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Tuberculosis in HIV positive individuals – risk factors, diagnostic methods and follow-up in a low-endemic country

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A dissertation for the degree of Philosophiae Doctor – July 2015

Tuberculosis in HIV positive individuals – risk factors, diagnostic methods and follow-up in a low-endemic country

PhD thesis

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Tromsø

July 2015

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ACKNOWLEDGEMENTS

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Pullar ND, Steinum H, Bruun JN, Dyrhol-Riise AM. HIV patients with latent tuberculosis living in a low-endemic country do not develop active disease during a 2 year follow-up; a Norwegian prospective multicenter study. BMC Infect Dis. 2014 Dec 17;14:667.

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Wergeland I, **Pullar N**, Assmus J, Ueland T, Tonby K, Feruglio S, Kvale D, Damås JK, Aukrust P, Mollnes TE, Dyrhol-Riise AM. IP-10 differentiates between active and latent tuberculosis irrespective of HIV status and declines during therapy. J Infect. 2015 Apr;70(4):381-91.

Paper 4

Pullar ND, Dyrhol-Riise AM, Bruun JN. Vitamin D levels and interferon-gamma responses correlate in HIV-infected persons with tuberculosis infection. Submitted July 2015.

ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ART	Anti-retroviral therapy
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette Guerin
CFP	Culture filtrate protein
CI	Confidence interval
CRP	C-reactive protein
CT	Computer tomography
DBP	Vitamin D binding protein
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
ESAT-6	Early secretory antigenic target-6
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
IGRA	Interferon-gamma release assay
IQR	Inter-quartile range
IL	Interleukin
INH	Isoniazid
IP-10	Interferon-gamma inducible protein
IRIS	Immune reconstitution inflammatory syndrome
LAM	Lipoarabinomannan
LTBI	Latent TB infection
MDR	Multidrug resistant
M.tb	Mycobacterium tuberculosis
QFT	Quantiferon-TB Gold In-Tube
RD1	Region of difference 1
SR	Erythrocyte sedimentation rate
TB	Tuberculosis
TNF	Tumor necrotic factor
TST	Tuberculin skin test
T-SPOT.TB	TSPOT
VDR	Vitamin D receptor
WHO	World health organization
XDR	Extensively drug resistant

SUMMARY

Tuberculosis (TB) is a diagnostic challenge in persons infected with human immunodeficiency virus (HIV). Furthermore, HIV positive individuals with latent TB infection (LTBI) have the highest risk of progression to active TB. WHO initiatives to reduce the global burden of HIV/TB co-infections include better surveillance, improved diagnosis of TB in HIV patients and implementation of TB preventive therapy in patients with LTBI. The last few years, interferon-gamma release assays (IGRA) have increasingly been used as a substitute or supplement to the tuberculin skin test (TST) for LTBI diagnosis. In this thesis we have assessed the performance of IGRA and TST in the diagnosis and follow-up of TB infection in HIV positive individuals living in Norway, a TB low-endemic country.

HIV positive individuals were included from seven clinics throughout the country and followed-up clinically and with repeat IGRA [QuantiFERON TB Gold (QFT)] for a period of two years. The median CD4 cell count was high in our cohort, and the majority of study participants received antiretroviral therapy (ART). In a smaller sample of this cohort we have also explored the potential of a number of cytokines as biomarkers to improve TB diagnosis. Further, we assessed the association between vitamin D status and its prognostic potential in TB infection in HIV positive persons.

We found that the prevalence of LTBI was higher that expected, consisting mainly of immigrants from TB endemic countries. Length of stay in Norway was inversely associated with IGRA positivity in immigrants from TB endemic countries, which may indicate waning of latent infection over time in a region of low TB infectious pressure. Fewer LTBI patients than expected received TB preventive therapy. Nevertheless, none of the study participants developed active TB during the 2-year duration of the study, implying a reduced risk of progression to active TB in HIV-infected persons on ART living in a region of low TB transmission. QFT was more practical and efficient for TB diagnosis in this low-endemic and high-income setting. However, QFT proved to be inadequate for monitoring purposes due to variability during longitudinal testing. Among the cytokines studied, IP10 performed best in differentiating between active TB and LTBI irrespective of HIV status. Furthermore, a cut-off level that provided excellent sensitivity and specificity for IP-10 could be set in HIV positive patients. We also observed that vitamin D levels were significantly lower in TB patients and there was a significant inverse correlation between 25(OH)D levels and TB-specific

interferon-gamma release. The significantly lower levels of $1,25(OH)_2D$ in active TB patients compared to LTBI patients suggests a protective function of this metabolite in LTBI.

Our findings contribute to the knowledge base for LTBI diagnosis and treatment guidelines for HIV positive patients living in TB low-endemic countries.

SAMMENDRAG

Det er utfordrende å diagnostisere tuberkulose (TB) hos personer med human immunsviktvirus (HIV). Dertil har HIV-infiserte personer høy risiko for at en latent TB (LTBI) går over til aktiv TB. Verdens Helseorganisasjon (WHO) sine globale strategier for å redusere HIV/TB koinfeksjon inkluderer bedre overvåkning, mer effektiv diagnostiske metoder for TB hos HIV-positive og implementering av forebyggende behandling hos personer med LTBI. Interferon gamma release assays (IGRA) har de senere år bli brukt i tillegg til eller i stedet for tuberkulin hudtest (TST) i diagnostisering av LTBI. I denne avhandlingen har vi sammenliknet IGRA og TST for diagnostisering og oppfølging av TB infeksjon hos HIV-positive bosatt i Norge, et land med lav endemisk forekomst av TB.

Studien inkluderer HIV-positive personer fra syv forskjellige sykehus. Samtlige er screenet for TB, fulgt opp klinisk og undersøkt med gjentatte IGRA [QuantiFERON TB Gold (QFT)]. Median CD4 tall var relativt høyt i vår kohort. De fleste deltakere sto på antiretroviralbehandling (ART). I et mindre utvalg av kohorten ble flere cytokiner analysert som potensielle biomarkører til forbedring av TB diagnostikken. Det ble også gjort analyser av den prognostiske betydningen av vitamin D status hos HIV-positive personer.

Resultatene viser at prevalensen av LTBI er høyere enn forventet, og at innvandrere fra TB-endemiske land utgjør den største andelen av affiserte. Prevalens av positiv QFT blant HIV-positive fra TB-endemiske land er signifikant høyere blant nylig innvandrede til Norge enn blant de som har bodd flere år i Norge. Dette kan indikere at TB-spesifikke immunrespons avtar etter flere år i et miljø med lav smitte. Færre HIV-positive med LTBI enn forventet fikk TB forebyggende behandling, men ingen deltakere utviklet aktiv TB i løpet av 2-års observasjonstid. Dette kan tyde på at HIV-positive på ART som bor i en setting med lav TB smitte har redusert risiko for at LTBI progredierer til aktiv TB.

Studien viser at QFT er en praktisk og effektiv metode for TB-diagnostisering i et samfunn som har råd til å bruke testen, der helsevesenet er velfungerende og der det er lav endemisk forekomst av TB. På grunn av stor variasjon i prøvesvar ved gjentatt testing er metoden ikke egnet til å vurdere effekt av pågående TB behandling. Av cytokinene som ble studert er IP-10 best egnet til å differensiere mellom aktiv TB og LTBI uansett HIV status. Vår studie viser også signifikante lavere vitamin D hos TB pasienter og en invers korrelasjon mellom 25(OH)D og quantitativ QFT resultat. Signifikant lavere nivå av $1,25(OH)_2D$ ble også observert hos personer med aktiv TB sammenliknet med LTBI pasienter. Dette antyder at vitamin D kan ha en beskyttende effekt mot utvikling av aktiv TB hos de med LTBI.

Studiens resultater kan bidra til retningslinjer for diagnostisering og behandling av LTBI hos HIV-positive personer bosatt i land med lav forekomst av TB.

1 INTRODUCTION

1.1 HIV

1.1.1 The Epidemic

An unusual and rapidly stigmatized illness presenting with pneumocystis carinii pneumonia (PCP) and Kaposi sarcoma in young homosexual males made headlines in the early 1980s. It soon became evident that also children and heterosexual men and women had contracted the disease. Since the identification of the retrovirus, human immunodeficiency virus (HIV), causing the illness coined as acquired immune deficiency syndrome (AIDS) in 1983, there has been major developments in the diagnosis and treatment of the infection. HIV is however the most common cause of death by a single infectious agent worldwide with an estimated 35 million peoples living with HIV, 2.1 million newly infected and 1,5 million deaths due to AIDS in 2013 [1]. Sub-Saharan Africa has the highest prevalence of HIV infected with 4.7% prevalence among adults compared to 0.3% in Central and Western Europe and North America. Norway is a HIV low-incidence country with 200-300 new HIV cases diagnosed yearly (figure 1). A total of 5622 cases were reported by the end of 2014 and an estimated 4500 persons are living with HIV in Norway [2,3]. A total of 156 (63%) of the 249 diagnosed with HIV had immigrant background in 2014, the majority (72%) acquiring the infection heterosexually before arriving in Norway.

Figure 1: HIV in Norway 1984-2014 (cases per 100000) [3]
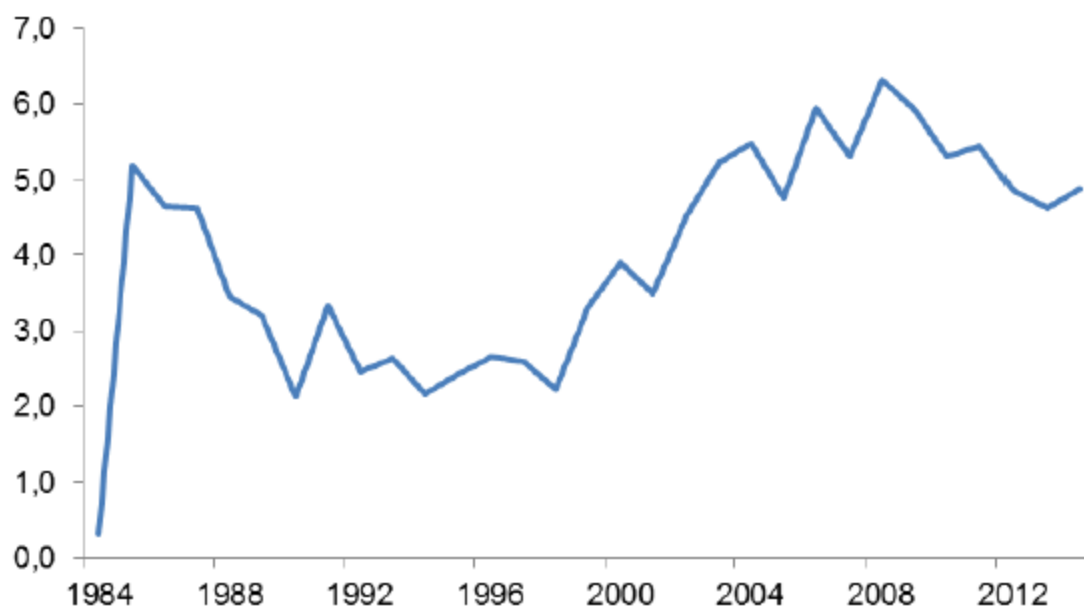
Source: The Norwegian Institute of Public Health

1.1.2 Pathophysiology

HIV belongs to the retroviridae class of viruses that have an RNA genome. There are two subtypes of HIV, HIV-1 and HIV-2. HIV-1 is the most virulent and causes the majority of infections worldwide. The virus spreads by entrance of contaminated bodily fluids such as blood or semen in the bloodstream. Sexual transmission is most common, followed by direct inoculation by needle sharing between intravenous drug users (IDU). However, transmission through blood transfusions and organ donation has become negligible due to rigid screening of donators. Mother-to-child transmission at birth and through breast-feeding still pose a challenge in resource-limited countries [4].

HIV targets mainly $CD4^+$ T cells, dendritic cells and macrophages [5]. After binding to specific receptors on these cells, the virus fuses with the cell and releases viral particles into the cell (figure 2).

Figure 2: Replication cycle of HIV [6]
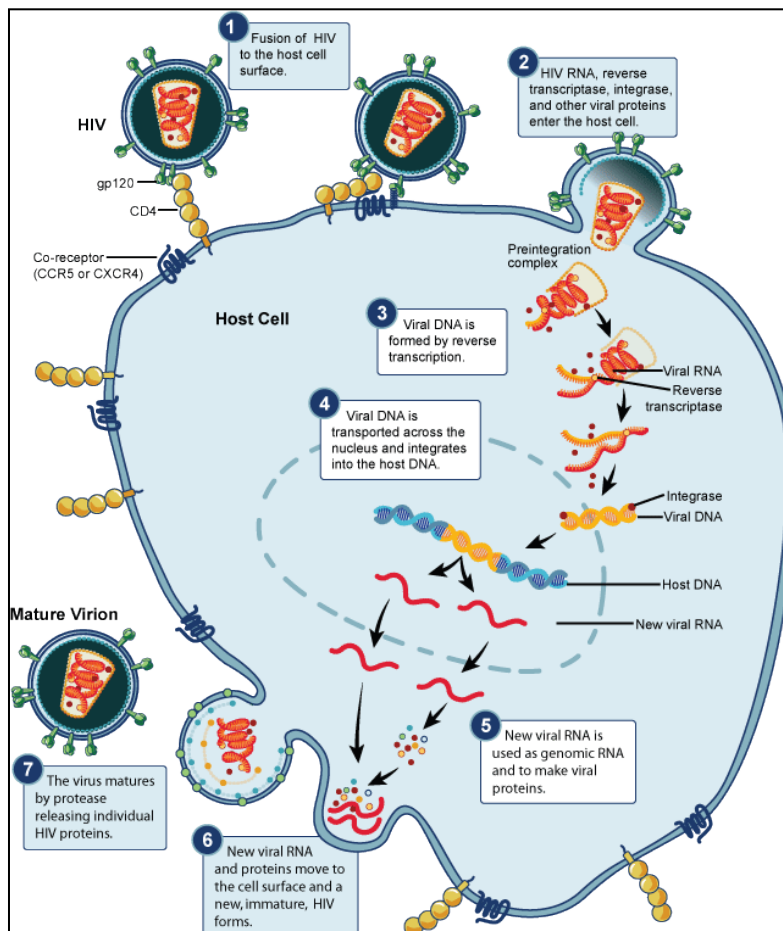
Adapted from the National Institute of Allergy and Infectious Diseases (NIAID) illustrations.

The HIV enzyme reverse transcriptase then converts viral RNA to DNA that enters the host cell's nucleus and integrates into the host cell's genome with the help of integrase enzyme. This viral DNA is called the provirus, which replicates whenever the host cell's DNA replicates. Replication produces the components necessary for creation of a new virus. The viral components are then assembled in the cytoplasm and released from the cell. Further viral maturation is propagated by protease-enzyme. Mature viruses then infect other cells and repeat this process that leads to gradual depletion of CD4$^+$ T cells.

Initially, HIV replication occurs unchecked by the host immune defenses due to the lack of HIV-specific responses. HIV RNA levels in the plasma can be very high in the acute phase of the infection. The development of HIV-specific CD8$^+$ cytotoxic cells results in reduction of viral levels and a chronic disease state. CD4$^+$ T cells are vital for combating infectious agents where cell-mediated immunity plays a central role. Persons living with HIV become susceptible to various opportunistic infections and malignancies that are otherwise not seen in immunocompetent individuals as their CD4$^+$ T cell levels deplete (figure 3). Some few HIV infected individuals can maintain low viral levels and normal CD4$^+$ T cell levels without treatment.

Figure 3: Relationship between HIV viral load and CD4+ T cell counts during the clinical course of HIV infection [7]
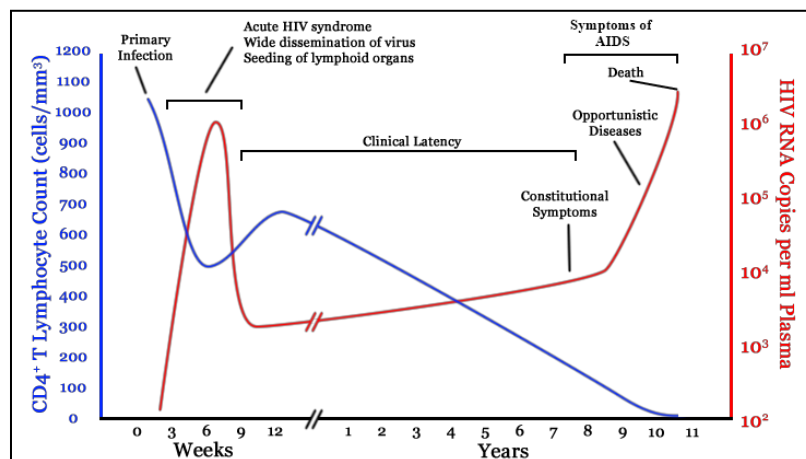
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1.1.3 Clinical manifestations and diagnosis

Nonspecific symptoms such as fever, lymphadenopathy and myalgia may be seen in patients with acute HIV infection, which usually develops 2-4 weeks after HIV exposure. Patients

may also have a generalized rash or may complain of sore throat. These symptoms usually resolve spontaneously after a period of days to weeks. Due to the likeness of symptoms to influenza, mononucleosis and various other self-limiting viral infections the diagnosis of acute HIV infection is often missed [8]. Opportunistic infections are seldom seen in the acute phase. Symptoms that may develop as chronic infection progresses are persistent lymphadenopathy, unexplained weight loss (wasting), fever, peripheral neuropathy and dementia. AIDS, the condition in which patients develop opportunistic infection or cancer, usually occurs after years of infection. The most common opportunistic infections and malignancy observed with progressive immune deficiency are mucosal fungal infections, herpes simplex, PCP, tuberculosis, bacterial pneumonia, Kaposi's sarcoma and lymphomas.

