subject, especially if the radiologic pattern could be related to the immunopathology of the disease at the site of the infection.

The pathology of TB of the lungs has been extensively studied.[21, 38-40,41] For us to understand TB of any organ, we need more in-depth understanding of the disease at the site of infection.[42] Diederichs, in a mini review of TB and HIV discusses that the TB granuloma is the unit that controls the spread of the disease.[23] The report discusses how both animal and clinical models are needed to confirm the hypotheses on containment of

TB in the presence or absence of HIV. This study would be such a clinical model. Of importance is that the authors state the need for research to be focused on the tissue that is diseased. They state that more work needs to be done on the events happening at the granuloma.

In our case, the research will be focused on the tissue that is affected, that is the granuloma around the prevertebral tissue.

Most of research on tuberculosis of the spine has been focused on purely clinical aspects, like the surgical approach, the type of bone graft to use to replace the diseased vertebral body, and the type of metallic fixation to use. [43, 44, 45, 46, 33] In South Africa there is very little research that involves application of the basic sciences in orthopaedics. This research will add to the few studies undertaken to date.

1.9 Clinical presentation

Patients may present with non-specific signs of general malaise. Constitutional signs of fever and night sweats are not common.

The clinical presentation of TB of the spine is that of local back pain, stiffness, and spasms of the muscles. The pain can be a dull ache, or constant debilitating pain. It is mechanical in nature, that is, it is made worse by activity. It can also be pathological in nature, presenting as a dull constant pain, that wakes the patient up from sleep. The prolonged period of chronic backpain is then followed by a gibbus, which is a prominence of a spinous process due to collapse of the vertebral body. A more obvious kyphosis may also

be noticeable.

Progression of the disease is slow and insidious and can vary between months and years. Most patients seek medical attention when there is already severe pain and severe deformity and/or neurological deficit. Neurologic symptoms depend on the level of spinal cord involvement and may vary between numbness and weakness in the extremities to tetraplegia or paraplegia.

It is not uncommon in the more remote areas of our country that a patient presents for the first time for medical treatment being carried to the hospital, because of paralysis of the legs. Indeed, the average time to diagnosis of TB of the spine is much higher than in other spine infections, like pyogenic infections. [37, 47]

The most serious complication of TB of the spine is paralysis. Paralysis or neurologic deficit in tuberculosis of the spine can be divided into two types:

(i) Early onset paraplegia: It occurs in active disease, usually within the first two years with active disease. The lesion found at surgery is referred to as a wet lesion because there is pus found at surgery in this lesion.

(ii) Late onset paraplegia: It occurs with late disease, usually many years after the apparent quiescence of the disease. It may also be from continued low grade grumbling activity in unhealed disease. The lesion at surgery is referred to as a dry lesion as there is no pus at surgery.

The neurologic deficit in active disease is caused by mechanical compression by abscesses, granulation tissue, necrotic pieces of bone or disc and pathological subluxation of the vertebrae. The spinal cord may have inflammatory oedema and myelomalacia. Rarely an infective endarteritis of the spinal vessels can also occur.[3, 44, 48] In late onset disease, the paraplegia occurs because of some intrinsic damage to the cord as in chronic vasculitis or is secondary to localized compression by a ridge of bone over a localized kyphosis (internal gibbus), or by a constrictive fibrotic band from scarring around the dura.

The spinal cord may demonstrate cord atrophy or even syrinx and interstitial gliosis secondary to stretching of the cord over the kyphosis. All these pathological features are demonstrated by MRI scan. These patients often present with signs of myelopathy.

1.10 Classification of neurologic deficit

Spinal cord compression in TB of the spine starts anteriorly over the anterior column. The earliest manifestation is a gradual increase in spasticity which may not be appreciated by the patient but will be observed by the clinician as exaggerated deep tendon reflexes and a positive Babinski reflex, shown by a toe extensor response when doing the plantar reflex.

As compression increases over the anterior column, the patient starts losing motor power. Compression progresses to affect lateral columns. This produces some reduction in sensation, mainly pain, crude touch and temperature. With a further increase in compression, posterior columns are affected leading to complete loss of sensation and

sphincter disturbance.[37] In long standing compression, spasticity is replaced by flaccidity and flexor spasms.

The Frankel classification was proposed to classify severity of neurologic deficit in acute spine trauma[49] and has been used extensively in classifying neurologic deficit in TB of the spine. In recent times it has fallen into disfavor because the level of injury is not incorporated into the classification, and because of its subjectivity in judging what the classification refers to as “useful” motor strength. It cannot measure subtle neurological changes during recovery.

Frankel Grading of Paraplegia:

A. Complete: The lesion is complete, with no motor or sensory function below the level involved.

B. Sensory only: There is some sensation present below the level of the lesion, but the motor paralysis is complete below that level.

C. Motor Useless: There is some motor power present below the lesion, but it is of no practical use to the patient.

D. Motor Useful: There is useful motor power below the level of the lesion. Patients in this group could move the lower limbs and many could walk, with or without aids.

E. Recovery: This implies that the patient was free of neurological symptoms, there is no weakness, no sensory loss, no sphincter disturbance. Abnormal reflexes may be present

The American Spinal Injury Association (ASIA) scale was introduced in 1982 and the international Medical Society of Paraplegia adopted it in 1992. [50, 51] The severity of neural deficit is reflected by a score that depends on the level of involvement, in addition to the severity of cord injury at the involved level.

The higher the score, the lower the level of involvement of the spinal cord.[41] This classification is now also used in non-trauma spinal cord injuries [52]

The ASIA classification identifies key muscle groups and sensory points that make it easy for clinicians to do a detailed reproducible examination and be able to determine if a spinal cord lesion is complete or incomplete. Appendix A shows the American Spinal Injury Association International Standards for Neurological Classification of Spinal cord Injury form used to evaluate spinal cord injury.

From this data, the ASIA Impairment Scale is graded as follows:

A. Complete: No motor or sensory function is preserved in the sacral segments S4-S5.

B. Incomplete: Sensory function preserved, but no motor function is preserved below the neurological level and includes the sacral segments S4-S5.

C. Incomplete: Motor function is preserved below the neurological level, and more than half of key muscles below the neurological level have a muscle grade less than 3.

D. Incomplete: Motor function is preserved below the neurological level, and at least

more than half of the key muscles below the neurological level have a grade of 3 or more.

E. Normal: Motor and sensory function are normal.

An ideal classification system should assess functional status of the patient and severity of cord compression. The classification system proposed by Tuli and modified by Jain seems to be ideal. However, it has not yet found common use in orthopaedics.[37] It classifies neurologic deficit into 5 stages:

Stage I: patient unaware of neural deficit, clinician detects plantar extensor and/or ankle clonus.

Stage II: the patient has spasticity with motor deficit but is a walker. The anticipated motor score in tetra paresis is between 60 and 100. In paraparesis, it is between 80 and 100. The sensory impairment is in the lateral column.

Stage III: bedridden spastic patient. Anticipated motor score for quadriplegic is 0–30, and for paraplegic it is 50–80. Sensory scoring is the same as in stage II.

Stage IV: bedridden patient with severe sensory loss, and/or pressure sores. Anticipated motor score in tetraplegia is 0 and in paraplegia it is 50. There is impairment of both lateral and posterior column sensations.

Stage V: same as stage IV and/or bladder and bowel involvement, and/or flexor spasms/flaccid tetraplegia/ paraplegia.

1.11 Radiology in tuberculosis of the spine

1.11.1 Radiographs

Although TB starts in the lungs, very rarely do we find an active pulmonary lesion in

patients with spine TB. There may be evidence of previous pulmonary diseases as shown by apical fibrosis of the lungs.

Early radiographic signs in the spine are a subtle decrease in a disk space height and localized osteopenia. [53] Later we get vertebral collapse, which is the typical lesion of collapse of adjacent vertebrae with reduced height of the intervening disc.

Although plain radiographs are usually the first investigation in patients with spine TB, early disease is not always detected with this method. For radiolucent lesions to be seen in the spine, there must be at least 30% bone mineral loss.[54]

1.11.2 Radionuclide bone scintigraphy

Radioactive isotope bone scanning has been used in endemic areas to diagnose musculoskeletal TB. Solav has used technetium-99 in methylene diphosphate as a radioactive tracer.[55,56] This modality is however not specific: an elderly patient with degenerative spine disease will have an increased uptake of Tc-99 even in the absence of TB because of high turnover of bone and blood supply in the degenerative process.

Positron Emission Tomography (PET) scan is a newer radioisotope scan. It promises to be more useful in TB than the conventional Tc-99 scan. In PET, the radioisotope tracer is fluorine-18-fluoro-2-deoxy-D-glucose (18F-FDG). It is a glucose analogue and is therefore avidly taken up by all metabolically active cells. In the TB granuloma, the macrophages and lymphocytes are highly metabolically active and therefore require and take up glucose. PET therefore shows high uptake in active tuberculosis. Combined with CT, it also gives anatomic definition of the lesion, which conventional Tc-99 imaging does

not give. [57]

FDG-PET/CT would therefore be useful in the imaging of TB of the spine. The problem is that it cannot differentiate between lesions of TB and tumors in the spine. It is superseded by MRI because MRI gives more anatomical definition of the abscess and can differentiate an abscess from a tumor. MRI is also much more available in our country than PET.

The main potential use of PET in TB of the spine would be in monitoring response to treatment. The XR appearance takes a long time to show changes related to healing, like sclerosis and subsequent remodeling. With PET, one would be able to see the decreased uptake, even in the presence of the metalware used to fix the spine.

1.11.3 Magnetic Resonance Imaging

The MRI scan is used extensively in the imaging of the spine for TB. In fact, it is the new gold standard for imaging in TB of the spine.[58] It shows bone changes before they are visible on XR. MRI gives a much better definition of the pathology of TB of the spine. Through MRI we can now say TB does not destroy the disc primarily as the disc is usually normal until late in the disease. What we see on XR is the peri-discal disease, causing collapse of the adjacent vertebrae around the disc, giving an impression of a destroyed disc. It is destruction of radiologic disc space rather than destruction of the disc.

Early involvement of the vertebra is seen on MRI as high signals on both T1 and T2 sequences. This bone oedema is seen well before destruction of bone is seen on XR.

With MRI we can accurately define how much bone is destroyed and thus are able to more accurately plan the length of the bone graft that will be required at surgery.

When interpreting the MRI scan, we look at non-contrast T1-weighted (T1W), T2-weighted (T2W) and short tau inversion recovery (STIR) sequences in axial, sagittal and coronal planes followed by contrast enhanced T1W sequences after intravenous administration of gadolinium contrast agent.[54]

With the aid of intravenous contrast agents, MRI is very accurate in distinguishing between granulation tissue and abscess.[54] The peripheral enhancement, also known as ring enhancement, is the main feature that differentiates tuberculosis from tumours. The size of the abscess can be approximated by multiplying the size of the abscess on coronal, sagittal and axial views. [25]

MRI shows us what is causing the compression of the spinal cord. The dura could be compressed by pus, by granulation tissue, by disc or by fragments of necrotic bone.

MRI also can show the extent of damage to the spinal cord for us to be able to prognosticate about the recovery of neurology.

In summary, in the radiologic assessment of TB of the spine, X-rays provide a general examination, while MRI shows the extent of spread of the disease into the soft tissues and the level of involvement of the spinal cord.[5]

1.12. Laboratory diagnosis of TB of the spine

Diagnosis of all infective diseases is by isolation and culture of the causative organism. Culture is of particular importance because it is required to extract genomic DNA that could be used for identification of genetic determinants of drug resistance.

There are several problems with the diagnosis of TB using isolation and culture. TB of the spine is a paucibacillary infection. This makes microscopic examination following acid fast staining not very reliable. *M. tuberculosis* is a slow growing bacillus. Culture may take up to six weeks, resulting in delay of the diagnosis and treatment.

In TB of the spine, sensitivity of the microscopic analysis is 33% and culture is 43%. The two combined give a sensitivity of 59%. [59]

Delay in diagnosis is responsible for more severe disease with more bone destruction and greater possibility of neurologic involvement.[60] Early diagnosis is important in TB of the spine so that appropriate treatment can be started early. This is particularly so as delayed treatment can lead to devastating neurologic consequences. [61]

In one population group in India, the delay in diagnosis was significantly less in patients with high educational status and high and medium income.[60] This reinforces the need to be more vigilant for TB of the spine in poorer communities.

1.12.1. GeneXpert

GeneXpert is an automated polymerase chain reaction (PCR) diagnostic test used in the diagnosis of pulmonary TB. It is now also widely used in extra pulmonary TB. PCR is a

non-culture molecular diagnostic test. It is a nucleic acid amplification test where the DNA of *M. tuberculosis* is amplified for identification.[53] It detects mutations in the rpoB gene that predict sensitivity to first line drug, rifampicin.[62] With use of amplification systems, nucleic acid sequences unique to *M. tuberculosis* can be detected directly in clinical specimens offering better accuracy than smear and greater speed than cultures. PCR is done on pus, on synovial fluid or on tissue like destroyed bone or granulation tissue, where the tissue is first homogenized in pestle and mortar before it is processed. The test can be done on a single specimen, without having to wait for a batch of specimens.

The results of the PCR test are available in 24 hrs. It has a sensitivity of 95.6% and a specificity of 96.2% for TB of the spine.[63] It is currently the most useful diagnostic test in TB of the spine.

1.12.2. Erythrocyte Sedimentation Rate

The main value of ESR in clinical medicine is to differentiate a radiologic lesion of TB spine from other mimickers of TB on XR, like fungal infections and tumors. ESR is markedly raised in patients with TB of the spine and declines to normal or near normal when an active lesion is controlled. Unlike in pyogenic infections, in TB the ESR is raised in the presence of a normal leucocyte count. This raised ESR in TB is a feature even in HIV-positive patients. ESR in HIV-positive patients with TB is raised to more than 100 ml/hr.[64]

1.12.3. Immunological diagnosis

Historically, immunological diagnosis of TB has been performed using the Mantoux test. In this test, an intradermal injection of a purified protein derivative of the pathogen is administered and 48-72 hours later the size of a raised wheal on the skin is measured. This delayed hypersensitivity reaction on the skin is a measure of the cell-mediated immune response.

More sensitive and specific immunologic assays designed for the immunologic diagnosis of TB have been developed. Interferon Gamma Release Assays (IGRA) measure the cytokine IFN-γ, released by T-cells obtained from a blood sample following re-stimulation with mycobacterial antigens. Effector T-cells can readily respond to antigenic stimuli by secreting cytokines.

They differ from memory cells in that memory T-cells require more time (>24hrs). Effector T cells are present only when the immune system is currently exposed to the antigenic stimuli, which in this case is *M. tuberculosis.* A positive IGRA therefore gives an indication of mycobacterial infection, but cannot distinguish between an active or latent TB, just like the Mantoux.

The Quantiferon TB Gold In Tube (QFT-IT) is an IGRA that measures the amount of interferon- gamma secreted following re-stimulation

These antigens are specific for *M. tuberculosis* complex and are not found in atypical mycobacteria or in the vaccine strain BCG, which lacks the region of difference (RD1), encoding these antigens in *M. tuberculosis* complex.[59]

Despite all the advances in the diagnosis of TB of the spine, the definitive diagnosis of

TB of the spine is still a major problem. One must have a high index of suspicion in endemic areas. Microscopy and culture for the detection of *M. tuberculosis* has a sensitivity of 59%. In cases where GeneXpert is used, the sensitivity for the detection of *M. tuberculosis* increases to 93%. Most patients with TB of the spine can be diagnosed based on typical MRI scan findings. 84% have typical MRI scan findings. The combined use of MRI scan and GeneXpert has a 97% sensitivity for the diagnosis of *M. tuberculosis* infection.[65]

1.12.4. Whole Genome Sequence (WGS)

A recent advance is to move away from phenotypic testing to genetic testing and also to move away from identifying one gene for drug resistance prediction, to characterizing the whole genome of the TB bacillus.[66, 67]

Whole genome sequencing is the process of determining the complete DNA sequence of the genome of an organism at a single time. This entails sequencing all the organism's chromosomal DNA as well as DNA contained in the mitochondria. This genome can then be used to diagnose TB, characterize mutations and predict drug resistance, not only to rifampicin, but to all first line and second line drugs.[68]

Coinfection with non-tuberculous mycobacteria (NTM) is not uncommon in endemic countries, up to 17% in some series. Accurate diagnosis of these NTM is important for correct effective treatment. Poor responses to *M. tuberculosis* treatment may be erroneously ascribed to drug resistance if these co-infecting mycobacteria have not been diagnosed.

Strategies that involve WGS in diagnosis and treatment of TB are therefore important.[69]

As WGS becomes more available and more affordable to poorer countries, it will be the method of choice for diagnosing TB.[68]

1.13. Treatment of TB of the spine

The overall goal of treatment is maintenance or recovery of mechanical spine stability and maintenance or recovery of neurological function, with the patient returning to activities of daily living. These goals can be achieved by multiple interventions, the main ones being anti-TB medication and surgery.

Physiotherapy and nutritional support play a big role in the treatment.

Medical management alone has been found useful in the management of TB. In the 1970's the Medical Research Council in the United Kingdom ran various trials in Africa and in the Far East to show the efficacy of TB medication alone in treatment of TB. [70, 71, 72] There have been reports of recovery of neurology in 38% of patients treated by anti-TB medication alone.[73]

Nowadays there is consensus that there are situations where surgery is required.

1.13.1. Medical treatment

In the medical treatment of TB of the spine, there is still controversy on the duration of treatment and the number of drugs to use. The World Health Organization recommends a category-based treatment for TB. Tuberculosis of the spine falls under category 1. The treatment in this category is divided into an intensive initiation phase, and a continuation phase.

The initiation phase is a two-month phase consisting of the four first line drugs, which are rifampicin, isoniazid, pyrazinamide and ethambutol. The continuation phase is a four-month phase consisting of two drugs, rifampicin and isoniazid.[5] For TB of the spine the continuation phase in most centers is for nine or 12 or 18 months.[29]

The National Guidelines for treatment of pulmonary TB in South Africa recommend a combination the four drugs (rifampicin, isoniazid, pyrazinamide and ethambutol) for two months, followed by a further four months of rifampicin and isoniazid.[74]

The four drugs have different properties. Rifampicin is bactericidal within an hour. It has high potency and is the most effective sterilizing agent. It works both intra- and extracellularly. Isoniazid is bactericidal in 24hrs. It kills more than 90% of bacteria within the first few days of treatment. It also works intra- and extracellularly. Pyrazinamide has low potency and achieves sterilization in 3 months. It acts only on intracellular bacilli in the macrophages. Ethambutol is bacteriostatic, has low potency and it minimizes the emergence of drug resistance. It works both intra- and extracellularly.[74] The treatment of drug-susceptible TB has recently been revised by the World Health Organization.[75]

In the management of TB of the spine in South Africa, most centers use the four drugs mentioned above on the National Guidelines for TB treatment. They are given as a combination tablet, Rifafour^{R} e-275. The tablet is given for 9 months, after which the treatment is stopped, or carried on for a further 3 months.[58] ESR and XR are used to

monitor effectiveness of the treatment.

The major problem with the prolonged treatment period is compliance. In a retrospective study done in Taiwan there was no benefit in prolonged periods of medical management.[75] The combination tablet decreases the number of tablets the patient must take per day. It is believed that this improves compliance. The method of Direct Observation of Treatment for TB medication (DOTS) also ensures compliance. This is a method where the patient is directly observed by a health worker to make sure that the patients take the TB medication.

Because of the length of time required for patients to be on anti-TB medication, the use of immunomodulators as an adjunct to TB chemotherapy has been investigated.[77] In this study, the immunomodulation used was intradermal BCG, oral levamisole and intramuscular diphtheria and tetanus vaccines, given to patients with osteoarticular TB who are not responding to regular anti-TB treatment. The results were promising in that there was an increase in the CD4 count in these patients, which is a reliable indicator of host immunity in patients with osteoarticular tuberculosis. The immunomodulators are, however, not used routinely in TB of the spine, where surgery is used as an adjunct to medical treatment.

1.13.2. Host-directed medical treatment strategies

Medical treatment is currently directed at the infecting organism. There is growing evidence that medical treatment can be directed at the host.

Inflammatory pathways taking place in the formation of a granuloma can be used to develop new host-directed therapies for the control of tuberculosis.[78]

As mentioned earlier, the granuloma is the hallmark of TB infection. It is composed of an arrangement of immune cells that contain the invading pathogen. It has an organized immune structure.[13, 30]

Macrophages within the granuloma develop into specialized cells, such as foamy macrophages and multinucleated giant cells. Mycobacterial lipids have been shown to trigger the differentiation of the macrophages. These foamy macrophages are found in the necrotic region in the granuloma. The center of the granuloma possesses a pro-inflammatory environment characterized by anti-microbial peptides and pro-inflammatory eicosanoids.

The tissue surrounding the caseum possesses a comparatively anti-inflammatory environment.[30] Disease driving processes are therefore compartmentalized within these granulomas. This again affirms that changes and variations within the granuloma can be associated with disease control or progression of the disease.

Dendritic cells are among the early arrivals at sites of infections. They engulf the bacteria, bacterial products and dying cells. In so doing they take a sample of the antigen, leave the site of infection, and migrate to the regional lymph nodes, where they trigger an acquired immunity response. This inflammatory dendritic cell migration from the granuloma may be the key to continued maintenance of the granulomatous lesion.[79]

The final step in the formation of the granuloma is the arrival of T- and B-Cells that form

a lymphocyte cuff around the granuloma, giving it a solid structure. The arrival of T-cells coincides with the arrest of bacterial proliferation by producing TNF-α and IFN-γ that enhances macrophage bactericidal activity. This is usually 2-3 weeks after initiation of infection.

TNF-α is thus one of the cytokines that may be useful in the treatment of TB, based on its activity in the formation of the granuloma. Production of TNF-α is required to induce macrophage killing.[30]

Excessive or low TNF-α production is detrimental to the host, causing tissue pathology. Blocking TNF-α activity is a feature that has been used to treat inflammatory disease like rheumatoid arthritis. The problem currently encountered is that some of the patients that have been put on TNF-α inhibitions for the treatment of rheumatoid arthritis, have had reactivation of latent TB.

Lipids may also be useful in the development of these host directed therapies. Lipids are an integral part of the granuloma, especially in the caseum, where we get the foamy macrophages. Lipid bodies within the foamy macrophages play a role in the reactivation of TB. Statins are already being used in medicine in the treatment of coronary artery diseases and are therefore already targeted in the search for host directed therapies.[78]

1.13.3. Surgery

Whenever there is a lesion in the spine, it is advised that the lesion be biopsied. This is

because in an area where TB is endemic, we may over-diagnose TB of the spine, and miss other infections like fungal infections and tumors. The biopsy is done through a needle biopsy or through major open surgery. Biopsy would therefore be the commonest indication for surgery in TB of the spine.

The role of surgery in treatment continues to be a subject of debate. A Cochrane database review assessing the role of surgery in patients being treated for TB of the spine with TB chemotherapy concluded there was insufficient evidence for routine surgical treatment. Indeed, surgery is used only in selected cases.[73]

Hodgson and Stock described a radical operation where the diseased bone and necrotic tissue and granulation tissue are removed, thus decompressing the cord and leaving a bed of well vascularized tissue.[80] The radical removal of all necrotic tissue allowed penetration of anti-TB drugs into the well-vascularized tissue, thus promoting healing of the disease. The vascular bed also helps with the incorporation of the bone graft. It has been shown that the sclerotic bone in the affected vertebrae blocks the penetration of TB drugs. [81]

The large defect in the anterior structures of the vertebral column is closed with a structural graft, using autogenous graft of ribs or fibula. All surgical procedures for TB of the spine are essentially a modification of this operation.

The disadvantage of the operation is that the patient requires to be in an intensive care environment post-operatively as the surgical procedure involves a transthoracic approach

to the thoracic spine or a retroperitoneal approach in cases that involve the lumbar spine.

The other disadvantage is that patients often must lie in bed for a long time while waiting for the bone graft to incorporate. With the development of more rigid spine fixation methods, the spine is now stabilized either with an anterior rod or plate, or posteriorly based pedicle screw fixation devices. This allows patients to be mobilized early without having to lie in bed until the graft has incorporated.

Several patients present with more extensive bone destruction. The rib graft proved to be too thin for adequate for stabilization of the spine. It is only useful if only the disc space is involved, which is a rare feature in tuberculosis. The fibula graft has been shown not to have a surface area wide enough to cover the endplate of the vertebral body. More than 3 fibula strut grafts would be required to cover a lumbar vertebral endplate.[82]

The inadequacy of the fibula and rib struts in supporting the spine, especially when there is more than one vertebral body involved, has led to the use of allografts.

The allografts have been found to incorporate very well into host bone, even in the presence of HIV co-infection.,[45,83]

The introduction of titanium to orthopaedic implants has made it possible to use vertebral cages made of this metal as strut grafts to replace the diseased bone. Mycobacteria have been shown not to adhere to titanium implants and do not form biofilm. It is therefore safe to use implants made of titanium in the presence of active tuberculous infection.[53]

One of the problems in surgical treatment of TB of the spine is that TB generally occurs

in poorly nourished communities, and in immunocompromised individuals. This makes surgery to be high risk for these patients, with increased pulmonary complications especially after a transthoracic procedure.