In previous years diagnosis of HIV consisted of enzyme immunoassay antibody testing with a confirmatory Western blot. Now, fourth generation assays that detect HIV p24 antigen are used followed by a confirmatory HIV-1/HIV-2 antibody differentiation immunoassay if positive. HIV RNA PCR is done if serological tests are inconclusive or when acute infection is suspected. HIV-provirus-DNA can also be measured in the early stages of infection.

1.1.4 Treatment

HIV infection is to date an incurable disease. Antiretroviral therapy (ART) reduces plasma viral load and in so doing allows normalization of $CD4^+$ T cell levels. However, when treatment is stopped a resurgence of virus counts occurs. Recommended ART consists of three or more drugs that target different steps in the HIV replication cycle. In general, ART is initiated at specified $CD4^+$ T cell levels, in hepatitis co-infection, when patients present with AIDS and to pregnant women [9]. Some guidelines also suggest initiation of treatment in cases of acute retroviral syndrome and for general prevention of sexual transmission of the infection to others.

1.2 Tuberculosis

1.2.1 The Epidemic

Despite the identification of *Mycobacterium tuberculosis* (*M.tb*) as the cause of tuberculosis (TB) in the 1800s, TB continues to be a global challenge. TB is the second most common cause of death by a single infectious agent worldwide with an estimated 9.0 million persons who developed TB and 1.5 million deaths in 2013 [10]. Underdeveloped countries, especially

on the Asian and African continent, bear the highest disease burden. Multidrug-resistant TB (MDR-TB) is an increasing challenge globally and accounted for 3.5% of newly diagnosed and 20.5% of previously treated TB in 2013. Norway was a TB high-burden country until the end of the second world war after which rates dramatically declined to low incidence rates in the 1990s [11,12]. Since 1995 there has been a gradual increase in new TB cases, with 80-90% occurring in immigrants (figure 4). In 2013 the incidence of TB among ethnic Norwegians was < 1 per 100000 population compared to 251/100000 for African born immigrants living in Norway.

Figure 4: Tuberculosis cases in Norway 1978 – 2012 [11]
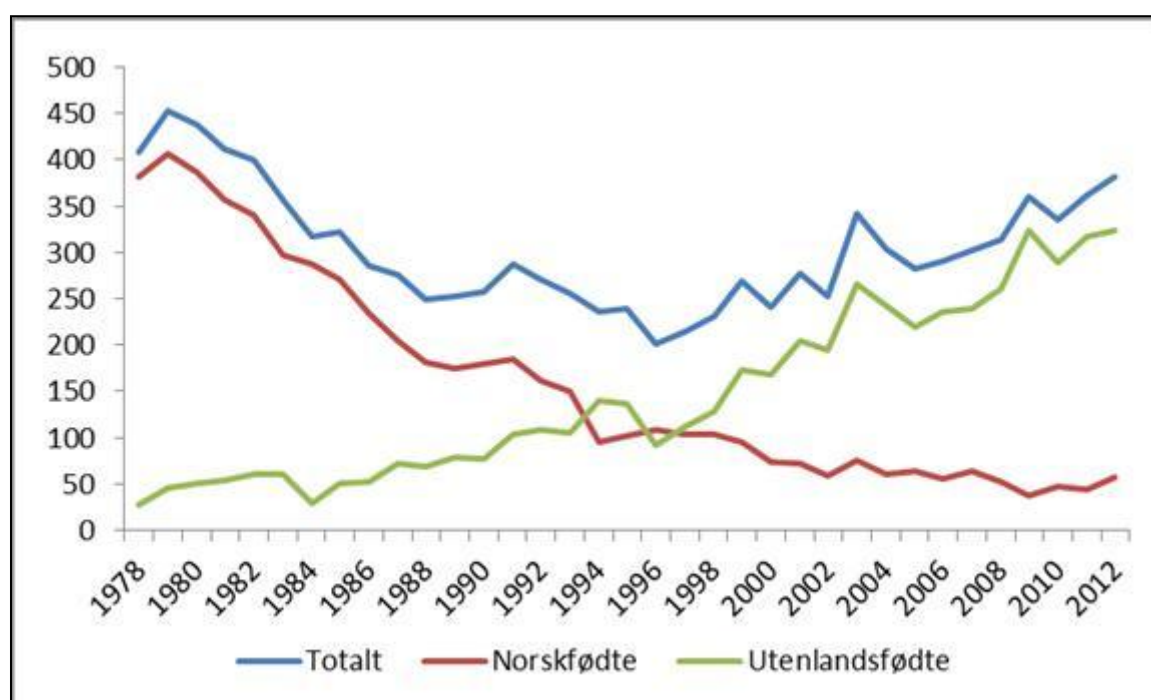
Norwegian-born (Norskfødte), foreign-born (utenlandsfødte) and total (totalt).
Source: The Norwegian Institute of Public Health

1.2.2 Pathophysiology

Humans are the only known natural reservoir for *M.tb*, which is transmitted by inhalation of aerosol containing bacilli from an individual with infectious pulmonary TB. *M.tb* is an intracellular pathogen that targets alveolar macrophages in the respiratory tract. Innate and adaptive immune responses, where T-cell mediated processes play a vital role, are triggered to eliminate or contain the infection. Formation of a granuloma or tubercle consisting of bacilli contained by monocytes, macrophages and neutrophils, classically observed in the upper lung lobes, is the hallmark of primary TB infection. Recent non-human primate studies have

revealed that there is a complex combination of immune responses that vary within each granuloma in a single subject irrespective of TB disease state [13]. An intricate balance of both pro- and anti-inflammatory responses was shown to be necessary for disease containment. If bacilli replication continues unchecked the tubercle increases with subsequent spread of the disease to lymph nodes or through the blood stream to other organs. The Ghon complex signifies primary TB infection and consists of calcified granuloma in the lung and involvement of hilar lymph nodes. In the natural cycle of the disease there is evidence that 50-70% of exposed individuals clear the infection completely, whereas approximately 5% of the remainder develop active disease within 2-5 years after being infected [14]. Latent TB infection (LTBI), whereby the infection is contained without symptoms or signs of active disease, develops in approximately 95% of the remaining individuals exposed (figure 5). Individuals with LTBI and an intact immune system have a 10% lifetime risk of developing active TB. The World Health Organization (WHO) estimates that a third of the world's population have LTBI [15].

Figure 5. The spectrum of *Mycobacterium tuberculosis* infection and the life cycle of *M. tuberculosis* [14]
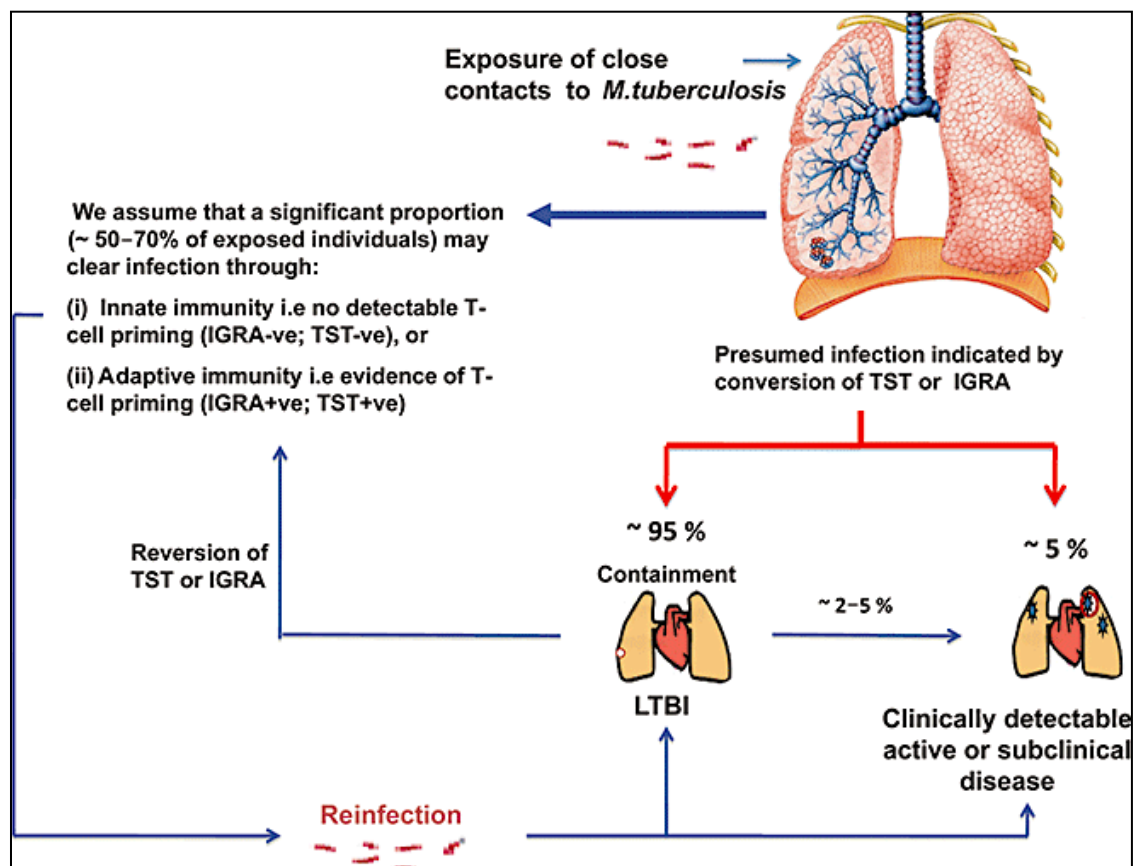
In the traditional understanding of TB infection active TB and LTBI represent two distinct clinical stages, with the former consisting of active bacterial replication and the later consisting of dormant bacteria [16]. However, animal, autopsy and PET-scan studies have revealed pathological findings in active TB varying from sterile lesions to lesions with high bacterial load. Furthermore, LTBI has been shown to include a spectrum from patients in which the infection is completely resolved to those who are asymptomatic despite having viable replicating bacteria [16-18]. Indeed, isoniazid (INH), which is known to only be active against replicating M.tb, is efficient treatment for LTBI, further challenging the traditional view of LTBI. These newer perspectives have led to an understanding of TB infection as a dynamic process ranging from clearance of infection to clinical active disease determined by the immune response (figure 6).

Figure 6: Newer understanding of TB infection reflecting a spectrum of immune responses [17]
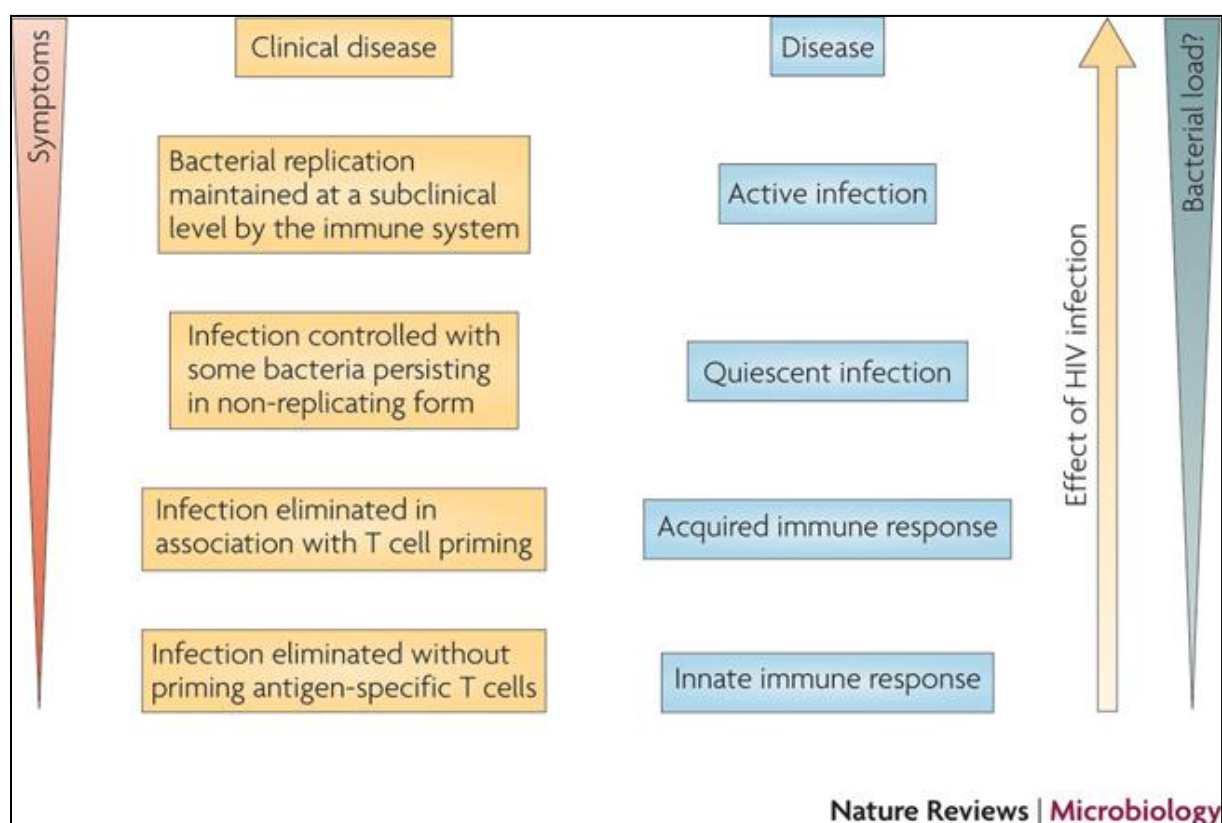
1.2.3 Symptoms and presentation

Symptoms of TB are nonspecific and include fever, cough, dyspnea, chest pain, haemoptysis, night sweats and weight loss. Cough lasting two weeks or more, fever, weight loss, drenching

night sweats or a combination of these is most often reported in patients with active TB [19-22]. Extrapulmonary TB may present with fever, weight loss, night sweats as well as pain in the affected area (for example bone affection and spinal affection in Pott's disease) or enlarged lymph node (lymphadenitis or scrofula).

TB can affect any organ in the body, however the majority of TB cases present as pulmonary TB. Among 5.4 million new TB cases reported in 2013, 85% had pulmonary TB (57% laboratory confirmed) and 15% had extrapulmonary TB [10]. The most common extrapulmonary presentation is lymphadenitis, predominantly cervical but affection of other peripheral and abdominal lymph node sites are described [23,24]. Abdominal focus with abscess-formation, central nervous system, bone and urinary system may also be affected. Miliary TB previously used to describe disseminated pulmonary TB with multiple diffuse nodular lesions in the lungs is often used to denote TB involvement of several organs. This condition is most often seen in immunocompromised patients.

Chest x-ray findings in pulmonary TB may vary from normal or diffuse interstitial changes (mostly in immunocompromised persons) to localized cavitary lesions. Lung infiltrates are often accompanied by ipsilateral hilar adenopathy. Cavitary lesions are easier seen on computer tomography (CT), which is also the preferred mode for investigation of peripheral and abdominal lymphadenitis and other abdominal involvement. CT scans can also reveal lung changes and pleural effusion in immunocompromised patients with a normal chest x-ray. TB disease of bone, spinal and CNS are better seen on magnetic resonance imaging [25].

1.2.4 Diagnosis

Direct microscopy and culture of sputum and other relevant specimens have for several years been the main tools in diagnosis of active TB. Microscopy is however an insensitive method, especially in children and immunocompromised individuals and it cannot distinguish between non-tuberculosis mycobacteria (NTM) and *M.tb* [26]. The gold standard for active TB diagnosis is positive culture, which can take several days with liquid media, or weeks if solid media are used, and thereby delay diagnosis. Development of PCR-based methods such as the GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, Ca, USA) has revolutionized diagnostics by providing more rapid identification of *M.tb* and microbial resistance [27]. However, GeneXpert MTB/RIF assay is expensive and currently only approved for sputum samples.

In contrast to active TB, there is no gold standard for LTBI diagnosis, which precludes accurate determination of sensitivity and specificity of available tests. The tuberculin skin test (TST) has traditionally been used for diagnosis of LTBI and is based on a delayed hypersensitivity reaction whereby T-cells previously exposed to TB recognize the antigen injected intradermally (figure 7).

Figure 7. Biological basis of the tuberculin skin test and interferon-assay [28].
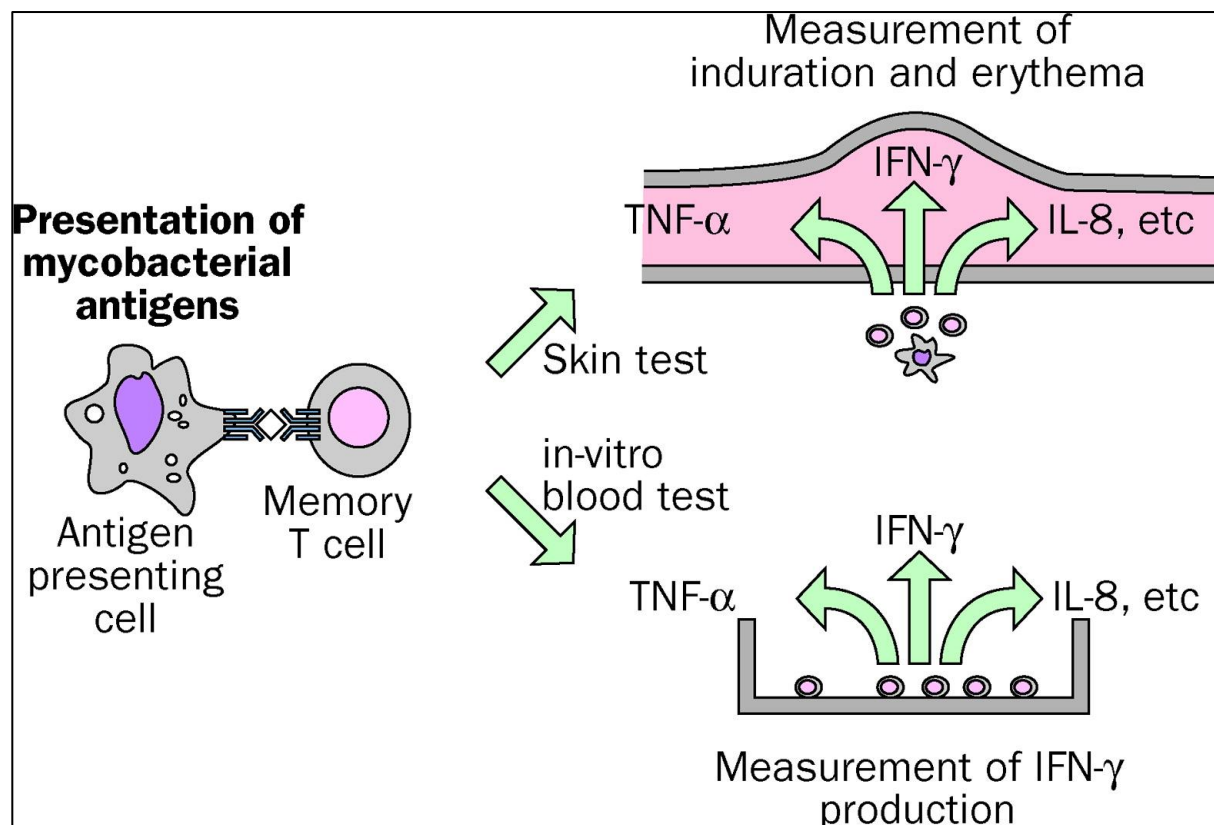
TNF α= tumor necrosis factor α; IFN-γ=interferon gamma; IL8=interleukin 8.
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The TST is however hampered by poor sensitivity in immunocompromised individuals and poor specificity due to cross reactivity with NTMs and the *Mycobacterium bovis* bacillus Calmette Guérin (BCG) vaccine [17]. In recent years Interferon-gamma (IFN-γ) release assays (IGRA) have replaced TST or been used as confirming tests after TST in certain patient groups [29]. IGRA tests measure IFN-γ released by CD4 cells after whole blood has been exposed to TB-specific antigens in vitro (figure 8). Two such tests are commercially available; T-SPOT.TB® (Immunotec, Oxford, UK) and QuantiFERON®TB Gold (Qiagen, Chadstone, VIC, Australia). T-SPOT.TB (TSPOT) is an enzyme-linked immunospot assay (ELISPOT) which measures the number of IFN-γ producing cells, QuantiFERON TB Gold

(QFT) is an enzyme-linked immunosorbent assay (ELISA) which measures the level of IFN-γ released in the plasma after stimulation.

Figure 8: Overview of the interferon-γ (IFNγ) assay technology [30]

These full-blood assays are expensive and require more laboratory infrastructure than the TST. IGRAs have proven to be more specific than the TST because the microbial antigens utilized,

early secretory antigenic target-6 (ESAT-6) and culture filtrate protein 10 (CFP10) are neither present in BCG nor in most NTMs. In addition, IGRA requires only one visit, compared to the TST, which requires visits both for administration of the test and for interpretation of the results. Still, the sensitivity of IGRA proves to be suboptimal in immunocompromised individuals, though better than TST [31-34].

1.2.5 Treatment of active TB

Good compliance and adequate duration and combination of anti-TB chemotherapy are important for the treatment of active TB, which is a curable infection. Guidelines typically suggest treatment regimens that include rifampicin, INH, pyrazinamide with or without ethambutol (depending on the risk of resistant TB) for two months followed by four months with rifampicin and INH [12,35,36]. Direct observed therapy (DOT) is strongly advised to assure patient compliance and in so doing avoid development of resistant TB. MDR-TB and extensively drug resistant TB (XDR-TB) require longer treatment duration with combinations of several older and newer drugs. MDR-TB is per definition bacilli that are resistant to at least INH and rifampicin. XDR-TB is resistant to at least INH, rifampin, fluoroquinolones and either aminoglycosides (amikacin, kanamycin) or capreomycin or both.

Sputum cultures after two months of therapy for pulmonary TB are performed to assess treatment efficacy. Sputum cultures are expected to be negative after two months of adequate therapy for drug-sensitive pulmonary TB. Self-reported symptoms are important and helpful in assessment for disease resolution and relapse as revealed in an international multicenter phase III trial, which reported a remarkable decline of fever and night sweats after two months therapy for drug-susceptible TB [19].

1.2.6 Treatment of LTBI

The main objective of screening for LTBI is to offer TB preventive treatment to persons at risk for TB reactivation. The rationale for providing preventive therapy is to prevent development of active TB on the individual level, thereby preventing spread of the disease on the community level. Due to the increased risk of adverse drug reactions such as hepatotoxicity and other side effects limiting compliance, only patients at risk should be offered TB preventive therapy [37]. Norwegian guidelines recommend preventive therapy to among others, persons with immune deficiency, before immune modulating therapy, and newly exposed individuals [12]. TB preventive therapy consists of either INH for six months

or INH, rifampicin for three months. Methods for monitoring the efficacy of preventive treatment are lacking, mainly due to the absence of a gold standard for LTBI diagnosis.

1.2.7 Vaccines

The BCG vaccine, which consists of live attenuated *Mycobacterium bovis*, has been in use since 1921 and is the only TB vaccine in use to date. The vaccine appears to be only protective against TB in childhood, and protection lasts approximately 10-15 years. Persons who are BCG vaccinated have strong TST reactions the first months after vaccination, but this reaction usually wanes over time. Several TB-low endemic countries have abandoned routine child vaccination, whereas childhood vaccination is the rule in middle to high-endemic countries. New types of vaccines are under development for therapeutic purposes as adjuncts to TB chemotherapy and for pre-exposure preventive purposes [38].

1.3 HIV and TB co-infection

1.3.1 The Epidemic

After an initial reduction in global TB rates there has been a new surge of cases corresponding to the emergence of the HIV epidemic. HIV is the most important risk factor for TB infection and TB is globally the most common opportunistic infection in HIV patients. According to WHO 13% of new TB cases globally in 2012 were HIV-positive, with the African continent bearing 75% of these cases. However, only 46% of notified TB patients had a HIV test performed which was though 15 times higher than the percentage tested in 2004 [15]. Higher mortality and morbidity complicate TB infection in HIV patients. Furthermore, HIV positive persons with LTBI have a yearly 10% risk of reactivation compared to a 10% lifetime risk for HIV negative individuals. Still, LTBI treatment reduces the risk of active TB by 32% in HIV patients [39].

To date we have no surveillance data on HIV/TB co-infections in Norway due to confidentiality considerations prohibiting identifiable registration of HIV status. However in 2012 co-infection with TB and HIV became a notifiable condition permitting data collection on prevalence, morbidity and mortality associated with double infections in Norway.

1.3.2 Pathogenesis

Cellular immune responses, particularly CD4$^+$ T cells, are central in controlling and containing *M.tb* infection. The selective depletion of CD4$^+$ T cells in HIV infection contributes to the increased risk of TB infection and reactivation of LTBI. Not only does HIV reduce the number of CD4$^+$ T cells but there is evidence suggesting that HIV also impairs *M.tb* specific T cell responses [40]. Further, it is apparent that HIV leads to an alteration of macrophage function, which renders them unable to combat the bacilli. Indeed, it is proposed that these factors lead to dysfunctional granuloma formation which may also explain the increased morbidity and mortality associated with TB in HIV infected patients. In turn, studies have shown that TB also promotes HIV replication by several mechanisms [40,41]. Activated T cells, generated due to TB infection, are preferred targets for HIV. In addition, cytokine production required for control of the TB infection promote replication of the HIV virus [41]. However, full knowledge of the pathogenesis of interaction between HIV and TB infection is not yet attained. Nevertheless, it is clear that treatment of HIV with ART reduces risk of TB [42].

1.3.3 Challenges of HIV/TB co-infection

Dual infection with HIV and TB poses several challenges related to clinical presentation, diagnosis and treatment.

1.3.3.1 Atypical symptoms and presentation

Symptoms of TB such as weight loss, fatigue and fever could overlap with symptoms of opportunistic infections in HIV infection. Indeed, HIV patients often lack typical symptoms associated with TB leading to missed cases [43-45]. Pulmonary TB may be seen at all levels of immunosuppression, but is more common at higher CD4$^+$ T cell levels. Extrapulmonary and miliary TB becomes more prevalent at lower CD4$^+$ T cell counts. Furthermore, as CD4$^+$ T cell counts decline granuloma formation is disrupted and cavitary lesions are not seen.

1.3.3.2 Drug interactions

To further complicate matters, treatment for HIV-associated TB has the potential of causing unfavorable drug interactions between ART and TB chemotherapy. In ART-naive HIV patients it is advised that they start ART within 2-8 weeks of TB treatment depending on the degree of immunodeficiency [9,12]. Rifampicin, an important component of TB therapy, is a potent inducer of the cytochrome P450 isoenzymes that control drug metabolism. The serum

concentration of the non- nucleoside reverse transcriptase inhibitor (NNRTI) and protease inhibitor (PI) classes of ART are reduced when combined with rifampicin as a result of increased metabolism [46]. Rifabutin, which is a less potent inducer of cytochrome P450, is an alternative to rifampicin, however its concentration may be reduced by the HIV medication efivarenz. Co-toxicity and increased side effects influence on patients' quality of life and HIV patients with TB end up taking a cocktail of pills for 6 months or more, which may affect motivation and compliance to therapy.

1.3.3.3 Immune reconstitution inflammatory syndrome

Immune reconstitution inflammatory syndrome (IRIS) that may be observed after initiation of ART consists of an apparent worsening of treated infections or unmasking of subclinical or latent infections. This reaction is an expression of the improvement of immune defenses in response to ART and is often seen in HIV patients with TB disease [41]. IRIS is usually self-limiting and manageable, but is potentially fatal, especially in cases of TB meningitis. Treatment of IRIS involves continuation of ART, specific therapy for the unmasked opportunistic infection and in some cases corticosteroid treatment to reduce inflammation.

1.3.3.4 Diagnostic challenges

Chest x-rays may be normal in up to 20% of HIV patients with pulmonary TB and sputum microscopy may be false negative [43-45,47]. Furthermore, extrapulmonary and disseminated TB disease that occurs often in HIV infected is difficult to diagnose. Several studies have been conducted to find effective algorithms for screening HIV infected for active TB that include routine chest x-ray despite the lack of symptoms and sputum analysis with GeneXpert MTB/RIF assay and urine samples for LAM which would increase case-detection [20,45,48-50]. According to the latest reviews the GeneXpert MTB/RIF assay had a pooled sensitivity of 79% (95% CI 70-86%) in HIV-infected persons [51].

Due to the increased risk of reactivation with resulting higher mortality and morbidity associated with HIV-associated TB, HIV patients should be screened for LTBI and offered prophylactic treatment if positive [9,12,35,36]. TST and in recent years IGRA have been used for this purpose. Neither TST nor IGRA can distinguish between active TB and LTBI and both tests can remain positive in persons previously treated for TB. Furthermore, both tests can be false negative due to anergy in patients with advanced HIV infection [34,52,53]. There is therefore still a need for efficient methods for diagnosing TB in HIV positive individuals.

1.4 Biomarkers for TB

It is estimated that one-third of new TB cases are undiagnosed each year due in part to the lack of accurate, inexpensive and rapid point-of-care diagnostic methods [27]. In the search for new TB diagnostics many studies have focused on biomarkers that can precisely predict TB disease, treatment outcome, and vaccine protection [54-56]. Biomarkers of a condition should ideally be present in the presence of the disease and should disappear when the condition is eliminated. Sputum culture after 2 months of active TB treatment is used to monitor treatment efficacy, however, it is inadequate in cases with immunodeficiency and extrapulmonary TB. In extrapulmonary TB, radiological evidence of resolution as well as reduction of inflammatory markers such as C-reactive protein (CRP) and the erythrocyte sedimentation rate (SR) are useful in monitoring treatment response [57,58]. Other diagnostics include detection of the lipoarabinomannan (LAM) in urine by commercially available enzyme-linked immunosorbent assay (ELISA). LAM is a lipopolysaccharide that is a major component of the mycobacterial cell wall. Its detection in urine is associated with disseminated TB and this test is therefore most useful in immunocompromised patients [59]. LAM has a reported sensitivity of 21-67% in HIV-infected patients compared to 6-21% in HIV negative TB patients. The sensitivity of LAM is higher in patients with advanced HIV disease due to higher mycobacterial load with a reported sensitivity of 56-85% in patients with CD4 counts <50 cells/μL. Additionally, reduction of LAM in urine after 1-2 months of combined TB and ART has been described in one study, however the utilization of LAM is restricted by its poor sensitivity in HIV patients with higher CD4 counts [60].

Several cytokines and chemokines are released as part of the immune response to TB infection and are candidates for biomarkers of TB infection stages (figure 9) [54]. The availability of rapid and simple multiplex assays for measuring plasma cytokine and chemokine levels has allowed for studies exploring their use as biomarkers. Most studies suggest an approach involving a combined panel of biomarkers adjunctive to IFN-γ for distinguishing between active TB and LTBI.

Figure 9. Schematic overview of key cells and cytokines involved in the immune response towards *Mycobacterium tuberculosis*-specific antigens [54]
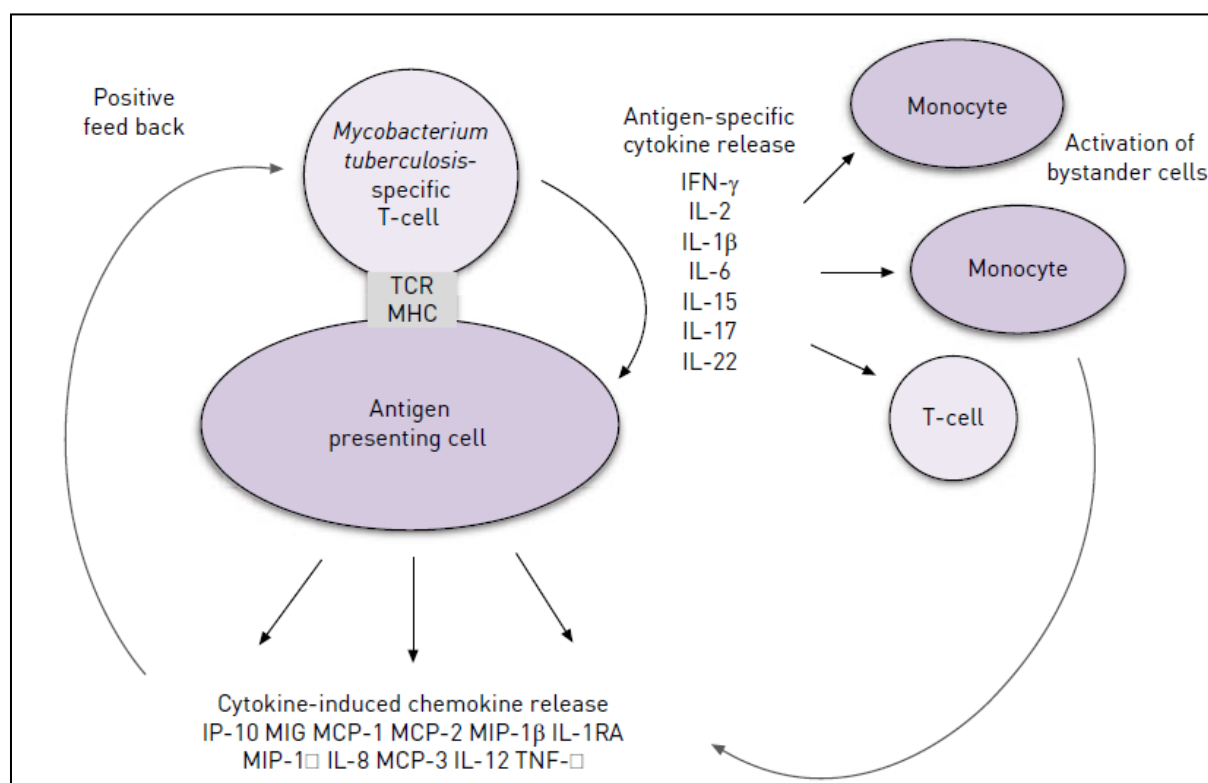
IFN: interferon; IL: interleukin; TCR: T-cell receptor; MHC: major histocompatibility complex; IP-10: IFN-γ-induced protein 10; MIG: monokine induced by IFN-γ; MCP: monocyte chemotactic protein; MIP: macrophage inflammatory protein; IL-1RA: IL-1 receptor antagonist; TNF: tumor necrosis factor.
Adapted from Chegou et al, Eur Respir J. Copyright ERS 2014. Public license Hyperlink: creativecommons.org
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1.4.1 Interferon-γ (IFN-γ)

IFN-γ is mainly produced by CD4+ T helper cells that are activated after recognizing antigen peptide presented by the antigen presenting cell (APC). This cytokine plays a central role in cell-mediated immunity by activating phagocytes and homing other immune cells to the site of infection. INF-γ induces production of other chemokines that are vital in raising adequate responses to infection. TST measures skin induration that correlates to INF-γ release *in vivo*, whereas IGRAs measure INF-γ release *in vitro*. Table 1 shows operational principles and test performance for TST, QFT and TSPOT. According to the latest reviews, IGRAs have superior specificity to TST in BCG-vaccinated populations, however sensitivity is comparable [61-63]. Some studies have also analyzed IFN-γ levels in BAL, pleural effusion, ascites fluid and cerebral spinal fluid using IGRA. According to a 2011 meta-analysis by Sester *et al* pooled sensitivity for TSPOT on such extrasanguinous samples in culture-confirmed cases

was 88% (95% CI 81-93%) compared to 52% (39-64%) for QFT [61]. QFT-plus, which also measures IFN-γ released as a result of CD8+ response has recently been launched [64].