The posterior-only approaches to the spine have improved treatment options for the patients who are otherwise poor contenders for surgery. With a posterior surgical approach, the patients can be operated on without requiring high care or intensive care post-operatively. We have found it particularly useful in the HIV-positive patients who are usually poor surgical risk. [84-86]

1.13.4 Indications for surgery.

Tuli came up with a regimen where the patients are put on TB medication and nutritional support before surgical treatment.[73] This approach has shown good surgical outcomes and redefined the indications for surgery.

The indications for surgery have therefore changed and surgery is now indicated for patients who:

- Have severe neurologic deficit on presentation
- Worsening or develop new neurologic deficit while on TB medication
- Show no neurologic improvement after 6 weeks of TB medication.
- Recurrence of disease while on TB medication.
- Lack of clinical response after 6 weeks of medication, as this implies that the medication is not reaching the site of infection in the necrotic tissue.

- Patients with a large prevertebral abscess also require surgery as it may cause respiratory distress in the cervical spine, or the TB medication may not reach the diseased area.

Surgery is also indicated in patients with:

- Loss of one vertebra in thoracic spine
- Loss of 1.5 vertebrae in lumbar spine.
- Kyphosis more than 30 degrees
- Posterior lesion with destruction of pedicle

The surgery in these cases is performed because of the risk of developing a kyphotic deformity and spine instability.[9] Rajasekaran described radiologic changes that predict the risk of collapse of the spine in children.[87] Presence of these signs in children is an indication for surgery. The signs are: Retropulsion of the vertebral body into the canal, subluxation of adjacent vertebrae, lateral translation of the spine at the site of the lesion and toppling of the proximal spine. This study will, however, exclude children.

1.14. Gaps in the body of knowledge

Most immunological research on tuberculosis is based on peripheral blood samples. These may not give a representative picture of which immune cells are involved at the organ affected or in the microenvironment of the granulomatous lesions.

In order to develop new strategies of treatment it may be useful to study the immune response at the site of the *M. tuberculosis* infection. [11]

This study will evaluate the spinal granulomas in HIV-negative and HIV-positive patients.

The micro-environment of these granulomas will be investigated for histopathology, as well as immunological markers and their concentrations.

Literature suggests that further progress in the management of TB will depend on knowing what happens at the site of infection. [17, 19] Many studies have concentrated on pulmonary tissue. [21-23] This study will show the effect of HIV on the stability of the TB granuloma in TB of the spine.

There are only a few articles that address the disease process in the spine. Danaviah, and Govender have investigated the TB granuloma in the Spine. [7, 16, 88]However, they were looking at the granuloma inside the confines of the spinal canal. They did not look at the granuloma in the wide space of the prevertebral tissue, where most of the disease expands and gives the radiological picture that we see.

This is where the focus should be if we want to know why the extent of spread of the disease is different in the HIV-positive and HIV-negative patients.

Their studies were done during the era where antiviral treatment was not available in the state sector in South Africa. Nowadays all the patients that are HIV-positive are on antiviral treatment. The TB granuloma may behave differently in this group of patients, hence the need to do the studies on the TB granuloma in this era.

Rajasekaran wrote that evidence relating to spine tuberculosis and HIV is lacking, making it difficult to recommend best optimal treatment.[43]

The main problem in treatment of TB of the spine is that the recommended period for taking TB drugs is long (9-24 months). This gives rise to poor compliance and resistance to medication. There is a lot of research being done on TB, looking at host directed therapies which may shorten the treatment period for TB.[78, 30] All this work has been done on pulmonary tissue. We believe that this work on the immune pathology on the spine granuloma will add to the information the world needs to make advancement in host directed therapies.

1.15. The research questions

Are there immunological factors responsible for the perceived difference in the distribution of TB spine in HIV-negative and HIV-positive patients?

The secondary research questions are:

1. What is the extent of spread of the disease in HIV-negative and HIV-positive patients?
2. Is there a difference in the cellular content and structure of the granuloma in TB of the spine in the two groups of patients?
3. Is there a difference in the cytokines from the granulomas of HIV-negative and -positive patients with TB of the spine?
4. What is the genome of the bacillus that causes TB of the spine?

1.16. Aim of the study

The aim of the study will be to identify immune factors responsible for local distribution of

tuberculosis of the spine in HIV-negative and HIV-positive patients, and to identify any organism-related factors that may be responsible for the spread of the bacilli to the spine.

1.17. Null Hypothesis

There is no difference in the granulomas and immune systems of HIV-negative and -positive patients that may lead to any difference in the distribution of the disease between the two groups of patients, and there are organism-related factors responsible for the spread of the disease from the lungs to the spine.

1.18. Study objectives

The objectives of this study will be to:

1. Measure and define the extent of destruction of the bone and extent of spread of the disease using plain XR and MRI scans of the affected area of the spine.
2. Grade the granuloma histologically from well-formed to poorly formed, according to the cellular composition of the granuloma.
3. Measure the expression of the cytokine TNF-α in the granuloma harvested at surgery from the site of infection
4. Determine the presence of acid-fast bacilli in the tissue, (colony forming units) using PCR, and determine the sensitivity of the bacteria to the commonly used drugs.
5. Perform whole genome sequencing on the genetic material from the bacilli that have been cultured.

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CHAPTER 2

CLINICO-RADIOLOGIC FEATURES AND EXTENT OF DISTRIBUTION OF TUBERCULOSIS OF THE SPINE IN HIV-NEGATIVE AND -POSITIVE PATIENTS

2.1 Introduction

In this chapter we study the radiologic features of tuberculosis of the spine in HIV-negative and HIV-positive patients. Extrapulmonary tuberculosis is estimated to account for up to 20% of tuberculosis infections.[1] The spine is affected in more than 50% of these extrapulmonary cases.[1, 2] TB of the spine accounts for 2% of all cases of TB.[3]

The thoracic spine is most commonly affected, followed by the lumbar spine.

The typical lesion of tuberculosis of the spine on plain radiographs is a disease affecting two contiguous vertebral bodies with narrowing of the intervening disc space. There are atypical lesions where the disease affects a single vertebral body or affects the posterior elements. This is referred to as atypical TB.[4]

TB co-infection with HIV is now very common, occurring in more than 50% of patients with TB of the spine in endemic areas. Indeed, one cannot talk about TB of the spine without mentioning the HIV status of the patient.

There is paucity of literature on the radiologic features of TB of the spine in HIV-positive patients.

In this chapter, we will look at the radiographs and MRI scans of patients with TB of the spine and see if there is any difference between the patients who are co-infected with HIV and those who are not.

2.2 Literature review

Tuberculosis spreads from the lungs via the blood stream to settle in several organs such as the kidneys, testis, and musculoskeletal system. This is referred to as extrapulmonary TB. Musculoskeletal TB occurs in 1%-13% of patients, and 50% occurs in the spine.[1, 2, 5] The thoracic spine is involved most often, followed by lumbar spine and then cervical spine.

There are four recognized patterns of vertebral involvement; paradiscal, anterior, central and neural arch.[3] The classical peri-discal disease starts in the subchondral bone of the vertebral endplate. This region is the metaphyseal-equivalent of a long bone. The arterioles in this region turn abruptly and have a sluggish blood flow. This permits the bacilli to lodge in this area and start to destroy bone and form pus.[6] Pus then spreads to the intervertebral disc space. Classically the disease will destroy two contiguous vertebral bodies with the disc collapsing within the vertebral bodies. The reason for the involvement of two contiguous vertebrae is that the segmental spinal artery bifurcates to supply two adjacent vertebrae.[3] With time, the disc will also be destroyed by the disease. The disease then spreads to the rest of the vertebral body causing collapse of the vertebral body.

The disease is characterized by pus formation. This pus tracks under the anterior longitudinal ligament to infect the adjoining vertebral bodies.

This route of spread may also be responsible for the spread of the disease to other vertebrae. The pus may track on other soft tissues like the psoas muscle to form a psoas abscess, which may be palpable in the groin at the region of the attachment of the

iliopsoas muscle to the lesser trochanter. The pus may also track down to form an abscess which points out through the triangle of Petit. This is a triangular space in the inferior lumbar spine bordered by the iliac crest inferiorly, latissimus dorsi muscle posteriorly, the external abdominal oblique muscle anteriorly and whose floor is the internal abdominal oblique muscle. The pus and destroyed bone may also spread posteriorly into the vertebral canal causing compression of the spinal cord and paraplegia. Neurologic deficit, as represented by paraplegia, is caused by mechanical compression of the spinal cord by pus, granulation tissue, fragments of necrotic bone or disc and lastly by a vascular phenomenon where we get vasculitis and thrombosis of the local vessels that supply the spinal cord. Paralysis from the vasculitis does not improve.

The disease rarely spreads to involve the posterior elements.[6] Also, it is uncommon for the disease to start primarily in the posterior elements. Marais et al. in their series of 274 patients with TB of the spine, reported a 28% incidence of posterior element involvement. It was, however noted that this posterior element involvement was by contiguous spread from the vertebral body. There were no isolated primary infections of the posterior elements. In posterior element disease there may be erosion of the head of the ribs and proximal part of the rib. The intervertebral space is often normal.[7]

In the central lesion, the disease starts in the center of the vertebral body.
This is followed by a concentric collapse of the vertebral body, forming what is seen as a vertebra plana on XR. The disc is not involved.[3]
The pathologic processes described above can be seen on radiologic investigations.

The plain radiograph is the first radiographic modality we use to investigate a patient presenting with symptoms suggestive of TB, *i.e*., long-standing back pain, deformity of the back and neurologic deficit. It is the least expensive imaging modality in tuberculosis. It is available in all institutions, including most rural hospitals. It provides one of the highest spatial resolution images. Plain radiography is limited by its poor contrast resolution, rendering it unsuitable for defining soft tissue changes. It is further hindered by its inability to obtain satisfactory lateral views in the upper thoracic spine, the so-called blind spot on spine XR.[8]

The first sign on plain radiographs is osteopenia along the endplates. This early sign is visible only when there is at least 30% loss of bone mineral. It explains why early disease may be missed on XR. As the disease progresses, we get erosion of the endplates. The endplates collapse and we get the radiologic narrowing of the disc space. It is worth noting that the disc is intact at this stage, but is prolapsed into the soft vertebral endplate, and may be seen on MRI. Only later will it be destroyed. The destruction of bone causes the lytic lesions we see in the vertebral bodies on XR. Because tuberculosis is a chronic disease, the body gets time to try to regenerate by forming new bone, seen as sclerotic lesions on XR. The vertebral body on XR will therefore have lytic and sclerotic lesions.
With time, the vertebral body will collapse, and at this stage we see the clinical sign of a gibbus, which is the prominent spinous process of the vertebral body. The classic radiologic lesion of TB of the spine is shown in figure 2.1 below.

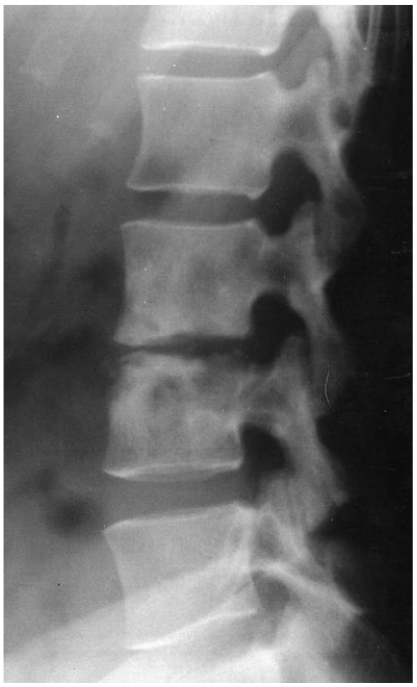

Figure 2.1. Classic radiograph of TB of the spine showing involvement of two adjacent vertebral bodies with lytic and sclerotic lesions and narrowing of the intervening disc space.

Unfortunately, even the most classic lesion of TB on XR is not pathognomonic of TB, as it can be seen in other chronic infections. This is the reason why biopsy is mandatory in TB-like lesions of the spine.

In the anterior lesion, the necrotic tissue and pus tracks beneath the anterior longitudinal ligament, because the TB has no proteolytic enzymes to destroy the ligament.

On XR of the thoracic spine, with the contrast of the air in the lungs, the abscess will be seen as a fusiform shadow on either side of the vertebral body on AP view, causing the so called ‘heart -in -heart’ appearance. The abscess can also be seen on an AP lumbar

spine as an asymmetry of the psoas outline. A long-standing anterior abscess will show on XR as a scalloping of the anterior vertebral bodies.

The loss of vertebral bone can be assessed on plain radiographs. Vertebral body collapse leading to a gibbus deformity is common in TB of the spine. The severity of the deformity is a measure of bone collapse. The angle of deformity can be measured by drawing a line along the superior end plate of the first normal vertebra cephalad to the lesion, and another line along the inferior endplate of the first normal vertebra caudal to the lesion. The two lines meet to form the angle of kyphosis.[9] This method is reliable and has been used by the Medical Research Council Working Party on Tuberculosis of the Spine in their studies in Korea, Hong Kong and in Zimbabwe.

The pathologic features seen on XR can be seen much earlier on MRI. MRI can show much more extensive involvement than XR. The sequences done in MRI are T1-weighted (T1W), T2-weighted (T2W) and Short Tau Inversion Recovery (STIR) sequences, in axial, sagittal and coronal planes. When infection is suspected, as in TB, these sequences are followed up by T1W sequences after injection with intravenous gadolinium contrast agent. The increased oedema of the vertebral body will show as reduced signal intensity on the T1W images, and high signal intensity on T2W, with a heterogeneous enhancement of the vertebral body. The STIR sequences help to differentiate between fluid and fat in non-contrast sequences and on T1W fat saturated post gadolinium sequences.[6,3] MRI will show the presence of skip lesions. Pus will show as ring enhancement in the post-gadolinium enhancement sequences. The post-gadolinium sequences make MRI to be

very good at differentiating between pus and granulation tissue or phlegmon.[10-12]. Compression of the spinal cord is seen much better on the T2W sequences. If there is oedema in the spinal cord, it will show as a low signal intensity on the T2W sequence, but if there is myelomalacia from vasculitis, the low signal intensity will also be present on the T1W images.

MRI is thus the preferred modality for investigating TB of the spine.[5] Signal changes occur early.[13] It is however an expensive modality and not available in the poorer communities, where this disease is endemic.[14]

MRI is reported to have a sensitivity of 96% and specificity of 92% and accuracy of 94%.[12] Bothara et al. in their series reported a sensitivity of 99% and that the MRI findings were not supported by histology in only 1% of cases.[15]

CT scan is another modality used in TB of the spine . It gives better detail of bone erosion especially in areas where plain XR may not be clear, as in cervical-thoracic spine and cranio-cervical spine. CT shows the bone changes much earlier than XR. It can be used to diagnose skip lesions. With post contrast CT, one can see the extent of pus formation and cord compression. CT can show calcification within the abscess, which sign is pathognomonic of TB.[3] Its biggest disadvantage is the amount of ionizing radiation to which the patient is exposed.

CT has been superseded by MRI in most centers, but its biggest use is in CT-guided needle biopsies.

Multiple level non-contiguous lesions (skip lesions) are not uncommon in TB. [16]They are defined as the presence of another lesion, in addition to the initial site of involvement, separated from the initial lesion by at least one vertebral level. In a series of 267 patients, Mohanty recorded an incidence of 10%.[10] In a rather small series on MRI of the spine, Kaila recorded an incidence of 71% (10 of 14). They recommended that whole spine MRI must be done on all suspected TB of the spine.[17]

Radionuclide imaging is a method of scanning that targets skeletal physiology. The conventional isotope is technitium-99 – methylene diphosphonate. (Tc-99m MDP).[18]

Newer isotopes have made radionuclide imaging to be more useful in inflammatory tissue. Positron emission tomography with the use of 18F-fluorodexygucose (18F-FDG PET/CT) is a noninvasive method that is used for differentiating malignant from non-malignant lesions. It has been reported to detect occult skip lesions in 63% of patients.[5] It is currently not being used in the diagnosis of TB of the spine, but reports show that it is useful in monitoring response to treatment.[19, 20]

There are reports that the radiologic changes in tuberculosis of the spine are different in HIV-negative patients to those found in HIV-positive patients.

In a large clinical series of 274 patients, Marais *et al* reported no difference in the number of vertebrae involved in spondylitis between HIV-positive and -negative patients. However, in the HIV-negative patients, there was more frequent loss of vertebral body height (81%) than in the HIV-positive group (50%). There were also more patients with

skip lesions in the HIV infected group. The destruction of the vertebral body was more common in in HIV-negative patients.[7]

Anley reported that HIV-negative patients have more total vertebral collapse, but no difference in overall size of abscess formation.[21] Sagane found that HIV-negative patients had a larger angle of kyphosis and more vertebral body destruction. Pus formation was significantly higher in the HIV-negative group.[22]

Very few articles address the radiologic differences difference between HIV-positive and -negative patients. This study will add to the few.

2.3 Aims and objective

The aims of the study are to assess the extent of distribution of TB of the spine in HIV-positive and HIV-negative patients and to define the clinical parameters in the two groups of patients.

The specific objectives are:

1. To determine whether the XR pattern is the classical pattern or an atypical XR pattern.
2. To examine the whole spine MRI looking for skip lesions.
3. To measure the extent of bone destruction as evidenced by extent of kyphosis on lateral XR and by loss of vertebral body height.
4. To measure the number of vertebrae involved in the disease process.
5. To measure the volume of pus as seen on T1 post gadolinium MRI sequences.
6. To determine the extent of cord compression.

2.4 Study design

This is a prospective multicenter study conducted over a 15-month period on 61 patients undergoing surgical treatment for TB of the spine.

2.5 Study material

We recruited all patients that were treated surgically for TB of the spine during the period May 2019 to August 2020. Surgically treated patients were chosen because they would provide a larger volume of biopsy tissue which would be sufficient for the tests we want to do on the specimens. The indications for surgery were:

1. Severe neurologic deficit on presentation.
2. Worsening of neurologic signs while on TB treatment.
3. No neurologic improvement after 6 weeks of TB treatment
4. Large prevertebral abscess.
5. Kyphosis of more than 30 degrees.

2.6 Inclusion criteria

1. Patients older than 18 years.
2. Diagnosis of TB made by a combination of clinical features, radiology, positive PCR, typical histologic lesion and microscopy and culture of the bacilli.
3. Patients who fulfilled the indications for surgery.

2.7 Exclusion criteria

1. Patients with sputum-positive active pulmonary tuberculosis

2. Patients with immunosuppressive disorders other than HIV, such as, but not limited to diabetes and rheumatoid arthritis
3. Patients on chronic steroid use.
4. Pregnant or lactating patients.

2.8 Methods

Ethical clearance for the study was obtained from the University of Pretoria, Faculty of Health Sciences Ethics Committee (Ref no. 73/2019) and from the National Health Research Database. (Ref no. GP_201904_004). Sixty-one (61) patients were recruited to the study.

We recorded the demographics of all the patients. Preoperatively we assessed the HIV status of the patients, CD4 count and viral load, ESR, s-albumin, chest XR and the neurologic status using the ASIA chart.

The XR of each patient was examined by an orthopaedic surgeon experienced in spine conditions. On the XR we recorded the anatomic area of the spine affected by the disease.

The angle of the gibbus was measured by drawing a line along the superior endplate of the proximal diseased vertebra and a line along the inferior endplate of the distal diseased vertebra, as seen in fig. 2.2. The angle subtended by the two lines is the angle of deformity.

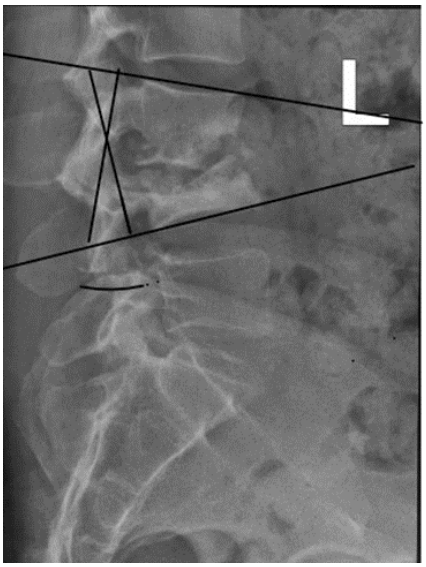

Fig 2.2. XR to show measurement of segmental kyphosis

The MRI was examined by an experienced Musculoskeletal Radiologist. The whole spine MRI was examined for skip lesions on the sagittal post contrast fat saturated T1 sequences (T1 FS post gad) and the site of the lesion was examined for involvement of the posterior elements. The number of vertebrae involved were counted on the T1 Fat Suppressed sequences. The extent of compression of the cord was measured by dividing the widest diameter of the extension of the pus and granulation tissue into the canal, by the diameter of the canal at that level (T1 FS post gad sequences). The volume of the abscess was measured by multiplying the following three values: On the transverse images, the width and AP extent of the pus, and the height of the lesion on the sagittal images. The loss of vertebral body height was measured by measuring the ratio of the height of the remaining uninvolved part of the proximal diseased vertebra to that of the adjacent normal vertebra. Similarly, with the distal vertebra the ratio of the height of the remaining uninvolved part of the distal diseased vertebra to the height of the adjacent distal vertebra.[7] These measurements were done on the T1 post gadolinium sequences.

1.8.1. Statistical methods

We used descriptive statistics which presented means, standard deviations, standard errors, proportions and associated 95 percent confidence intervals. Chi-square test was used to evaluate the degree of association between factors categories. Furthermore, 2 sample Welch t- test for unequal variances was used to compare proportions. A non-parametric equivalent for two-sample Wilcoxon (Mann-Whitney) rank-sum test was used in the comparison of the HIV-negative and HIV-positive groups to achieve the objectives. All tests were performed at an alpha level of 0.05. Stata 16 was the tool used for analysis

2.9 Results

Sixty-one patients were enrolled in the study. Thirty-one of the patients were male and 30 females and 18 patients were HIV-negative and 43 were HIV-positive.

The diagnosis of TB was confirmed by a positive GeneExpert test in 85.45% of the specimens, Histology showed granulomas, Langerhans giant cells and necrosis in various combinations in all specimens. Culture was positive in 48,3% after an average of 18 days of incubation. The Ziehl-Neelsen stain was positive in 15.5%. The combination of all these tests and the MRI scan confirmed the diagnosis of TB.

The age range of the entire group was 25-64 years, with a mean age of 42.8 years.

The mean age of the 18 HIV-negative patients was 43.3 years and that of the 43 HIV - positive patients was 42.6 years. Figure 2.3 is a bar graph showing the age distribution of the patients by gender and HIV status.

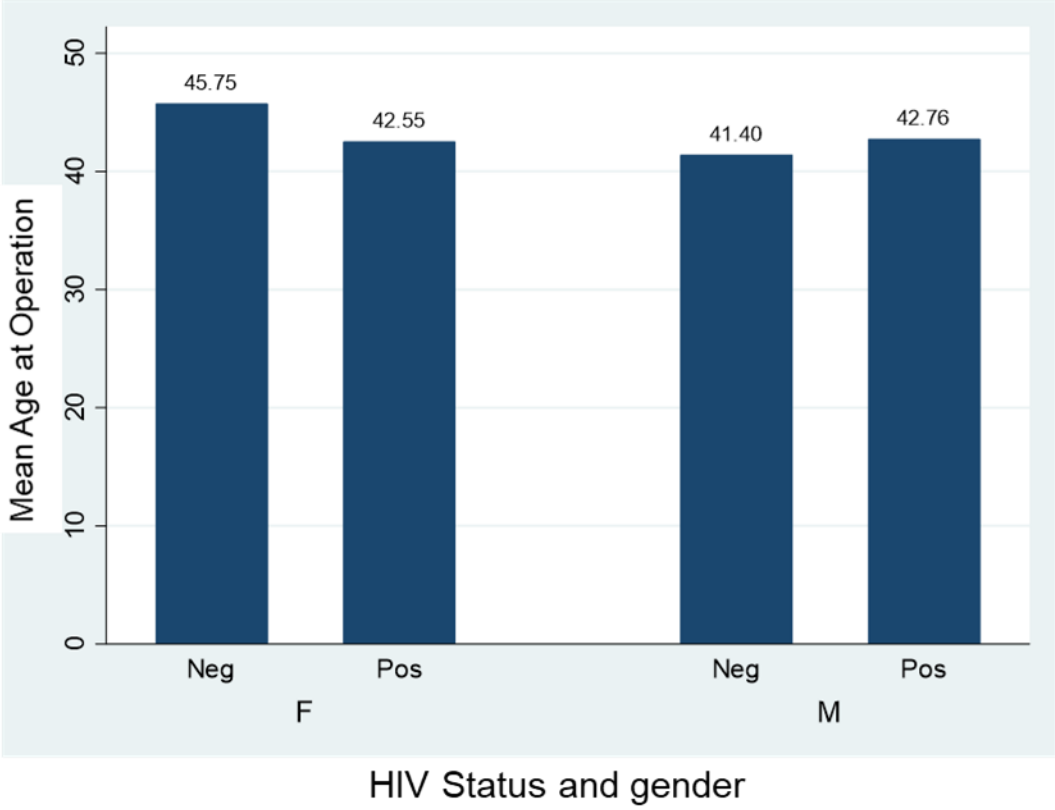

Fig 2.3. Bar chart of age distribution of patients by gender and HIV status

ESR

The ESR ranged from 2-129 mm/h in the HIV-negative patients, with a mean of 56.17 mm/hr. One patient (5.5%) had ESR of less than 20 mm/hr. Only three patients in HIV -negative group had an ESR of 100 mm/hour or more. In the HIV-positive group, the ESR ranged from 2-115 mm/hour, also with a mean of 56.17 mm/hr. In two patients (4.6%) it was less than 20mm/hr. Only three patients had an ESR of 100 mm/hour or more. In the whole study group, only 6 of 61 patients (9.83%) had an ESR of 100 or more.