Table 1: Comparison of test characteristics of TST, QFT and TSPOT [33,34,61]

Characteristics	TST	QFT	TSPOT
Need for laboratory infrastructure	No	Yes	Yes
Test format	In-vivo (Intradermal)	In-vitro (ELISA)	In-vitro (ELISPOT)
Test antigens	PPD	ESAT-6, CFP-10, TB 7.7	ESAT-6, CFP-10
Test substrate	Skin	Whole blood	Peripheral blood mononuclear cells
Need for return visit	Yes	No	No
Test measurement (units)	Induration after 72 hours (mm)	IFN-γ concentration (IU/ml)	Number of IFN-γ producing cells (spot forming units – SFU)
Time to result	72 hours	16-24 hours	16-24 hours
Cross reactivity with BCG	Yes	No	No
Cross reactivity with NTM	Yes	Less likely (possible with *M.kansasi*, *M.marinum*, *M.szulgai*)	Less likely (possible with *M.kansasi*, *M.marinum*, *M.szulgai*)
Boosting	Yes	No	No
Inter-reader variability	Yes	No	Possible (manual counting)
Sensitivity (95% CI)	General: 77% (71-82%) HIV-positive: 60% (34-82%)	General: 80% (75-84%) HIV-positive: 61% (54-67%)	General: 81% (78%-84%) HIV-positive: 65% (56-74%)
Specificity (95% CI)	BCG vaccinated: 59% (46-73%) Non-vaccinated: 97% (95-99%)	BCG vaccinated: 96% (94-98%) Non-vaccinated: 99% (98-100%)	BCG vaccinated: 93% (86-100%)

1.4.2 Interferon-γ inducible protein (IP-10)

IP-10 is a chemokine that has been most studied as a surrogate immunodiagnostic biomarker. APCs release IP-10 in response to stimulation by several cytokines, but mainly IFN-γ and TNF-α. IP-10 is released in much higher amounts than IFN-γ which makes it ideal for use as a biomarker [65]. Studies comparing IP-10 and IGRA reveal similar sensitivity and specificity, and it is suggested that combining the tests could increase sensitivity for active TB [54]. In addition it seems apparent that IP-10 is less affected by CD4^{+} cell levels than IGRA [66]. IP-10 is however not specific for TB, but is increased in other inflammatory conditions such as bacteremia, hepatitis C and HIV-associated opportunistic infections and malignancy [67-70].

1.4.3 Other chemokines and cytokines

Monokine induced by IFN-γ (MIG-γ), macrophage inflammatory protein (MIP-1α, MIP-1β), monocyte chemotactic protein (MCP-1, MCP-2, MCP-3) are all chemokines that have been studied as potential biomarkers, however with variable outcomes [54].

Tumor necrosis factor alpha (TNF-α) is a cytokine that play a central role in macrophage activation and in increasing cytokine and chemokine production during mycobacterial infection [71]. TNFR1 and TNFR2 are two cell surface receptors mediating TNF-α functions. The shedded extracellular portions of these receptors circulate in plasma as soluble TNFr1 (sTNFr1) or sTNFr2 which have inhibitory effect on TNF-α by competing with TNF receptors. The protective effect of TNF-α is illustrated by the increased risk of TB reactivation in patients receiving anti-TNF or sTNFr treatment for chronic inflammatory disease [72,73].

Interleukins (IL) are cytokines (except for IL-8 which is a chemokine) produced in general by activated T-cells, macrophages, natural killer cells and dendritic cells. Several cytokines in the interleukin group have been explored as potential biomarkers for TB. IL-2 plays a central role in T-cell proliferation and in regulating T-cell production of IFN-γ. Some studies have revealed comparable performance of IL-2 to IFN-γ and IP-10 as a biomarker for active TB and a recent meta-analysis revealed a pooled sensitivity of 81% and specificity of 95% for detection of LTBI [54,74]. The pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 have both been noted to increase after BCG vaccination [75]. In a recent study IL-10 levels were increased during TB treatment and subsequently decreased at the end of treatment [76]. Lower levels of the pro-inflammatory cytokine IL-17 have been reported in active TB patients compared to contacts or LTBI patients [77]. It appears that IL-17 is produced at an early stage of the immune reaction to TB infection as supported by findings of a higher IL-17 levels preceding TST conversion [78].

1.4.4 PTX3 and CRP

The proteins CRP and PTX3 belong to the pentraxin family and are part of the acute-phase reaction in response to inflammation. The short pentraxin CRP is produced in the liver in response to mainly IL-6, whereas the long pentraxin PTX3 is produced by many different cell types, predominantly macrophages and dendritic cells. Serum CRP concentration has for

years been a standard test for analyzing the extent of inflammation in disease. A study conducted among HIV-infected patients in South Africa revealed that CRP had a 100% negative predictive value at very low CRP levels (<1.5mg/l) and 100% positive predictive value at very high levels (>400mg/l) [79]. However, these cut-off values included only a minority of patients tested and identified only a minority of patients with active TB suggesting inadequacy of CRP in screening. Nevertheless, CRP values $\geq$ 50mg/l were strongly associated with a poor prognosis in this study and a previous study (among HIV-negative patients) by the same author suggests that CRP is a good correlate of TB treatment response [57]. The use of PTX3 plasma levels for active TB diagnosis and treatment monitoring in HIV-uninfected patients was examined by Azurri *et al* who found that levels were higher in active TB patients compared to healthy household controls [80]. Furthermore, PTX3 levels decreased with successful TB treatment and increased in patients with treatment failure.

1.5 Vitamin D and tuberculosis

Research the last few years have revealed that vitamin D is not only vital for bone homeostasis but also plays an important role in chronic disease processes such as chronic infections, cardiovascular disease and cancer [81]. Vitamin D is a fat-soluble vitamin primarily derived by the action of sunlight on the skin, whereby ultraviolet B radiation convert 7-dehydrocholesterol in the skin to cholecalciferol (vitamin D3). Vitamin D can also be obtained from dietary sources as cholecalciferol and ergocalciferol (vitamin D2). With the help of vitamin D-binding protein (DBP), cholecalciferol and ergocalciferol are transported in the bloodstream to the liver which converts both into 25-hydroxyvitamin D [25(OH)D]. The active form of the vitamin, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] is produced by the kidney which converts 25(OH)D to $1,25(OH)_2D$ by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1). CYP27B1 is also present in macrophages which can also produce $1,25(OH)_2D$ when activated by IFN-γ [81]. Unlike renal production of $1,25(OH)_2D$ which is regulated by calcium and phosphorus via parathyroid hormone (PTH), macrophage production is regulated by inflammatory cytokines. Both 25(OH)D and $1,25(OH)_2D$ circulate bound to vitamin D-binding protein (DBP) and the binding of the active metabolite to the vitamin D receptor (VDR) on target cells initiates vitamin D-mediated processes [82-85].

1,25(OH)₂D also has a self-regulatory role by activating the production of 24-hydroxylase (CYP24) which catabolizes both vitamin D metabolites.

Despite being the inactive form of vitamin D, 25(OH)D is the main circulating form in the blood and is thought to best reflect vitamin D status. This is due to the fact that $1,25(OH)_2D$ has a shorter half-life and circulates at lower levels than 25(OH)D [86]. Furthermore, $1,25(OH)_2D$ can be normal or increased in vitamin D deficiency as a result of secondary hypoparathyroidism. Therefore, $1,25(OH)_2D$ is only recommended in monitoring acquired and inherited disorders of vitamin D and phosphate metabolism [86,87]. According to The Endocrine Society guidelines, 25(OH)D levels < 20 ng/ml (50 nmol/l) are defined as vitamin D deficiency and levels of 21-29 ng/ml (50-75 nmol/l) are defined as vitamin D insufficiency, whereas normal values range from 30 to 50 ng/ml. Causes of vitamin D deficiency include skin pigmentation, low exposure to sunlight and malnutrition. Studies have shown that genetic polymorphisms of BDP and VDR can affect bioavailability of, and responsiveness to $1,25(OH)_2D$, respectively [81,82].

The actions of vitamin D in immune regulation is incompletely understood, but studies have shown that $1,25(OH)_2D$ play a role in innate and adaptive immunity with VDRs being expressed in most proliferating cells of the immune system [82,82,83,88,88]. The antimycobacterial potential of vitamin D was suggested by the historical use of cod-liver oil in the treatment of TB [89]. Early experiments revealed that $1,25(OH)_2D$ inhibited the growth of *M.tb* in macrophages, an effect that is enhanced by IFN-γ [81,88]. It has since been discovered that $1,25(OH)_2D$ has mainly an immunosuppressive effect, but also has immunostimulatory effect important for controlling *M.tb* infections [81,85,88,90]. The immunostimulatory effects include the production of the antimicrobial protein cathelicidin and the enhancement of phagocytosis. Immunosuppressive effects include suppression of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokines, which may contribute to limiting tissue damage.

During the last decade a variety of studies, mostly from TB endemic countries, have shown that 25(OH)D levels are low in patients with TB compared to controls suggesting an association between vitamin D deficiency and TB susceptibility [82,84,88]. Furthermore, vitamin D status may have prognostic potential in HIV, as deficiency is associated with progression [91-93]. Studies of the association between vitamin D and HIV/TB co-infection conducted in African countries with regular sunlight have however reported both higher,

lower and comparable vitamin D levels compared to controls [94-98]. Still, intervention studies with vitamin D supplementation to TB treatment have been inconclusive [99-102].

2 AIMS OF THE THESIS

The main goal of this study was to examine the prevalence of active TB and LTBI among HIV-infected persons living in Norway and explore diagnostic methods and biomarkers for TB disease in this low-endemic setting.

2.1 Specific aims

2.1.1 Paper 1

To measure the prevalence of positive IGRA and TST, evaluate concordance between tests and correlate findings to clinical, demographical and epidemiological background factors in HIV-positive persons living in a low-endemic country.

2.1.2 Paper 2

To observe for development of active TB in LTBI patients and study changes in INF-γ responses over time in HIV-infected patients with LTBI (treated and untreated), active TB, prior active TB and no TB infection over a 2-year period.

2.1.3 Paper 3

To compare levels of cytokines and chemokines in plasma from HIV-negative and HIV-positive persons with and without TB infection, and explore their potential use as biomarkers for TB diagnosis and treatment responses.

2.1.4 Paper 4

To compare 25(OH)D and 1,25(OH)$_2$D levels in HIV positive patients with active TB, LTBI, prior active TB and no TB infection, and correlate vitamin D levels to *M.tb*-specific INF-γ levels.

3 MATERIAL AND METHODS

3.1 Study design

Table 2 summarizes the study design utilized for each paper.

Table 2: Overview of study design for each paper

Paper	Study design	Data collected
1	Cross-sectional study of HIV infected persons recruited in the period January 2009 until October 2010	Demographics, clinical, immunological and microbial data on HIV and TB, IGRA, TST.
2	Prospective, comparative cohort study with follow-up over 24 months	Follow-up QFT, CD4, TB exposure, clinical follow-up.
3	Comparative retrospective study of HIV infected persons from the above cohort and HIV negative with and without TB.	Plasma cytokines, chemokines and growth factors. Pre- and post-treatment results in active TB patients.
4	Cross-sectional study of a subset of HIV patients from the above cohort	Vitamin D and QFT in LTBI, active TB prior active TB and no TB patients

3.2 Data collection

3.2.1 Inclusion and follow-up (Paper 1 & 2)

The follow-up and treatment of HIV-infected persons in Norway is centralized to Infectious diseases or Internal Medicine clinics, usually one in each municipality or county. HIV testing is routinely performed in pregnant women, before surgical procedures, blood and milk donations, and before fertility treatments. Otherwise, HIV tests are performed based on clinical suspicion or in persons otherwise at risk for acquiring the infection. The study population in paper I and II consists of individuals ≥ 18 years of age with known HIV-infection that attended routine follow-up at seven infectious diseases clinics spread throughout Norway. Participants were recruited during the period of January 2009 to October 2010 from the following clinics: University Hospital of Northern Norway, St. Olavs University Hospital in Trondheim, Haukeland University Hospital in Bergen, Haugesund

Hospital, Vestre Viken Hospital in Drammen, Aker University Hospital in Oslo, Sørlandet Hospital in Kristiansand. Exclusion criteria were missing QFT (figure 11).

All HIV infected persons attending the seven participating clinics were informed about the study. The attending clinician filled out a questionnaire for each person that agreed to participate in the study. The questionnaire (appendix I) at inclusion consisted of demographical detail, background information on HIV infection obtained from clinical records, background information on TB such as BCG vaccination and previous close contact with a contagious active TB patient.

All participants were questioned about TB-like symptoms such as fever, weight loss, cough, hemoptysis, night sweats and swollen glands. This was followed by a clinical evaluation and chest x-ray. A sputum sample was obtained and blood was taken for IGRA and other tests including CD4 and CD8 cell counts, HIV virus load, SR, CRP. All participants were then referred to Mantoux testing.

Participants with a positive IGRA or suspected TB infections underwent intensified screening for active TB with an additional induced sputum sample or bronchoalveolar lavage (BAL). Organ specific scans and biopsies were obtained in selected patients with symptoms or signs indicating TB disease in extrapulmonary sites. Based on the results of this screening participants were assigned to one of the five study groups.

During the follow-up phase of the study QFT was taken at 3, 6, 12 and 24 months after starting therapy in active TB patients and treated LTBI patients. For untreated LTBI patients, QFT was taken at the same time intervals. CD4 cell counts were obtained simultaneously and participants were questioned about symptoms and any travel to TB endemic countries or new TB exposure between testing points. QFT and CD4 cell counts were taken at 12 and 24 months and information about symptoms and possible TB exposure were registered for participants in the untreated groups "prior active TB", "prior latent TB" and "no TB" groups.

Figure 11: Paper 1 & 2: Flowchart showing study method for inclusion, clinical evaluation and follow-up of study participants

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┌─────────────────────────────────────────┐
│        HIV positive ≥18 yrs             │
│  Period of inclusion: Jan 2009 – Oct 2010│
│              7 hospitals                 │
└─────────────────────────────────────────┘
                    │
┌─────────────────────────────────────────┐
│  Questionnaire: Demographics, HIV, TB    │
│          risk, TB symptoms               │
│          Clinical evaluation             │
└─────────────────────────────────────────┘
                    │
┌─────────────────────────────────────────┐
│ Chest x-ray, induced sputum, routine     │
│ blood tests (including CD4, HIV virusload),│
│ QuantiFERON-TB Gold, TSPOT.TB (1         │
│ hospital), TST (after IGRA and within 3  │
│ months after inclusion)                  │
└─────────────────────────────────────────┘
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Active TB	Latent TB +/- treatment	Prior active TB	Prior latent TB	No TB

Routine blood tests and QFT at 3, 6, 12 and 24 months	Routine blood tests and QFT at 12 and 24 months

3.2.2 Data from TB nurse coordinators (paper 2)

Since surveillance data on TB/HIV co-infections in Norway were lacking when this study was initiated, we contacted TB care nurses who have an administrative role in the follow-up of patients on TB treatment. TB care nurses from municipalities in Norway filled out a questionnaire with the following questions: 1) Are TB patients routinely tested for HIV in your municipality, 2) For years 2003-2010: number of persons treated for active TB, numbers co-infected with HIV, numbers with unknown HIV status, 3) For years 2003-2010: number of persons treated for LTBI, numbers co-infected with HIV, numbers with unknown HIV status. Systematic information was lacking for 2003 and 2004; therefore only data from 2005-2010 was used. As a quality control numbers of active TB patients each year were checked up against data from the Norwegian Surveillance System for Communicable Diseases (MSIS).

3.2.3 Inclusion and follow-up (paper 3)

The HIV positive participants in paper 3 were included from the cohort in paper 1 and 2 above. Results from multiplex analysis were registered for randomly selected patients with LTBI, active TB and negative QFT. Plasma from HIV negative patients with active TB from a previous study was also analyzed [103]. These patients had been prospectively included at the Oslo University Hospital, Norway and followed up during a period of 24 weeks of anti-TB chemotherapy. Blood samples were taken before initiation of chemotherapy and at 2-4 weeks, 6-12 weeks and 12-24 weeks of therapy. Plasma was immediately harvested from these blood samples, frozen and stored at $-80°C$ until analysis. Plasma from HIV negative patients with LTBI from another Norwegian study was analyzed [104]. These patients were recruited from the Haukeland University Hospital in Bergen where they were referred for evaluation for active TB or LTBI due to a positive TST and/or suspected TB exposure. Only plasma from patients with a positive QFT and no indication of a previous or current active TB were analyzed. Controls consisting of age-matched QFT negative/HIV negative individuals were Norwegian employees with negative TST and no known TB exposure recruited from the Haukeland University Hospital.

3.2.4 Inclusion paper 4

HIV positive patients were included from the cohort in paper 1-3. Analysis of 25(OH)D and 1,25(OH)$_2$D were performed on frozen serum samples from these patients. *M.tb*-specific IFN-γ was measured using QFT.

3.3 Study population

3.3.1 Definition of study groups

- Active TB

The international case definition for active TB was used in this study [105]. Accordingly, active TB diagnosis was given when the presence of *M.tb* was confirmed by acid-fast bacilli on microscopy, growth in culture or by a nucleic acid amplification test (PCR). In addition, a clinical case of active TB was defined as the presence of a positive TST or IGRA, and/or clinical or radiological signs of active disease with complete diagnostic evaluation followed by the initiation of treatment with standard anti-TB therapy.

- Latent TB

There is no gold standard for the diagnosis of LTBI. Due to evidence that IGRAs have better specificity than TST, especially in BCG-vaccinated populations LTBI diagnosis was given at baseline to participants with a positive IGRA (QFT and/or TSPOT) and no indication of a current or previous active TB that could explain positivity.

- Prior active TB

The "prior active TB" group consisted of participants that were diagnosed and treated for active TB before inclusion and had no indication of current active TB or LTBI. Information about TB diagnosed and treated in a country outside of Norway was based on self-report, as it was not possible to gain access to medical records of these participants.

- Prior latent TB

Participants who had previously received preventive TB therapy were included in the "prior latent TB" group.

- No TB

Participants with a negative IGRA and no previous or current TB were assigned to the "No TB" category.

3.3.2 Study population paper 1 and 2

A total of 304 HIV positive individuals were enrolled in the study, however QFT was missing for six persons who were therefore excluded (figure 12). Of the remaining 298 participants, 160 (54%) were female and the majority (72%) originated from a TB-endemic country. The majority of participants from TB-endemic countries (n = 215) originated from the African continent (76%), followed by South-East Asian (16%). Eastern Europeans contributed with only 5%. The median age was 37 years (range 19-61) and median years of stay in Norway 6 years (range 0-38) for individuals from TB-endemic countries. Females constituted 64% of this group. In contrast, the majority of HIV positive persons from TB low-endemic country were male (73%), and the median age was 50 (range 25-73). Nadir CD4 counts for the entire study population was 190 cells/µl (range 0-1160), however median enrolment CD4 count was high (427 cells/µl, range 3-1870) and 67% were on ART. 79% of the study population was BCG vaccinated and 32 individuals had been diagnosed and treated for active TB previously.

Figure 12: Study population paper 1 and 2

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                    ┌────────────────────────────────────────┐
                    │       HIV positive included (N=304)     │
                    └────────────────────────────────────────┘
                                        │
  ┌──────────────────────────┐          │
  │ 6 excluded due to missing │──────────┤
  │       QFT results         │          │
  └──────────────────────────┘          │
                    ┌────────────────────────────────────────────────────────┐
                    │ TST (N=217)        QFT (N=298)        TSPOT (N=117)      │
                    │ TST+: 52 (24%)     QFT+: 77 (26%)     TSPOT+: 29 (25%)   │
                    │ TST-: 165 (76%)    QFT-: 218 (73%)    TSPOT-: 84 (72%)   │
                    │                    3 indeterminate (1%) 4 Borderline (3%)│
                    └────────────────────────────────────────────────────────┘
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| LTBI (N=64) | Active TB (N=7) | Prior active TB (N=26) | Prior LTBI (N=4) | No TB (N=197) |

TB preventive treatment
Treated: 39 (61%)
Untreated: 25 (39%)

Available follow-up QFT
LTBI: 47 (73%)
Active TB: 5 (71%)
Prior active TB: 21 (81%)
Prior LTBI: 3 (75%)
No prior or current TB: 138 (70%)

3.3.3 Study population paper 3

Figure 13 shows the study population in paper 3. Among the HIV positive participants, median CD4 counts were 286 cells/µl (range 50-425) for active TB patients, 475 cells/µl (range 110-1870) for QFT+ LTBI patients and 394 cells/µl (range 3-1270) for QFT- controls. A total of 51 (63%) of the HIV patients were on ART at inclusion in the study. All the six HIV positive patients with active TB had pulmonary TB whereas among HIV-uninfected TB localization was pulmonary in 39 (66%), extrapulmonary in 18 (31%) and disseminated in two (3%). There were 11 MDR-TB cases (1 HIV+, 10 HIV-) and five mono-resistant TB (1 HIV+, 4 HIV-). Active TB diagnosis was based on positive *M.tb* culture in 60 (92%) whereas five (1 HIV+, 4 HIV-) had clinical indications of active TB disease. The majority 52 (88%) of HIV uninfected active TB patients (88%) and LTBI patients (73%) came from TB-endemic countries, whereas 92% of HIV-uninfected QFT-negative controls were from TB low-endemic countries.

Figure 13: Study population paper 3
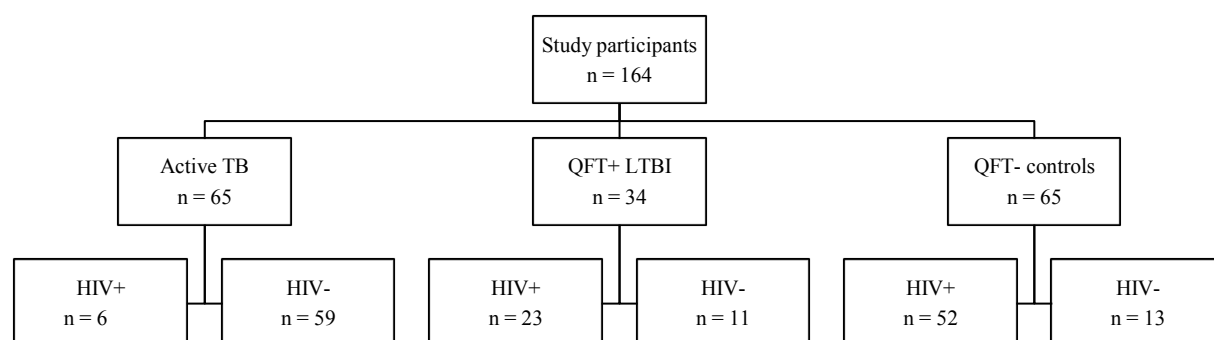
3.3.4 Study population paper 4

A total of 60 HIV-infected with available frozen serum samples taken at inclusion were included from the University Hospital of Northern Norway cohort presented in paper 1-3. The majority (89-100%) of individuals in the three TB groups and 50% in the "no TB" group were dark-skinned immigrants from TB-endemic countries. Furthermore, Caucasian participants had significantly higher vitamin D levels compared to dark-skinned participants. Therefore, to achieve appropriately matched data only dark-skinned individuals from TB endemic countries (N=43) were included in paper 4 (figure 14). There were no significant differences in gender, age and ART between the TB groups.

Figure 13: Study population paper 4
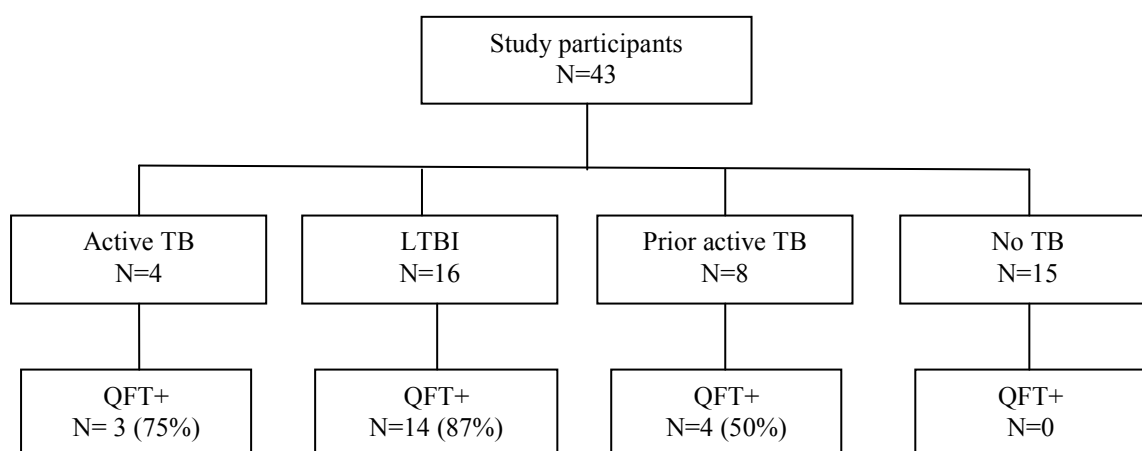
3.4 Test procedures

3.4.1 Tuberculin Skin Test (TST)

TST by the Mantoux method was administered only once at inclusion to HIV-infected participants. To avoid the possibility of boosting, the TST was administered after blood samples for IGRA were taken [106]. Trained TB-nurses performed this test whereby 0,1 ml tuberculin (2 tuberculin units PPD RT23, Statens Serum Institute, Copenhagen, Denmark) was injected intradermal on the forearm. Reactions on the skin were measured 72 hours later using calipers. An induration of ≥5 mm was considered a positive test according to guidelines for HIV-infected persons [107].

3.4.2 Interferon-γ release assays (IGRA)

3.4.2.1 QuantiFERON TB Gold In-Tube

QFT was performed at the certified laboratories at each of the participating hospitals according to manufacturer instructions. For each participant 1ml of blood was collected in the three provided tubes: QuantiFERON Nil tube, TB antigen tube and QuantiFERON Mitogen tube. Each tube was then shaken appropriately, labeled and put in a 37°C incubator within 16 hours of collection. After 16-24 hours of incubation the tubes were centrifuged, plasma was extracted and the level of IFN-γ (IU/ml) was measured by ELISA. Both the quantitative INF-γ results (TB antigen minus Nil) and the qualitative results (positive or negative) were registered. QFT was considered indeterminate if IFN-γ in Nil was > 8 IU/ml or if Mitogen minus Nil < 0.5 IU/ml). The test was considered negative if TB antigen minus Nil was < 0.35 IU/ml (or ≥ 0.35 and < 25% of Nil value). A positive QFT was defined as TB antigen minus Nil ≥ 0.35 IU/ml and ≥ 25% of Nil value (despite the results of Mitogen minus Nil).

3.4.2.2 T-SPOT.TB

TSPOT was performed according to manufacturer instructions at the certified microbiology laboratory at the University Hospital in Northern Norway. From each participant at this hospital, 8 ml of blood was drawn in Vacutainer sodium citrate CPT tubes (Beckton Dickinson Diagnostics, Franklin Lakes, New Jersey) and processed within eight hours of collection. The CPT tubes were centrifuged to isolate peripheral blood mononuclear cells (PBMCs). The extracted PBMCs were then washed twice by centrifugation with RPMI

medium (Invitrogen, Auckland, New Zealand) and resuspended in AIM-V culture medium (Invitrogen, Auckland, New Zealand). Following manual counting of viable cells a standardized cell suspension of 2.5×10^5 cells/100μL. The TSPOT assay consists of four wells for each patient sample: a Nil Control (no antigen), Panel A (ESAT-6), Panel B (CFP10) and a positive control (phytohaemagglutinin). 50 μL AIM V culture medium and 100 μL PBMC suspension was added to each of the four wells for each patient sample. The wells were then incubated in a humidified incubator at 37°C with 5% CO_2 for 16-20 hours. Following washing, spot development and drying, spots were counted manually and by an ELISPOT plate imager. A test was considered indeterminate if the Nil Control spot count was > 10 spot forming units (sfu) or if the Positive Control spot count was < 20 sfu. Positive results were defined as >7 sfu, negative results as <5 sfu and borderline values were set at 5, 6 or 7 sfu according to manufacturers' recommendations.

3.4.3 Multiplex analysis of cytokines, chemokines and growth factors

Plasma samples were analyzed using a multiplex cytokine assay (Bio-Plex Human Cytokine 27-Plex Panel; Bio-Rad Laboratories Inc., Hercules, CA) containing assays for interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (bFGF), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), IFN-γ, eotaxin/CCL11, IP-10/CXCL10, macrophage chemoattractant protein 1 (MCP-1)/CCL2, macrophage inflammatory protein 1 alpha (MIP-1α)/CCL3, MIP-1β/CCL4, regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5, tumor necrosis factor (TNF), platelet-derived growth factor -BB (PDGF-BB) and vascular endothelial growth factor (VEGF).

The samples were analyzed on a Multiplex Analyzer using Bio-Plex Manager 6.0 (Bio- Rad Laboratories) according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation were <12% for all analytes. Nine of the markers evaluated (IL-1β, IL- 2, IL-4, IL-5, IL-9, IL-10, IL-13, IL-15 and GM-CSF) were below the lower detection level (LDL) or detected at very low levels in all samples (> 80% below the LDL of the assay), and were therefore excluded from further statistical analysis. The remaining occasional values (<20%) below the LDL were replaced by a defined common value below the LDL (0.001), which allowed non-parametric statistical analysis.

3.4.4 Enzyme immunoassays (EIA)

Plasma levels of CXCL16, pentraxin 3 (PTX3), soluble TNF receptor 2 (sTNFr2), Fas Ligand (FasL), thymus and activation regulated chemokine (TARC)/CCL17,), osteoprotegerin (OPG), activated leukocyte cell adhesion molecule (ALCAM), IL-23, secreted frizzled-related protein 3 (sFRP3) and C-reactive protein (CRP) were measured by EIAs (R&D Systems, Stillwater, MN). Intra-and inter-assay coefficients of variation were <10% for all except FasL (<15%). MD-2 was analyzed by an in-house ELISA whereby-MD-2 was captured on immune plates coated with a TLR-4-Fc fusion protein and detected with digoxygenin labeled MD-2 mAb 5D7 or digoxigenin labeled IIC1 and anti-digoxygenin- HRP (Roche). The MD-2 standard was purchased from R&D Systems, Stillwater, MN.

3.4.5 Vitamin D analyses

25(OH)D and 1,25(OH)$_2$D were analyzed at the Hormone Laboratory of Endocrinology, Oslo University Hospital, which serves as the reference laboratory for hormone analysis in Norway. The concentration of 25(OH)D was measured using liquid chromatography-mass spectrometry and 1,25(OH)$_2$D levels were measured using enzyme immunoassay (EIA) (IDS Nordic, Herlev, Denmark). 25(OH)D < 50 nmol/l was defined as vitamin D deficiency, 25(OH)D levels of 50-75 nmol/l is defined as vitamin D insufficiency according to the Endocrine Society Clinical Practice Guidelines [87]. It should be noted that there is a lack of consensus concerning 25(OH)D levels which define hypovitaminosis D, however we chose to use the above cut-offs which are most often used in the literature [81,86,87,108,109].

3.5 Statistic analysis

Information from completed questionnaires including blood test results were coded and entered into Microsoft Office Excel 2007 and crosschecked. For QFT, IFN-γ results ≥ 10 IU/ml were recorded as 10 IU/ml due to the uncertainty of test results above that level. IFN-γ < 0.35 was recorded as 0.34 IU/ml.

All analyses done in paper 1, 2 and 4 were performed using STATA 12 software (STATA Corporation, College Station, Texas, USA). Descriptive data were presented as medians with ranges or interquartile range (IQR) or as mean or numbers (percentages). Differences between groups were assessed using the χ^2 test for categorical variables and the Kruskal-Wallis rank-

sum test for continuous variables in paper 1. Concordance between QFT and TSPOT and between TST and each IGRA were determined by calculating Cohens kappa (κ) coefficients (paper 1). Univariate and multivariate odds ratios (ORs) were obtained using logistic regression models. Quantitative QFT trends over time for different patient groups in paper 2 were analyzed using linear mixed models with random intercept. Correlations between vitamin D and IFN-γ levels were analyzed using Spearman rank correlation coefficients. Linear regression models were applied to study the association between vitamin D and IFN-γ. Statistical analyses in paper 3 were performed using IBM SPSS statistics 21, Matlab 7.10, or GraphPad Prism 6. The Mann-Whitney U-test was used to detect pairwise differences between the HIV positive and HIV negative TB groups at baseline. It was also used to compare HIV negative MDR-TB versus drug-sensitive TB, as well as extrapulmonary versus pulmonary active TB cases at time points where the sample size in each group was n ≥ 5. Binary logistic regression was used to assess whether each of the biomarkers could differentiate between active TB, LTBI and QFT negative controls pairwise after adjusting for age, sex and HIV-status. Wilcoxon signed rank test was used to evaluated changes in biomarker levels over time in active TB patients undergoing treatment. The relationship between CD4 counts and selected biomarker in the HIV positive group was assessed using Spearman's correlations. Receiver operator characteristic (ROC) curve analyses were performed for the biomarkers that seemed to differentiate the most between active TB and LTBI.

3.5.1 Sample size (Paper I and II)

We assumed a situation of pairwise data (QFT positive, QFT negative) before and after treatment (after 3 months). Applying McNemars test with the null-hypothesis that the probability of a change from positive to negative is the same as for a change from negative to positive. Power was calculated based on a situation in which 35% of the populations reverted from a positive to a negative test and 15% converted from a negative to a positive test, using the significance level of 2,5% and power of 80%. The sample size would have to be 103 persons.

3.6 Ethical considerations

The study was approved by the Regional Committee for Medical and Health Research Ethics (REK-Vest, REK-Nord and REK-Sør-øst) and the Norwegian Social Science Data Services (NSD), Bergen, Norway. A written informed consent was obtained from each participant.

3.7 Discussion of the study methods

3.7.1 Internal validity

Internal validity refers to whether the findings in a study are correct for the population in study. Errors such as selection bias, information bias and confounding limit internal validity. Appropriate study design, study setting and methods are crucial for the validity of a study. The cross-sectional design and longitudinal design used in this study is inherently limited in that only associations between factors and outcomes can be described rather than causal relationships [110].

- Selection Bias

Selection bias occurs when study participants are not representative for the population in question. For this study it would occur if the HIV patients that participate were not representative of HIV positive persons generally in Norway. HIV care is centralized to internal medicine/infectious diseases clinics from which we recruited participants to the study. The main HIV clinic in the country capital of Oslo did not participate in this study and one could suspect that this could introduce a bias in the population. However, the demographical profile of participants in the study is comparable to the profile in HIV surveillance statistics from the Norwegian Institute of Public Health suggesting that the study population is representative.

Selection bias could occur due to an automatic exclusion of HIV-infected persons that are unaware of their diagnosis or HIV-positive persons that choose not to be followed up. HIV is often diagnosed by random voluntary testing. Persons that are at risk (prostitutes, intravenous drug users, MSM, immigrants from endemic countries) may choose to test themselves more than others who do not have traditional risk profiles. In addition, there may be some that are aware of their HIV positive status that choose to not go to follow-up because they feel healthy or due to the fear of stigmatization. The health system in Norway is unlike other countries

public and HIV patients have the right to free follow-up (including fully refunded travel expenses, clinic appointments, medication). There is therefore little chance of selection bias due to economic status.

Missing values may have introduced selection bias in this study. Poor compliance is a well-known disadvantage of TST. A total of 79 patients did not show up for administration of the TST or for reading after 72 hours despite repeat reminders. There was however no significant difference in the characteristics between individuals with missing TST and those with available test results.