We looked at patients with active pulmonary disease as seen on XR and compared the ESR between the HIV-negative and -positive patients. There were 4 HIV-negative and 9

HIV-positive patients. The mean ESR in the HIV-negative group was 62±12.13 mm/hour, 95%CI [23.37-100.62] and in HIV-positive group it was 40.77±11.8 mm/hour, 95%CI [13.56- 67.99]. The ESR was generally lower in HIV-positive group of patients with active chest disease, but not significantly so as seen in the table I below.

Table 2.i: Comparison of ESR in patients with active pulmonary disease, using the two-sample t-test with unequal variances

Group	n	Mean	Std. Err.	[95% Conf.	Interval]
HIV -Neg	4	62	12.13809	23.37117	100.6288
HIV -Pos	9	40.77778	11.80252	13.56113	67.99443

s-Albumin

The serum albumin ranged between 28- 47 g/l in the HIV-negative patients. Only one patient (0.05%) had an albumin of less than 30 g/l in this group. In the HIV-positive patients, the serum albumin ranged between 23 – 43 g/l. Four patients had an albumin of less than 30 g/l.

In the whole study group, the serum albumin was less than 30 g/l in 5 of 61 patients (8.1%).

Platelets

The platelet count ranged from 157-539 $x10^9$ g/L in HIV-negative patients and from 154-551 x 10^9 g/L.

CD4 cell count

The CD4 count in HIV-positive patients was <200 cells/µl in ten patients, 201-500 cells/µl in 23 patients and > 501 cells/µl in the remaining ten patients.

Pre-operative treatment and viral load

Preoperatively, the HIV-negative patients had a duration of symptoms ranging from 1- 60 months with a mean of just less than a year, at 11.76 months. Eleven of the 18 patients had been on TB drugs before the operation for a period of 1-9 months, an average of 2.6 months per patient.

The HIV-positive patients had a pre-operative duration of symptoms also ranging from 1-60 months, with a mean of 11.56 months. Twenty-seven of the patients were on TB treatment for an average of 3.7 months at the time of surgery. Of the 43 HIV-positive patients, 35 were on antiretroviral treatment for a period of an average of 33.25 months before entering the study. Their viral load was undetectable in 31 patients (72.09%), less than 20 copies / ml of blood in a further four patients and ranged from 100 to 113324 copies / ml of blood in eight patients.

Table 2.ii below shows the preoperative blood results. There is no statistical difference between HIV-negative and HIV-positive patients on all the blood tests that were done.

Table 2. ii. Pre-operative blood results comparing HIV-positive and -negative patients

Group Summary and Comparison of Blood Parameters								
	HIV-Negative			HIV Positive				
	Mean	SE	95% C.I.	Mean	SE	95% C.I	Pr(|T|	Significance
ESR (mm/hr)	56.17	8.07	[38.2 73.2]	56.17	16.2	[26.2 53.4]	0.87	Non-sig
CRP (mg/l)	42.94	9.13	[23.7 62.2]	41.50	5.9	[29.6 53.4]	0.89	Non-sig
Albumin (g/l)	34.44	1.43	[31.4 37.5]	34.91	0.87	[33.2 36.7]	0.78	Non-sig
Platelets (10^9/l)	341.78	27.92	[82.9 400.7]	351.37	18.1	[314.8 387.9]	0.78	Non-sig
Hb (g/dl)	12.12	0.58	[11.0 13.2]	12.14	0.27	[11.6 12.7]	0.97	Non-sig
WCC (10^9/l)	6.79	0.52	[5.8 8.0]	5.89	0.41	[5.1 6.7]	0.21	Non-sig
CD4 (cells/µl)	-			385.00	38.0	[308.1 461.9]	n/a	

Chest XR

On assessing the pulmonary involvement, 46 (75.4%), had a clear chest XR. Thirteen patients (21.32%) had active disease and two patients (3.28%) had evidence of fibrosis, compatible with healed pulmonary TB.

The radiologic investigations of the spine show the extent of the distribution of TB in the spine.

The disease affected the thoracic spine in 27 patients (44.2%), lumbar spine in 24 patients (39.3%), thoraco-lumbar junction in six patients (9.8%), cervical spine in three patients (4.9%) and one patient had the disease in the lumbosacral junction (1.64%). This is shown in the bar graph on fig 2.4 below.

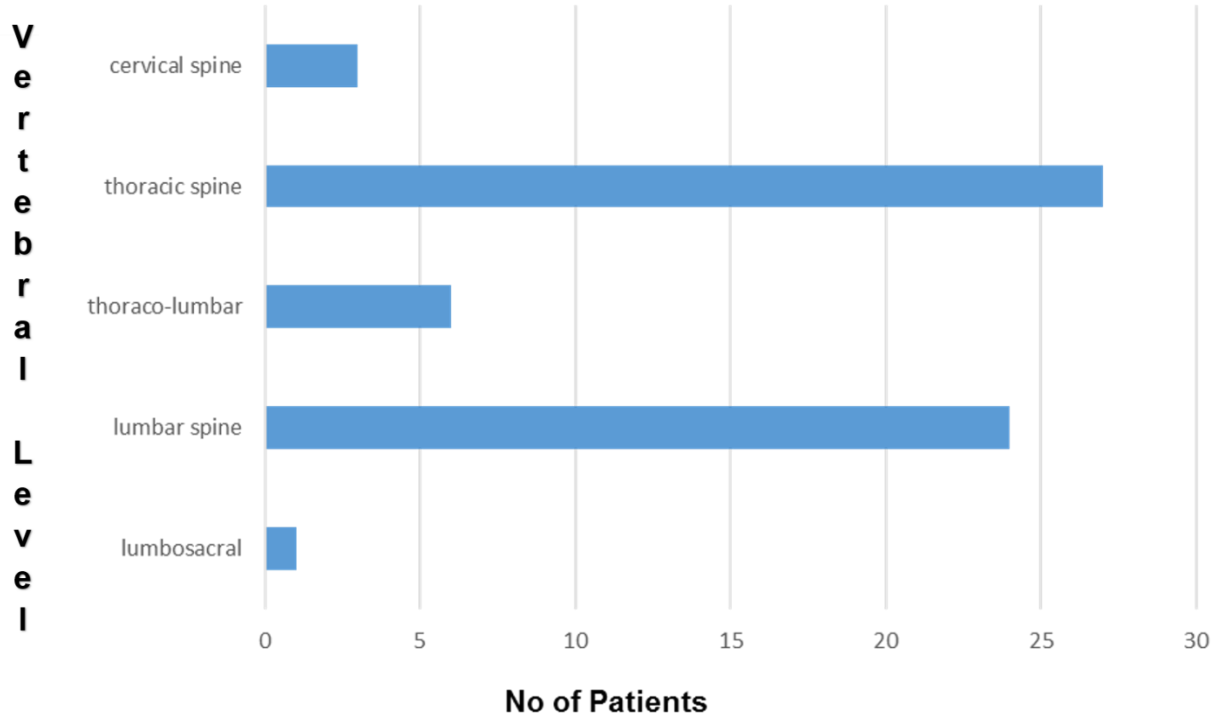

Fig. 2.4. Distribution of TB in the various areas of the spine

Radiologic features of TB of the spine in HIV-negative and HIV-positive patients

1. Typical or atypical XR appearance

The typical radiologic appearance of a two-body disease with narrowing of the intervening disc space as seen in fig. 2.5 (left) was seen in 45 patients (73.77%), and the atypical pattern, represented here on the right by the vertebra plana pattern, was seen in 16 patients (26.23%).

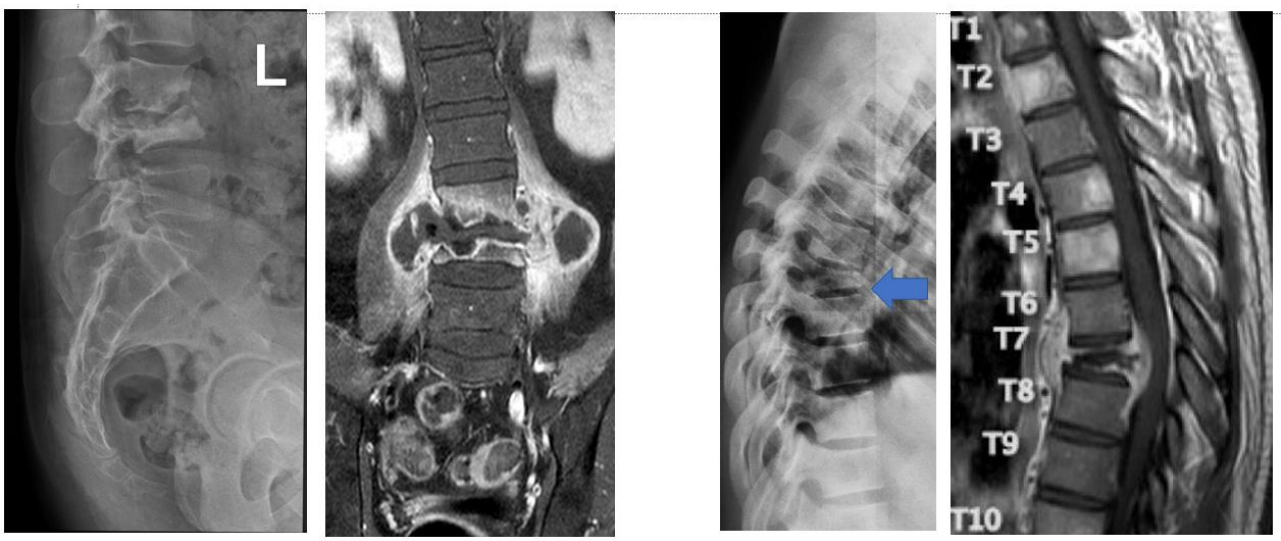

Fig. 2.5 :(left) Typical appearance of TB spine on plain radiographs, showing involvement of the adjoining vertebral bodies of L3 and L4 with narrowing of the intervening disc space. MRI shows the ring enhancement of the abscess. (Right) Atypical lesion of TB spine with symmetrical involvement of the vertebral body (blue arrow). MRI again shows ring enhancement to confirm TB.

Table 2. iii shows the relationship between this radiologic pattern and the HIV status of the patients. Of the 45 patients with a typical XR pattern, 14 (77.78%) were HIV- negative and 31 (72.09%) were HIV-positive. Of the 16 patients with an atypical XR pattern, 4 (22.22%) were HIV-negative and 12 (27.91%) were HIV-positive. This means that 6.56% of the patients (4 of 61) were HIV-negative with atypical XR, and 19.67% (12 of 61) were HIV-positive.

Table 2.iii. Comparison between XR appearance and HIV status of the patient

XR appearance	HIV-Neg	HIV-Pos	Total
Atypical(n)	4	12	16
Row %	25.00	75.00	100.00
Column %	22.22	27.91	26.23
Typical(n)	14	31	45
Row %	31.11	68.89	100.00
Column %	77.78	72.09	73.77

Although there are more patients in the HIV-positive group with atypical radiologic pattern (19.67%), Table 2.iv below shows that there is no evidence of statistical significance. (p=0.645)

Table 2.iv. Summary of typical and atypical XR appearance

	Proportion	Std. Err.	Logit [95% Conf.	Interval]
Atypical HIV -Neg	.2222222	.0979908	.0841829	.4703596
Atypical HIV -Pos	.2790698	.068402	.1639459	.4331515
Typical HIV -Neg	.7777778	.0979908	.5296404	.9158171
Typical HIV -Pos	.7209302	.068402	.5668485	.8360541

P=0.645

2. Skip lesions

Four patients did not have a whole spine MRI. We therefore could not assess those patients for skip lesions. As shown in table 2.v below, skip lesions were found in 6 patients (10.53%) in this series. There were no skip lesions in 51 patients (89.47%). Analyzing these results further, one of 15 patients (6.25%) in the HIV-negative patients and five of

36 (12.20%) HIV -positive patients had skip lesions. Although there is no statistical evidence of association of skip lesions with HIV status of patients (p=0.66), there is a higher incidence of skip lesions in HIV-positive patients.

Table 2.v. Association of occurrence of skip lesions with HIV status of the patients

Skip Lesions	HIV-Neg	HIV-Pos	Total
NO (n)	15	36	51
Row %	29.41	70.59	100.00
Column %	93.75	87.80	89.47
YES (n)	1	5	6
Row %	16.67	83.33	100.00
Column %	6.25	12.20	10.53

P-value = 0.66

3. Angle of kyphosis

The mean angle of kyphosis in the HIV-negative group was measured to be 25.38± 2.90 degrees, 95%CI: [19,26-31.50] and for the HIV-positive group it was 25.09±2.37degrees, 95%CI: [20.29-29.89], giving a probability value of p=0.3264, which is not significant.

These results show that there is no difference between the angle of deformity and the HIV status of the patient. The results are summarized in table 2.vi below.

Table 2.vi. Two-sample t-test with unequal variances for comparison between angle of kyphosis and HIV status

Group	Obs	Mean	Std. Err.	[95% Conf. Interval]
HIV -Neg	18	25.38889	2.900461	19.26945 - 31.50833
HIV -Pos	43	25.09302	2.379037	20.29193 - 29.89411

p-value = 0.3264

4. Loss of vertebral body height

The mean loss of vertebral body height in the HIV-negative group was 0.96 ± 0.10, 95%CI [0.73 -1.19]. In the HIV-positive group, the loss of vertebral body height was 1.33 ± 0.10, 95% CI [1.12 – 1.55]. There is therefore a significant difference in bone loss between the HIV- and HIV+ patients. The HIV-positive patients lost more bone from the destructive effect of the disease, as seen in table 2.vii below.

Table 2.vii: Two-sample t-test with unequal variances to compare vertebral body height between HIV-negative and HIV-positive patients

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf. Interval]	
Neg	16	.96875	.107517	.4300678	.739583	1.197917
Pos	41	1.337805	.1066219	.6827134	1.122314	1.553296

P -value= 0.0190

5. Number of affected vertebrae

The mean number of affected patients in the HIV-negative group of patients as seen on MRI was 2.56±0.245 and 95%CI [2.03-3.07] and of affected HIV -positive patients was 3.28±0.306 and 95%CI [2.66-3.90]. This shows that there is a marginal difference between the number of affected vertebrae in the two groups of patients. (p= 0.07) The table below summarizes the relationship between the number of vertebrae involved and the HIV status of the patient.

Table 2. viii. Two-sample t-test with unequal variances to show number of vertebrae involved in HIV-negative and HIV -positive patients

Group	Obs	Mean	Std. Err.	[95% Conf.	Interval]
Hiv -Neg	18	2.555556	.2455116	2.037571	3.07354
Hiv -Pos	43	3.27907	.3055876	2.662369	3.895771

P-value = 0.07

6. Volume of pus

The mean volume of pus in HIV -negative patients was 63.30 ± 19.11mm^3 ,95% CI [22.5-104.04] and in HIV -positive patients it was 109.92 ± 28.36mm^3 ,95%CI [52.60-167.24]. There is generally almost double the volume of pus in HIV-positive patients than in HIV -negative patients, but no evidence of statistical significance. (p= 0.1635).

Table 2. ix. Two-sample t-test with unequal variances to compare pus in HIV – negative and HIV -positive patients

```
------------------------------------------------------------------------------
   Group |     n          Mean     Std. Err.      [95% Conf. Interval]
---------+--------------------------------------------------------------------
HIV -Neg |      16     63.30313     19.11541        22.55959    104.0467
HIV -Pos |      41     109.9239     28.36253        52.60108    167.2467
---------+--------------------------------------------------------------------
p-value = 0.1635
```

7. Cord compression

The extent of compression of the spinal cord was graded as follows:

A: No compression = 8 patients
B: 1-25% compression = 16 patients
C: 26%-50% compression = 14patients
D: 51%-75% compression = 8 patients
E: 75%- 99% compression = 7 patients
F: 100% compression = 4 patients.

Statistical analysis showed no difference in the extent of compression of the spinal cord in the HIV- and HIV+ patients as shown in table 2.x below, with p value = 0.810. There was also no difference in the ASIA scale for neurological impairment between the two groups of patients.

Table 2.x: Extent of compression of the spinal cord in HIV- and HIV+ patients

Extent of Cord Compression				
Grade of Compression		**HIV -**	**HIV +**	**Total**
A	(n)	1	7	8
	Row %	12.50	87.50	100.00
	Column %	6.25	17.07	14.04
B	(n)	4	12	16
	Row %	25.00	75.00	100.00
	Column %	25.00	29.27	28.07
C	(n)	5	9	14
	Row %	35.71	64.29	100.00
	Column %	31.25	21.95	24.56
D	(n)	2	6	8
	Row %	25.00	75.00	100.00
	Column %	12.50	14.63	14.04
E	(n)	2	5	7

	Row %	28.57	71.43	100.00
	Column %	12.50	12.20	12.28
F	(n)	2	2	4
	Row %	50.00	50.00	100.00
	Column %	12.50	4.88	7.02
Total		16	41	57
		28.07	71.93	100.00
		100.00	100.00	100.00

Fisher's exact = 0.810

Figure 2.xi summarizes the radiologic results.

Table 2.xi: Radiographic and MRI features of spine tuberculosis in HIV-negative and -positive patients

	HIV-Negative			**HIV-Positive**				
Radiographic /MRI parameter	**Mean**	**SE**	**95% C.I**	**Mean**	**SE**	**95% C.I**	**PR (|T|**	**Statistical significance**
Kyphosis (degrees)	25.38^0	2.9^0	[19.26-31.50]	25.09^0	2.37^0	[20.29-29.89]	0.32	Non sig
No vertebrae involved	2.55	0.24	[2.03-3.07]	3.27	0.30	[2.66-3.89]	0.07	Non sig
Volume of pus mm^3)	63.30	19.11	[22.5-104]	109.92	28.3	[52.60-167]	0.16	Non-sig
Vertebral body height (ratio)	0.968	0.10	[0.73-1.1]	1.33	0.10	[1.2 – 1.5]	0.01	Sig

2.10 Discussion

Tuberculosis of the spine is the commonest form of skeletal tuberculosis, accounting for 50% of skeletal tuberculosis.[10, 23] With the increase in HIV in infection the world over, and especially in poor communities, HIV co-infection of TB is increasing. In South Africa, HIV co-infection of tuberculosis is as high as 70 %.[7]

In this series of patients with TB of the spine operated over an 18- month period, 43 of the 61 patients were HIV-positive, which is 70.49% of the patients. This is the largest ratio of HIV-negative to HIV-positive patients with TB of the spine in a prospective study reported in South Africa.

In a series of 50 patients with tuberculosis of the spine in a South African setting, Anley reported that 40% of the patients were HIV -positive. In a large series of 274 patients, Marais *et al* reported that 76% of the patients were HIV-positive[7]. These were both retrospective studies. These figures show that there is a steady increase in the number of HIV-positive patients with spine TB, from 40% in Anley's study in 2012 to the 70.8% in this study done 7 years later. Because of this increasing ratio of HIV-positive to HIV-negative patients, it is becoming difficult to get a series of patients with a comparable number of patients. In this study we used the t-test with unequal variances to be able to compensate for the unequal numbers.

Our results as shown on figure 2.3 show that there is no difference in age distribution and gender in HIV-positive and -negative patients. This is a similar finding to the study by Marais et al. In some series the HIV-positive patients tended to be younger.[21]

The disease affected the thoracic and lumbar spine in 79% of the patients as seen on fig. 2.4. This is because of the rich arterial blood supply of the thoracic spine and the blood reflux in the valveless Batson's plexus in the lumbar spine. This pattern of distribution has been reported by various other authors.[21]

Table 2.ii shows the blood results of the patients taken preoperatively. There is no statistical significance between the blood results in HIV-positive and HIV-negative patients. There are, however, some interesting findings with regards to nutritional status. Tuberculosis is a disease of poverty, and malnutrition. In this series, the serum albumin, which is a measure of protein nutrition, was less than 30 g/l in only 8.1% of the patients

(5 of 61). This is perhaps because a number of the patients had been on treatment for TB for at least for 2.6 months in 11 of the HIV-negative patients, and for 3.7 months in 27 of the HIV-positive patients. During this period the patients are also put on a high protein diet as advocated by Tuli.[24]

The ESR is an investigation commonly used to differentiate a spine lesion caused by TB from a lesion of non-tuberculous origin. TB should be suspected if the ESR is 100 mm/hr. An ESR result of more than 20 mm/hour has a sensitivity of 60% to 90% but is not specific. It does, however, have a great prognostic value in that its serial values show a gradual decrease after initiation of effective therapy.[25] In this series, the ESR was a mean of 56mm/hour. Only six patients had an ESR above 100 mm/hour. Other authors also did not find high ESR in patients with TB of the spine.[4, 26, 27,] Nwabuko reported an ESR above 100 mm/hour in HIV-positive patients who have active pulmonary TB.[28] In our patients only one of the six patients with ESR above 100 mm/hour had an active chest infection on XR. There is no statistical difference in the ESR values between the two groups. This is perhaps a reflection of effective antiviral treatment on the HIV-positive group as seen in the low or undetectable viral load in 72.09% of the HIV-positive patients.

Typical/atypical radiologic appearance

The typical radiologic picture of TB of the spine, being involvement of two contiguous vertebrae and an intervening disc space, is a result of the pathologic process of the disease. TB starts in the anterior and peri-discal area. This results in the typical lesion of involvement of two contiguous vertebral bodies and narrowing of the adjoining

intervertebral disc space.

Pande [29, 30] wrote about the atypical lesion of TB of the spine and reported the incidence to be 2.1%. These were lesions that do not fit in the typical pattern. He described these atypical lesions as: a concentric collapse of a vertebra, an ivory vertebra, involvement of the neural arch and multiple vertebral disease, either in continuity or not in continuity. Those not in continuity are also referred to as skip lesions. The authors described these lesions as being difficult to associate with TB, leading to delay in diagnosis and treatment. In this series, the typical radiologic pattern was seen in 73.77% of the patients (45 patients) and the atypical pattern in 26.23% (16 patients). The atypical pattern in HIV-negative patients was seen in 6.6% of the patients (4 of 61), and the atypical pattern in HIV-positive patients was seen in 19.6% of the patients (12 of 61). Although there is no statistical significance ($p>0.05$), the results show that the atypical radiologic pattern is seen mostly in HIV-positive patients.

In a series of 30 patients, Sagane found no difference between the HIV-negative and -positive patients, with both groups showing a two body disease in 73,3% and 66.7% of patients respectively.[22]

Skip lesions

Multiple non-contiguous spine lesions (skip lesions) represent one of the atypical features of TB of the spine[4, 31] Skip lesions were found in 10.53% of patients in this series. Skip lesions were found in one in 6.25% in HIV-negative patients and in 12.20% in HIV-positive patients. There is no statistical difference in the distribution of skip lesions between HIV-negative and HIV-positive patients ($p= 0.0665$).

Sagane[22] also found no statistical difference in skip lesions between the two groups of patients(p=0.09). Anley[21] found skip lesions in seven (14%) of their patients. They also could not statistically associate them with the HIV status of the patients. In a small series of 14 cases , Kaila reported the incidence of skip lesions to be 71.4% (10 out of 14).[17] Wu attributes this reported increase in skip lesions to the practice of doing full spine MRI for suspected TB of the spine.[17]

From these studies, skip lesions are not infrequent in TB. Although we could not show any statistical significance, we tend to see them in HIV-positive patients. We postulate that because of the decreased immune status in the HIV-positive patients, the disease can disseminate wider to other areas of the spine. TB paralysis is a devastating disease, it is therefore important not to miss skip lesions. Whole spine MRI should be requested when TB of the spine is suspected.[31, 32] From these results we can add that this is particularly important if the patient is HIV-positive.

Skip lesions of tuberculosis must be differentiated from other causes of multiple non-contiguous spine lesions, like lymphoma, multiple myeloma and metastatic disease.

Number of vertebrae involved

The mean number of vertebrae involved in the disease in HIV-negative patients as seen on MRI was 2,55 ±1.04, 95%CI [2.03-3.07] and in HIV-positive patients 3.27± 2.00. 95% CI [2.66-3.89]. There is a marginal difference between the two groups, with p= 0.0351. Sagane reported no difference in the number of vertebrae involved between the two

groups of patients (p=0.53) [22]

Anley reported a mean of 3.5 vertebrae in the HIV-negative patients and 3.6 vertebrae in the HIV-positive patients. They concluded that there was no statistical difference between the two.