Multicenter studies depend on the dedication and commitment of representatives from participant hospitals for both including patients in the study and to ensure follow-up of study parameters. Strategies to motivate representatives included regular contact by email and telephone with reminders and academic updates. Nevertheless, follow-up QFT results were missing in 84 (28%) patients (paper 2). Despite there being no statistically significant differences between patients with results and those without, these missing values are an important limitation to this study that may have allowed for unknown sources of bias.

Loss to follow-up may also be a source of selection bias. Longitudinal studies of this kind are prone to lose participants to follow-up. To minimize this, follow-up time-points in the study coincided with standard visits to the clinic. Patients that missed a visit were contacted and given new appointments. Five participants were deported out of Norway and were therefore lost to follow-up.

- Information bias

Information bias occurs when information obtained in the study is inaccurate. There may be inaccuracy in information about the illness and/or exposure factors. This inaccuracy can be intentional or non-intentional and may be generated by the study participants and/or the interviewer/researcher.

To avoid this, background information about HIV infection and previous TB infection were retrieved from patient journals and not only by self-report. However, information on prior TB infection diagnosed and treated outside of Norway was based on self-report, which could have resulted in recall bias.

- Confounding

Repeated TB exposure after inclusion in the study would be a confounding factor in the study of the dynamics of IFN-γ during treatment and its fluctuations in participants without TB. We should avoid this confounding factor in Norway where TB transmission rates are low. However, travel to high burden areas, especially immigrants visiting their home countries could have posed a problem in interpretation of IGRA results. Participants were asked about travel and possible new exposure at each consultation and these were controlled for in statistical analysis of IGRA dynamics.

- Misclassification bias

As previously mentioned there is no gold standard for LTBI. Studies on the diagnostic accuracy of TST and IGRA for LTBI have determined sensitivity by test positivity in active culture positive TB disease, whereas specificity has been based on how the tests perform (negativity) in persons with no exposure to TB. IGRAs may be negative in patients with active TB and LTBI and may be positive in atypical mycobacterial infections with *M. kansasii*, *M. szulgai* or *M. marinum*. As a result, misclassification error may have occurred by defining LTBI according to IGRA positivity. To date, however, IGRA is the most reliable test available for LTBI diagnosis, especially in HIV patients.

The fact that QFT analysis was performed at each participating hospital is a potential source for bias. However, internationally recommended and validated commercial tests with standardized procedures were used at all hospitals.

TSPOT were positive in two participants with negative QFT whom were diagnosed with LTBI based on this result. TSPOT was only performed in participants from one clinic, and although the concordance between TSPOT and QFT was very good, it is feasible that a few LTBI diagnoses may have been missed.

- Other study limitations

Underpowered studies increase the probability of type II errors (false-negatives). The sample size of TSPOT analyses was too small to explore risk factors associated with positive TSPOT or QFT/TSPOT discordance. There were also too few active TB/HIV co-infected patients to adequately address cytokine dynamics in this group during therapy. However, to attain an optimal sample size one would have to recruit patients from other neighbouring countries as

the prevalence of active TB is low in HIV patients in Norway. Furthermore paper 4 was also limited by small sample size.

3.7.2 External validity

External validity refers to the generalizability of study findings to the general public. Despite limitations mentioned above we believe that the internal validity of the study is adequate which is a prerequisite for external validity. Our study can be generalized to HIV positive persons living in TB low-endemic countries with satisfactory HIV healthcare.

4 SUMMARY OF MAIN RESULTS

4.1 Paper 1

We studied the prevalence and risk factors for positive IGRA in a HIV cohort. The prevalence of positive tests was 26% for QFT, 25% for TSPOT and 24% for TST. In the multivariate analysis, origin from a TB-endemic country (OR 7.59, 95% CI 2.94-19.60, p = 0.001), previous active TB (OR 4.50, 95% CI 1.83-11.06, p = 0.001) and contact with a TB patient (OR 4.10, 95% CI 1.77-9.51, p = 0.001) were associated with a positive QFT. 33% of HIV positive persons from TB-endemic countries were QFT+. Excluding those previously treated for active TB, 49% of immigrants living only 0-3 years in Norway had a positive QFT compared to 12% of those who had lived ≥ 10 years (p=0.002). In the multivariate analysis there was a significantly lower odds for a positive QFT with ≥ 10 years of stay in Norway (OR 0.17, p=0.002) compared to those with 0-3 years of stay. The OR for linear trend for years of stay in Norway was 0.87 (95% CI 0.79-0.96, p = 0.007).

Overall agreement between TSPOT and QFT results was 91% (κ 0.77, p < 0.001) and between TSPOT and TST was 80% (κ 0.52, p < 0.001). Overall agreement between QFT and TST results was 80% (κ 0.47, p < 0.001). Median INF-γ was higher in participants with QFT+/TST+ results (4.38 IU/ml, range 0.5-10 IU/ml) compared to those with QFT+/TST- results (0.81 IU/ml, range 0.39-10 IU/ml) (p < 0.001).

4.2 Paper 2

According to the survey among TB care nurses only 2-7% of active TB and 1-5% of LTBI patients were confirmed HIV-infected, but 40% of active TB and 50% of LTBI patients had not been tested for HIV during the period 2005-2010.

Four of the seven HIV-infected patients diagnosed with active TB in our study had been treated for active TB abroad and one had developed MDR-TB. During follow-up there was a significant reduction of INF-γ levels already at 6 months (coefficient -3.10 IU/ml, p = 0.020) and further at 12 months (coefficient -2.59 IU/ml, p = 0.012).

A total of 64 HIV patients were diagnosed with LTBI, 60 from TB-endemic countries and four patients were Norwegians. Median INF-γ was 2.00 IU/ml (IQR 0.56-6.20) in LTBI

patients from TB-endemic countries compared to 0.91 IU/ml (IQR 0.49-5.63) in LTBI patients from Norway. Only 39 (61%) LTBI patients received preventive treatment. Median INF-γ value at baseline for treated LTBI was 3.48 IU/ml (IQR 0.94-8.91) compared to 1.13 IU/ml (IQR 0.47-4.25) for untreated LTBI patients (p = 0.029). Neither treated nor untreated LTBI patients developed active TB during the two years of follow-up.

In the two-year follow-up with repeated QFT, there was no significant difference in the trend of INF-γ over time between treated and untreated LTBI patients. Reversion rates were 23% for treated LTBI and 44% for untreated LTBI. In LMM analyses LTBI patients with a positive TST had higher INF-γ results over time compared to TST negative patients (coefficient 2.887 IU/ml, p < 0.001), irrespective of preventive therapy.

38% of "prior active TB" patients tested QFT positive at baseline. Despite fluctuations in IFN-γ levels the majority maintained qualitative baseline QFT results during the two-year follow-up with a conversion rate of only 9%. In LMM analysis IFN-γ levels increased with increasing CD4 counts (coeff. 0.008 IU/ml per cell/μl, p = 0.003). Mean INF-γ values over time were significantly lower in patients with a previous pulmonary TB (mean 1.42 IU/ml, SD 1.97) compared to patients with extra-pulmonary TB (mean 3.99, SD 4.29, p = 0.02).

Conversion rates to positive QFT in the no-TB group were 7% at 12 months and 4% at 24 months. No patients reported exposure to TB during the study period.

4.3 Paper 3

We identified cytokines and chemokines that may improve TB diagnosis and serve as biomarkers of TB treatment efficacy in HIV positive and negative persons. In HIV positive participants, the levels of IP-10 and sTNFr2 were significantly higher in active TB patients compared to those with LTBI and QFT negative controls (p < 0.005). Applying ROC curve analyses an IP-10 cut-off level of 2547 pg/mL distinguished active TB from LTBI with 100% sensitivity and specificity in HIV positive patients. In HIV negative patients, a maximal sensitivity of 71% and specificity of 82% was attained with cut-off level of 689 pg/ml. PTX3 was the only cytokine that differentiated between active TB and LTBI in HIV negative patients with significantly higher levels in the former (p < 0.005). IP-10 levels decreased during anti-TB chemotherapy in HIV uninfected patients, with significant reduction after 12-24 weeks (p = 0.002) where levels were similar to levels in the LTBI and QFT negative

control group. Applying the optimal IP-10 cut-off level of 674 pg/ml for distinguishing between active TB and QFT negative controls, 43/59 (73%) of active TB patients were positive at baseline and 9/20 (45%) were positive at 12-24 weeks.

4.4 Paper 4

We studied the association between vitamin D and TB infection in HIV-infected individuals. The prevalence of vitamin D deficiency (25(OH)D < 50nmol/l) and insufficiency (25(OH)D 50-74 nmol/l) was 56% and 27% respectively, the majority occurring in patients with a present or past TB infection. Median 25(OH)D levels were lower in ATB (39 nmol/l, IQR 21-47, p=0.03), LTBI (41 nmol/l, IQR 29-54, p=0.03) and in prior ATB patients (36 nmol/l, IQR 28-49, p=0.03) compared to HIV patients with "no TB" (60 nmol/l, IQR 51-99 nmol/l). Median 1,25(OH)2D levels were lower only in ATB patients (41 pmol/l, IQR 39-53) when compared to both the LTBI (115 pmol/l, IQR 77-133, p=0.005), prior active TB (111 pmol/l, IQR 101-131, p=0.007) and the "no TB" groups (112 pmol/l, IQR 85-158, p=0.019). Median 25(OH)D level was lower in patients with a prior ATB (38 nmol/l, IQR 33-58) compared to those with "no TB" (70 nmol/l, IQR 53-99, p = 0.015).

There was a significant negative correlation between 25(OH)D and IFN-γ levels measured by QFT (Spearmans rho -0.41, p = 0.007). Applying linear regression each unit increase in IFN-γ corresponded to a 5 nmol/l decrease in 25(OH)D level (p = 0.046). There was also a significant negative correlation between 25(OH)D and TST size (spearmans rho -0.41, p=0.03).

5 DISCUSSION OF MAIN RESULTS

We have conducted a study of TB diagnosis and follow-up of a cohort of HIV positive persons living in Norway. None of the participants developed active TB during the 2-year follow-up. Our emphasis has been on the performance of IGRA in this low-endemic setting, as well as the potential of other cytokines as biomarkers for TB diagnosis and treatment efficacy. We also conducted a pilot study exploring the relationship between vitamin D status and TB, including TB-specific IFN-γ responses, in HIV positive individuals. The results of these studies are presented in the following section.

5.1 Prevalence of TB among HIV-positive persons in Norway

The prevalence of HIV/TB co-infections in Norway is unknown and therefore we initially performed a pilot survey among out- and in-patient clinics throughout Norway where TB care nurses reported low routine HIV testing of TB patients. The prevalence of confirmed HIV infection among TB cases was however low and comparable to WHO estimates of 0-4% [10]. Surprisingly however, the prevalence of positive immunodiagnostic tests for TB among HIV positive individuals in this study was higher than expected considering a previous study from the similarly TB low-endemic Scandinavian country of Denmark [111]. In that study Brock et al reported a prevalence of positive QFT of 4.6% among HIV positive patients living in Denmark. The higher prevalence of positive tests in our study (24-26%) could be due to the higher proportion of participants from TB-endemic countries (72%) compared to Brock et al's study consisting of 74% ethnic Danish participants. Nevertheless, the prevalence of positive QFT was also higher among patients from TB-endemic countries in this study (33%) compared to the Danish study (16%). Following complete screening of all participants in the study, seven patients were diagnosed with active TB and 64 with latent TB, giving a prevalence of 2% and 21% respectively. The majority of TB infections were diagnosed in persons from TB-endemic countries (100% and 94% respectively). Studies from TB low-endemic countries reveal a high proportion of TB patients from TB-endemic countries reflecting migration patterns [112-116]. The overrepresentation of HIV positive persons from TB-endemic countries warrants vigilance among clinicians for TB screening even in low TB-endemic countries.

5.2 Factors associated with a positive IGRA and TST

Factors associated with IGRA and TST positivity were origin from a TB-endemic country, previous active TB and contact with a TB patient, consistent with other studies [111-114]. Higher nadir CD4 cell counts were noted among patients with a positive IGRA compared to those with negative results, whereas CD4 counts at enrolment were comparable. This finding is in contrast to other studies which reveal a relationship between CD4 counts and IGRA positivity, reflecting the tests' dependency on a relatively intact immune system and poorer performance in HIV patients with advance immunosuppression reflected by a higher number of indeterminate results [34]. The lack of this association between CD4 counts and IGRA positivity may be due to the higher baseline CD4 counts among the participants in this study compared to others. Both nadir and baseline CD4 counts were however significantly higher in participants with a positive TST. Furthermore, younger age as well as a previous active TB and higher baseline CD4 counts were independently associated with a TST ≥15 all probably indicative of a more robust immune response.

In our study we found that QFT+ HIV patients from TB-endemic countries had resided in Norway for a shorter period of time than those with a negative QFT test. Adjusting for baseline CD4 counts and ART, patients living in Norway ≥10 years had significantly lower odds for a positive QFT compared to persons living <3 years. This finding may reflect a waning of TB-specific immune responses even in HIV patients over time in an environment of low infectious burden. It has been hypothesized that some LTBI patients may clear the infection without preventive treatment and some studies have provided indirect evidence of this phenomena [14,117-119]. Specifically, Wiker et al. analyzed TB registry data in Norway following birth cohorts over 10-year periods before and after TB was under control in the country [119]. Using a model based on decreasing rates of reactivated TB as a proxy for waning of latency, the authors suggested a half-life of 8.8 years for LTBI in settings of low disease transmission. Ewer et al found that TB-specific responses measured by TSPOT disappeared in some untreated TST-/TSPOT+ TB contacts [117]. In another study, Mori et al. found that TB prevalence measured by QFT in an older Japanese population with previous TB exposure was much lower than the predicted TB prevalence suggesting a waning of latency over time [118]. HIV status was not accounted for in these studies. Though an intriguing theory, extra caution has to be taken in its interpretation as HIV patients are most vulnerable for TB reactivation and immunodiagnostics tend to have lower sensitivity in these patients [34]. However, higher CD4 cell counts were noted in patients with a prolonged stay in

Norway and one would therefore not expect false negative IGRA in this group. Preventive TB treatment reduces the risk of TB reactivation by 32% in HIV infected patients, however poor compliance and serious medicament interactions are a concern [39]. If a time-dependent decline in TB-specific IFN-γ responses indicates clearance of LTBI without treatment this could have policy implications for preventive TB treatment guidelines in low-endemic countries. It may be that preventive TB treatment, which carries the risk of adverse effects and drug interactions is not necessary in stable HIV patients on effective ART. To further explore this theory we conducted a longitudinal study to observe for incident TB and to assess the dynamics of TB-specific immune responses and the predictive value of IGRA reversion over a two-year period, details of which will be discussed in the following section.

5.3 TST and IGRA: Test performance and concordance

Norwegian guidelines has until recently recommended IGRA as a supplement to or instead of TST for TB screening of HIV-infected persons [12]. There is however a paucity of studies on the performance of these tests in general in low-endemic countries and specifically no study has so far been performed in the HIV positive population in Norway. For screening purposes the TST was wrought with poor attendance with 27% failing to show up for administration of the test or reading of the results after 72 hours despite free services and repeat reminders. Due to the lack of a gold standard for LTBI, sensitivity estimates are generally based on the performance of the test in active TB patients. All active TB patients in this study tested positive on one or both IGRAs. QFT had a sensitivity of 85.7% for all TB cases (6/7 positive) and 100% for laboratory confirmed TB. TST was performed on four patients and only one was positive (10mm, sensitivity 25%). This study is underpowered to perform adequate analysis of test sensitivity, however previous studies from TB low-endemic countries have reported higher sensitivity of IGRA compared to TST in HIV patients with culture-confirmed TB [34,120].

The agreement between TST and both IGRAs was moderate and concordance between both IGRAs was good in this study, in line with other studies [34,114,121,122]. However, in contrast to some studies in low-endemic setting, BCG status did not explain TST+/QFT- discordance in our study possibly due to the high numbers of BCG-vaccinated individuals in our study [123,124]. TST-/QFT+ discrepancies were also observed, but were not related to CD4 cell counts. There was however higher IFN-γ levels in patients with concordant

TST+/QFT+ results compared to patients with TST-/QFT+ results. This finding implies that TSTs are more often negative at QFT values close to cut-off, which could be an indication of higher sensitivity of QFT [122]. In contrast to other studies of IGRA in HIV positive individuals there were few indeterminate QFT in this study likely due to high CD4 cell counts in our study population [53,114,125-127]. Hence, in this high-income TB low-endemic setting QFT proved to be more practical and efficient than TST for the diagnosis of TB in HIV patients.

5.4 IGRA in the diagnosis and follow-up of TB infection

- **Active TB**

IGRA was designed for the diagnosis of LTBI and not active TB, which is a microbiological and clinical diagnosis. However, due to the lack of a gold standard for LTBI diagnosis, sensitivity estimates of IGRA are often based on its performance in active TB cases. There were too few active TB patients in this study to make valid conclusions on IGRA performance in this patient group. However, the characteristics of these patients illustrate some of the challenges in TB diagnosis in HIV patients. Of the seven patients diagnosed with active TB, five had pulmonary TB and two had extrapulmonary TB. Five met laboratory definitions whereas two had strong clinical indications of a pulmonary TB with characteristic findings on chest x-ray and laboratory signs of inflammation combined with a positive IGRA. Four patients with pulmonary TB had no symptoms and two with positive sputum culture had no pathology on chest x-ray (only one of these had symptoms). As part of the initial screening algorithm in our study a sputum sample was obtained for all participants to identify active TB patients missed by the lack of symptoms, a negative IGRA and normal chest x-rays, a diagnostic approach that is done at some national hospitals. However, sputum examination did not exclusively reveal additional cases of active TB. Also of note, four of the seven patients diagnosed with active TB were previously treated for active TB in their home country, including one patient that had developed MDR-TB. This illustrates the importance of full clinical evaluation in HIV patients with a prior active TB, particularly if the prior treatment and follow-up is unknown.

To assess IFN-γ dynamics during and after anti-TB treatment repeat QFTs were taken and results were available in five active TB patients. There was a significant reduction of QFT results during treatment and only one patient with baseline positive QFT remained positive at 24 months. Significant reductions in IFN-γ responses during and after anti-TB therapy have been reported in studies with larger cohorts [128-130]. Nevertheless several patients maintained qualitative positive IGRA after effective anti-TB treatment. In a study conducted in Italy, Sauzullo et al. found that 30.7% of HIV patients with active TB retained QFT positivity after anti-TB therapy and there was overall no significant decrease in IFN-γ response before and after treatment [130]. Despite the lack of adequate studies from TB low burden settings it is reasonable to conclude that IGRAs are suboptimal for the monitoring of treatment effect in active TB patients [131].

- **Prior active TB**

An important drawback to the performance of IGRA is the occurrence of persistently positive tests in patients adequately treated for a prior active TB. In our study 38% of patients previously successfully treated for TB had positive QFTs. This is in line with other studies from TB low-endemic countries [111-115]. To explore possible mechanisms for this finding we compared the characteristics of previous active TB patients with and without a positive IGRA. We found that patients with a prior extra-pulmonary TB maintained higher IFN-γ levels throughout the 2-year follow-up compared to those with pulmonary TB. This finding suggests that a stronger T-cell response is retained after successful treatment of extrapulmonary TB, reflecting pre-treatment response levels. In addition higher CD4 cell counts were associated with persistently positive QFT in these patients which may reflect higher numbers of circulating TB-antigen specific effector T-cells. Length of time since treatment did not have an influence on IFN-γ responses.

- **LTBI**

There is no gold standard for the diagnosis of LTBI and the knowledge of host-pathogen interactions in TB infections is incomplete. Due to the compromised specificity of TST in BCG-vaccinated populations we chose to define LTBI based on positive IGRA followed by a full clinical examination to exclude active TB. A total of 62 patients received an LTBI diagnosis based on that definition. In addition, two patients with negative IGRA received the

diagnosis by their clinicians as they had recent documented close contact with an infectious TB patient and received preventive therapy. One could hypothesize that the lower mycobacterial load in LTBI would be reflected in a lesser immune response compared to active TB that would decline more rapidly in response to treatment. Furthermore, that a consistent decline is more likely in an environment of low TB transmission. A randomized, controlled study would be the optimal study design to explore IFN-γ changes with and without TB preventive treatment in LTBI patients. However, due to ethical concerns with giving placebo versus TB preventive treatment in HIV patients that are at most risk for TB reactivation we chose an uncontrolled study approach. Nevertheless, as many as 39% did not receive TB preventive treatment allowing for comparison of both treated and untreated LTBI patients.

QFTs were repeated over a 2-year period and our data showed reversion rates of 23% for treated and 44% for untreated LTBI. Treated LTBI patients had significantly higher baseline IFN-γ results compared to untreated (3.48 IU/ml vs. 1.13 IU/ml), which could explain the higher reversion rates in the latter. There was no significant difference in the trend of IFN-γ levels over time between treated and untreated LTBI patients. Our data therefore show that QFT cannot be used to monitor the effects of TB preventive therapy, a conclusion that has also been reached in other studies [104,132,133]. It should also be emphasized that the clinical significance of reversions are uncertain. Indeed, in ART-naïve patients with low CD4 counts a reversion should cause concern as it may indicate failing immune defenses in the presence of active TB. Randomized controlled studies of longer duration in HIV patients are necessary to assess the prognostic value of reversions and conversion observed with and without treatment.

To further explore variations in IFN-γ response we compared patients that reverted with those that maintained positive IFN-γ levels. Our study revealed that positive baseline TST and higher nadir CD4 counts were significantly associated with maintained QFT positivity. Indeed, study participants with concordant QFT and TST results at baseline maintained higher IFN-γ levels throughout the 2-year observation period regardless of preventive treatment. Similar findings were reported in a study of health care workers, of TB contacts in a school outbreak and a study of serial IGRA after rifampicin prophylaxis [117,134,135]. Concordant TST and IGRA responses associated with persistently high IFN-γ levels on serial testing may indicate a stronger and more stable immune response, which may have prognostic significance. A

stronger immune response could reflect more effective protection against developing active TB, but could also reflect response to a higher mycobacterial load. Evidence for the latter was seen in a study conducted in Taiwan in which HIV patients with concordant TST and TSPOT results had a 7.8-fold increased risk of developing active TB if left untreated [136]. This finding suggests that dual testing could more accurately identify patients that would benefit from TB preventive treatment.

5.5 Performance of IGRA during longitudinal testing: conversions and reversions

Repeat QFT in HIV positive participants with baseline negative IGRA and no indication of a present or past TB infection allowed us to study the reliability and consistency of this assay for longitudinal testing. The setting of low TB disease transmission was also ideal for studying the natural fluctuations of IFN-γ. Conversions and reversion were observed in this group despite no reported TB exposure during the study period although IFN-γ values of positive QFT at 12 and 24 months were low. Only one of those who converted at 24 months had a positive TST at baseline. Boosting of IFN-γ responses following a TST is a known phenomenon, however there was no difference in IFN-γ variation over time in patients with missing TST and those who had a TST performed in our study [106]. Possible causes of variation in IFN-γ levels in serial testing may be technical attributes of the assay itself or may represent real changes in immune responses. Such changes in immune responses may be reflected in CD4 cell counts. CD4 cell counts were analyzed concurrently with each repeat QFT and there was no relationship between concurrent CD4 cell counts and IFN-γ fluctuations over time in this group of patients. Other factors that may alter immune responses such as stress or other infections were not controlled for in this study.

Our study revealed high reversion rates among untreated LTBI patients who had median IFN-γ close to the established cut-off of ≥ 0.35 IU/ml. Similar fluctuations of IFN-γ values close to cut-off have been described in longitudinal studies of varying duration in both HIV negative and positive individuals [106,137-140]. Metcalfe et als study in U.S.A. of 543 individuals revealed that QFT results < 0.59 IU/ml were more likely to revert to negative on retesting due to the inherent variability of the test [139]. The study population consisted of individuals referred for routine LTBI screening, however, nationality, TST status, prior active TB and HIV status were unknown. In another study conducted in a TB low-endemic country, 846

HIV positive individuals underwent serial testing with QFT [137]. Reversion rates were 33% and conversion rates was 9%, but reduced to 6% after applying a cut-off of >0.70 for follow-up results. Our results and these reports suggest that QFT is inadequate for longitudinal testing and an adjustment of cut-off or establishment of a borderline zone is necessary to improve the reliability of this assay.

5.6 Preventive treatment of LTBI in Norway

According to Norwegian guidelines HIV-infected persons meeting the following criteria should receive TB preventive therapy: 1) a positive IGRA, 2) TST >5mm and negative IGRA with a high risk of TB or 3) close contacts (CD4 < 200 cells/μl) to persons with infectious active TB regardless of IGRA or TST results [12]. Nevertheless, in this study only 61% of LTBI patients who met these criteria received TB preventive treatment. Preventive TB therapy was decided upon and implemented by the responsible clinician without input from the study researchers. Still, the study setting may have led to higher implementation of therapy than usual. Poor implementation of TB preventive treatment in Norway is also revealed in a study among asylum seekers where none of the seven HIV-infected LTBI patients were treated [141]. A recent survey among physicians in the TB low-endemic country of Germany revealed that 58.4% were reluctant to start TB preventive treatment due to a fear of adverse drug effects [142]. In that survey only 36% considered preventive therapy necessary in HIV infected patients with a positive TST or IGRA.

Interestingly, untreated LTBI patients in our study had lower IFN-γ levels compared to treated patients, indicating a tendency for clinicians to make decisions based on the magnitude of IFN-γ response. In addition, clinicians refrained from treating the few QFT positive patients from TB low-endemic countries with no known TB exposure. Three out of four of these patients reverted to negative when QFT was repeated. This was also described in a study of US-born HIV patients at low risk for TB in whom 80% with initial positive QFT reverted to negative when retested after a median time of 40 days [143]. It is therefore likely that these represent false positive tests and we recommend repeating QFT in HIV patients with no TB exposure risks to avoid unnecessary TB preventive therapy. Aichelburg et al also arrived to the same conclusion in a study in Austria in which one-third of baseline IGRA positive patients tested negative on follow-up at 24 months [144].

There is a paucity of studies that examine the consequences of not treating LTBI in HIV positive persons living in TB low-endemic countries [31]. Neither treated nor untreated LTBI patients, nor patients with a positive TST considered uninfected by TB, developed active TB during the 2-year observation period. This is in contrast to the two longitudinal studies of HIV patients in a low-endemic country presented in a review by Diel et al. [31]. In Aichelburg et al's study from Austria, 8.3% of QFT positive HIV patients who did not receive preventive therapy developed active TB within a follow-up period of 19 months [120]. A total of two out of 20 TSPOT positive patients (10%) developed active TB at 3 and 10 months follow-up respectively in a London based study [145]. In a Swiss study where LTBI diagnosis was based on TST, none of the treated LTBI patients but 6.5% of untreated LTBI patients developed active TB during the follow-up of 52 months [146]. This corresponded to a numbers-to-treat (NNT) of 15 persons to prevent one case of active TB (NNT = 8 for immigrants from TB endemic countries, NNT = 33 for patients from TB low-endemic regions). In that study it was also apparent that whereas low CD4 counts are associated with an increased risk of TB reactivation, the risk is less among HIV patients receiving efficient ART. The discrepancy between the above studies and our study may be due to the fact that CD4 cell counts and the proportion of patients receiving efficient ART were higher in our study. Furthermore, patients with active TB in the other studies may have been misclassified as LTBI, whereas active TB patients were meticulously identified at inclusion in our study. In a recent publication from Denmark, HIV positive individuals with positive QFT who did not receive TB preventive treatment were followed-up for a period of 6 years [147]. The study participants had high CD4 cell counts and the majority were receiving ART. Two participants among the 28 with positive QFT developed active TB at 11 and 54 months after testing, resulting in a PPV of 7%. One patient was from the Philippines and the other was Danish with risk factors such as alcoholism and IVDU, but it is not stated whether these patients were on ART and if possible new exposure to TB was controlled for. It is nevertheless clear from all studies that the majority of IGRA or TST positive HIV patients do not develop active TB. Furthermore, the early initiation of ART to reduce the risk of ATB should be emphasized [148,149]. There is a need for prognostic biomarkers to identify those who will progress to active TB. Longitudinal studies of longer duration and regular clinical evaluation are necessary to assess TB risk in HIV patients on efficient ART and high CD4 counts in low-endemic countries.

Based on our findings we advocate a policy of retesting HIV patients with low INF-γ levels as well as to consider deferring preventive therapy in patients expected to have low risk of progression to active TB due to efficient ART and high CD4 count. This is in accordance to newer British guidelines which recommend IGRA testing and TB preventive therapy to the following in the event of a positive test: 1) patients from sub-Saharan Africa who have used ART < 2 years, 2) IGRA positive patients from medium TB incidence countries with < 2 years on ART and CD4 cell count < 500 cells/μl, and 3) patients for low-incidence countries if < 6 months on ART and CD4 cell counts < 350 cells/μl [150]. In addition, HIV patients with close and prolonged contact with patients with infectious active TB should be tested and receive TB preventive therapy regardless of test results, CD4 count or ART.

5.7 Surrogate biomarkers for TB diagnosis

Neither TST nor IGRA can distinguish between active, latent or previous active TB as both tests measure an adaptive immune response to TB, which does not necessarily correlate to the presence of live *M.tb* in the body. Furthermore, both can be negative in the presence of active TB emphasizing the importance of clinical parameters and the need for adjunctive biomarkers to identify patients with TB infection and to distinguish between stages of TB infection. Our study provides valuable data since we compare cytokine levels in both HIV positive and negative individuals with and without TB infection.

IP-10 proved to be the most consistently differentiating cytokine in our study. Plasma levels of IP-10 differentiated between active TB and LTBI irrespective of HIV co-infection and in HIV positive individuals excellent sensitivity and specificity was attained at a cut-off level of 2547 pg/ml. Suboptimal sensitivity and specificity were achieved for HIV negative participants, equivalent to results from a recent meta-analysis which reported a pooled sensitivity of 72% (95% CI 69-74%) and specificity of 82% (79-85%) for IP-10 in blood samples for the diagnosis of TB [151]. Interestingly, we also observed a significant negative correlation between CD4 cell counts and plasma IP-10 levels in HIV positive individuals, indicating that the sensitivity of IP-10 (in contrast to IGRA tests) is maintained in advanced HIV infection although specificity may be compromised.

IP-10 levels decreased during active TB treatment in HIV negative patients in our study [80,152-155]. Due to low sample size, analyses were not performed during treatment of active TB in patients co-infected with HIV. In a study conducted in Ethiopia, Mihret et al. reported

significantly decreased levels of IP-10 after TB anti-TB therapy in HIV negative patients, but no significant difference in pre- and post-treatment levels in HIV positive patients [154]. Riou et al reported significant reduction in IP10 levels after TB treatment irrespective of HIV status. However, the decline was more profound in HIV negative patients compared to HIV positive patients [155]. The fact that IP-10 is not *M.tb* specific but is also associated with HIV infection may explain the lack of, or the lower decline of this chemokine in response to therapy [68]. Nevertheless, IP-10 combined with IGRA have the potential to improve active TB diagnosis in HIV positive patients. Studies have also explored IP-10 in other clinical samples such as pleural effusion and its utility as a point-of-care test using dried blood and plasma spots [151,156-158].

sTNFr2 proved also to be valuable in distinguishing between active TB, LTBI and no TB participants regardless of HIV status. To the best of our knowledge the only other study that explored the potential of sTNFr2 as a biomarker for TB disease was performed by Juffermans et al who observed increased levels of both sTNFr1 and sTNFr2 in active TB patients irrespective of HIV status [159]. Furthermore, both declined during treatment. Though a decline in sTNFr2 was observed after active TB treatment in HIV negative patients in our cohort, levels fluctuated during treatment limiting its value as a biomarker of treatment efficacy.

As in the study by Azzuri et al, PTX3 levels declined during treatment of drug sensitive TB in our study [80]. Interestingly, a transient increase was observed before decline after 24-48 months in MDR-TB patients, possibly reflecting a delayed response to treatment in this group of patients.

5.8 Vitamin D status in HIV patients with and without TB

Epidemiological and cross-sectional studies have shown an association between low vitamin D levels in HIV and in TB infections, however a causative role cannot be established by such studies. It is however of interest to identify clinical parameters in TB and HIV infection that are associated with hypovitaminosis D to develop hypothesis of causality. We were particularly interested in whether there is a correlation between TB-related inflammation and

vitamin D status. To explore this we compared vitamin D levels and TB-specific IFN-γ release in paper 4.

We found that low vitamin D levels was significantly associated with positive TST and IGRA tests and there was a significant negative correlation between TST size and quantitative IFN-γ values and 25(OH)D. This novel finding underpins the association of vitamin D with inflammatory processes involved in TB infection. Other studies have revealed high levels of other inflammatory biomarkers such as IL-6, IL-8, TNF and CRP in HIV positive persons with vitamin D deficiency supporting an association between immunological activation and vitamin D status [93,160,161]. However, high levels of IFN-γ production could also reflect preserved protective TB-specific immunity rather that high level inflammation.

The prevalence of combined vitamin D deficiency and insufficiency in our study was comparable to that seen in dark-skinned participants in other studies conducted in western countries [162,163]. We also found significantly lower levels of 25(OH)D in active TB, LTBI and prior active TB patients compared to participants with no present or past TB [88,164]. Some studies have reported no significant differences in vitamin D levels, whereas a Greenland study reported both high and low levels in TB patients and a Tanzanian study reported higher 25(OH)D levels in pulmonary TB patients compared to non-TB patients [88,94,95,98,164,165]. Economic, environmental/seasonal and nutritional factors affect vitamin D levels and may contribute to the inconsistency in study results. Hypothesis for low 25(OH)D in TB infections include overconsumption or defects in metabolism as a result of inflammatory processes [84,88]. Our study however also revealed that HIV positive persons previously treated for active TB had significantly lower 25(OH)D levels compared to participants in the "no TB" group in line with a previous study with HIV negative subjects [166]. Early studies have shown that the TB drugs rifampicin and isoniazid can cause a decrease in 25(OH)D levels [167]. "Prior ATB" patients in our study had been treated a median of 10 years before inclusion and it seems unlikely that the effect of anti-TB treatment should last so long. It may be that post-treatment levels reflect levels before treatment, which may be associated with other factors such as race.

Interestingly however, $1,25(OH)_2D$ levels was significantly lower in active TB patients whereas levels were comparable in the LTBI, prior active TB and "no TB" group in our study. Few studies have reported on $1,25(OH)_2D$ levels likely because it is not considered a reliable

marker for vitamin D status [87]. A previous Norwegian study found comparable 25(OH)D levels in HIV positive and negative individuals whereas $1,25(OH)_2D$ levels were significantly lower in HIV positive participants [168]. Another paper reported that previous AIDS and higher CD4 counts were associated with low levels of $1,25(OH)_2D$ levels, but not with 25(OH)D levels in a Swiss population [169]. The Norwegian study included a few patients with atypical mycobacterial infection but none of the studies had patients with TB. Two studies have shown conflicting result in $1,25(OH)_2D$ levels in patients with active TB (HIV negative) compared to healthy controls, but none compared levels in LTBI patients [170,171].

$1,25(OH)_2D$ has both immunosuppressive and immunostimulatory effects which are important for controlling *M.tb* infection [81,82,85,88]. Despite being considered an inadequate measure for vitamin D status, it is interesting to note that $1,25(OH)_2D$ is depleted in active TB patients, whereas levels are maintained in LTBI patients in our study. We hypothesize that the immune regulatory functions of $1,25(OH)_2D$ levels contribute to successful containment of TB infection with its depletion marking a failure of these functions, initiating the development of active TB. Our findings imply that 25(OH)D is an insufficient biomarker of vitamin D status and effect in HIV/TB co-infection and studies should include measurements of 1,25(OH)D.