There is no difference in the number of vertebrae involved in all the three studies quoted above. The reason for this could be the control of the HIV disease as shown in this study. Our patients were treated at the time when HIV-positive patients had access to ARV drugs in our country. In this cohort, 35 of the 43 HIV-positive patients were on ARV treatment for an average of 33.25 months at the time of surgery. The viral load was not done in 3 patients. In 31 of the 43 HIV-positive patients (72.09%), the viral load was not detectable. This shows a good control of the retroviral disease on antiretroviral drugs. This would make the HIV-positive patients behave like HIV-negatives, to some extent or in some of the parameters that one elects to measure.

Angle of kyphosis

The typical lesion starts in the anterior paradisical area and spreads posteriorly. As the involved vertebral bodies lose bone anteriorly, a kyphotic deformity then develops.[33] There are various methods used to measure the kyphosis in TB spine. One can measure the Cobb angle using the upper end plate of the proximal involved vertebra and the lower endplate of the inferior vertebra.[34] This is the method we used in this study. The second method is to measure the Cobb angle using the superior and inferior endplates of the first normal vertebra proximally and distally.[9, 33] The disadvantage of this method is that it tends to underestimate the amount of bone loss by showing a lower level of kyphosis.

Figure 2.6 shows the angle of kyphosis in the same patient using the two different methods.

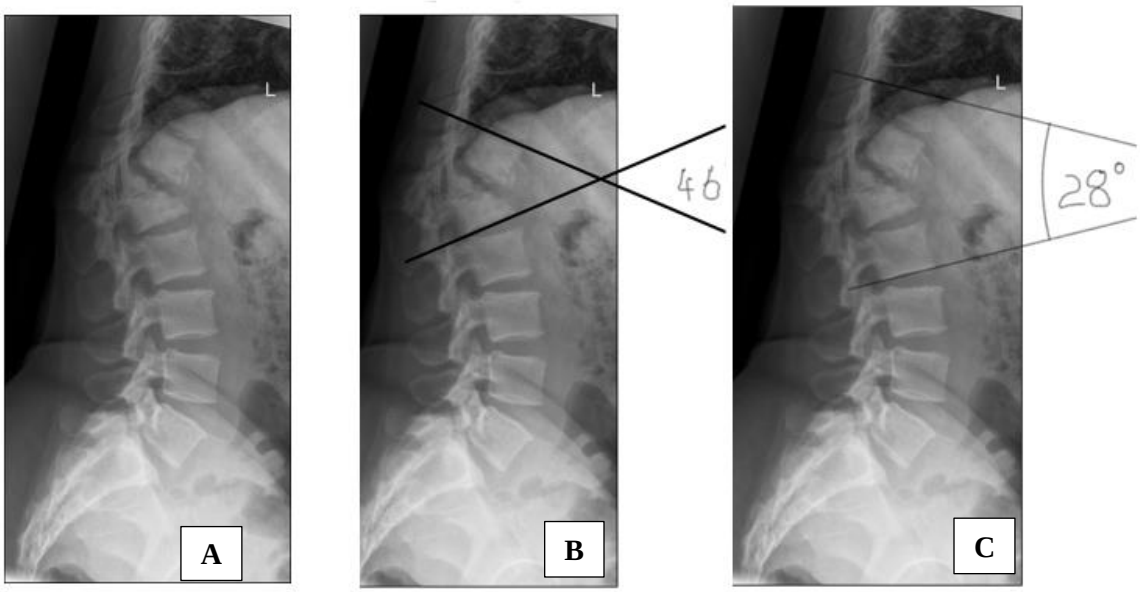

Fig. 2.6. Measurement of angle of kyphosis using two different methods

When the first method is used, we get an angle of 46 degrees (Fig 2.6 B). When the second method is used, we get a smaller angle of kyphosis, due to the compensatory hyperextension of the intervertebral disc, as seen on the L1-2 disc space in Fig 2.6 C above.

A third method is to measure the angle subtended by the posterior vertebral body line of the first normal vertebra caudally or distally[35] This can also be done on MRI[21]. The angle so measured may not be representative of the amount of bone loss as these patients are recumbent on the MRI scanner.

In this study the mean angle of kyphosis in the HIV-positive group was 25.38^0 ±2.90,

95%CI [19.26-31.50] and in HIV-negative patients it was 25.09^0 ±2.37, 95%CI [20.29-29.89]. There is no difference in kyphosis between the two groups of patients (p=0.326). Sagane in their study found a statistically significant difference in the degree of kyphosis between the two groups. The degree of kyphosis was greater in HIV-negative than HIV-positive patients.[22] Similarly, Anley reported that the average kyphosis in HIV-negative patients was 19.2 degrees and in HIV-positive patients was 9.5 degrees., which was statistically significant.[21] They attributed this difference to be due to reduced immune response of the type 4 hypersensitivity reaction caused by HIV infection. Their measurements were however, on MRI, meaning that the patients were supine. This may have had a bearing on the true results of the kyphosis. In our study we measured kyphosis using plain radiographs because this is what we use in clinical medicine to measure kyphosis.

Volume of pus

The mean volume of pus in the HIV-negative patients was 63.30 mm^3 ± 19.11, 95%CI [22,5-104.04] and in HIV-positive patients it was 109.92 mm^3 ±109.92, 95%CI [52.60-167.24]. It is not statistically significant (p=0.1635) but the mean volume of pus is almost double in the HIV-positive patients.

In the study by Sagane, the mean size of abscess was 1.8 cm^2 in HIV-negative and 7.9 cm^2 in HIV-positive patients. There was a statistical significance between the groups at p<0.0001.[22] They attributed this to decreased cell-mediated immunity and induction of inflammatory responses by the TB infection. Anley in their series showed no statistically

significant difference in the size of abscesses between the two.[21]

Aboobakar in their series of 20 patients with tuberculous psoas abscesses, reported that 14 of these patients were HIV -positive.[36]

The fact that there is more pus in the HIV-positive patients may have a bearing on the surgical procedure that the surgeon may elect to do in these patients. It suggests that decompression as in costo-transversectomy may be sufficient in these patients, rather than the extensive debridement that is advocated in the Hong Kong procedure. [22, 37] This treatment method, initially proposed by Sagane,[22] and now being suggested by the results in this study, needs to be explored further in a clinical study.

Loss of vertebral body height

In this study there is significantly more bone destruction in the HIV-positive patients, compared to the HIV-negative patients, as seen in table 2.vii (p= 0.01).

These findings are different to those of Sagane et al who showed that the amount of bone destruction was greater in HIV-negative, and Anley et al who found more collapse in HIV -negative patients.[21, 22]

The new and most important findings in this study that are very different from previous studies in the literature relate to vertebral body loss and extent of pus formation. This study shows that there is significant vertebral bone loss in HIV-positive patients (p=0.01) and though not statistically significant, marginally more pus in HIV-positive patients. This

is the direct opposite of the study by Anley who found more vertebral bone loss in HIV-negative patients and no difference between the two groups in pus formation. Sagane found more vertebral bone loss and more pus formation in HIV-negative patients, and Marais described more vertebral bone loss in HIV-negative patients. They all postulated that their findings were based on the type IV hypersensitivity reaction which is suppressed in HIV-positive patients, hence less bone destruction in HIV-positive patients.[7,21,22]

Our radiologic findings of more bone destruction and pus formation in HIV-positive patients may be explained by the body's response to the infection by the TB bacilli and by the human immunodeficiency virus. The bone tissue damage we see in TB of the spine, leading to vertebral bone collapse is caused primarily by the granuloma formation and subsequent necrosis.[38, 39]

TB of the spine is however a paucibacillary disease. The extent of tissue damage that we see in this disease cannot be explained only by the effect of the few bacilli found in tuberculosis of the spine. The tissue damage is mostly due to locally produced proinflammatory and anti-inflammatory cytokines.[40]

The immune control of *M. tuberculosis* is mediated by multiple cell types that all contribute to the formation of a granuloma. Specific immune cells such as macrophages and some CD4 T-cells secrete pro-inflammatory cytokines, notably TNF-α, and others like IL-1, IL-12, IFN-γ. The inflammation can be suppressed by anti- inflammatory cytokines which are secreted by some immune cells like regulatory T-cells and

macrophages. The anti-inflammatory cytokines include IL-4, IL-10 and transforming growth factor beta (TGF-β). Cicchese describes that there is a fine balance between the up regulation and down regulation of this immune response, which determines the amount of tissue damage, as seen in figure 2.7.[41]

HIV is involved in disturbing this balance by various mechanisms, which include decreasing CD4 cell count and depletion of CD4 T-cells in the granuloma, poor granuloma formation, suppression of cellular immune reactivity and impaired TNF-α-mediated apoptosis of *M. tuberculosis* by the macrophages.[40] All these have an effect in determining how much bone is destroyed by the disease.

In an immune histochemical analysis in bone of 30 patients with TB of the spine, Izawa[42] found that the receptor activator of nuclear factor-κB (RANK-L) pathway was strongly activated in TB of the spine. The activation of the RANK-L pathway was caused by cytokines, especially TNF-α and IL-6. The expression of these cytokines is increased in HIV infection.[40] It is known that in severe pulmonary TB, there is an intense immune reaction leading to tissue destruction. The intense immune reaction in TB of the spine, as found in the presence of HIV infection, leads to the bone destruction we see in TB of the spine patients with HIV co-infection.

Furthermore, in support of these findings of vertebral bone loss in HIV-positive patients, Bezuidenhout[43] examined pulmonary tissue in HIV-negative and -positive patients with pulmonary TB. She found that necrotic granulomas were more evident in HIV-positive patients and that HIV co-infection because of decreased macrophages, showed poor

granuloma formation. There was also a close association between TNF-α and necrosis.

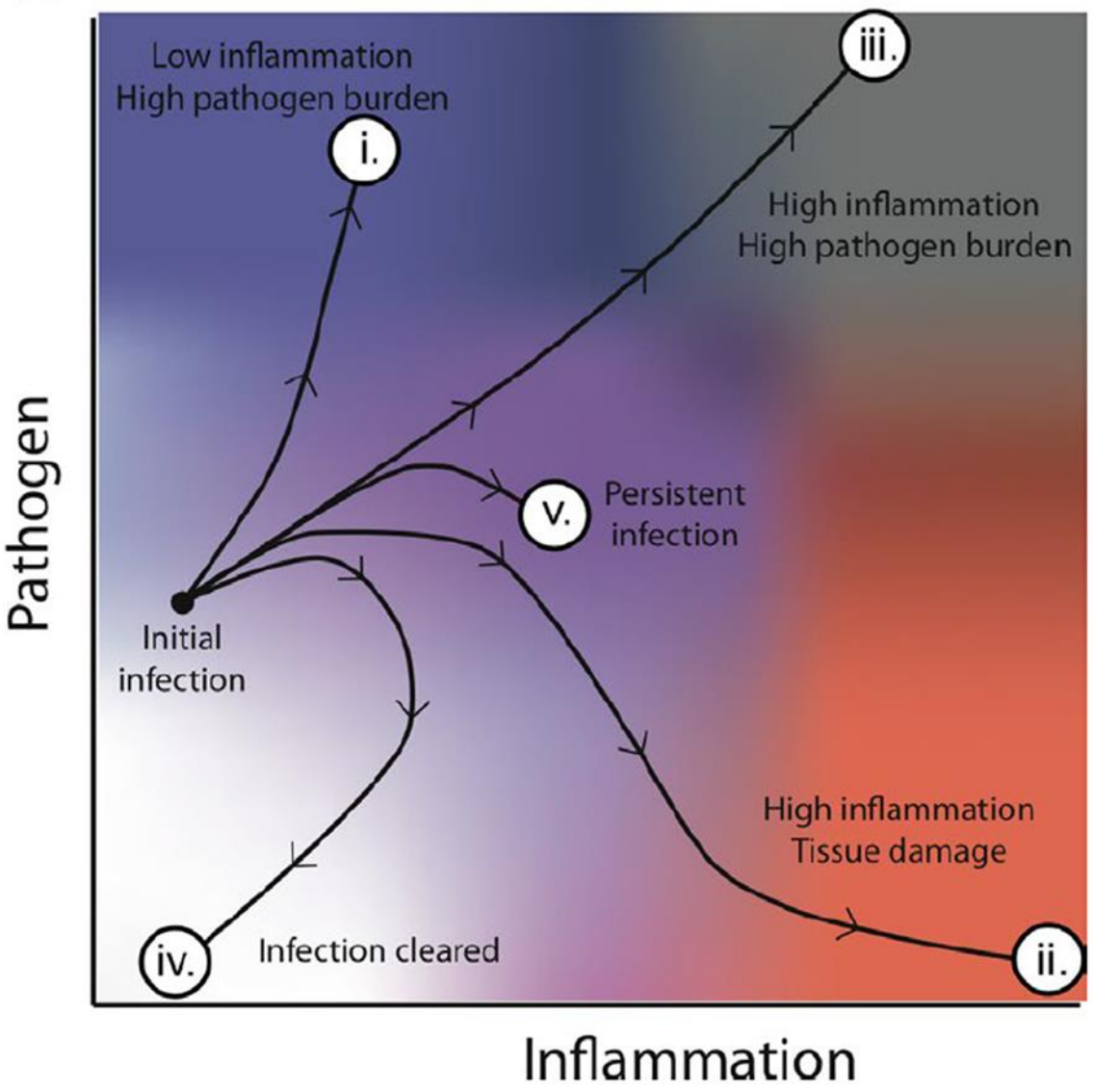

Fig 2.7. Dynamics of pro- and anti-inflammatory immune responses steers disease projection along various paths. The outcomes are (i) high pathogen burden; (ii) tissue damage; (iii) high pathogen burden along with large amount of tissue damage; (iv) cleared infection and return to base line level of inflammation; (v) dynamically balanced immune response to control pathogen growth and limit host pathology. (adapted from Cicchese et al. Immun. Reviews.2018;285:147-167)

It is for this reason that in the next chapter we will be looking at the granulomas and cytokine expression, particularly TNF-α in this cohort of spine TB patients.

2.10.1 Limitations of the study

The study was on surgically treated patients only and may not be representative of all patients with TB of the spine. The fact that the HIV-positive patients had been on treatment and well controlled may make comparison between the two groups difficult.

2.11 Conclusion

HIV-positive patients show more vertebral body destruction and marginally more pus formation. This bone destruction is due to the activation of the RANK-L pathway by cytokines, which process is more pronounced in HIV co-infection. These findings may have a bearing on the type of surgery we do in TB of the spine in that we may elect to tailor the surgery for HIV-positive patients to surgical drainage of the pus and stabilization of the spine only, without doing the extensive decompression currently practiced.

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CHAPTER 3

THE TB GRANULOMA AND CYTOKINE EXPRESSION IN HIV-NEGATIVE AND HIV-POSITIVE PATIENTS

3.1 Introduction

In this chapter, the TB granuloma and its role in the immune response in tuberculosis of the spine will be discussed. It is hoped that the structure of the granuloma and cytokine expression may be able to explain the findings we made on the radiologic differences of TB of the spine in HIV-negative and -positive patients.

3.2 The TB granuloma

The TB granuloma is a complex cellular structure that can interact immunologically with the invading pathogen. Its formation is firmly linked to the delayed type hypersensitivity reaction, which is an adaptive immunity mediated by activated T-cells and cytokines.[1] It is an organized compact aggregate of macrophages. The macrophages are transformed into epithelioid cells in response to *Mycobacteria tuberculosis* infection. The infected macrophages can also fuse with each other to form Langhan's giant cells or accumulate lipid to be called foamy cells. Infected macrophages arrange themselves into a concentric ring, around a lipid-rich core of central necrosis.

The granuloma macrophages produce a large range of cytokines that result in recruitment of a diverse range of cells, responsible for the production of cytokines and the recruitment of new cell types.[2] These cells, which include neutrophils, dendritic cells, T-cells, B-cells, natural killer cells and fibroblasts, form a concentric ring around the granuloma.

Macrophages also promote dissemination of the disease by leaving the primary granuloma to establish new granuloma sites. Macrophages can adapt[3] to kill engulfed microbes but are also involved in regulating early organism development and co-ordinate tissue repair. [3] The granuloma provides a safe place in which mycobacteria can infect the newly recruited macrophages and protect the host against the mycobacteria.[4]

The center of the granuloma consists of caseum, a coagulative form of tissue necrosis. If the host can control the bacterium, necrosis stops and the caseum is replaced by calcification or fibrosis.[2] Caseative necrosis is the breakdown of participating cells, especially macrophages. It is a sign of active tuberculosis in an immunocompetent host. With progression of the disease, there is expansion of the caseum and conversion from coagulative to liquefactive necrosis with erosion to surrounding tissues. In the spine the liquefactive necrosis forms the characteristic cold abscess which may be inside the spinal canal, or in the surrounding tissues, like the psoas muscle.[5] Spread of liquefactive material causes disease transmission in the lung and facilitates the distribution of the disease to other areas of the spine.

The bacilli induce production of lipoxins by the host, which are anti-inflammatory eicosanoids that induce macrophage necrosis and dissemination of the bacilli.[6] Because of the lipoxins, the source of carbon inside the granuloma is therefore cholesterol, not glucose. This leads to lack of carbon nutrients, hypoxia and a high concentration of nitric

acid. The significance of cholesterol in the survival of mycobacteria inside the granuloma is evidenced by the negative role that statins play against mycobacteria.[7]

In addition, it is thought that an inflammatory storm, driven by CD4 cells, drives a rapid neutrophil response which causes rapid tissue destruction.[8] The caseous necrosis is a consequence of apoptosis of infected macrophages and activated T-cells. TNF-α is a known activator of apoptosis.[9] There is a very active interaction between T- and B-cell response, with CD4 T-cells playing a critical role in shaping the B-cell response.[10]

3.3 The TB granuloma and cytokines

At the beginning of infection, the mycobacteria require an inflammatory environment to develop the granuloma. Subsequently, survival is linked to an environment that has no inflammation. This switch is caused by the protein early secretory antigen target (ESAT-6), secreted by the mycobacteria. This ESAT-6 transforms macrophages from a phenotype that produces pro-inflammatory cytokines IL-6, IL-12, TNF-α and INF-γ , to a phenotype that produces anti-inflammatory cytokines IL-10 and TGF-β.[7,11] The balance between the release of pro-inflammatory and anti-inflammatory cytokines is required for the establishment of infection.[12]

Pro-inflammatory cytokines exhibit bactericidal factors at the expense of generating a potent pro-inflammatory environment.[13] TNF-α preserves granuloma structure and integrity by inhibiting mycobacterial growth and preventing macrophage death. If there is no TNF-α, the granuloma disintegrates.[14] The pro-inflammatory environment is found

more in the center of the granuloma. The tissue surrounding the caseum has a predominantly anti-inflammatory trait.[15] The cytokines have also been found to be the major cause of tissue damage in pulmonary tuberculosis. [16]

The low oxygen tension in spine tissue as opposed to pulmonary tissue explains the pauci-bacillary nature of spine TB.[6,17] *M. tuberculosis* responds to this anoxia by entering into a state of non-replicating persistence.[2] The other reason for the spine tissue to be pauci-bacillary is that epithelial cell-derived matrix metalloprotease 9 (MMP9) is deficient in the spine. This enzyme not only promotes granuloma formation, but also promotes *M. tuberculosis* replication.

The destructive nature of TB of the spine suggests that *M. tuberculosis* is not the only cause of the extensive tissue damage. The extensive destruction in TB of the spine is likely due to chronic inflammation triggered by a relatively low number of bacteria, perpetuated by bacterial derived components. *M. tuberculosis* sheds cell wall components known as exosomes, which contain immunogenic cell wall lipids and proteins. The exosomes are taken up by macrophages and other immune cells. Uptake of these *M. tuberculosis*-derived exosomes by uninfected macrophages inhibits INF-gamma mediated activation and upregulation of mycobactericidal pathways. These processes help to extend the influence of the bacterium to non-infected cells and thus helps to explain the phenomenon of extensive host cell involvement in the granuloma, despite the low number of local bacteria.[2]

3.4 The TB granuloma in HIV infection

HIV negatively affects granuloma formation and TB specific immunity. This leads to

decreased ability of the granuloma to contain *M. tuberculosis* and killing of *M. tuberculosis*-infected cells. This all leads to more extensive disease progression. It weakens the host's response to *M. tuberculosis,* due to insufficient macrophages , and insufficient activated CD4 T-cells which are key components of protective immunity against TB.[18] There is a reduced number of T-cells in granulomas of patients with extrapulmonary TB.[19]

Histology of TB granulomas and HIV co-infection is however a story of contrasts and contradictions. Danaviah found that macrophage activation and differentiation in spine TB were similar in HIV-positive and -negative patients and that granulomatous architecture was similar in both groups of patients.[5] In a systematic review, Diedrich summarized the histologic findings in HIV-positive patients as showing poorer granuloma formation and increased bacterial load. There was however a large amount of heterogeneity among the studies that they reviewed.[20]

The cytokine system is also affected. Granulomas in HIV-positive patients have high levels of TNF-α and are more necrotic.[9] de Norhona had found the opposite in HIV-positive patients.[21] Other authors write that the TNF-α mediated macrophage apoptotic response to *M. tuberculosis* is impaired in HIV.[22]

The histological pattern of TB granulomas has been graded into four types according to the formation of the cells and their arrangement in the granuloma.[23] Poorly formed granulomas are immune suppressed granulomas. Immuno-compromising conditions

include deficiency of TNF-α, IFN-γ and IL-12.[6]

HIV increases the risk for TB in several ways:[5]

- HIV replication is increased at site of mycobacterial infection
- HIV produces primary or reactivation of TB by killing CD4 T-cells within the granuloma. HIV positive patients have fewer CD4 T-cells
- HIV manipulation of macrophage function prevents killing of the bacilli.
- HIV co-infected macrophages release less TNF-α than TB-only infected cells and also induce less TNF-α-dependent apoptosis.
- HIV induces changes in mycobacterial specific T-cells that decrease their ability to contain the mycobacteria

When patients with HIV are treated with anti-retroviral drugs (ARVs), the plasma viral load is reduced and the CD4 T-cell count is restored. Patients on ARV's are still more susceptible to TB than HIV-negative patients. This is dependent on the number of CD4 T-cells.

Patients with TB spine and co-infected with HIV have a risk of developing the Immune Reconstitution Inflammatory Syndrome (IRIS). It is a paradoxical worsening of the TB after initiating ARV's. There are two types:

(i) Paradoxical TB-associated IRIS

The patient is on TB treatment and is then started on ARV's

(ii) Unmasking TB-associated IRIS

Patient is not on TB treatment, not known to have TB, and then starts ARV's.

Both forms of IRIS result from rapid recovery of mycobacterial immune responses, resulting in inflammatory responses to *M. tuberculosis* antigens.[24]

3.5 Host-directed therapy

The major problem in the treatment of TB is that the duration of treatment is long, leading to poor compliance.

Host-directed therapy (HDTs) is a new and emerging concept where in the treatment of TB, the host response is modulated by various treatments. It is promising to identify effective adjuvants for the treatment of TB. HDTs have gained considerable interest as they target the host immune mechanisms. [25] HDT candidates would include modulators of pathologic inflammation and drugs for maintenance of homeostasis in the cells.[26, 27]

Some of the HDTs include:

- Vitamin D: It inhibits proliferation of *M. tuberculosis* inside macrophages through stimulation of innate immune responses during the infection. It also helps in the differentiation of naïve T cells to regulatory T cells. It can therefore be given in the treatment of TB.[28,29,30]
- Non- steroidal anti-inflammatories: The inflammatory response in active TB leads to active destruction of tissue during the stage of the liquifying caseum. Non-steroidal anti-inflammatories, like diclofenac can be used to control the inflammation, and hence reduce tissue damage.

- TNF-α: TNF-α plays a key role in the formation and maintenance of the integrity of the granuloma. Inhibition of TNF-α through inhibitor drugs may be helpful in controlling the disease.[29 ,31, 25]

3.6 Motivation for the study

HIV co-infection of patients with TB of the spine makes their treatment more complicated. There is therefore a need to investigate the immune response caused by HIV on the TB granuloma, especially the type of granuloma and cytokine expression. We decided to investigate this immune response at the site of the disease as the cytokine response at the site of infection does not necessarily mimic the response in plasma. This is possibly due to redistribution of antigen specific cells to the site of disease, a phenomenon referred to as immune compartmentalization.[32]

3.7 Aim

The aim of this part of the study is to investigate the histological appearance and cytokine expression of TB of the spine granulomas in HIV-negative and HIV-positive patients. This may then explain the difference in the distribution of the disease in HIV-negative and HIV-positive patients as seen in the previous chapter.

3.8 Objectives

1.To investigate the relationship between the HIV status and the histologic type of the granuloma.

2. To investigate the relationship between the grading of the granuloma and cytokine

expression of the granuloma.

3. To investigate the relationship between cytokine expression and the HIV status of patient.

4. To investigate the relationship between the expression of TNF-α and the extent of pus formation or bone destruction.

3.9 Material

Sixty-one patients had surgery for tuberculosis of the spine. Tissue was taken for microscopy and culture, GeneXpert analysis, histology and immunohistochemistry.