6 CONCLUSIONS AND RECOMMENDATIONS

- The prevalence of LTBI is high among HIV positive persons in Norway, boosted by the high proportion of individuals from TB endemic countries. Clinicians need to be aware of this and improve HIV screening in TB patients as well as TB screening of HIV positive persons. For screening purposes IGRA performed better than TST due to the lack of compliance issues and higher sensitivity. There was however evidence of stronger IFN-γ responses in concordantly positive TST/QFT which may have prognostic significance advocating dual testing with IGRA and TST.

- HIV positive persons from TB endemic countries have a reduced risk of a positive QFT the longer they live in Norway. This may indicate a waning of latent TB infection in a setting with low TB transmission. ART, which reduces the risk of reactivation, should be prioritized in these patients.

- In a low endemic setting, HIV patients on ART and with adequate CD4 cell counts have a low risk of progression from LTBI to active TB. Fluctuations of IFN-γ levels close to the established cut-offs also justify that retesting and follow-up without preventive therapy is appropriate for selected HIV patients. Furthermore, deferment of TB preventive therapy in HIV patients on efficient ART appears to be safe in low-endemic settings with optimal HIV care facilities.

- QFT is unsuitable to monitor the efficacy of TB treatment, as there is a high degree of intra-assay variation even in a TB low-endemic setting.

- Plasma IP-10 differentiates between active TB and LTBI in HIV positive persons and is a valuable adjunct to TB diagnosis. IP-10 also holds promise as a biomarker for active TB treatment efficacy.

- Both 25(OH)D and 1,25(OH)$_2$D are significantly reduced in HIV positive patients with active TB and 1,25(OH)$_2$D levels may have prognostic significance in patients with LTBI. Furthermore, there is a significant correlation between *M.tb*-specific INF-γ responses and 25(OH)D reinforcing the association between vitamin D and the immune response to TB.

- Larger longitudinal studies of longer duration are necessary to provide adequate immunological profiling to rapidly and correctly distinguish between patients with active TB and LTBI. Such studies are also needed to better identify LTBI patients who would benefit from TB preventive therapy in TB low-endemic countries. In addition, the role of vitamin D as a prognostic marker for TB progression could be further explored.

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Appendices

Hiv og Tuberkulose i Norge:
Studier av nye diagnostiske metoder og immunitet hos hiv-positive personer med latent og aktiv tuberkulose

Sykehus: _____ **Pasientkode:** _____ **Initialer:** _____

Utfylt av: _____ **Dato**: _____

Stilling: ☐ **Lege** ☐ **Sykepleier** ☐ **Annet** (beskriv): _____

Del A: Personopplysninger

Kjønn: ☐ **Kvinne** ☐ **Mann** **Fødselsår:** _____

Fødeland: _____ **År ankomst Norge**: _____

Mors fødeland: _____ **Fars fødeland**: _____

Del B: Hiv sykdom

1. **Smitteår**: _____ ☐ Ukjent

2. **Smittested**: ☐ Norge ☐ Utlandet. Land: _____ ☐ Ukjent

3. **HIV-diagnose**: År: _____ ☐ Ukjent

4. **Laveste CD4 celle tall** (celler/μL): _____ Dato:_____ ☐ Ukjent

5. **Høyeste HIV-RNA** (kopier/ml): _____ Dato: _____ ☐ Ukjent

6. **AIDS-diagnose**: ☐ Ja År: _____ ☐ Nei

    AIDS-definerende diagnose(r):_____

    _____

7. Andre kroniske sykdommer (beskriv): _____

_____

8. Serologi: **Pos**   **Neg**   **Ukjent**

HBs antistoff :   Dato:_____   ☐   ☐   ☐

HBs antigen: Dato:_____   ☐   ☐   ☐

HCV antistoff   Dato:_____   ☐   ☐   ☐

HCV-RNA   Dato:_____   ☐   ☐   ☐

Toxoplasma IgG   Dato:_____   ☐   ☐   ☐

CMV IgG   Dato:_____   ☐   ☐   ☐

9. Antiretroviral terapi: ☐ Ja, år:_____   ☐ Nei

Indikasjon: ☐ AIDS   ☐ CD4 tall   ☐ HIV RNA

                 ☐ Primær HIV   ☐ Svangerskap

CD4 celle tall før behandling (celler/µL):   _____

HIV-RNA før behandling (kopier/ml):   _____

Del C: Tuberkulose bakgrunn/risiko

1. BCG-vaksine: ☐ Ja, år:_____   ☐ Nei   ☐ Ukjent   ☐ Arr

2. Tidligere Mantoux-test   ☐ Ja. Land: _____   År:_____   Indurasjon (mm): ____

                                     ☐ Nei   ☐ Ukjent

3. Rtg. thorax (tatt tidligere):   ☐ Ja, år:_____   ☐ Nei

        Forandringer forenlig med TB: ☐ Ja   ☐ Nei   ☐ Ukjent

4. **Tidligere behandlet for tuberkulose**: ☐ Ja År:_____ ☐ Nei   ☐ Ukjent

☐ Pulmonal   ☐ Ekstrapulmonal   ☐ Disseminert

☐ IRIS   ☐ Ukjent

Behandlingslengde: _____mnd

Medikamenter: _____

Behandlingsresultat:

☐ Kurasjon (mikroskopi/dyrkning negativ).

☐ Behandlingssvikt (mikroskopi/dyrkning positiv eller manglende klinisk respons).

☐ Avbrutt behandling

☐ Ukjent behandlingsresultat

5. **Tidligere forebyggende behandling**:   ☐ Ja   ☐ Nei   ☐ Ukjent

År: _____ Behandlingslengde (mnd): _____

Medikamenter: _____

6. **Opphold i land utenom Vest Europa**:   ☐ Ja   ☐ Nei

Land: _____   År: _____ Antall mnd:_____

_____   År: _____ Antall mnd:_____

_____   År: _____ Antall mnd:_____

7. **Nærkontakt med person med tuberkulose**:   ☐ Ja   ☐ Nei   ☐ Ukjent

Land: _____   År: _____

Hvem: ☐ Husstand ☐ Familie   ☐ Arbeidsplass   ☐ Skole   ☐ Omgangskrets

Tidsperiode: ☐ < 1 dag   ☐ < 1 uke   ☐ 1 uke-3 mnd   ☐ > 3 mnd

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Hiv og Tuberkulose i Norge:
Studier av nye diagnostiske metoder og immunitet hos hiv-positive personer
med latent og aktiv tuberkulose

Sykehus: _____   **Pasientkode**: _____ **Initialer**: _____

Utfylt av: _____   **Dato**: _____

Stilling: ☐ **Lege**   ☐ **Sykepleier**   ☐ **Annet** (beskriv): _____

Del A: Utredning ved inklusjon

1. Kliniske symptomer:

☐ Ingen

☐ Feber

☐ Vekttap   Antall kg: _____   Tidsperiode:_____

☐ Hoste

☐ Ekspektorat   Blodig: ☐ Ja   ☐ Nei

☐ Nattesvette

☐ Andre symptomer, beskriv:_____

Symptomvarighet: ☐ < 1 mnd   ☐ 1-3 mnd   ☐ > 3 mnd   ☐ ukjent

2. IGRA-tester:

QFT TB Gold (dato): _____ ☐ Pos Verdi*)_____IU/ml ☐ Neg   ☐ Inkonklusiv
*) (Verdi: TB antigen minus Nil)

T-SPOT.TB (dato): _____ ☐ Ikke tatt ☐ Pos   ☐ Neg   ☐ Inkonklusiv

3. Mantoux (siste 3 mnd):   ☐ Pos   Indurasjon (mm): _____   ☐ Neg

☐ Ikke utført

4. **Rtg. Thorax (siste 3 mnd):** ☐ Ja ☐ Nei

    Forandringer forenlig med TB: ☐ Ja ☐ Nei

5. **Mikroskopi:** ☐ Ja ☐ Nei

    Indusert Sputum (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Annet (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Spesifiser materialet: _____

6. **Dyrkning**: ☐ Ja ☐ Nei

    Indusert Sputum (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Annet (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Spesifiser materialet: _____

7. **PCR** ☐ Ja ☐ Nei

    Indusert Sputum (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Annet (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Spesifiser materialet: _____

8. **Laboratorieprøver**:

CD4 celle tall (celler/µL): _____ Dato:_____

HIV-RNA (kopier/ml): _____ Dato:_____

Senkningsreaksjon (mm/t): _____ Dato:_____

CRP (mg/L) _____ Dato:_____

Leukocytter (10^9/L) _____ Dato:_____

Lymfocytter (10^9/L) _____ Dato:_____

ALAT (U/L) _____ Dato:_____

Hemoglobin (g/dl) _____ Dato:_____

Del B: Konklusjoner av screening med eventuelle tiltak

☐ Ikke latent eller aktiv tuberkulose

☐ Tuberkulose (pulmonal)

☐ Tuberkulose, ekstrapulmonal (hvilket organ):_____

☐ Tuberkulose, disseminert (> 2 organ eller milliær): _____

☐ IRIS

☐ Sannsynlig tuberkulose (klinisk diagnose uten positiv mikroskopi eller dyrkning)

☐ Latent tuberkulose (basert på Mantoux, Interferon-test og/eller risikovurdering)

Profylaktisk behandling: ☐ Ja   ☐ Nei

      Hvis ja: Dato: _____   ☐ Isoniazid   ☐ Rifampicin

Evt kontraindikasjoner mot behandling: _____

Inkluder protokoll 'latent TB'

Aktiv behandling: ☐ Ja   ☐ Nei

      Hvis Ja,   Dato: _____

      Medikamenter   ☐ Isoniazid   ☐ Rifampicin   ☐ Rifabutin

                         ☐ Ethambutol   ☐ Pyrazinamide

                         ☐ Andre, spesifiser: _____

Evt kontraindikasjoner mot behandling: _____

Inkluder protokoll 'aktiv TB'

Antiretroviral behandling (ART):   ☐ Ja   ☐ Nei

ART medikamenter: _____

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Oppfølgingsskjema: Aktiv tuberkulose   **Konsultasjon #** _____

Sykehus: _____   **Pasientkode:** _____

Dato for siste konsultasjon:_____

Utfylt av:
Navn: _____   **Dato**: _____

Stilling: ☐ **Lege**   ☐ **Sykepleier**   ☐ **Annet** (beskriv): _____

Del A: Aktiv tuberkulose behandling

Oppstartsdato: _____

Medikamenter   ☐ Isoniazid   ☐ Rifampicin   ☐ Rifabutin
                 ☐ Ethambutol   ☐ Pyrazinamide
                 ☐ Andre, spesifiser: _____

ART medikamenter: _____

Del B: Oppfølging

1. Kliniske symptomer:

☐ Ingen symptomer
☐ Feber
☐ Vekttap   Antall kg/tidsperiode:_____
☐ Hoste
☐ Ekspektorat   Blodig: ☐ Ja   ☐ Nei
☐ Nattesvette
☐ Andre symptomer, beskriv:_____

2. IGRA-tester:

QFT TB Gold (dato): _____   ☐ Pos Verdi*)_____IU/ml ☐ Neg   ☐ Inkonklusiv

T-SPOT.TB (dato): _____   ☐ Ikke tatt ☐ Pos   ☐ Neg   ☐ Inkonklusiv

*) (Verdi: TB antigen minus Nil)

3. **Rtg. Thorax** (dato):_____ ☐ Ja ☐ Nei

    Regress av tb-forandringer: ☐ Ja ☐ Nei ☐ Normal rtg

4. **Annen rtg undersøkelse** (dato):_____ ☐ Ja ☐ Nei

    Spesifiser:_____

    Regress av tb-forandringer: ☐ Ja ☐ Nei ☐ Normal rtg

5. **Mikroskopi:** ☐ Ja ☐ Nei ☐ Ikke utført

    Sputum (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Annet (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Spesifiser materialet: _____

6. **Dyrkning**: ☐ Ja ☐ Nei ☐ Ikke utført

    Sputum (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Annet (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Spesifiser materialet: _____

7. **PCR** ☐ Ja ☐ Nei ☐ Ikke utført

    Sputum (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Annet (dato): _____ ☐ Pos ☐ Neg ☐ Ikke utført

    Spesifiser materialet: _____

8. **Laboratorieprøver**:

CD4 celle tall (celler/µL): _____ Dato:_____

HIV-RNA (kopier/ml): _____ Dato:_____

Senkningsreaksjon: _____ Dato:_____

CRP _____ Dato:_____

Leukocytter _____ Dato:_____

Lymfocytter _____ Dato:_____

ALAT _____ Dato:_____

Hemoglobin _____ Dato:_____

Del C: Konklusjoner/tiltak

1. Aktiv behandling: ☐ Forlenget, årsak spesifiser:_____

☐ Endret, årsak spesifiser:_____

☐ Ferdigbehandlet (dato):_____

☐ Seponert (dato):_____
                    Årsak:_____

2. Behandlingsresultat ved avsluttet behandling (dersom aktuelt):

☐ Kurasjon (mikroskopi/dyrkning negativ).

☐ Behandlingssvikt (mikroskopi/dyrkning positiv eller manglende klinisk respons).

☐ Avbrutt behandling

☐ Ukjent behandlingsresultat

☐ Ukjent behandlingsresultat

3. ART: ☐ Residiv

☐ endret,
spesifiser:_____

☐ Seponert (dato):_____

Årsak:_____

4. ☐ **Evt. andre tiltak**:

spesifiser:

Hiv og Tuberkulose i Norge:
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Oppfølgingsskjema: Latent tuberkulose	**Konsultasjon #**

Sykehus: _____   **Pasientkode:** _____

Dato for siste konsultasjon:_____

Utfylt av:
Navn: _____   **Dato**: _____

Stilling: ☐ **Lege**   ☐ **Sykepleier**   ☐ **Annet** (beskriv): _____

Del A: Latent tuberkulose

Profylaktisk behandling: ☐ Ja   ☐ Nei

Hvis Ja: Dato: _____   ☐ Isoniazid ☐ Rifampicin

ART medikamenter: _____

Del B: Oppfølging

1. Kliniske symptomer:

☐ Ingen symptomer
☐ Feber
☐ Vekttap   Antall kg/tidsperiode:_____
☐ Hoste
☐ Ekspektorat   Blodig: ☐ Ja   ☐ Nei
☐ Nattesvette
☐ Andre symptomer, beskriv:_____

2. Nytt opphold i land utenom Vest Europa: ☐ Ja   ☐ Nei

3. Ny nærkontakt med person med tuberkulose: ☐ Ja   ☐ Nei

4. IGRA-tester:

QFT TB Gold (dato): _____   ☐ Pos Verdi*)_____IU/ml ☐ Neg   ☐ Inkonklusiv

T-SPOT.TB (dato): _____   ☐ Ikke tatt ☐ Pos   ☐ Neg   ☐ Inkonklusiv

5. Laboratorieprøver:

CD4 celle tall (celler/µL):   _____   Dato:_____

HIV-RNA (kopier/ml):   _____   Dato:_____

Senkningsreaksjon: _____   Dato:_____

CRP   _____   Dato:_____

Leukocytter   _____   Dato:_____

Lymfocytter   _____   Dato:_____

ALAT   _____   Dato:_____

Hemoglobin   _____   Dato:_____

6. Evt. andre undersøkelser:_____

_____

Del C: Konklusjoner/tiltak

Profylaktisk behandling: ☐ uendret

☐ endret, spesifiser:_____

☐ ferdigbehandlet (dato):_____

☐ seponert (dato):_____
Årsak:_____

Endret til aktiv behandling: Dato: _____
(inkluderes videre som aktiv TB)

Årsak: ☐ Aktiv tuberkulose

☐ IRIS (immunrekonstitusjonssyndrom)

ART: ☐ uendret

☐ endret, spesifiser:_____

☐ seponert (dato):_____
Årsak:_____

☐ **Evt. andre tiltak**:

Hiv og Tuberkulose i Norge:
Studier av nye diagnostiske metoder og immunitet hos hiv-positive personer med latent og aktiv tuberkulose

Oppfølgingsskjema: Ikke tuberkulose   Konsultasjon

Sykehus: _____   **Pasientkode:** _____

Dato for siste konsultasjon:_____

Utfylt av:
Navn: _____   **Dato**: _____

Stilling: ☐ **Lege**   ☐ **Sykepleier**   ☐ **Annet** (beskriv): _____

Del A: Oppfølging

1. Kliniske symptomer:

☐ Ingen symptomer
☐ Feber
☐ Vekttap   Antall kg/tidsperiode:_____
☐ Hoste
☐ Ekspektorat   Blodig: ☐ Ja   ☐ Nei
☐ Nattesvette
☐ Andre symptomer, beskriv:_____

2. Nytt opphold i land utenom Vest Europa: ☐ Ja   ☐ Nei

3. Ny nærkontakt med person med tuberkulose: ☐ Ja   ☐ Nei

4. IGRA-tester (12 og 24 mnd etter screening):

QFT TB Gold (dato): _____   ☐ Pos Verdi*)_____IU/ml ☐ Neg   ☐ Inkonklusiv

T-SPOT.TB (dato): _____   ☐ Ikke tatt ☐ Pos   ☐ Neg   ☐ Inkonklusiv

5. Laboratorieprøver:

CD4 celle tall (celler/μL):   _____   Dato:_____

HIV-RNA (kopier/ml):   _____   Dato:_____

Senkningsreaksjon: _____   Dato:_____

Senkningsreaksjon: _____ Dato:_____

CRP _____ Dato:_____

Leukocytter _____ Dato:_____

Lymfocytter _____ Dato:_____

ALAT _____ Dato:_____

Hemoglobin _____ Dato:_____

4. Andre undersøkelser:_____

Del B: Konklusjoner/tiltak

☐ Verken latent eller aktiv tuberkulose

☐ Indikasjon for ny utredning latent tuberkulose

☐ Indikasjon for ny utredning aktiv latent tuberkulose