3.9.1 Exclusions

We excluded from the various laboratory investigations all specimens:

1. that were compromised.
2. that did not reach the specific laboratory on time.
3. that were insufficient for a particular test.

3.10 Method

In the patient's clinical data, we looked for the duration of anti-viral and of anti-TB treatment, and viral load at time of surgery.

Tissue samples collected during surgery were sent to the National Health Laboratory Services.

3.10.1 Collection and processing of tissue for histology

Tissue for histology was fixed in formalin (41% formaldehyde and 0.9% NaCl, 1:8 v/v) and transported to the lab within one hour of collection. Samples were embedded in

paraffin wax, sectioned to 3-5 microns slices, stained in haematoxylin and eosin and Ziehl-Neelsen stain. These sections were then examined under light microscopy. The pathologist was blinded to the HIV status of the patient.

The granulomas were classified into four histologic grades based on the arrangement and types of cells found at microscopy, and necrosis.

Grade 1: lymphohistiocytic aggregates

no epithelioid cells

no necrosis

no Langhan's giant cells

Grade 2: lymphohistiocytic aggregates

with single lying epithelioid cells

no necrosis

no Langhan's giant cell

Grade 3: Clusters of epithelioid cells

no necrosis

no Langhan's giant cells

Grade 4: Clusters of epithelioid cells

either necrosis

or Langhan's giant cells

3.10.2 Processing of the specimen for immunohistochemistry

The parrafin-embeded blocks were sent to the laboratory of Dr Hlumani Ndlovu at the

University of Cape Town to detect the expression of the cytokine TNF-α in the tissues, using a standard immunohistochemistry protocol and confocal microscopy.

Briefly, the blocks were cut into 5µm sections and baked overnight in an oven. The sections were deparaffinized by dipping them twice in xylene for 5 -minutes. This was followed by dipping in 100% ethanol and 95% ethanol for 2 minutes twice. The sections were rehydrated by dipping in tap water for 1 minute before boiling in a pressure cooker for 2 minutes in antigen retrieval buffer (2.4 g Tris Base, 0.24g EDTA, 0.05ml Tween 20, pH 9.0).

The pressure cooker was then placed in a sink with running water to allow it to cool until pressure had gone and valves opened. The slides were then taken out and allowed to cool for 30 min. They were then rinsed once in 1× phosphate-buffered saline (PBS).

The slides were then covered with 100µL of blocking buffer (2% BSA in 1× PBS), and left in a humidified container for 1 hour.

The blocking buffer was flicked off and a primary antibody cocktail (1:50 dilution of mouse anti-human TNF antibody, Abcam) was added, and incubated overnight at 4°C. The primary antibody cocktail was flicked off and the sections were washed 3 - 4 times with PBS. A secondary antibody cocktail (1:1000 dilution of goat anti-mouse Cy3) was added and the slides were incubated in a humidified chamber for 1 hour at room temperature. The secondary antibody cocktail was flicked off and the slides were washed 3 - 4 times with PBS. A drop of mounting fluid containing DAPI (Prolong Gold with Antifade, Sigma-Aldrich) was added, and a cover slip was gently set down to prevent bubbles from forming. The images were kept in the dark until they were examined on a Carl Zeiss 880 LSM Confocal microscope with Fast Airyscan technology.

Image analysis:

The confocal images were uploaded on Image J software and mean fluorescence intensity was quantified to estimate the expression of TNF-α. Consensus between three observers who are experienced in in examining microscopy images was also used in estimating the TNF-α expression. The images were given a score ranging from 0 – 3; where zero denotes no background, 1 denotes signal in one cell, 2 denotes signal in one or more cells, and 3 denotes signal in many cells with clear outlines.

3.10.1 Statistical methods

We used descriptive statistics which presented means, standard deviations, standard errors, proportions and associated 95 percent confidence interval. Chi-square test was used to evaluate the degree of association between factors categories. Furthermore, 2 sample t- test was used to compare proportions. A non-parametric equivalent for two-sample Wilcoxon (Mann-Whitney) rank-sum test was used in the comparison of the HIV-negative and HIV-positive groups to achieve the objectives. All tests were performed at an alpha level of 0.05. Stata 16 was the tool used for analysis

3.11 Results

The forty-five patients who had tissue taken for histochemistry, had been on anti-TB treatment for an average of 1.5 months for the HIV-negative and 1.8 months for the HIV -positive participants. They had been on anti-viral medication for a period of 0-120 months (average=33.6 months). The viral load at the time of surgery was not detectable in 70% of the patients (23 patients), less than 20 viral copies/ml of blood in four patients, less

than 100 copies/ml blood in one patient, and in four patients it ranged from 660- 113324 copies/ml of blood.

Fifty-eight specimens were received at the pathology laboratory. Three specimens could not be examined for histology as the tissue was compromised. Fifty-five specimens were therefore entered into the study. Seventeen were from HIV-negative patients and 38 from HIV-positive patients.

The granulomas were graded from 1-4, according to the cellularity and necrosis of the granulomas. Figures 3.1 to 3.4 are examples of the granuloma grading

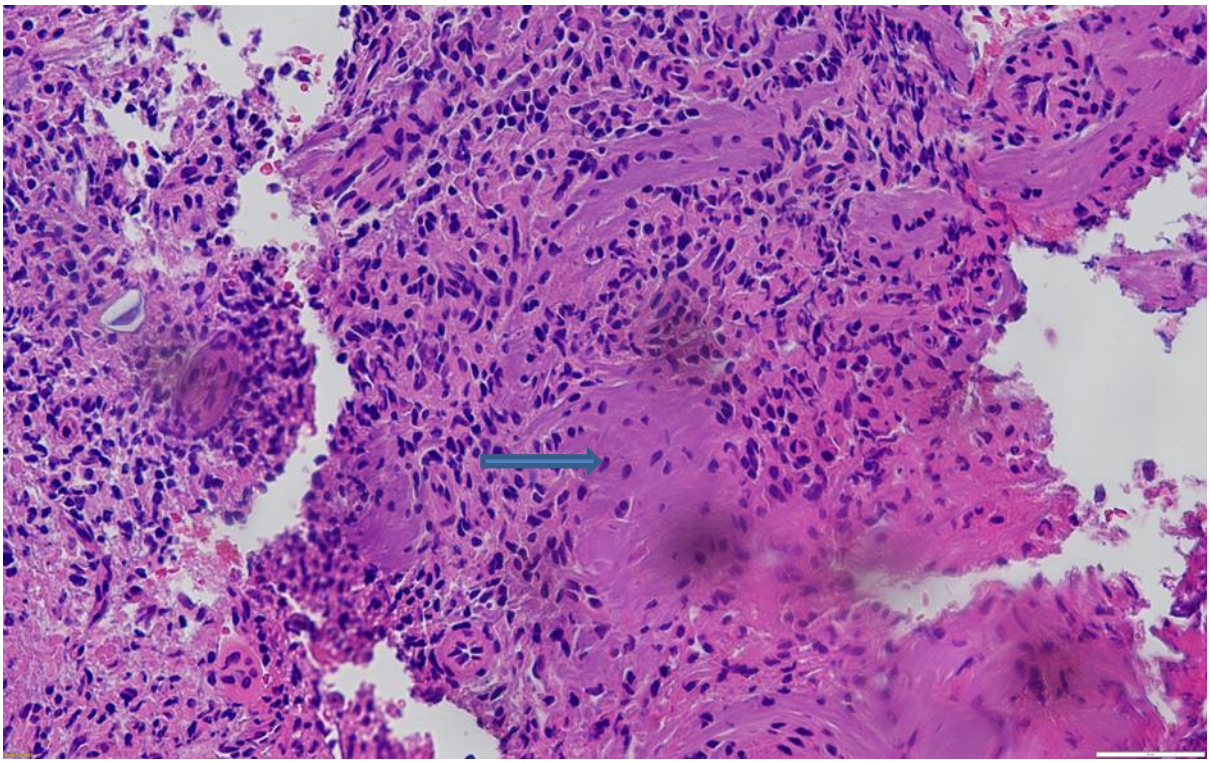


Figure 3.1: A photomicrograph of grade 1 granuloma, x 20 magnification. Note a loose aggregate of macrophages with a single lying epithelioid cells (blue arrow).

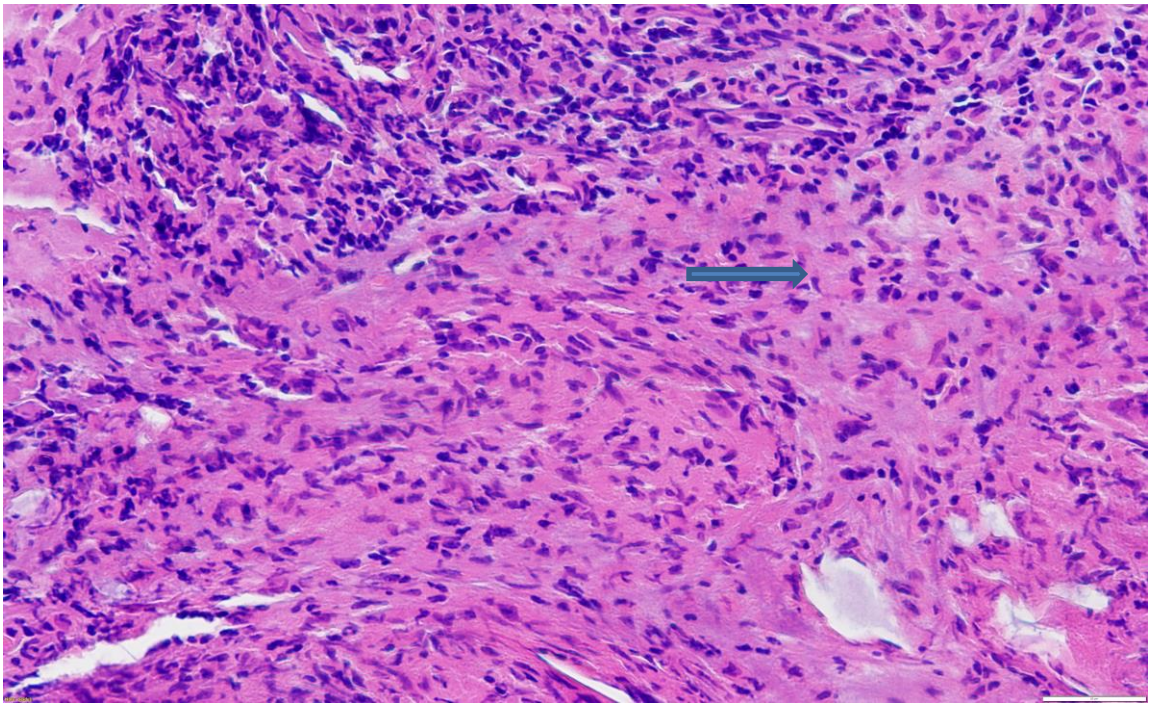


Figure 3.2: A photomicrograph of grade 2 granuloma, x 20 magnification. Note a zonal distribution of central epithelioid cells with a peripheral rim of macrophages (blue arrow). There are no giant cells or central necrosis present yet.

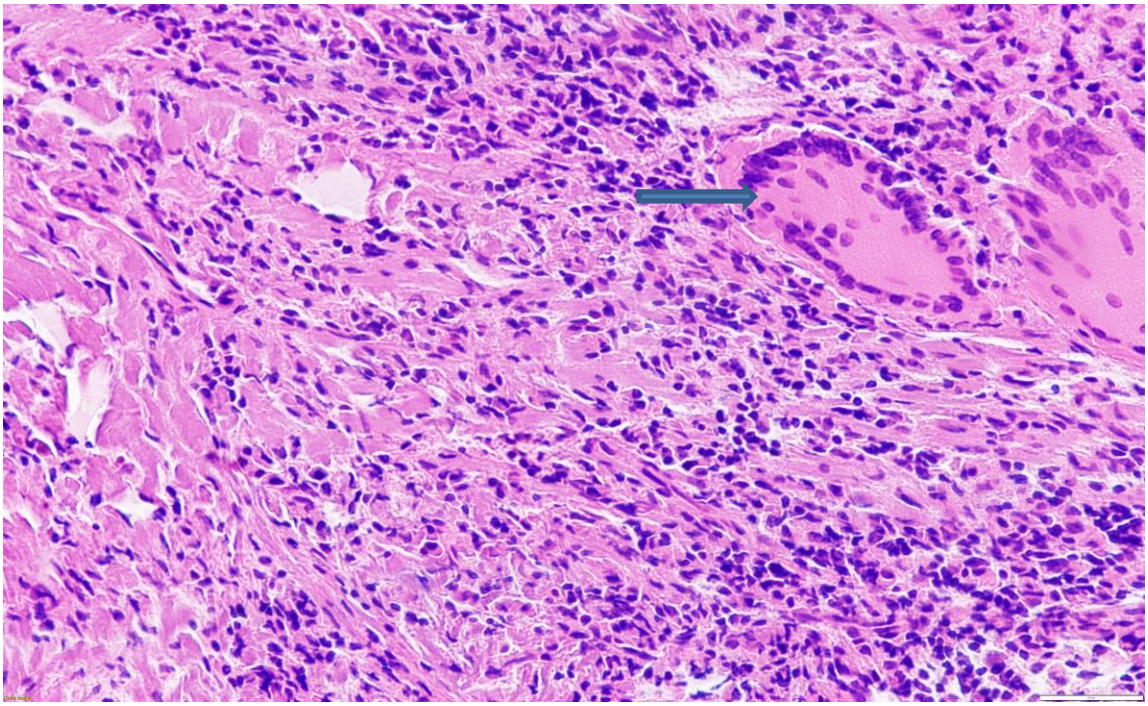


Figure3.3: A photomicrograph of grade 3 granuloma, x 20 magnification. Note a tight aggregate of macrophages with a many epithelioid cells, as well as Langhan's type giant cells (blue arrow)

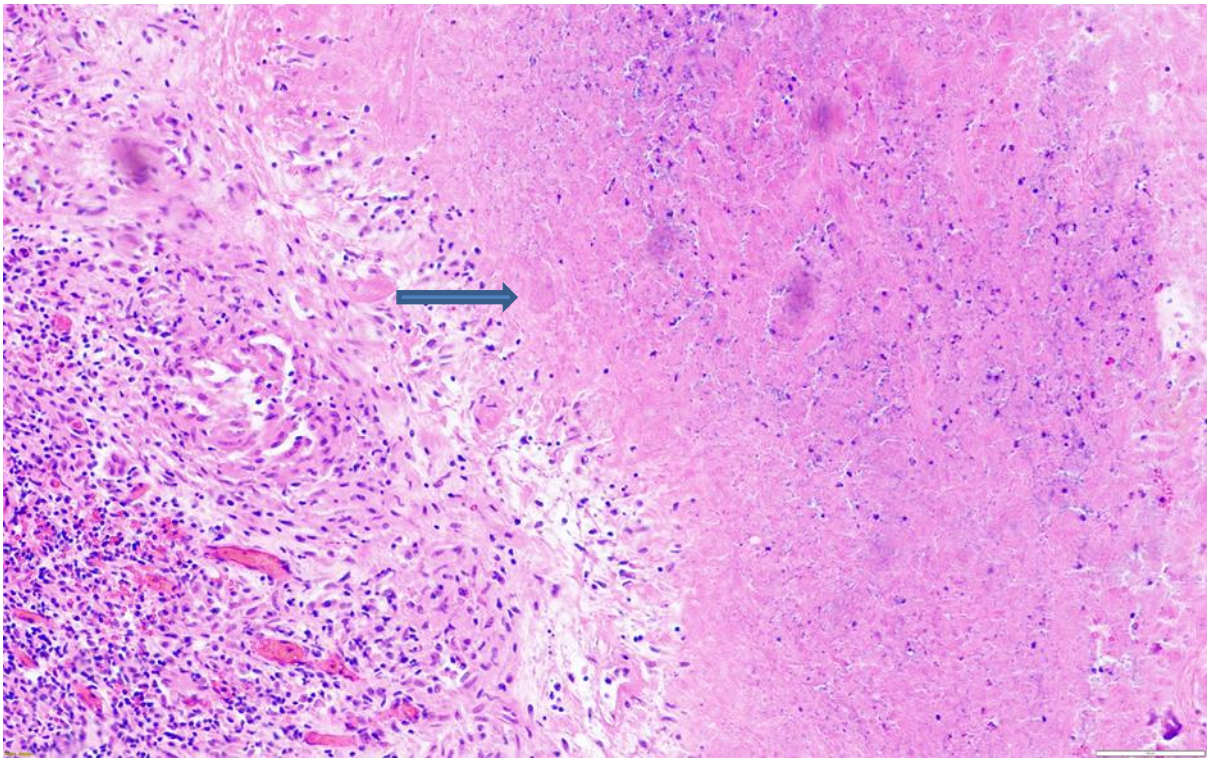


Figure3.4: A photomicrograph of grade 4 granuloma, x 20 magnification. Note an extensive central zone of necrosis with a palisade of epithelioid cells (blue arrow). The peripheral rim of macrophages is thinned out as more and more macrophages are transformed in to epithelioid cells.

The table 3.i summarizes the grading of the granulomas and distribution according to the HIV status of the patient. There were no grade 2 granulomas in the HIV-negative group.

Table 3.i: Distribution of grading of granulomas and HIV status of the patient

Grading of Granuloma	HIV -Neg	HIV -Pos	Total
1	9	6	15
2	0	10	10
3	2	7	9
4	6	15	21

In table 3.ii the granuloma grades 1 and 2 have been grouped together, and grades 3 and 4 also grouped together. There are 25 (45.5%) granulomas in grades 1 & 2 and 30 (54.56%) in grades 3 & 4. There were more patients belonging to the HIV+ group in both grades of granulomas, with 16 (64%) in grades 1 & 2 and 22 (73%) in grades 3 & 4.

Table 3.ii: Relation between the HIV status of the patient and the type of granulomas

Gran. grading	HIV -Neg	HIV -Pos	Total
Grades 1&2(n)	9	16	25
Row %	36.00	64.00	100.00
Column %	52.94	42.11	45.45
Grades 3&4(n)	8	22	30
Row %	26.67	73.33	100.00
Column %	47.06	57.89	54.55

The table of proportions, table 3.iii shows no statistically significant difference in the distribution of the two groups of granulomas between HIV-negative and -positive patients – the Mann-Whitney test shows a p -value of 0.64

Table 3.iii: Proportions between the granuloma groups and HIV status

	Proportion	Std. Err.	Logit [95% Conf.	Interval]
_Grading @ group HIV				
Grades 1 & 2 HIV -Neg	.5294118	.1210578	.2980893	.7487542
Grades 1 & 2 HIV -Pos	.4210526	.0800933	.2734506	.5842581
Grades 3 & 4 HIV -Neg	.4705882	.1210578	.2512458	.7019107
Grades 3 & 4 HIV -Pos	.5789474	.0800933	.4157419	.7265494

p= 0.6494

The Ziehl-Neelsen stain for acid-fast bacilli was done in all 58 specimens that reached the lab. The Z-N test was positive in 9 (15.52%) of the specimens.

Table 3.iv shows the results of the Ziehl-Neelsen stain.

Table 3.iv: Ziehl -Neelsen staining of the granuloma

Z-N stain (Neg/Pos)	Grading of Granuloma 1-4					
	1	2	3	4	No granuloma	Total
Neg (n)	14	10	6	17	2	49
	28.57	20.41	12.24	34.69	4.08	100.00
	93.33	100.00	66.67	80.95	66.67	84.48
Pos (n)	1	0	3	4	1	9
	11.11	0.00	33.33	44.44	11.11	100.00
	6.67	0.00	33.33	19.05	33.33	15.52
Total	15	10	9	21	3	58
	25.86	17.24	15.52	36.21	5.17	100.00
	100.00	100.00	100.00	100.00	100.00	100.00

Table 3.v relates the Z-N staining of the granulomas to their histologic groupings. It shows that there were 7 granulomas in grades 3&4 that were positive for acid fast bacilli and only one granuloma was positive for acid fast bacilli in grades 1&2. This was statistically significant, with the 1-sided Fischer's exact test showing a p-value of 0.047.

Table 3.v: Comparison between the two groups of granulomas and Z-N stain for acid fast bacilli

	Z- N stain (Neg/Pos)		
Cat_grading	Neg	Pos	Total
Grades 1 & 2	24	1	25
	96.00	4.00	100.00
	51.06	12.50	45.45
Grades 3 & 4	23	7	30
	76.67	23.33	100.00
	48.94	87.50	54.55
Total	47	8	55
	85.45	14.55	100.00
	100.00	100.00	100.00

1-sided Fisher's exact = 0.047

Forty-five specimens were received and processed at the immunology laboratory. Tables 3.vi and 3.vii show the relationship between TNF-α expression and the HIV status using the consensus method and the MFI method respectively. There is no statistically significant relationship between TNF-α expression and HIV status as reflected by the p-value of 0.67 in table 3.vi and 0.97 in table 3.vii.

Table 3.vi. Relationship between TNF-α (consensus method) and HIV status

TNF(using consensus between observers)	Group/HIV+/- Neg	Pos	Total
No TNF	0	1	1
	0.00	100.00	100.00
	0.00	3.03	2.22
Hazy background	5	9	14
	35.71	64.29	100.00
	41.67	27.27	31.11
TNF in Cell/incomplet	3	6	9
	33.33	66.67	100.00
	25.00	18.18	20.00
Well formed cell with	4	17	21
	19.05	80.95	100.00
	33.33	51.52	46.67
Total	12	33	45
	26.67	73.33	100.00
	100.00	100.00	100.00

Fisher's exact = 0.674

Table 3.vii. Relationship between TNF-α (MFI values) and HIV status

	Grp	N	Mean	S.Err	95% C.I		pr	
TNF (MFI)	HIV -ve	12	42.74	4.9	31.9	53.5	0.97	NS
	HIV +ve	33	42.54	2.16	38.1	46.9		

On examining the expression of the cytokine TNF-α in relation to the grading of the granulomas, we find that the mean expression of TNF-α using mean fluorescence intensity is 39.79 in grade 1&2, which is marginally lower than the 43.89 in grades 3&4 granulomas. This his however not statistically significant, with a p-value =0.37. (Table 3.viii)

Table 3. viii. Relationship between granuloma grades and TNF-α

	Grp	N	Mean	S.Err	95% C.I		pr	
TNF (MFI)	Grade1&2	18	39.79	3.37	32.67	46.91	0.37	NS
	Grade3&4	25	43.89	2.06	39.15	47.63		

In Fig 3.5 we find that the grades 3&4 granulomas are scattered throughout the MFI range, whereas grades 1&2 granulomas are mainly localized in the lower end of the MFI scale. This we interpret as showing less TNF-α in the group 1&2 granulomas.

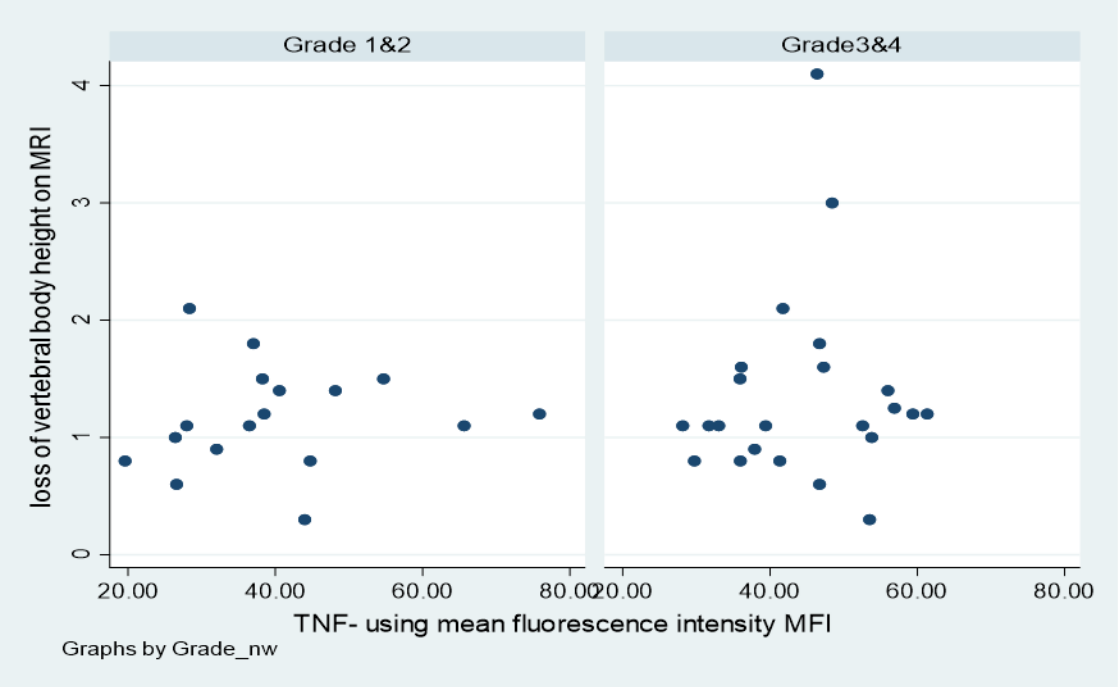


Fig. 3.5: Grade 1&2 granulomas and 3&4 granulomas in relation to TNF-α expression

Fig 3.6 is a combined scatter graph of the two groups of granulomas, again showing that the groups 1&2 are scattered more on the lower end of the mean fluorescence intensity scale.

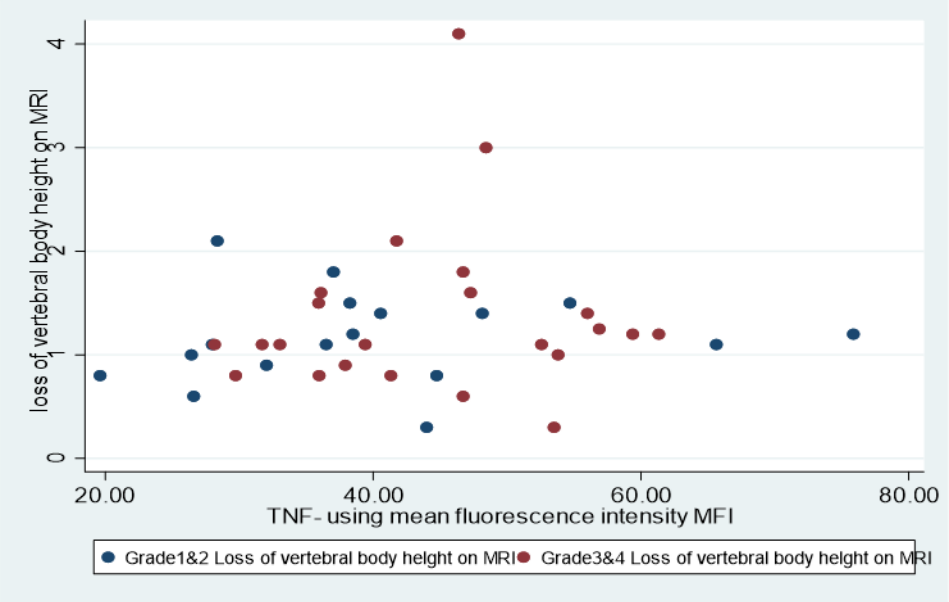


Fig. 3.6: Graphs of granulomas 1&2 (blue dots) and granulomas 3&4 (red dots) in relation to TNF-α

We went on to compare the severity of the disease as expressed by the CD4 count, with the grading of the granuloma and TNF-α expression. Table 3.vix below shows the grading for the severity of the disease.

Table 3.vix: Classification of the HIV-positive patients according to CD4 count

CD4 catergory	Frequency	Percent
Below 200	9	21.43
201 - 300	12	28.57
301 - 400	7	16.67
401 - 500	4	9.52
Over 500	10	23.81
Total	42	100.00

The table shows that 21% (9) of the patients had severe disease, with CD4 count less than 200 cells/µl, and a further 28.6% (12) with a CD4 count of 200-300 cells/µl. .

The CD4 count was compared with the grading of the granuloma as seen in table 3.x.

Table 3.x: CD4 count compared with grading of the granuloma.

Grading of Granuloma 1-4	CD4_category: Below 200	201 - 300	301 - 400	401 - 500	Over 500	Total
1	1	3	0	1	1	6
Row %	16.67	50.00	0.00	16.67	16.67	100.00
Column %	11.11	25.00	0.00	25.00	14.29	16.22
cell %	2.70	8.11	0.00	2.70	2.70	16.22
2	2	2	2	1	3	10
Row %	20.00	20.00	20.00	10.00	30.00	100.00
Column %	22.22	16.67	40.00	25.00	42.86	27.03
Cell %	5.41	5.41	5.41	2.70	8.11	27.03
3	3	0	1	1	1	6
Row %	50.00	0.00	16.67	16.67	16.67	100.00
Column %	33.33	0.00	20.00	25.00	14.29	16.22
Cell %	8.11	0.00	2.70	2.70	2.70	16.22
4	3	7	2	1	2	15
Row %	20.00	46.67	13.33	6.67	13.33	100.00
Column %	33.33	58.33	40.00	25.00	28.57	40.54
Cell %	8.11	18.92	5.41	2.70	5.41	40.54
Total	9	12	5	4	7	37
Row %	24.32	32.43	13.51	10.81	18.92	100.00
Column %	100.00	100.00	100.00	100.00	100.00	100.00
Cell %	24.32	32.43	13.51	10.81	18.92	100.00

Fisher's exact = 0.687

The table shows that 24.3% (9) of the specimens that were examined had a CD4 count of <200 cells/µl. Six of these (66.6%) had grade 3&4 granulomas. A further 32.43% (12) had a CD4 count of 200-300 cells/µl, and 58.3% (7) of them had grade3&4 granulomas. There was no significant difference between the grading of the granuloma and the CD4 cell count (p= 0.687).

The results of the relationship between CD4 count and TNF-α, using both observer consensus method (Table 3.xi) and Mean Fluorescence Intensity (Figure 3.7) are shown below.

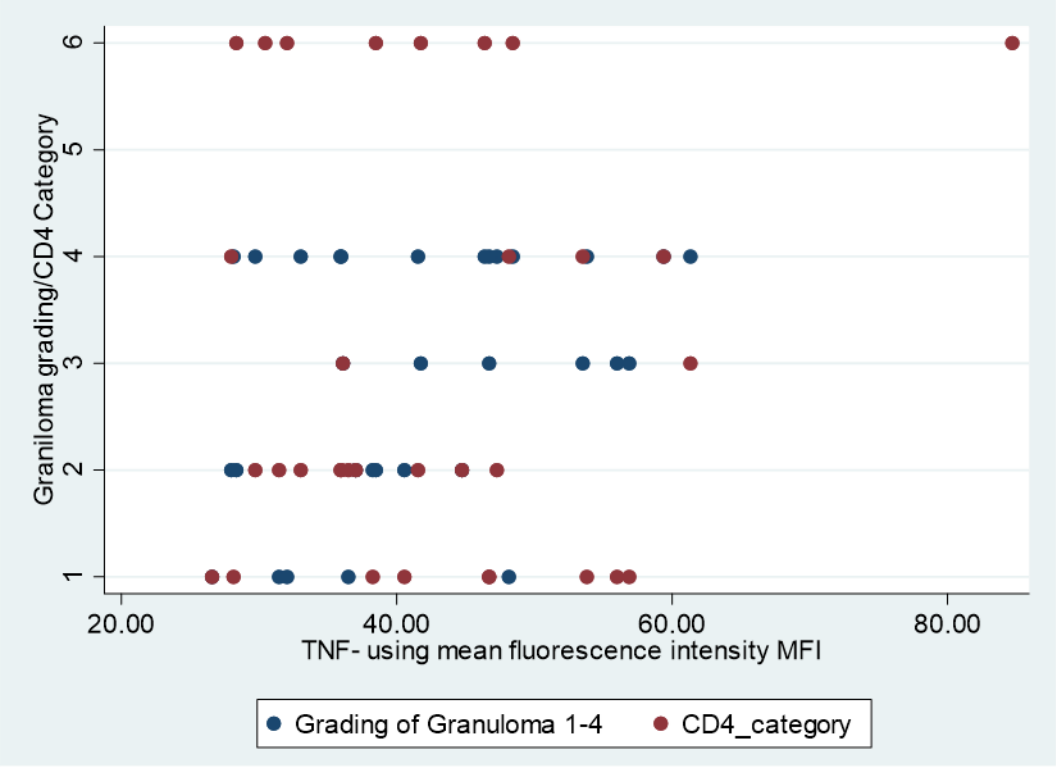
Table 3.xi: CD4 count compared with TNF-αexpression (consensus between observers)

TNF(using consensus between observers)	CD4_category					Total
	Below 200	201 - 300	301 - 400	401 - 500	Over 500	
0	0	1	0	0	0	1
Row %	0.00	100.00	0.00	0.00	0.00	100.00
Column %	0.00	10.00	0.00	0.00	0.00	3.03
Cell %	0.00	3.03	0.00	0.00	0.00	3.03
1	2	3	1	0	3	9
Row %	22.22	33.33	11.11	0.00	33.33	100.00
Column %	22.22	30.00	50.00	0.00	37.50	27.27
Cell %	6.06	9.09	3.03	0.00	9.09	27.27
2	1	1	0	1	3	6
Row %	16.67	16.67	0.00	16.67	50.00	100.00
Column %	11.11	10.00	0.00	25.00	37.50	18.18
Cell %	3.03	3.03	0.00	3.03	9.09	18.18
3	6	5	1	3	2	17
Row %	35.29	29.41	5.88	17.65	11.76	100.00
Column %	66.67	50.00	50.00	75.00	25.00	51.52
Cell %	18.18	15.15	3.03	9.09	6.06	51.52
Total	9	10	2	4	8	33
Row %	27.27	30.30	6.06	12.12	24.24	100.00
Column %	100.00	100.00	100.00	100.00	100.00	100.00
Cell %	27.27	30.30	6.06	12.12	24.24	100.00

Fisher's exact = 0.687

In this table 3.xi, 27.3% (9) of the specimens examined had CD4 count below 200 cells/µl. There was consensus between the observers that 6 of the specimens (66.6%) had a strong signal for TNF-α, recorded as grade 3 in the table. There was no difference

between CD4 count and TNF-α using this method, with p= 0.687.



p=0.039

Fig 3.7: CD4 compared with TNF-α (mean fluorescent intensity)

The graph in fig 3,7 also showed no difference between TNF-α expression using mean fluorescence intensity and CD4 count (p=0. 039)

3.12 Discussion

The granuloma is a confluence of macrophages that attempt to contain the invading mycobacteria. In response to the infection, the macrophages transform into a number of immune cells, like epitheloid cells and foamy cells and also fuse to form giant cells. HIV infection affects the cellular components of the granuloma in a way that negatively affects the ability of the granuloma to contain and kill the mycobacteria.

HIV and granuloma

In our study we found no difference in the cellular composition of granulomas as measured by the grading of the granulomas in table 3.ii and table 3.iii. There is no difference between the poorly-formed granuloma grades 1&2 and well-formed granulomas grades 3&4 in their distribution between HIV-negative and -positive granulomas (p= 0.64). This finding is probably because the HIV-positive patients had been on treatment for HIV, for an average of 33.6 months at the time of surgery. The treatment had been so effective that in 70% (23 patients) of them, the viral load was undetectable. In a further four (12%) it was less than 20 viral copies/ ml blood.

These findings are like those in the study by Danaviah et al, also performed on TB spine tissue. They found that macrophage differentiation was similar in HIV-negative and -positive patients and that the granuloma architecture was the same in both groups of patients.[5]

However, Bezuidenhout [9] and De Norhona[21] found that there was more necrosis in HIV -positive granulomas and that these granulomas were poorly formed.

The cellular composition of the HIV granuloma is complex. Diedrich, in a systematic review that involved nineteen studies with a sample size totaling 899, concluded that there was a large heterogeneity in the cellular content of the HIV granuloma. It was difficult to ascribe any pattern to HIV co-infection.[20]

Bacterial concentration in the granulomas

The Z-N stain in this study was positive in 15.5% of the patients, confirming the paucibacillary nature of TB in the spine. In further analyzing this result, 87% of the HIV

positive granulomas belonged to groups 3&4, and this was statistically significant. This means that there are more bacilli found in the well- formed granulomas. We expect this to be so as the bacilli are in their resting phase inside these granulomas.

These findings are different to those reported by other authors, but these were on lymph node aspirates.[33] They attributed this to the fact that well formed granulomas have the capsule of the layers of cells intact and the bacilli are thus not readily stained by the Z-N stain. However, the Z-N is able to stain bacilli irrespective of where they are on the pathology section.

Diedrich also reported a bigger bacillary load in HIV-positive granulomas.[20]

TNF and HIV status and granuloma

In our study we found no difference in the expression of TNF-α between HIV-positive and -negative patients. This is different from the study of a much smaller series of patients by Bezuidenhout. They studied a total of 12 patients with equal numbers of HIV-positive and -negative patients. They found that the HIV-positive granulomas have a higher expression of TNF-α.

However, De Norhona found that TNF-α expression was suppressed in HIV-positive granulomas. Both studies were performed in pulmonary tissue. These findings are again in keeping with the findings of heterogeneity between the various studies on the composition of TB granulomas. Diedrich[20] blames this heterogeneity of results on poor or no standardization of the methods used for measuring the expression of TNF-α in the granulomas.

Although this study shows no statistically significant difference between the TB granuloma grading and their expression of TNF-α, figures 3.5 and 3.6 show that the grade 1&2 granulomas were scattered on the lower end of the mean fluorescence intensity range. This suggests that the poorly formed granulomas have less TNF-α.

One of the functions of TNF-α is to stimulate resting macrophages and prevent dissemination of the disease. TNF-α plays a key role in the maintenance of granuloma.[15,25] In the context of host directed therapies, the findings of less TNF-α in poorly formed granulomas needs to be investigated further, perhaps with a study with a larger sample size. This is important as the current host directed therapy uses systemic anti TNF-α medication, with one of its side effects being reactivation of TB.[31] It is worth looking at TNF-α treatment directed locally to the site of infection, as extrapulmonary TB, like TB of the spine, tends to be localized at the site of infection.

Severity of HIV infection (CD4 count) and granuloma grading and cytokine expression

The results also show that there is no relation between the severity of the disease and the immune response of the granulomas. It could be that if one were to use some of the other cytokines involved in the TB immune process, one could find some difference. Alternatively, these results could be an indication of how well the HIV status was controlled in this cohort.

3.12.1 Limitations of the study

The patients had been on HIV treatment for an average of 33 months at the time of surgery and the viral load was not detectable in 70% of the patients. This may affect the

cytokine expression and distribution of immune cells in the granulomas of these patients.

The sample size ended up being smaller than calculated, because of some specimens being rendered unsuitable in the various laboratories.

3.13 Conclusion

We have not been able to define any specific granuloma pattern that is specific for HIV-positive and -negative patients.

There is no difference in TNF-α expression between HIV-positive and HIV-negative granulomas.

There is a suggestion that the poorly formed granulomas have marginally less TNF-α expression than the well-formed granulomas. This may have a bearing on host directed therapies.

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CHAPTER 4

PATHOGEN-RELATED FACTORS IN THE SPREAD OF THE DISEASE TO THE SPINE – THE LABORATORY DIAGNOSIS OF TB SPINE AND THE ROLE OF WHOLE GENOME SEQUENCING IN DIAGNOSIS AND IDENTIFICATION OF MUTATIONS THAT MAY BE RESPONSIBLE FOR THE BACILLI SETTLING IN THE SPINE

4.1 Introduction

In this chapter, we look at the pathogen related factors that may be responsible for the spread of the disease from the lungs to the spine. The *M. tuberculosis* bacilli seem to be in a very friendly environment in the lungs. They seem to thrive very well as evidenced by the abundance of bacilli in the sputum at microscopy under the Ziehl-Neelsen stain. There must be something that makes them move from the lungs and survive in the hostile hypoxic environment of the spine. By doing whole genome sequencing (WGS) on the bacilli that we culture, we aim to investigate the pathogen-related factors that lead to the bacilli settling in the spine. In the process, we will also investigate the role of WGS in the diagnosis and treatment of TB of the spine.

4.2 Literature review

Laboratory diagnosis of TB is complex, with various stages aimed at isolating the organism and testing for its resistance profile to commonly used drugs.

When a specimen gets to the laboratory, smear microscopy under Ziehl-Neelsen stain to look for acid-fast bacilli is performed. The method identifies most infectious patients. The results are quick, available in 24hrs, but the method has a low sensitivity and cannot differentiate between *M. tuberculosis* and non-tuberculosis mycobacteria. It is particularly not very useful in TB of the spine as the lesions in this disease are paucibacillary.

The specimen is also sent for culture. Culture is done in the Lowenstein-Jensen solid medium Culture can also be done in a tube that contains mycobacteria-selective culture liquid medium that is coupled to an automated instrument to read the results, the mycobacterial growth indicator tubes (MGIT's). The culture is incubated for a maximum of 6 weeks, and if the mycobacteria are present, they will be cultured in that 6-week period. Once growth is flagged in liquid culture, a DNA line probe assay is used to confirm the presence of mycobacteria, as well as to determine the susceptibilities to anti-tuberculous drugs should the organism be confirmed to be in the MTB complex. The line probe assay has probes for susceptibility testing to first- and second- line anti-tuberculous drugs. From liquid culture, phenotypic drug susceptibility testing can also be done for drugs that are not represented on the line probe assay eg. bedaquiline , linezolid etc.[1]

The drawback of this process of phenotypic drug resistance testing is that it takes a number of weeks, because the specimen has to be cultured first.

Done simultaneously with this workflow is the nucleic acid gene amplification test. This GeneXpert test detects the presence of *M. tuberculosis* DNA and mutations in the *rpoB* gene that predicts resistance to rifampicin. The results in our hospital are available within 24 hrs but can be available in under 2 hrs. It has a 98% specificity for detecting *M. tuberculosis* but sensitivity varies depending on the site and type of sample.[2, 3] It is the

fastest and most useful diagnostic tool in our environment. It has replaced AFB microscopy in most protocols for TB diagnosis.

Commercial genotyping assays, like Xpert® MTB/Rif Ultra are faster and useful for diagnosis of TB. However, these can only screen a small number of genetic loci that are associated with drug resistance.

What GeneXpert has introduced is a shift away from the phenotypic testing methods to genetic testing of *M. tuberculosis*. It has introduced a need for more aggressive and comprehensive interrogation of the *M. tuberculosis* genome beyond the *rpoB* gene, to the complete genome of the *M. tuberculosis*.

Whole genome sequencing is a further development in the diagnosis and antimicrobial susceptibility testing of TB.[4] It is the process of determining the complete DNA sequence of the genome of an organism at a single time. This entails sequencing all the organism's DNA and DNA contained in the mitochondria. Whole genome sequencing enables the screening of resistance-associated loci and can identify other loci as predictors of resistance. Applied to clinical practice, WGS would give complete results more rapidly than the currently available methods

WGS still requires that the bacteria be cultured in the normal method used in phenotypic drug resistance testing. Accordingly, WGS takes a long time to produce results. The culture ensures enough bacterial DNA as the method relies on sufficient good quality DNA to provide adequate depth of coverage for providing the genome. There is a move in clinical medicine to do WGS directly from the specimen, which is the sputum in

pulmonary tuberculosis. Sputum is however contaminated with genetic material from the host's resident microbial flora. This will complicate bioinformatics and depth of coverage of the results. The method is not yet generally used to diagnose TB in the clinical setting, but big strides are being made towards this, notably by the National Institute for Communicable Diseases in our country.[5] Targeted enrichment of the sputum is currently being done to bypass the culture step.[6, 7]

There is no doubt that WGS will eventually get into the clinical space, and all doctors who treat TB, including orthopaedic surgeons who treat TB of the spine will need to be familiar with it.

WGS starts with extraction and purification of genomic DNA.[8] DNA is fragmented into shorter pieces, which are then sequenced in 'reads' of 100-500 base pairs (bps)for bench top sequencers. The Illumina MiSeq (Illumina San Diego, CA, USA) is the sequencing platform mostly used. Based on these reads, a variety of tools can be used to identify the organism. One commonly used tool compares reads with existing microbial DNA databases. This is often the *M. tuberculosis* strain H37Rv. Using this reference-based approach, single nucleotide polymorphisms (SNP) which is a difference in a single base in the genome compared to the reference, and insertions or deletions (indels) present in the test isolate can be identified and compared with the reference strain.

This genome can be used to:[9]

- diagnose TB, especially differentiate TB from non-tuberculous mycobacteria.
- predict resistance, not only to rifampicin, but to all the first- line drugs and some second -line drugs.

- to characterize mutations, for subspecies and lineage identification and surveillance.

4.2.1 Drug susceptibility

Diagnosing drug resistance is a big problem in the control of tuberculosis.

Multi-drug resistant TB (MDR TB)is estimated to be found in 4.1% of all new TB patients in the world and 19% of previously treated TB patients.[10] This level of drug resistance is even higher in the poorer communities.[11] TB organism resistance is caused, among others, by inadequate drug treatment. One of the contributors to the inadequate drug treatment is the long treatment period in TB of the spine, especially in poor communities where the patients must travel long distances to the treatment sites. [12]

The resistance to treatment is a characteristic of the TB bacterium. It is a slow growing organism. It is an obligate intracellular organism and has a complex highly hydrophobic cell envelop acting as a barrier. Most of its resistance determinants are however encoded in its genome.[13] Walker et al used WGS to clinically predict drug resistance and to identify drug phenotypes that could not yet be identified as predictors of drug resistance.[9]

With targeted enrichment of the sputum, results of antibiotic susceptibility could be predicted within five days.[6]

4.2.2. Surveillance

WGS also shows the genetic heterogeneity of the strains. This is useful in determining the origin of the strain, which factor would be particularly useful in periods of outbreak

of disease.[14]Certain strains are prevalent in some countries, like lineage 1 (Indo-Oceanic) and lineage 3 (East-African-Indian) are prevalent in pulmonary TB in India.[15] In this study we wanted to know which strain is prevalent in TB of the spine in South Africa.

4.2.3 Gene polymorphism in TB.

It is known that the hosts genetic factors are important in controlling the infection and indeed the extent of spread of the disease. Li and Zhou found that among a Chinese population that had TB of the spine and pulmonary TB, there was a difference in the genes responsible for production of INF-y, a key cytokine in the host's defence against TB. [16] In this study we hypothesized that there is a gene polymorphism in the bacterial genome that is responsible for the bacteria to settle in the spine and cause TB of the spine. We hypothesized that there is a mutation that would make these bacteria to settle in the vertebral bone. We therefore decided to compare the genome of the strains causing TB of the spine to that of known pulmonary strains.

4.3 Material

Sixty-one patients were operated for TB of the spine because of progressive weakness of the legs or paraplegia, poor response to TB medication, deformity and radiologic features that were regarded as being diagnostic of TB of the spine.

At surgery tissue was taken for histology, microbiology, and whole genome sequencing.

4.4 Method

Tissue for histology (granuloma tissue and bone) was transported in a cooler bag at 4 degrees centigrade in formalin (41% formaldehyde and 0.9% NaCl) within one hour of collection. Samples were then embedded in paraffin wax, sectioned (3-5 micron), stained in Haematoxylin and Eosin and examined under light microscopy. The tissue was also stained with Ziehl-Neelsen stain (Z-N stain) for mycobacteria.

4.4.1 Histology

According to haematoxylin and eosin staining, the granulomas were classified into four categories, based on the arrangement and types of cells found at microscopy.

Grade 4 granulomas are well-formed, characterized by clusters of epithelioid cells, and caseous necrosis.

Grade 3 granulomas are also well-formed, but do not have caseous necrosis.

Grade 2 granulomas are poorly formed. They have a single lining of epithelioid cells and no caseous necrosis.

Grade 1 granulomas are also poorly formed granulomas, have no epithelioid cells but have foamy macrophages.

4.4.2 Microbiology

Specimen types that were sent for microbiology were pus and tissue. Samples were transported to the laboratory in sterile saline. Upon receipt in the lab, the specimens were divided into two equal aliquots. One aliquot was used to process on the Xpert MTB Rif Ultra, while the second specimen was submitted for culture. All specimens were

processed using the Xpert® MTB/Rif ultra (Cepheid, Sunnyvale, USA) assay or GeneXpert. All specimens were processed in a biological safety level 2 cabinet using appropriate personal protective equipment.

Tissue samples were cut into smaller pieces using a sterile scissor. Two millilitres of phosphate buffer were added to suspend the specimen. The sample was then ground using mortar and pestle. A volume of 0.7ml of the homogenised suspension was then transferred to a sterile tube. A volume of 1.4 ml of Sample Reagent (SR) that is provided with the Xpert® MTB/Rif ultra-kit was added to the tube making a volume of 2ml.The tube was vortexed for 10-20 seconds, repeated after 10 minutes of incubation at room temperature for 5 minutes. Using a sterile pipette, the contents of the tube were transferred into the Xpert® MTB/Rif ultra-cartridge. The cartridge was then loaded into the GeneXpert module following the manufacturer's instructions.

Pus specimens were transferred to a sterile tube and mixed with the Sample Reagent at a ratio of 1:2. Specimen volume of less than 0.1 ml were deemed insufficient. Two millimetres of the mixture were loaded into the GeneXpert module following the manufacturer's instructions.

In addition, all tissue specimens were cultured for *M. tuberculosis.* Specimens were incubated for a maximum of 42 days using liquid culture. Once culture was positive, the specimen was subjected to a Ziehl Neelsen stain to confirm the presence of cords on the stain. An MPT 64 antigen was also done on the specimen to confirm MTB complex. This was then followed by antimicrobial susceptibility testing using the Hain Line Probe Assay (Hain Life Sciences).

The Line Probe Assay (LPA) is a molecular genetic assay for identification of resistance of *Mycobacterium tuberculosis* complex to rifampicin and/or isoniazid. Phenotypic susceptibility testing was performed for ethambutol and pyrazinamide. Phenotypic isoniazid susceptibility testing was also done to confirm the isoniazid susceptibility result of the LPA. If resistance to rifampicin was detected, a second LPA (Hain MTBDRsl assay) was done. The isolate was tested for susceptibility to the second line drugs (fluoroquinolones and aminoglycosides). These specimens were also referred to a referral laboratory for phenotypic susceptibility testing to other second line agents such as bedaquiline and linezolid.

4.4.3 Whole genome sequencing

The genomic material from cultured isolates was sent to the National Institute for Communicable Diseases (NICD) for whole genome sequencing.

For the investigation of whole genome sequencing, care was taken to confirm purity of each isolate. Only a single isolate unique to a patient was included in this study. DNA was extracted using the Nuclisens easyMAG DNA extraction system (BioMérieux, Marcy-l'Étoile, France) from a concentrated 1 ml volume of positive culture MGIT medium. The DNA extracts were quantified using the Qubit 4 Fluorometer (Life Technologies, Carlsbad, California, United States). Libraries were prepared using the Illumina Nextera DNA Flex Library Preparation kit (Illumina, San Diego, California, United States). Libraries were sequenced on the Illumina NextSeq 550 sequencing system using the NextSeq 500/550 High Output Kit v2.5 (300 Cycles). Sequencing analysis was completed using CLC Genomics Workbench v11 (Qiagen, Venlo, the Netherlands) to detect any variants (single nucleotide polymorphism, insertions and deletions) against the *Mycobacterium*

tuberculosis reference genome (NC000962.3). Lineages were assigned using the SNP bar coding as described by Coll et al.[17] Maximum Likelihood trees were constructed using a SNP based approach as described previously.

4.5 Results

Smear microscopy on the Ziehl-Neelsen stain was positive in nine patients and negative in fifty. The test could not be done in three specimens as these were deemed not suitable due to delay in getting the specimen to the laboratory. The Z-N stain was thus positive in only 15.51% (9 of 58) of cases in whom the test was done. Table 4.i shows the results of the Ziehl-Neelsen stain.

Table 4.i: Results of Ziehl-Neelsen staining

Z- N stain |

(Neg/Pos)	Freq.	Percent
Neg	49	84.49
Pos	9	15.51
.	(3)	
Total	58	100.00

Microscopy under the H&E stain showed that there were 15 patients with group I granulomas, ten in Group 2, nine in group 3 and 21 in group 4. Six specimens could not be processed or graded because they were not suitable for processing (4), no inflammation (1) and no granules (1). The results show that the diagnosis of TB could be confirmed or strongly suspected in all the 55 cases on whom the test was done. The results are summarized in table 4.ii below.

Table 4.ii: Grading of the granulomas on microscopy

Granuloma Grading 1-4	Hiv Negat	Hiv Posit	Total
1	9	6	15
2	0	10	10
3	2	7	9
4	6	15	21
Tissue Compromised	1	5	6
Total	18	43	61

The Xpert® MTB/Rif was not done in 6 specimens (specimen insufficient 2, specimen rejected 2, test did not reach the microbiology work bench,2). The test was therefore done in 55 patients. The test was positive in 47 patients and negative in 8 patients. The test was thus positive in 85.45% of patients on whom it was done. In the 23 patients where the Xpert® MTB/Rif was done in both pus and necrotic tissue, the test was positive in both specimens.

Table 4.iii shows further analysis of the Xpert® MTB/Rif negative patients and those on whom the test was not done. This shows that the diagnosis of TB could be suspected based on a combination of histology, XR features and typical ring enhancement and pus formation on post contrast MRI scan. In only two patients these tests could not prove the diagnosis of TB conclusively. Both patients were HIV-positive, the XR appearance was atypical, there was no ring enhancement of the lesion on MRI, histology showed no inflammation or granules, and TB culture was negative. With the combination of XR, MRI,

Histology, Xpert® MTB/Rif and culture we could confidently diagnose TB of the spine in 96.7% of the patients (59 out of 61 patients) in this series.

Table 4.iii: Other diagnostic modalities on Xpert® MTB/Rif negative patients

Study No	HIV Status	Xpert® MTB/Rif	TB Culture	Z-N stain	Histology: Granuloma Grade	XR Typical / Atypical	MRI Pus formation mm^3
1	Pos	Rejected	Not Done	neg	2	Typical	3.2
12	Pos	Insufficient	Neg	neg	1	Typical	84
14	Pos	insufficient	Not Done	neg	Not suitable	Typical	1
15	Pos	Rejected	Neg	neg	2	Atypical	991
17	Neg	Not in lab	Neg	neg	1	Typical	6.4
22	Neg	Not in lab	Neg	neg	Not suitable	Typical	6.4
26	Pos	Neg	Pos	neg	4	Typical	No pus
40	Neg	Neg	Neg	neg	1	Typical	9.1
41	**Pos**	**Neg**	**Neg**	**neg**	**No Granules**	**Atypical**	**No pus**
45	Pos	Neg	Neg	neg	1	Atypical	68
50	Pos	Neg	Not done	POS	No inflamatn	Atypical	No pus
55	**Pos**	**Neg**	**Neg**	**Neg**	**2**	**Atypical**	**12.9**
56	Pos	Neg	Neg	neg	2	Typical	1.1
58	Pos	Neg	Neg	neg	3	Typical	40

With regards to drug resistance, four patients were Rifampicin resistant on Xpert® MTB/Rif.

TB culture was not done in 3 specimens. There was no growth after 42 days of incubation in 30 patients, that is, we could not culture the bacteria in 51.7% of patients. Culture was positive in 28 patients (48.3%). The time to culture was an average of 18.7 days (range 11-32 days).

Table 4.iv further analyses the phenotypic drug sensitivity of the specimens on whom bacteria were cultured. Four specimens were INH mono-resistant, while two were RIF

mono-resistant, but were sensitive to fluoroquinolones and aminoglycosides. (2nd line drugs). All the four that were mono-resistant to INH on culture, were sensitive to Rif on Xpert® MTB/Rif and the two that were Rif mono-resistant on culture were also Rif resistant on Xpert® MTB/Rif. There were no cases of MDR TB or XDR TB.

Table 4.iv: Drug sensitivity on the cultured specimens

Study No	GeneExpt Xpert® MTB/Rif	Culture (Line Probe Assay)
1	Rif Sens	INH Mono-res
6	Rif Res	Rif Mono-res (Sens to FQ + AG)
28	Rif Sens	INH Mono-res
33	Rif Sens	INH Mono-res
54	Rif Res	INH Mono-res
61	Rif Res	Rif Mono-res (Sens to FQ + AG)

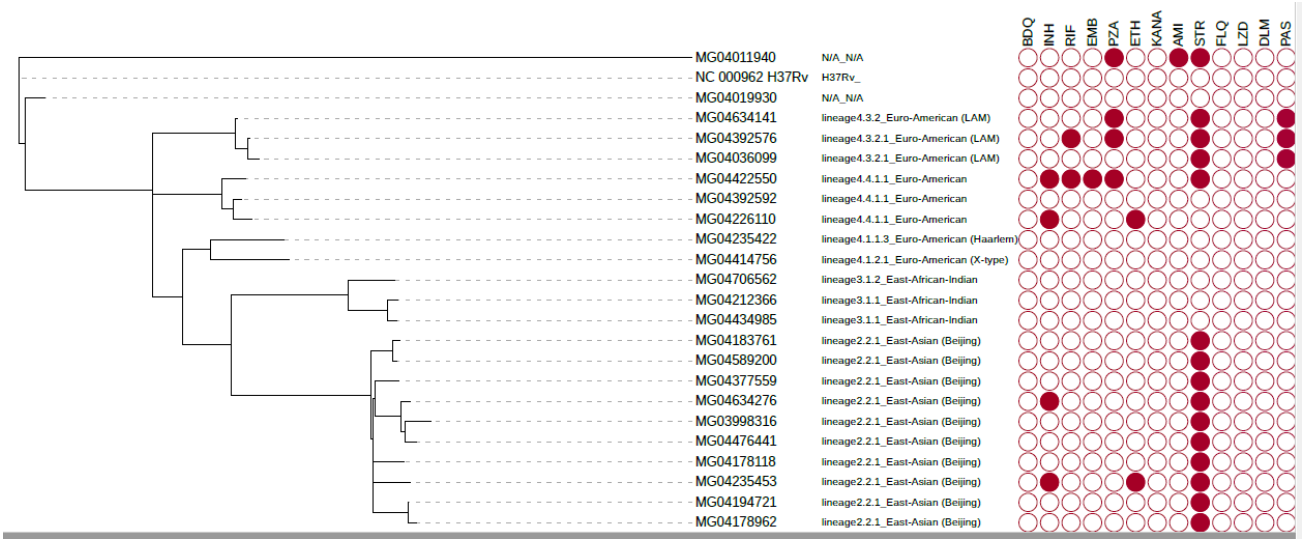
Whole genome sequencing was done on 23 of the 28 isolates that were received from the cultured specimens. The 5 samples excluded were either contaminated or had insufficient material for sequencing.

The lineage and phylogenetic relatedness of the isolates are summarized in table 4.v. Ten out of the 21 strains (47.62%) belonged to lineage 2, the East-Asian (Beijing) strain. 14.28% belonged to each of East-Africa-Indian, Euro-American and Euro-American (LAM) strains, and 4.76% belonged to each of Euro-American (X-type) and Euro-American (Haarlem)

Table 4.v: Distribution of strain types

Strain type	*Frequency*
East-African-Indian	14.29%
East-Asian (Beijing)	47.62%
Euro-American	14.29%
Euro-American (Haarlem)	4.76%
Euro-American (LAM)	14.29%
Euro-American (X-type)	4.76%
Grand Total	**100.00%**

Fig 4.I is a graphic representation of the lineage and phylogenetic relatedness of the 23 isolates. The first three are two non-tuberculous mycobacteria and the reference strain of *M. tuberculosis* H37Rv.18 The remaining 21 are the isolates under investigation. WGS thus helps in making the correct diagnosis of the pathogen. It identified the non-tuberculous mycobacteria in this group of 23 isolates.



BDQ,Bedaquiline. DLM, Delamanid. FLQ, Fluoroquinolones. LZD, Linezolid. INH,Izoniazid. RIF, Rifampicin. EMB, Ethambutol. PZA,Pyrazinamide. ETH,Ethionamide. KANA, Kanamycin. AMI,Amikacin. STR, Streptomycin. PAS, Para-aminosalicylic acid.

Fig 4.1. Maximum likelihood tree of isolates showing lineage and genotypic resistance profile (filled circles indicate resistance to the drug tested)

We then looked for drug resistance profile as provided by the WGS. WGS tests for resistance to most of the drugs used to treat TB. There is a high frequency of resistance to Streptomycin. One isolate (MG04422550) shows resistance to multiple drugs that are first line drugs in the treatment of TB.

We looked at the clinical diagnostic tests done on the specimens on which WGS was done. The results are reflected in table 4.vi. Of interest is that one of the specimens (study no. 26,) was Xpert® MTB/Rif negative, but when we did the whole genome sequencing, it showed that the patient had *M. tuberculosis* and is sensitive to all the drugs. Also, study no.36 (MG04422550) shows resistance only to INH, and on WGS it shows that the patient is resistant to all first- line drugs, making it a multidrug resistant isolate (MDRTB).This patient was HIV-positive, and persisted to have a high ESR despite adequate TB medication. When the results of WGS came out and showed that he had MDR-TB, he was admitted to a TB hospital where he was treated with second line drugs and made a good clinical improvement, ESR decreased, and good incorporation of the bone graft started to show over a 6-month period.

A few of the isolates that show a positive diagnosis on WGS, are negative on the Z-N stain, once again showing that smear microscopy is not a good test for diagnosing TB of the spine.

Table 4.vi: Comparing the results of whole genome sequencing to the tests currently done in clinical assessment of TB of the spine.

Study No.	WGS: Lineage & Strain	WGS: Drug Resistance	Xpert® MTB/Rif	Culture Sensitivity (LPA)	Histology: Granuloma Grading	Ziehl-Neelsen stain
6	2.2 East-Asian (Beijing)	STR	Rif sens	sensMTB	4	**Pos**
8	4.3 Euro-Amer (LAM)	STR, PAS	Rif sens	sensMTB	4	**Pos**
19	2.2 East-Asian (Beijing)	STR	Rif sens	Sens MTB	2	Neg
20	2.2East-Asian (Beijing)	STR	Rif sens	sensMTB	3	**Pos**
21	2.2 East-Asian (Beijing)	STR	Rif sens	SensMTB	4	Neg
23	3.1 East-Africa-Indian	nil	Rif sens	sensMTB	4	Neg
24	4.4 Euro-American	INH, ETH	Rif sens	**INH res**	1	Neg
26	4.1 Euro-Amer-Harlem	nil	**Neg**	sensMTB	4	Neg
27	2.2 East-Asian (Beijing)	INH, ETH, STR	Rif sens	**INH res**	1	**Pos**
29	2.2 East-Asian (Beijing)	STR	Rif sens	sensMTB	4	Neg
30	4.3 Euro-Amer (LAM)	RIF,PZA,STR,PAS	Rif sens	sensMTB	4	Neg
31	4.4 Euro-American	nil	Rif sens	SensMTB	4	Neg
34	4.1 Euro-Amer(X-type)	nil	Rif sens	SensMTB	4	Neg
36	4.4 Euro-American	INH,RIF,ETH,PZA, STR,PAS	Rif sens	**INH res**	4	Neg
37	3.1 East-Africa-Indian	nil	Rif sens	SensMTB	4	Neg
39	2.2 East-Asian (Beijing)	STR	Rif sens	sensMTB	3	**Pos**
43	2.2 East-Asian (Beijing)	STR	Rif sens	sensMTB	4	Neg
49	2.2 East-Asian (Beijing)	STR	Rif sens	sensMTB	2	Neg
51	4.3 Euro-Amer (LAM)	PZA,STR,PAS	Rif sens	sensMTB	1	Neg
52	2.2 East-Asian (Beijing)	INH, STR	Rif sens	**Rif sens INH res**	3	Neg
57	3.1 East-Africa-Indian	nil	Rif sens	sensMTB	4	Neg

In further assessing the role of whole genome sequencing, we went on to identify unique genetic markers that may be associated with infection in the spine.

Mutations generated against the reference genomes of *M. tuberculosis* H37Rv occurring at a frequency of 90% associated with these genomes were concentrated into a single file resulting in 28550 mutations across all 21 genomes of TB of the spine. Fig. 4.2 shows a sample of the mutations.

	Episodeno	Reference Position	Lineage	Type	Allele	Frequency
2	MG03998316	1849	East-Asian (Beijing)	SNV	A	100
3	MG03998316	1977	East-Asian (Beijing)	SNV	G	98,630137
4	MG03998316	4013	East-Asian (Beijing)	SNV	C	100
5	MG03998316	7362	East-Asian (Beijing)	SNV	C	100
6	MG03998316	7585	East-Asian (Beijing)	SNV	C	100
7	MG03998316	9304	East-Asian (Beijing)	SNV	A	100
8	MG03998316	11820	East-Asian (Beijing)	SNV	G	100
28543	MG04706562	4390580	East-African-Indian	SNV	T	100
28544	MG04706562	4393178	East-African-Indian	SNV	G	100
28545	MG04706562	4397736	East-African-Indian	SNV	T	100
28546	MG04706562	4400661	East-African-Indian	Deletio	-	92,3913044
28547	MG04706562	4401509	East-African-Indian	SNV	T	99,0384615
28548	MG04706562	4407588	East-African-Indian	SNV	C	99,1525424
28549	MG04706562	4408923	East-African-Indian	SNV	T	98,9247312
28550	MG04706562	4409954	East-African-Indian	SNV	C	99,0825688
28551	MG04706562	4410306	East-African-Indian	SNV	C	100
28552						

Fig 4.2: Extract from the spreadsheet to show the 28550 mutations in the 21 isolates

From these mutations we removed all known phylogenetic mutations and all synonymous mutations as these have no effect on protein synthesis. We also removed all mutations associated with Proline-Glutamic acid (PE) and Proline-Proline-Glutamic acid (PPE) regions of genome. These families of genes encode proteins carrying PE and PPE motifs found near the N-terminal end. They are poorly sequenced when using short read sequencing technology such as Illumina.

A concentrated file of mutations found in pulmonary samples was created, these included the pulmonary samples sharing the same lineage as samples of TB of the spine. This mutation list was used to remove all mutations between TB of the spine and pulmonary TB samples.

We were then left with 1272 mutations uniquely associated with TB of the spine isolates.

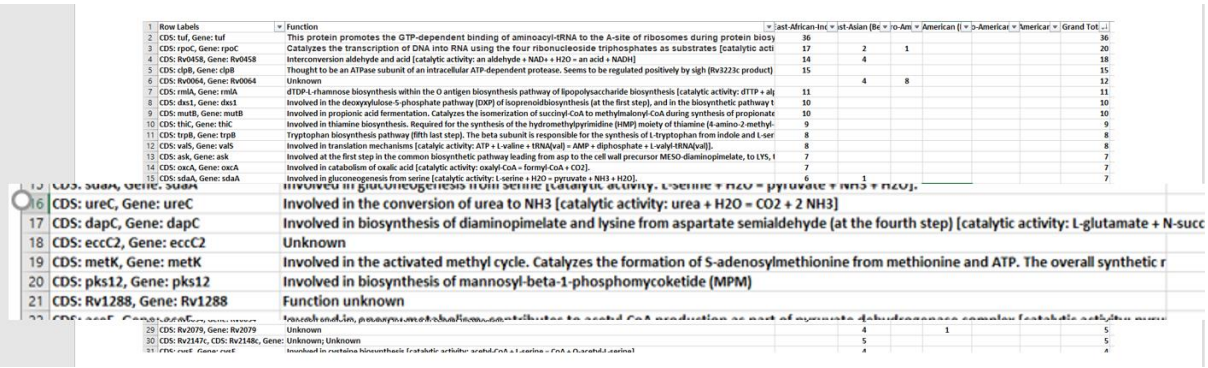
Fig 4.3 shows a sample of the mutations uniquely associated with isolates of TB of the spine.

	Episodeno	Reference F	Lineage	Type	Allele	Zygosit	Frequency	Unique_toTBSPINEset
2	MG04414756	4080	Euro-American (X-ty	SNV	T	Homozygo	100	CDS: recF, Gene: recFNP_214517.1:c.801G>T
3	MG04235422	9767	Euro-American (Haa	SNV	G	Homozygo	94,3820225	CDS: gyrA, Gene: gyrANP_214520.1:c.2466T>G
4	MG04226110	14467	Euro-American	SNV	C	Homozygo	100	CDS: Rv0012, Gene: Rv0012NP_214526.1:c.379G>C
5	MG04414756	20834	Euro-American (X-ty	SNV	G	Homozygo	96,9072165	CDS: rodA, Gene: rodANP_214531.1:c.807G>C
6	MG04706562	26748	East-African-Indian	Deletion	-	Homozygo	95,7446809	CDS: Rv0021c, Gene: Rv0021cNP_214535.1:c.134delG
7	MG04706562	29392	East-African-Indian	SNV	T	Homozygo	99,1304348	CDS: Rv0025, Gene: Rv0025NP_214539.1:c.148C>T
8	MG04235422	32351	Euro-American (Haa	Insertion	G	Homozygo	100	CDS: Rv0029, Gene: Rv0029NP_214543.1:c.294_295insG
9	MG04235422	32362	Euro-American (Haa	Insertion	A	Homozygo	100	CDS: Rv0029, Gene: Rv0029NP_214543.1:c.305_306insA
10	MG04235422	33516	Euro-American (Haa	SNV	A	Homozygo	100	CDS: Rv0030, Gene: Rv0030NP_214544.1:c.293C>A
1267	MG04476441	4395725	East-Asian (Beijing)	SNV	G	Homozygo	100	CDS: Rv3909, Gene: Rv3909NP_218426.1:c.1534A>G
1268	MG04235422	4401611	Euro-American (Haa	SNV	C	Homozygo	100	CDS: Rv3912, Gene: Rv3912NP_218429.1:c.742A>C
1269	MG04377559	4406495	East-Asian (Beijing)	SNV	T	Homozygo	100	CDS: parA, Gene: parANP_218434.2:c.1037G>A
1270	MG03998316	4407620	East-Asian (Beijing)	SNV	G	Homozygo	100	CDS: gid, Gene: gidNP_218436.2:c.583T>C
1271	MG04476441	4407620	East-Asian (Beijing)	SNV	G	Homozygo	100	CDS: gid, Gene: gidNP_218436.2:c.583T>C
1272	MG04634276	4407620	East-Asian (Beijing)	SNV	G	Homozygo	98,8372093	CDS: gid, Gene: gidNP_218436.2:c.583T>C
1273	MG04706562	4410306	East-African-Indian	SNV	C	Homozygo	100	CDS: Rv3922c, Gene: Rv3922cNP_218439.1:c.110T>G
1274								

Fig.4.3: 1272 Mutations uniquely associated with isolates of TB of the spine

We analysed relationships of the known pulmonary samples with samples of TB of the spine at nucleotide level. These mutations were stratified by Lineage, in the hope of finding a unique mutation found across all lineages specific to samples of TH of the spine. This was not observed – the most frequent unique mutation was found in the East-Asian (Beijing) lineage, however, at a 50% frequency amongst the Beijing lineages. There were 864 unique mutations by lineage.

We repeated the analysis at the gene level to see if a specific pattern could be observed related to a specific gene across all lineages. This too was not observed – there were 590 unique genes in which mutations were found. We compared variants within the set and against the pulmonary sample variants to identify any variant exclusive to the study samples. No variant was found to be unique to these spine isolates.



Row Labels	Function	ast-African-Inc	ist-Asian (Be	o-Am	American (I	o-Americar	Americar	Grand Tot
CDS: tuf, Gene: tuf	This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosy	36						36
CDS: rpoC, Gene: rpoC	Catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates [catalytic acti	17	2	1				20
CDS: Rv0458, Gene: Rv0458	Interconversion aldehyde and acid [catalytic activity: an aldehyde + NAD+ + H2O = an acid + NADH]	14	4					18
CDS: clpB, Gene: clpB	Thought to be an ATPase subunit of an intracellular ATP-dependent protease. Seems to be regulated positively by sigh (Rv3223c product)	15						15
CDS: Rv0064, Gene: Rv0064	Unknown		4	8				12
CDS: rmlA, Gene: rmlA	dTDP-L-rhamnose biosynthesis within the O antigen biosynthesis pathway of lipopolysaccharide biosynthesis [catalytic activity: dTTP + alp	11						11
CDS: dxs1, Gene: dxs1	Involved in the deoxyxylulose-5-phosphate pathway (DXP) of isoprenoidbiosynthesis (at the first step), and in the biosynthetic pathway t	10						10
CDS: mutB, Gene: mutB	Involved in propionic acid fermentation. Catalyzes the isomerization of succinyl-CoA to methylmalonyl-CoA during synthesis of propionate	10						10
CDS: thiC, Gene: thiC	Involved in thiamine biosynthesis. Required for the synthesis of the hydromethylpyrimidine (HMP) moiety of thiamine (4-amino-2-methyl-	9						9
CDS: trpB, Gene: trpB	Tryptophan biosynthesis pathway (fifth last step). The beta subunit is responsible for the synthesis of L-tryptophan from indole and L-ser	8						8
CDS: valS, Gene: valS	Involved in translation mechanisms [catalytic activity: ATP + L-valine + tRNA(val) = AMP + diphosphate + L-valyl-tRNA(val)].	8						8
CDS: ask, Gene: ask	Involved at the first step in the common biosynthetic pathway leading from asp to the cell wall precursor MESO-diaminopimelate, to LYS, t	7						7
CDS: oxcA, Gene: oxcA	Involved in catabolism of oxalic acid [catalytic activity: oxalyl-CoA = formyl-CoA + CO2].	7						7
CDS: sdaA, Gene: sdaA	Involved in gluconeogenesis from serine [catalytic activity: L-serine + H2O = pyruvate + NH3 + H2O].	6	1					7
CDS: sdaA, Gene: sdaA	Involved in gluconeogenesis from serine [catalytic activity: L-serine + H2O = pyruvate + NH3 + H2O].							
CDS: ureC, Gene: ureC	Involved in the conversion of urea to NH3 [catalytic activity: urea + H2O = CO2 + 2 NH3]							
CDS: dapC, Gene: dapC	Involved in biosynthesis of diaminopimelate and lysine from aspartate semialdehyde (at the fourth step) [catalytic activity: L-glutamate + N-succ							
CDS: eccC2, Gene: eccC2	Unknown							
CDS: metK, Gene: metK	Involved in the activated methyl cycle. Catalyzes the formation of S-adenosylmethionine from methionine and ATP. The overall synthetic r							
CDS: pks12, Gene: pks12	Involved in biosynthesis of mannosyl-beta-1-phosphomycoketide (MPM)							
CDS: Rv1288, Gene: Rv1288	Function unknown							
CDS: Rv2079, Gene: Rv2079	Unknown		4		1			5
CDS: Rv2147c, CDS: Rv2148c, Gene:	Unknown; Unknown		5					5

Fig. 4.4: A sample of the 590 unique genes by lineage, showing their function. Note that a number of genes have unknown function

504 genes occurred in less than 10% of isolates. There are however a number of genes with unknown function. It is not certain if these are the genes that may be responsible for certain functions that will make these bacteria survive in the vertebral bone. In a further study the remaining 86 genes will be reviewed to understand the function of these genes and a possible relationship to the samples of TB of the spine.

4.6 Discussion

The laboratory diagnosis of TB is done by both direct and indirect methods. The direct methods include microscopy, culture, antigen detection and nucleic acid detection. Indirect methods include immune response assessment with tuberculin skin test (Mantoux) and interferon-gamma release assays (IGRAs).

Smear microscopy is cheap and easy to perform, but its sensitivity and specificity are poor. Culture is very reliable, but it takes weeks for the results to come out. The sensitivity of tuberculin skin tests and IGRAs is suboptimal and does not distinguish between active and latent disease. The molecular methods have the advantages of simplicity, rapidity

and accuracy. These include polymerase chain reaction, such as Xpert MTB/RIF Ultra and line probe assay (LPA).[19]

The laboratory diagnosis of tuberculosis of the spine is not easy. In this series the diagnosis of TB was made using a combination of clinical presentation, radiologic findings, and laboratory tests. We covered the radiologic findings in the previous chapter. The histologic changes in this series were all supportive of the diagnosis of TB.

AFB smear microscopy was positive in only 15.51% of specimens. Unlike in pulmonary TB where the sputum is often teeming with bacteria, TB in the spine is a paucibacillary disease. In other extrapulmonary sites (pus, ascites fluid, urine, csf) smear microscopy was found to be accurate in 68.4%[20] . Because of the pauci-bacterial nature of TB of the spine, smear microscopy will miss the diagnosis in a lot of patients with TB of the spine**.** It is not as useful as in pulmonary TB**.** Even for pulmonary tuberculosis, the World Health Organization endorses the Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) real-time PCR platform to diagnose TB[21, 22]

PCR was positive in 85.5% of specimens. Where PCR was performed in both tissue and pus, it was positive in both specimens.

In a study of 74 patients, Pandey found a sensitivity of 73.07% and a specificity of 93.75% (95% CI, 62.97-83.17).[23]As the specificity for PCR is high, a negative PCR should rule out TB if the histology is also negative. In our series all those with negative PCR had histology supportive of TB. In a study done in South Africa[24] on biopsy tissue for TB of the spine, the test had a sensitivity of 95.6%, specificity of 96.2% positive predictive value of 97.7 % and negative predictive value of 92.6%. The test needs a smaller quantity of

bacteria, 130 colony forming units per ml as compared to 10 000 in culture to make a diagnosis. The combined use of PCR and MRI has 97% sensitivity in the diagnosis of TB.[25]

Whole genome sequencing is a newer molecular method that investigates the whole genome of the organism. It thus gives a complete assessment to include diagnosis, drug sensitivity to a wide variety of drugs, trace the origin of the species and is thus useful during disease outbreaks.

In this study we have also used WGS to see if the genomic material of the TB bacilli causing disease in the spine is the same as those causing pulmonary disease. We subjected 23 specimens to WGS.

The diagnosis of TB could be made on all those isolates. Of interest is that WGS picked up two isolates that were non-tuberculous mycobacteria. One isolate in this series was Xpert® MTB/Rif negative. These two examples in this small series demonstrate how accurate WGS is in diagnosing TB of the spine. This is because WGS looks at the whole genome of the organism, and not only at one region of the genome as Xpert® MTB/Rif does.

Multidrug resistance TB (MDRTB) is defined as resistance of *M. tuberculosis* to the two main first-line drugs in therapy.[26] Drug resistance has been poorly reported upon in TB of the spine, to such an extent that Pawar alerted us to the need to include drug sensitivity protocols in treatment of TB of the spine. [27] Since then there have been a few reports on drug resistance in TB of the spine, especially in centres where genotyping is done.[28, 29] In TB spine it is caused by a multiplicity of factors, including monotherapy, suboptimal

drug ingestion and inadequate duration of treatment.[30] Most patients with TB of the spine come from poor socioeconomic background, usually staying far from treatment centres,[31] This results in patients not completing their treatment. In some parts of South Africa MDR TB of the spine is as high as 4% .[32] It is of particular importance that the diagnosis is made early so that appropriate MDRTB treatment be started on time. The mortality for MDRTB is as high as mortality for TB in the pre antibiotic era.[30]

In this study, one of the 21 isolates (4.7%) our isolates showed MDRTB on WGS. This is within the reported range for TB of the spine in the literature.[33] . As our WGS study was done some months after the diagnosis was made on the available laboratory examinations, we could only diagnose this MDRTB isolate 12 months after treatment had commenced. We could not diagnose it by our phenotypic methods of drug resistance testing. Had WGS been a method used in clinical medicine, we would have diagnosed MDR TB at the beginning of the diagnosis process and started the patient on second line of drug treatment.

Drug resistance in *M. tuberculosis* is a function of chromosomal mutations in a few genes.[34] WGS of culture isolates generates a complete genetic drug resistance profile. To generate this drug resistance profile is however, delayed by the need for the essential step of culture. WGS directly from sputum is now possible with targeted enrichment of the sputum.[7] This then provides rapid detection of drug resistance within a clinically short time.

Doyle et al. report on using this method on 43 sputum samples.[6] They sequenced *Mtb* from sputum samples after targeted DNA enrichment using the Agilent SureSelectXT kit.

Drug sensitivity could be determined from the WGS samples within 5 days, 24 days earlier than MGIT culture. This improved turnaround time enables prompt appropriate treatment.

This targeted enrichment programme has not yet been done on tissue from TB of the spine.

While several pulmonary isolates have been sequenced, availability of whole genome sequencing from extrapulmonary sites is limited. There are suggestions that genetic variations in *M. tuberculosis* might contribute to the disease presenting in extrapulmonary sites. In their work on 5 extrapulmonary isolates**,** Sharma could not find any common genetic mutations in the isolates and recommended that more work be done to map the genetic diversity of MTB.[35]

Sarkar postulated that the development of extrapulmonary tuberculosis depends on the ability of the pathogen to have affinity for cells other than those of pulmonary tissue.[36] They found that different strains have different invasion capacity for different cell types. For examples, some strains showed a propensity for osteoblasts.

Li et al looked for genetic differences that may be responsible for the development of pulmonary TB or TB of the spine. They found that some genotypes were significantly associated with pulmonary TB.[16]

We also looked for pathogen- related factors in the spread of the disease from the lungs to the spine. Our results comparing the genome of the 21 samples with that of *M. tuberculosis* H37Rv showed no mutations were specific to spine *M. tuberculosis*. Spine isolates have a mixed lineage, just as we find in pulmonary TB. Most of them (45%), were lineage 2, East-Asian (Beijing) type. The distribution of the lineages of these bacteria are

similar to those found in pulmonary TB.[37,38] At genome level we found some genes encoding unknown protein function. Further work will still need to be done to find out if these genes are not in fact altering the metabolic function of the *M. tuberculosis* to be able to adjust to the hypoxic environment of the spine.

4.7 Conclusion

Whole Genome Sequencing suggests that the bacilli that settle in the spine have the same genetic makeup as those in the lung, there are no mutations that select themselves to be more viable in spine tissue. However, further research needs to be done on the genes with no known function. They could be the ones that make the bacilli settle in the vertebral bone.

WGS allows more accurate diagnosis of the organism, including identification of non-tuberculous mycobacteria.

Introduction of WGS in the clinical setting will assist in early diagnosis of MDRTB in the spine and early treatment.

Smear microscopy is not useful for the diagnosis of tuberculosis of the spine as this is a paucibacillary condition.

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CHAPTER 5
PERSPECTIVES

5.1 Perspectives

In this multicentre study of sixty-one consecutive patients with tuberculosis of the spine, we find that 70% of our patients are HIV-positive. This is a marked increase from a previous study reported eight years ago where only 40% of patients were HIV positive.[1] This is also in keeping with the worldwide increase in HIV infection. iIt is however satisfying to note that 70% of the patients on HIV treatment in this series have suppressed viral loads, where the viral load is recorded as less that detectable . This is in keeping with UNAIDS figures that show that 72% of people living with HIV and on treatment in Southern Africa, 65% have a suppressed viral load.[2] Human immunodeficiency virus (HIV) is a major global health problem that has disproportionately affected sub-Saharan Africa. This region has about two thirds [1] (64%) of the world's burden of HIV infections . South Africa has the highest burden of HIV infections in the world[3] with about 7.9 million people living with HIV. Thus, SA contributes approximately 20% of HIV infections to the global HIV pandemic, despite having about 1% of the world's population.

It is therefore not surprising that the incidence of HIV in patients with TB of the spine in South Africa is 70%. Indeed, you cannot talk about a diagnosis of TB of the spine without mentioning the HIV status of the patient.

The typical lesion of TB of the spine is a disease affecting two contiguous bodies with narrowing of the intervening disc space. In this study we have shown that the atypical pattern of TB of the spine, particularly the pattern that involves non-contiguous vertebrae, is found in 83% of patients who are HIV-positive. It is important therefore that when we investigate for TB of the spine, we not only request MRI scan of the affected area of the spine, but that we do an MRI scan of the whole spine so that we do not miss skip lesions. This practice is supported by other authors.[4, 5]

The major finding of this study is that there is more vertebral bone loss (p=0.01) and marginally more pus formation in HIV-positive patients with TB of the spine. This is a direct opposite to the findings of other researchers who reported on this subject.[1,6, 7] All report that there is more bone loss in HIV-negative patients. Anley explained their findings by suggesting that this is due to repression of the type IV hypersensitivity reaction, where there is decreased cytokine production and thus less bone formation. The other authors simply cited the reasons given by Anley[1].

Both South African studies[1, 6] were retrospective studies with data collected during the period when anti-viral medication was not available to the public sector in South Africa. In this study, at the time of surgery, 70% of the HIV-positive patients had a viral load of less than detectable, thus showing good control of the HIV infection. Furthermore, our findings are supported by literature on the pathology of Tuberculosis of the spine. Izawa[8] has shown that there is increased RANK-L activity, which is mediated by cytokines, especially TNF-α. He mentions that in severe pulmonary disease there is an intense immune reaction that causes increased destruction of lung tissue. He extrapolates that

this intense immune reaction is the cause for the increased bone destruction in HIV. Bezuidenhout[9] examined tissue from HIV-positive and HIV-negative pulmonary TB patients. She found that in HIV-positive patients there were more necrotic granulomas, decreased macrophages leading to poor granuloma formation, and that there was close association between TNF-α and tissue necrosis. Both these studies support our results.

The clinical significance of this finding is that if there is more pus in the HIV-positive patients, it suggests that we can modify the surgical procedure in these patients by only doing a wide costo-transversectomy to remove the pus and stabilize the spine posteriorly, without having to do the extensive Hong Kong procedure.[10] This approach in HIV -positive patients will improve the morbidity associated with surgery for TB of the spine.

Rajasekaran[11] writes that the real pathology in this disease will be found by examining the tissue at the site of the disease, that is examine the granuloma in TB of the spine. For that reason, we examined the granulomas histologically and immunologically to see if there are any differences between the two groups of patients.

The granulomas were graded according to their cellularity and on presence of necrosis. There is no granuloma pattern that is specific to HIV-negative and HIV-positive patients. This finding is in keeping with the findings of other authors as well.[12] Diedrich, in a systematic review addressing this subject, concludes that there is marked heterogeneity in the granulomas and that it is difficult to ascribe one pattern to HIV-positive granulomas.[13] We also found no statistically significant difference between the expression of the cytokine TNF-α and the HIV status of the patients. Furthermore, although there is a rather marginal suggestion that the poorly formed granulomas have less TNF-α, the relationship is not statistically significant. This study is therefore not conclusive in showing

the immunologic basis of our findings of more bone destruction in HIV-positive patients. The study is also not conclusive on the use of the cytokine TNF-α in host directed therapies. A study with a larger sample size might shed more light. Also, perhaps other cytokines will behave differently.

Lastly, we looked at the whole genome sequencing to explore its utility in TB of the spine and specifically to see if it can tell us if the bacilli that cause TB in the spine are the same bacilli we find in pulmonary TB or they different mutants. We have not been able to find any mutants among these isolates that are specific to the spine isolates. Other authors working in this field have also not been able to do so.[14] The interesting finding in this study is that there are some genes with unknown function. It could be that some of these genes are responsible for some of the characteristics of the spine bacilli, like the ability to survive in the hypoxic environment of the spine. We have cultivated some interest at National Institute for Communicable Diseases and as such, more work will be done on these spine isolates.

We have shown that in South Africa, the mycobacterial strains causing TB of the spine are the same strains that cause Pulmonary TB . The lineage 2 East- Asian (Beijing) type is the most predominant at 47%. This is similar to the strains causing pulmonary TB in South Africa. **[15, 16].** We have shown that WGS is useful in detecting drug resistance. Drug resistance in TB of the spine is a big cause of mortality.[17] Its incidence in South Africa is estimated at 4.0%.**[18, 19]**.

Orthopaedic surgeons are generally poor at monitoring drug resistance. In TB of the spine, the main cause of the drug resistance is prolonged drug ingestion by patients who come from poor socio-economic environments, who usually cannot afford even the

transport to get to the treatment centres. It is therefore important that we diagnose drug resistance and add it to our protocol of treatment of TB of the spine. [17,20] In this series WGS has helped us diagnose one MDR-TB patient whom we missed when using our normal phenotypic drug testing. This gives us a diagnosis of MDR -TB of 4.7%. Unfortunately, even with WGS, MDR-TB of the spine can only be diagnosed after the bacteria have been cultured. There are moves to do WGS directly on the clinical specimen, *ie* sputum in pulmonary TB. This will speed up the diagnosis of drug resistance. Unfortunately, the process is currently quite costly, but it will eventually come to be a clinical tool in TB of the spine one day.

In summary, this research thesis has shown us that:

1. HIV infection in TB of the spine patients in South Africa has increased to 70%. This is in keeping with the global increase in HIV infections
2. Contrary to the current literature, there is more bone destruction in HIV-positive patients and marginally more pus formation. This finding will enable us to modify the surgical treatment for HIV-positive patients to the surgically less traumatic procedure of draining the pus and decompression, without having to do extensive debridement.
3. It is important do whole spine MRI when investigating HIV-positive patients with TB of the spine, so that we do not miss skip lesions.
4. Histology and immunology studies do not show a specific pattern that can be attributed to the HIV status of the patient.

5. WGS will be a useful tool in early detection of MDR-TB once it becomes available in clinical medicine.
6. The strains of *M. tuberculosis* causing disease in the spine are the same as those causing pulmonary TB in South Africa.
7. We have not been able to find any mutations responsible for the bacilli settling in the spine, but this study points to the area in where more work needs to be done.

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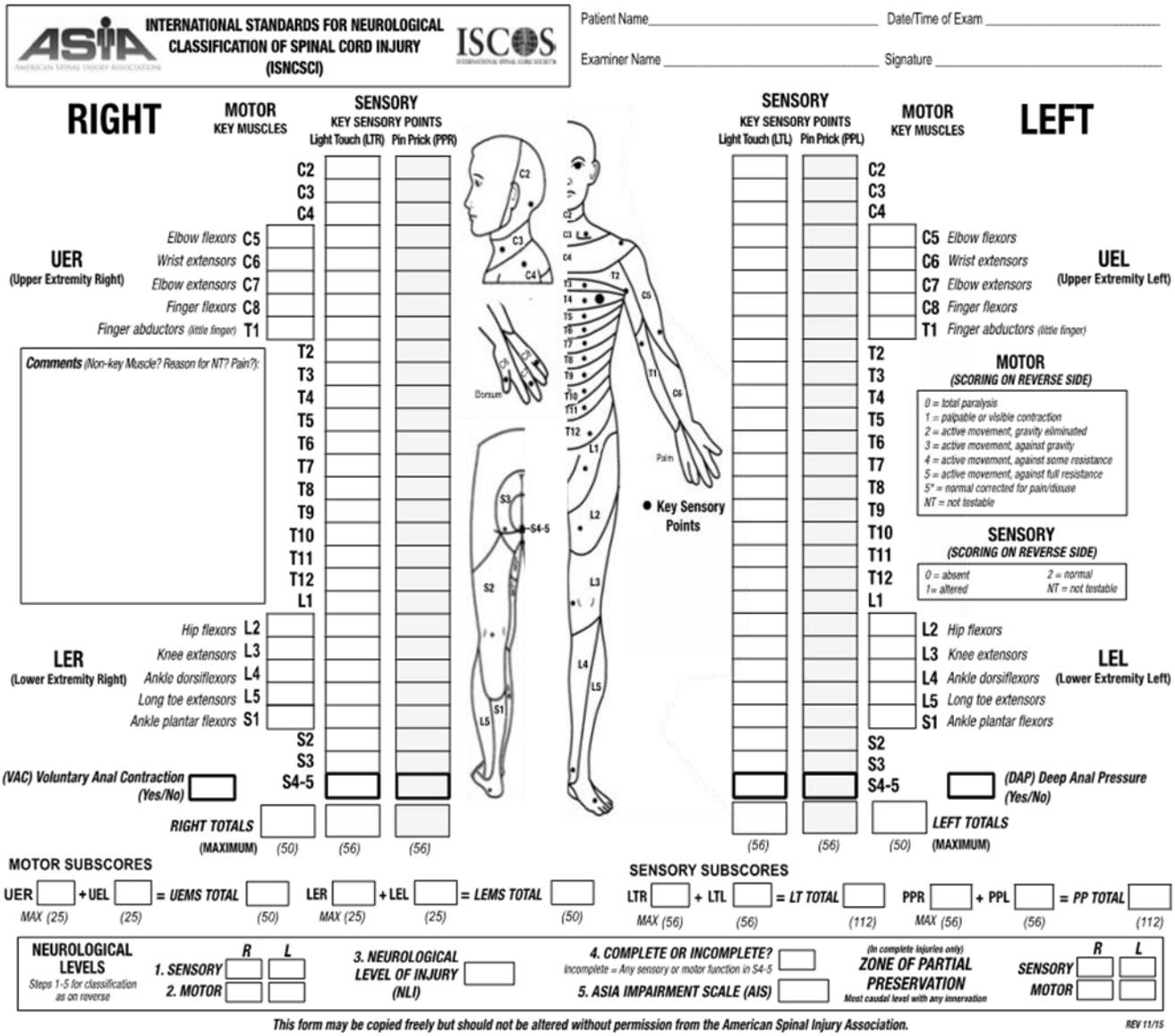
APPENDICES

Appendix A: ASIA score chart



Appendix B: Informed Consent Document

Name of Patient: _____

Hospital: ----------

Hospital Number: _____

Date of Birth: _____ /Age: ____

Gender: _____

Contact number: _____ or _____

Research Number: _____

Title: Tuberculosis of the spine in HIV positive and negative patients: immune factors in local distribution of the disease.

Principal Investigator: Prof M Ngcelwane.

Local investigator at the site…….

About this form:

This form gives you important information about a research study. Please read it carefully. In addition to you reading it, one of our staff members will explain it to you and will be able to answer any questions you may have about the study.

If you are willing to participate in the study, you will be requested to sign this form.

Introduction to the research study.

TB of the spine is caused by gems that infect the bone and destroys the bone of the spine. It also causes formation of pus around the spinal cord. The pus and the destroyed bone compress the spinal cord and make the patient unable to move the lower limbs and thus unable to walk. In other words, the patient gets paralysed.

Purpose of the research project.

The extent of destruction on the bone and pus formation differs from person to person. We think this bone destruction and pus formation is more extensive in people who are co-infected with HIV. We also know that the body tries to fight the spread of the disease.

In this research we are trying to find out how the body contains the disease, ie. how it it controls the extent of destruction of bone and pus formation. We are particularly interested in the effect of HIV infection in the distribution of the disease.

The reason you are being chosen for this study is that you have been diagnosed with TB of the spine and you will be treated by having an operation to your spine.

Procedure:

Your treating doctors have decided that they will treat you by operating on your spine. In this study we will take blood for tests to see how strong your body in fighting infection is. We will also take the dead bone and pus for additional tests that will help us to find out the cause of the extent of destruction of bone and formation of pus.

Benefits of the study.

The benefits of the study is that it will allow doctors to know how the body contains TB infection in patients with and without HIV infection. We hope that the tests we have done will be able to help in developing new medicines that may shorten the length of time patients with TB spine have to take medication.

Risks and discomfort.

There are no extra risks to you in taking part in the study. We normally will take blood from you before we do an operation to the spine. We will take an extra tube of blood for the tests we want to do. You will not feel that an extra 5 cc of blood has been taken.

In theatre, we normally remove all the dead bone and pus, and take it for tests to prove that it is indeed TB that is causing the disease. In this study, instead of throwing away the extra dead bone and pus, we will send it for more tests to help find out how your body has been fighting the infection.

The procedures you will be subjected to are therefore the same as when you would be having the operation without the study being done.

Alternatives to participation.

If you do not want to participate in the study, your treatment will not be affected. The treatment of operation that has been described to you by your doctors will continue. The only difference is that they will not do the extra tests from the blood and the dead bone and pus.

Refusal or withdrawal from the study.

You may withdraw from the study at any time, it will not affect your treatment.

How long will I take part in this study?

After the specimens have been taken in theatre and the operation is over, the study is over for you. You will then have the follow-up examination following a spine operation every 3 months for a year.

Confidentiality

You will be allocated a research number. We will take all measures to protect your privacy when participating in the research. All information is kept in secure place. Only the principal researcher will know about the results that are specific to you. The results of the study will be published in medical literature, but there will be no reference to your person.

Request for more information.

You may ask any questions about the study or discuss participation at any time.

The people to ask is the specialist who is discussing this research with you. He/she is the specialist in your hospital who is the investigator.

You may also elect to ask the principal investigator, Prof M Ngcelwane from the University of Pretoria, who is contactable at 021 354 2851 or at Mthunzi.ngcelwane@up.ac.za.

Injury statement.

There is no risk of injury to yourself. The blood we will be taking is as we do for the operation. You will feel some pain from the needle prick, which is the same prick you will have had for taking preoperative blood. The tissue we are taking for the research is the tissue we would otherwise be throwing away.

The risks that are there are those related to any spine operation, which would be the same as when you are not taking part in the study. These risks will have been discussed by the specialist in your hospital.

What are the possible benefits for being in the study?

There will be no benefits to yourself really, but the research results will add more valuable information in the medical treatment of TB of the spine. It will help us understand how the disease spreads to destroy more bone and compress spinal cord. This may help us find other medicines that we can use together with TB medication to shorten the time it takes to use TB medication. Future patients with TB spine may stand to benefit from your participation.

We request your permission to take the dead tissue that is removed during the operation for further examination to find more about TB spine, and to take blood to examine for further tests.

Please tick one below.

Granted/Agree Not Granted/Disagree

Statement of person giving consent

I have read this consent form. The consent form has also been explained to me.

This research study has been explained to me, including risks and benefits.

The opportunity to ask question relating to the research was offered. I give my consent to participate in this study.

I also participate with the understanding that my confidentiality will be protected.

Signature of participant Date:

_____

Contact:

Signature of witness Date:

_____

Contact:

Statement of the investigator.:

I have explained the research to the study subject

I have answered all questions about this research study to the best of my ability.

Investigator: Date:

Appendix C: Biostatistician's letter

saMRC advancinglife

BIOSTATISTICS UNIT

Date: 19/06/2018

LETTER OF CLEARANCE FROM THE BIOSTATISTICIAN

This letter is to confirm that the student, with the Name(s) Mthunzi V Ngcelwane , a PhD candidate at Department of Orthopedics, University of Pretoria and Steve Biko Academic Hospital discussed the Project with the title "***TUBERCULOSIS OF THE SPINE IN HIV POSITIVE AND NEGATIVE PATIENTS: IMMUNE FACTORS IN LOCAL SPREAD OF THE DISEASE***" with me.

I hereby confirm that I am aware of the project and also undertake to assist with the Statistical analysis of the data generated from the project.

This is a prospective study to determine the immune factors responsible for wider spread of tuberculosis of the spine in HIV positive patients. The primary aim is to define the immune factors responsible for the destruction of the tissue at the local site of infection in TB spine in HIV positive and negative patients. The project consists of several phases/hypotheses which require use of descriptive summaries and presentation of proportions and associated 95% confidence interval for HIV positive and HIV negative participants. Comparison of proportions using independent t-test will be undertaken between the two groups in with respect to the series of hypotheses of interest and for types of tests. Thereafter, multivariable logistic regressions may be used to identify the factors influencing. Stata 15 will be the tool to be used for the analysis.

SAMPLE SIZE

No prevalence figure is available for a formal sample size calculation. It is recommended that the Researcher uses a window period of 9 – 12 months for the recruitment of participants that meet the inclusion criteria anticipating that a minimum of 30 – 45 participants/arm in order to undertake the research.

Name Dr. S.A.S Olorunju

Signature _____

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Appendix D :Research Ethics Committee Approval Certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

28 March 2019

Approval Certificate
New Application

Ethics Reference No.: 73/2019
Title: Tuberculosis of the Spine in HIV negative and HIV positive patients: Immune and pathogen-related factors in the local distribution of the disease

Dear Prof MV Ngcelwane

The **New Application** as supported by documents received between 2019-02-18 and 2019-03-27 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-03-27.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-03-28.
- Please remember to use your protocol number (73/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 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, Second Edition 2015 (Department of Health)

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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo