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**Relationship of immunodiagnostic assays for
tuberculosis and numbers of circulating
CD4+ T-cells in HIV-infection**

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Abbreviations

ACD	Acid Citrate Dextrose
AFB	Acid-Fast Bacilli
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen-Presenting Cells
BAL	Broncho-Alveolar Lavage
BCG	Bacille Calmette-Guérin
CCR5	Chemokine (C-C motif) Receptor 5
CD 4	Cluster of Differentiation molecule 4
CFP-10	Culture Filtrate Protein-10
CXCR4	Chemokine (C-X-C motif) Receptor 4
DNA	Deoxy-Ribonucleic Acid
DTH	Delayed-Type Hypersensitivity Reaction = Type IV Reaction according to Gell and Coombs
EDTA	Ethylene-Diamine-Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunospot
ESAT-6	Early Secretory Antigenic Target-6
FACS	Fluorescence Activated Cell Sorting
FSC	Forward Scattered Light
Gp41/120	Glycoprotein 41/120
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus, Species 1
HIV-2	Human Immunodeficiency Virus, Species 2
IFN- γ	Interferon- γ
IGRA	Interferon- γ Release Assay
IQR	Interquartile Range
LTBI	Latent Tuberculosis Infection
MTB	<i>Mycobacterium tuberculosis</i>
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTI	Nucleoside Reverse Transcriptase Inhibitors
NTM	Non-Tuberculous Mycobacteria
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PI	Protease Inhibitors
PPD	Purified Protein Derivate
RD1	Recombinant <i>M. tuberculosis</i> region of difference 1
RNA	Ribonucleic Acid
RT23	Tuberculin created by the Statens Serum Institute, Denmark
ROI	Reactive Oxygen Intermediate
SFC	Spot-forming Cell
SPSS	Statistics program (=Statistical Package for the Social Sciences)
SSC	Side Scattered Light
SSI	Statens Serum Institute, Denmark

TB	Tuberculosis
Th1	T Helper Cells Type 1
Th2	T Helper Cells Type 2
THT	Tuberkulin-Hauttest
TST	Tuberculin Skin Test
WBA	Whole Blood Assay
WHO	World Health Organisation

1. Introduction

1.1 Epidemiology of tuberculosis and HIV-infection

Tuberculosis and Human Immunodeficiency-virus (HIV)-infection range among the leading public health problems today (1). Developing countries are predominately affected, especially in sub-Saharan Africa (2).

The World Health Organisation (WHO) estimates that approximately one third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis. The majority of individuals infected with *M. tuberculosis* does not develop active tuberculosis due to a successful host immune response (3, 4). Latent infection with *M. tuberculosis* (LTBI) is usually defined as infection with *M. tuberculosis complex*, manifested by a pre-defined tuberculin skin test (TST) (**see chapter 1.4.1**) reaction and/or a positive interferon- γ release assay (IGRA) result (**see chapter 1.4.2**) without signs of active disease (5). In the year 2008, 9.4 million new cases of tuberculosis and 1.8 million tuberculosis-related deaths occurred, of which 0.5 million cases were HIV-positive (1). The AIDS-epidemic is the predominant reason for the rising numbers of tuberculosis cases in Africa (6). The interaction of these two diseases leads to increased morbidity and mortality in dually infected subjects (7). In fact, tuberculosis continues to be the leading cause of death in individuals with HIV-infection (8).

In the year 2008, it was estimated that approximately 33.4 million people were living with HIV-infection globally and approximately 2.0 million died of AIDS. More than two thirds of all HIV-infected patients live in sub-Saharan Africa, with nearly 1.9 million new cases in the same year (9). Infection with HIV doubles the risk of tuberculosis reactivation early after HIV-seroconversion (10), and people with HIV-infection are 6 – 50 times more likely to convert to active tuberculosis than people without HIV-infection (11). The prevalence of HIV in tuberculosis patients in Africa is 40 % (12), the incidence rises by 3 % per year (13).

In Uganda, the clinical site of this study, 98.000 new tuberculosis cases occurred in 2008 with the incidence of 310 per 100.000 people. In the year 2007, when the last data on HIV-infection were collected, altogether one million HIV-seropositive people lived in Uganda. The prevalence among adults (15 – 49 yrs.) was 6.1 % and 89.000 patients died of AIDS or opportunistic diseases (14).

Tuberculosis and HIV-infection in Africa, 2006

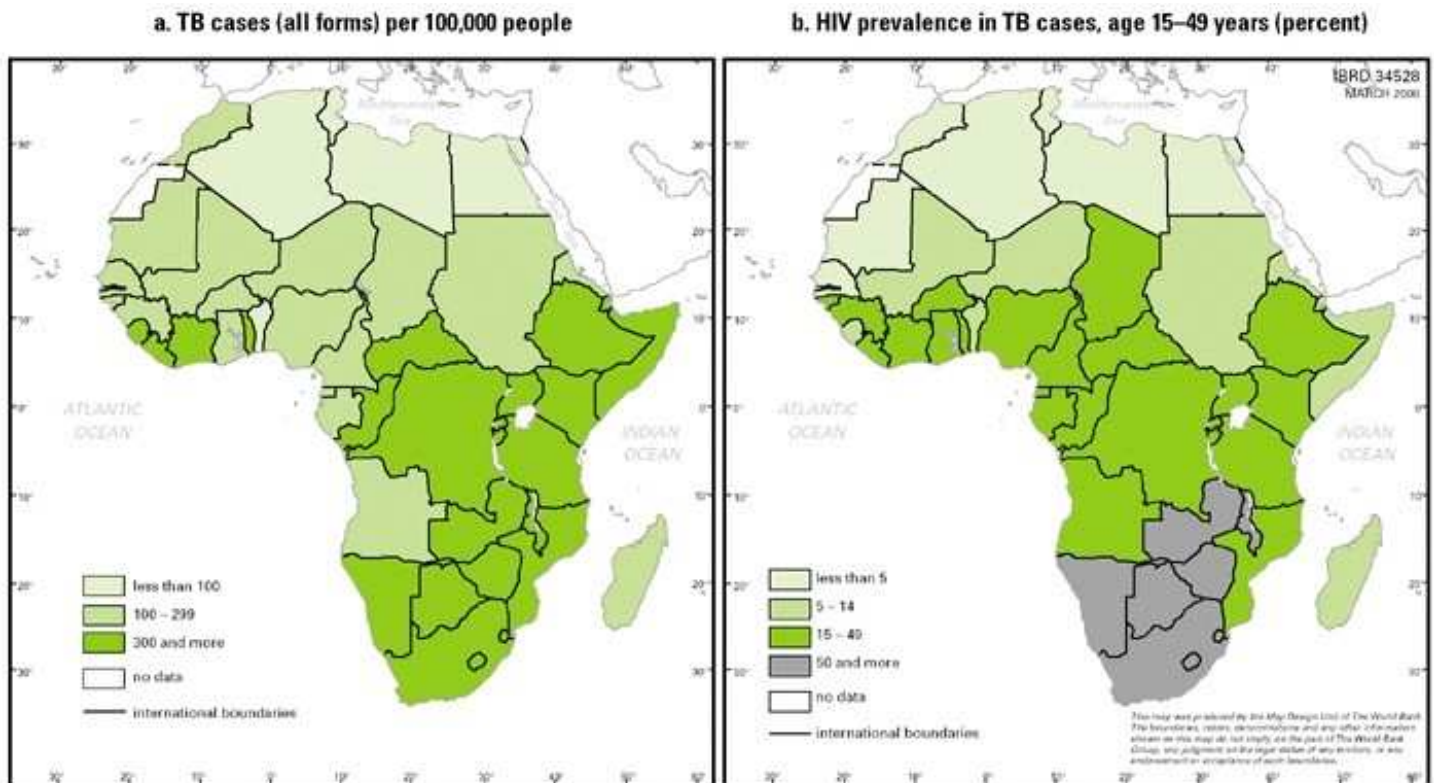


Figure 1.1: a) Tuberculosis cases (all forms) per 100.000 people. b) HIV-prevalence in tuberculosis cases, age 15 - 49 (percent) (15)

1.2 Pathogenic features of HIV-infection, opportunistic diseases and co-infection with *M. tuberculosis*

The Human Immunodeficiency-virus (HIV) is part of the genus of Lentiviridae, family of Retroviridae (16). Two human pathogenic species of HIV have been discovered: HIV-1 and HIV-2 (17). HIV-1 (group M, subgroup A to K; group N; group O) was the first one to be described and is more virulent, relatively easily transmitted, and causes most of HIV-infections worldwide. HIV-2 (subgroup A to F) is less infectious and largely confined to Western parts of Africa.

The virus' entry pathway into its primary target cells – especially CD4+ helper T-cells, but also macrophages and dendritic cells – can be divided into three major steps: binding, activation and fusion. A fundamental determinant for these events is the expression of the CD4+ receptor on the cellular membrane. The viral envelope

protein consists of a surface subunit, glycoprotein 120 (gp120), which contains a binding domain specific for the human CD4+ receptor and a transmembrane subunit, gp41, which contains a hydrophobic peptide essential for the viral infection of the human cells. High affinity binding between CD4+ and gp120 and binding of chemokine co-receptors, such as CCR5 (= chemokine [C-C motif] receptor 5) and CXCR4 (= chemokine [C-X-C motif] receptor 4), cause fusion of the viral and the cellular membranes and release the viral core into the target cell to initiate its own replication (18):

Lentiviruses are transmitted as single-stranded, positive-sense, enveloped RNA-viruses. After entry of the target CD4+ T-cell, the viral RNA-genome is converted into double-stranded DNA by the virally encoded enzyme Reverse Transcriptase present in the virus particle, after which the viral DNA is integrated into the cellular DNA by the virally encoded Integrase additionally to different host cellular co-factors. Then the genome is transcribed (19).

Now, two further pathways are possible: the virus may become latent and the infected cell continues functioning, or the virus may become active and starts replicating, and a large number of virus particles are liberated with the potential to infect other cells (20). The crucial characteristic of infection with HIV is the destruction of the acquired cellular immune system following the initial massive immune activation (21). Fifty to 70 % of persons with primary HIV-infection show the clinical, nevertheless non-specific symptoms of fever, sore throat, skin rash, lymphadenopathy, splenomegaly, myalgia, arthritis, or sometimes even meningitis. The symptoms of primary HIV are often misinterpreted as influenza-like-illness. So, the lack of specificity and the variable severity of these symptoms may partially explain, why most patients usually do not associate them with a newly acquired HIV-infection (22).

At the beginning of the infection, typically the HI-virus is widely disseminated and the CD4+ T-lymphocyte count in the peripheral blood declines persistently (21, 23). After 4 – 6 weeks, this highly active phase of virus replication is then contained by an HIV-specific cell-mediated and also a humoral immune response and leads to a significant down-regulation of the virus in the patient's blood (21). The cell-mediated immune response consists predominately of HIV-specific cytotoxic T-lymphocytes that fight virus-expressing cells (24, 25), whereas the humoral antibodies

(seroconversion) are directed against different HIV-proteins and consist of immune complexes of virus particles, immunoglobulin, and complement (22).

In most cases, however, the virus is not completely eliminated from the body, and a state of chronic, persistent viral replication ensues, which is unique among viral infections in humans and suggests that certain mechanisms of viral escape from the immune response may be operative (21, 22, 26). The primary set-point of viral load prevents the disease from progressing and at the same time reflects both HIV-virulence and host immune responses (21, 27). After a latency period of 10 years on average, progression to AIDS occurs. Still, there are less than 5 % of HIV-infected persons who do not show progression of HIV-disease, but have stable CD4+ T-cell counts even after years of infection. These persons are called long-term non-progressors (28).

During the latency period, HIV-infected individuals generally do not develop symptoms of opportunistic infections, e.g., pneumococcal or candida infections, despite the fact that the incidence of some of these infections is already increased at this stage of HIV-disease (29). Unless effective suppression of the viral replication is provided, a continuous loss of CD4+ T-cells follows which finally leads to the stage of AIDS (30).

While HIV cannot be eradicated from the human body, recent achievements in therapeutic options have drastically changed the HIV-associated morbidity and mortality. With the introduction of Highly Active Antiretroviral Therapy (HAART) in 1996, consisting of three different classes of drugs to inhibit progression of the infection, the prognosis of people living with HIV and AIDS has improved significantly in the developed world (31-33).

The three classes of HAART are the Nucleoside Reverse Transcriptase Inhibitors (NRTIs, e.g., Zidovudin, Lamivudin), Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs, e.g., Nevirapin, Efavirenz) and Protease Inhibitors (PIs, e.g., Ritonavir, Saquinavir). The treatment regimen itself involves one Protease Inhibitor (PI) in combination with two Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (N(t)RTIs), or one Non-nucleoside Reverse Transcriptase Inhibitor (NNRTI) in combination with two NRTIs (34). Several new drugs are now available in these classes (second generation NNRTIs and novel PIs), as well as new classes of drugs, Integrase Inhibitors, CCR5-antagonists and fusion inhibitors (Enfuvirtide) (31, 34).

The disadvantage of HAART-therapy is that current antiretroviral agents are not virucidal, thus eradication of viremia cannot be achieved and successful therapy often results in latently HIV-infected CD4⁺ T-cells and various other cellular reservoirs with stable plasma levels of HIV-1 RNA below the limits of detection of commercial assays. Thus, when HAART-therapy is stopped, viral loads return to pre-treatment levels (32, 33, 35).

In 80 % of cases, AIDS is diagnosed because of HIV-related complications, especially opportunistic infections, but also other illnesses and malignancies. In immunodeficiency, most opportunistic infections caused by parasites, fungi, viruses, mycobacteria or bacteria present with atypical manifestations, are more complicated and require a more deliberate treatment compared to other immunosuppressed or non-immunocompromised patients (36).

Infections with *Candida spec.*, *Cryptococcus neoformans*, Cytomegalovirus, Epstein-Barr-virus, Herpes simplex virus, *M. avium intracellulare*, *M. tuberculosis*, *Pneumocystis jirovecii*, *Salmonella spec.*, *Toxoplasma gondii* or Varicella-zoster-virus belong to the AIDS-defining illnesses and are part of the most common opportunistic infections, as well as Cryptosporidia, JC-virus and Microsporidia. With progressing HIV-infection and decreasing CD4⁺ T-cells, these pathogens cause various illnesses which usually do not arise human diseases (37, 38).

In contrast to tuberculosis, infections with other opportunistic pathogens are generally dependant upon the level of immunodeficiency (39). By counting the number of CD4⁺ T-lymphocytes in the blood, which normally ranges between 500 – 1200 cells per microliter (µl), and measuring the viral load, the extent of immunosuppression can be quantified. HIV-infected patients with numbers of circulating CD4⁺ T-lymphocytes below 200 or even below 100 per µl are most vulnerable for the development of opportunistic infections (39, 40). Yet, different from other opportunistic infections, where there is a selective range of CD4⁺ T-cell count in which the disease occurs, tuberculosis arises throughout a broad range of CD4⁺ lymphocyte count (11, 41, 42), and the risk of rapid tuberculosis progression soon after infection or re-infection with *M. tuberculosis* is highly increased in HIV-positive individuals: In persons co-infected with *M. tuberculosis* and HIV, the annual risk can exceed 10 % (10, 11, 43).

1.3 Infection with *M. tuberculosis*

1.3.1 Pathogenesis of the infection

Mycobacterium tuberculosis is an obligate aerobe, slow-growing, acid- and alcohol-fast bacillus (AFB) without an outer cell membrane, and can be stained by Ziehl-Neelsen or Kinyoun method (44). Robert Koch was the first to discover and describe *M. tuberculosis* in 1882 (44), and received the Nobel Prize in Physiology or Medicine for this discovery in 1905.

M. tuberculosis spreads directly from person to person by inhaling aerosol droplets containing infectious particles with approximately three tubercle bacilli each. These particles normally are released from the lungs of patients with cavitary pulmonary tuberculosis through coughing, with the lung being the most common and most important primary site of the infection (45).

The mycobacterial cell wall consists of glycolipids, lipoproteins and several other molecular components, which all possess immunomodulatory activity (46). Once inhaled, the bacilli enter the alveoli of the lung and are phagocytosed by resident alveolar macrophages (47). The tubercle bacilli, instead of being destroyed, are able to reside and multiply in the macrophage's phagosomes. These early phagosomes containing virulent *M. tuberculosis*, so called parasitophorous or (mycobacterial) vacuoles, are inhibited from fusion with lysosomes, which normally leads to maturation into hydrolytic phagolysosomes and degradation of bacterial proteins providing peptides for antigen presentation to lymphocytes (48). Yet, inhibition of phagosome maturation by *M. tuberculosis* is not complete and some bacteria are killed or at least prohibited from replication by antibacterial mechanisms including acid pH, reactive oxygen intermediates (ROI), lysosomal enzymes, and toxic peptides (48-51). The induction of apoptosis of the macrophage by lymphocytes, however, can be prevented and the mycobacteria are able to escape from the host's immune response. Persisting in the macrophage, the tubercle bacilli elicit the production of soluble effector molecules which attract monocytes, lymphocytes, and neutrophils, and regulate the development of the host's cellular immune response that controls the infection in the majority (90 %) of immunocompetent individuals (51, 52).

In the lung, bacterial containment is focused on granulomatous lesions, where different T-cell populations participate in the protective immune response. The granuloma is composed of a central core of macrophages together with multinucleated giant cells, surrounded by macrophages and lymphocytes. The CD4+ T-lymphocytes produce interferon- γ (IFN- γ) and hence are of the T-helper 1 (Th1) type, while some of the CD8+ T-cells also secrete perforin and granulysin which directly kill mycobacteria-infected macrophages (50, 53). The flow of information through IFN- γ and several other cytokines activates the macrophages so that they are capable of controlling mycobacterial replication and granuloma formation. This adaptive immune response also leads to the development of a delayed-type hypersensitivity reaction (DTH) manifested by a positive TST (**see chapter 1.4.1**).

Granuloma are an effective means of containing the spread of the bacteria, nevertheless, may leave some of the bacilli dormant, but alive for decades in a non-replicating hypometabolic state (50). As long as the balance between cells and cytokines is not impaired, granuloma develop containing bacteria and persist throughout a person's life in an asymptomatic and non-transmissible state (50, 51, 53). When the balance is damaged by only one element in this complex interplay, be it that the infected person cannot control the initial infection in the lung (10 % of apparently immunocompetent persons) or that a latently infected person's immune system becomes weakened by immunosuppressive drugs, HIV-infection, malnutrition, aging or other factors, granuloma tend to disintegrate. Thereby, the infection can be reactivated. By dissemination of *M. tuberculosis* within the lungs (active pulmonary tuberculosis), to other tissues or organs (miliary or extrapulmonary tuberculosis) active disease occurs and uncontrolled growth of *M. tuberculosis* can be associated with extensive lung damage (50-52).

1.3.2 Clinical aspects

In tuberculosis, one can distinguish between primary infection and post-primary infection. Primary infection refers to the above explained phenomena that take place, when an individual comes into contact with the tubercle bacillus for the first time, shown by TST-positivity (**see chapter 1.4.1**) after 3 to 8 weeks (51). It remains asymptomatic or presents only with minimal clinical symptoms similar to those seen in common cold. Post-primary infection occurs, when a patient who has already been infected with the tubercle bacillus in the past develops tuberculosis.

The ability of the host to respond to the organism may be reduced by certain diseases like silicosis, diabetes mellitus, and diseases associated with immunosuppression. In these circumstances, the likelihood of developing tuberculosis is greater (54). As most cases of tuberculosis are initiated by the respiratory route of exposure, pulmonary tuberculosis is the most common site, leading to severe wasting. In addition, by hematogenous or lymphoid dissemination of the bacilli from infected lungs extrapulmonary manifestations can arise, such as spinal tuberculosis, cervical lymphadenitis, tuberculosis of the central nervous system (CNS), and cutaneously as lupus vulgaris (51, 54).

In pulmonary tuberculosis, cough is the most common symptom. Early in the course of the illness it may be non-productive, but subsequently, as inflammation and tissue necrosis ensue, sputum is usually produced and is key to many diagnostic methods. Inflammation of lung parenchyma adjacent to a pleural surface may, as well, cause chest pain, whereas dyspnea only occurs in extensive tuberculosis disease. Further constitutional symptoms can be fever, anorexia, weight loss and night sweats, as well as pallor, cyanosis, jaundice, pedal oedema, and lymph node enlargement (54, 55).

A life-threatening disease is the haematogenous spread of *M. tuberculosis* causing miliary tuberculosis and depending predominately on the balance between mycobacterial virulence and host immune defence. Clinically significant miliary disease affects between 1 % and 7 % of patients with all forms of tuberculosis (56). In 85 %, the typical radiographic findings of evenly distributed diffuse small 2 – 3 mm nodules can be found (57).

In most cases, the onset of clinically manifest tuberculosis is insidious and not particularly alarming so that months can go by before the diagnosis is established. Thus, a comprehensive diagnostic approach for a patient with possible tuberculosis includes a detailed medical history and clinical examination, as well as the results of radiological, microbiological and immunological methods (58). The diagnosis can be confirmed by sputum smear microscopy and/or Loewenstein-Jensen culture, which relies on the detection of AFB in sputum, culture or bronchial secretions obtained by bronchoalveolar lavage (BAL). Smear should be stained by Ziehl-Neelsen for conventional microscopy or Auramine-Rhodamine for fluorescence microscopy. Yet, culture on Loewenstein-Jensen medium is still much more sensitive, but takes 6 – 8 weeks of time. However, when tuberculosis and HIV are co-morbid, tuberculosis is often sputum-smear negative (2, 41, 59).

1.4 Diagnosis of tuberculosis

1.4.1 The Tuberculin Skin Test (TST)

The TST (Mendel-Mantoux-Test) has been the only method available for diagnosing latent infection with *M. tuberculosis* in the past century. Even today this test remains a widely used technique, although it contains some major disadvantages (60). The history of the TST goes back to Robert Koch who discovered tuberculin, initially with the purpose of curing the disease. Yet, tuberculin proved to be a possibility to detect a past or present tuberculous state, a finding which Koch presented at the 10th International Congress of Medicine in Berlin in 1890 (61). Tuberculin is obtained from steamed tuberculous bacterial cultures and consists of a precipitate of non-species-specific molecules. Therefore, it is also called Purified Protein Derivate (PPD).

In 1905, Clemens von Pirquet first introduced a tuberculosis screening test, while in 1907 Charles Mantoux and Felix Mendel developed the current test based on the intradermal delayed-type hypersensitivity reaction (62-64).

The mechanism of the TST relies on the observation that sensitised T-lymphocytes proliferate in regional lymph nodes following the presentation of PPD by antigen-

presenting cells (APC, e.g., macrophages, dendritic cells, Langerhans cells) (64). After clonal expansion, the antigen specific T-cells are recruited to the site of antigen inoculation, where they produce an inflammatory reaction of mild-to-moderate intensity, normally lasting only a short period of time. In persons who have been sensitised by prior mycobacterial infection, however, the inflammatory response intensifies and a lymphomonocytic perivascular infiltration develops due to recruitment by pro-inflammatory cytokines. The cellular infiltrates predominately consist of activated lymphocytes, especially T-lymphocytes with a CD4+ phenotype, and monocytes (64-66). Because of expression of adhesion molecules and alteration of the permeability of vessels stimulated by the secreted cytokines, the inflammatory reaction gives rise to a visible and palpable induration and is accompanied by oedema and erythema. Intense reactivity may even be associated with fever, more general swelling of the limb, regional lymphadenopathy, or rarely lymphangitis (67).

The host response to tuberculin begins within 5 to 6 hours, usually peaking in intensity after 48 to 72 hours and persisting for several days. The tuberculin PPD RT-23 by the Statens Serum Institute (SSI) is used in most countries. The standard skin test dose for RT-23 is 2TU of which 0.1 ml solution is injected intracutaneously at the volar aspect of the forearm with a tuberculin syringe, the needle bevel facing upward. Creation of a visible pale wheal is crucial, as subcutaneous administration will result in a false-negative test. The skin test reaction should be read between 48 and 72 hours after administration, preferably by the ballpoint pen and ruler method (68).

The interpretation of the TST depends on different factors, and knowledge of tuberculin test sensitivity and specificity, as well as positive predictive value, is required to interpret skin test reactions properly (69). TST-sensitivity in persons with LTBI and normal immune responsiveness is heterogeneous with a pooled estimate of 77 %, whereas specificity is low and very heterogeneous in BCG-vaccinated populations, but reaches high estimates of 97 % in non-vaccinated populations (70). Besides, positive tuberculin tests also occur in persons who have been infected with non-tuberculous mycobacteria. These reactions result in a lower specificity and a low positive predictive value in persons who have a low probability of LTBI. Even if the test has a specificity approaching 99 %, testing of persons in such low-prevalence

groups would result in most positive tests being false-positive (71), or can be obtained due to previous BCG-vaccination, incorrect method of TST-administration, incorrect interpretation of reaction or incorrect bottle of antigen used (72).

However, the specificity of the test is also dependent on the criterion used to define a “positive” test. It can be improved by progressively increasing the reaction size that separates positive from negative reactors (at the expense of decreasing test sensitivity) (73). **Table 1.1** summarises criteria for appropriate interpretation of TST-reactions among different risk groups (54).

In addition, the TST can produce false-negative results. These may occur in cutaneous anergy (inability to react to skin tests because of a weakened immune system, e.g., in HIV-infection), recent tuberculosis infection (within 8 – 10 weeks of exposure), very old tuberculosis infection (many years), very young age (less than 6 months old), recent live-virus vaccination (e.g., measles and smallpox), overwhelming tuberculosis disease, some viral illnesses (e.g., measles and chicken pox), incorrect method of TST-administration, and incorrect interpretation of reaction (72). Immunosuppressed individuals, including HIV-infected persons, especially at later stages of immunosuppression, are often unable to mount appropriate delayed-type hypersensitivity responses (DTH) to PPD despite probable LTBI (74, 75). As these persons are at high risk to develop tuberculosis, it is very important to reliably detect LTBI and subsequently offer preventive anti-tuberculosis drug therapy to those tested positive (76-79). Therefore, as alternative screening tools in vitro interferon- γ release assays (IGRAs) using enzyme-linked techniques (**see chapter 1.4.2**) have been developed which are highly specific for *M. tuberculosis* and unaffected by prior BCG-vaccination.

Table 1.1: Guidelines for determining a positive skin test reaction, stratified by risk groups according to the American Thoracic Society (ATS) (54)

Classification of the Tuberculin Skin Test Reaction		
Induration of 5 or more millimetres considered positive	Induration of 10 or more millimetres considered positive	Induration of 15 or more millimetres considered positive
<ol style="list-style-type: none"> 1. HIV-positive persons 2. Recent contacts to TB-case 3. Fibrotic changes on chest radiograph consistent with old TB 4. Patients with organ transplants and other immunosuppressed patients (receiving the equivalent of > 15 mg/d Prednisone for > 1 mo) 	<ol style="list-style-type: none"> 1. Recent arrivals (< 5 yr) from high-prevalence countries 2. Injection drug users 3. Residents and employees of high-risk congregate settings: prisons and jails, nursing homes and other health care facilities, residential facilities for AIDS patients, and homeless shelters 4. Mycobacteriology laboratory personnel 5. Persons with clinical conditions that make them high-risk: silicosis, diabetes mellitus, chronic renal failure, some hematologic disorders (e.g. leukemias and lymphomas), other specific malignancies, weight loss of >10 % of ideal body weight, gastrectomy, jejunioileal bypass 6. Children < 4 yr of age or infants, children, and adolescents exposed to adults in high-risk categories 	<p>Persons with no risk factors for TB.</p>

1.4.2 Interferon- γ Release Assays (IGRA)

Although the Tuberculin Skin Test used to be the only test for detecting latent tuberculosis infection for a long time, advances in mycobacterial genomics in the late 1990s identified a genomic segment in *M. tuberculosis* that is encoded by the Region of difference-1 (RD-1) (80) and is deleted from most environmental mycobacteria, apart from *M. kansasii*, *M. marinum*, and *M. szulgai* (81-83), as well as from all strains of BCG-vaccine. Thus, BCG-vaccination does not yield false-positive results here. Two proteins of this stretch of DNA, early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10), are strong stimuli of T-helper cells type 1 (Th1) in patients with *M. tuberculosis*-infection (84, 85), and during the past decade, interferon- γ release assays (IGRAs) have emerged as attractive diagnostic alternatives taking advantage of this fact.

Two IGRAs are currently commercially available, the QFT-G and its newer version QFT-G-IT (Cellestis, Carnegie, Australia) both relying on the enzyme-linked immunosorbent assay (ELISA) technique, and the T-SPOT.TB (Oxford Immunotec, Oxford, United Kingdom) relying on the enzyme-linked immunospot (ELISPOT) technique, first developed by Lalvani et al (86).

The rapid *ex vivo* ELISPOT assay counts individual antigen-specific T-cells. T-cells from persons with *M. tuberculosis*-infection become sensitized to ESAT-6 or CFP-10 *in vivo*. When the T-cells re-encounter these antigens *ex vivo* in the overnight ELISPOT assay, they release the cytokine IFN- γ . By the next morning, each T-cell gives rise to a dark spot, which is the “footprint” of an individual *M. tuberculosis*-specific T-cell (85, 87). The readout is thus the number of spots that are counted using a magnifying lens or automated reader (88). The ELISA (QFT-G) assay, on the other hand, measures the IFN- γ concentration in the supernatant of a sample of diluted whole blood after 24 h of incubation with ESAT-6 and CFP-10 (89), and QFT-G-IT enables blood to be collected directly into tubes containing the TB-specific antigens. New blood tests have an internal positive control (i.e., a sample well stimulated with a potent non-specific stimulator of IFN- γ production by T-cells); this controls the results of the test for technical errors, including failure to add viable functioning cells to the well. Although a negative TST in immunosuppressed individuals can be false-negative, the failure of the positive control in the blood tests provides the important information that the test’s result cannot be reliably interpreted, because it may reflect an underlying *in vivo* immunosuppression, negatively affecting T-cell function in the *in vitro* stimulation (90).

The sensitivity of these tests in active tuberculosis ranges from 76 to 100 %, whereas the specificity exceeds 88 %. This improved specificity of IGRAs over the TST has been confirmed by multiple studies, reviewed by Diel et al. (91), Menzies et al. (92), Mori et al. (93), Pai et al. (70), and Richeldi (90), which also underline that IGRAs are more sensitive than the TST in immunosuppressed patients. Yet, although these tests are unaffected by prior BCG-vaccination, they cannot distinguish between active tuberculosis and LTBI, when performed on PBMCs (70).

Interferon- γ release assays have several further important advantages over the TST: Testing requires only one patient visit and the assays are *ex vivo* tests, which reduce

the risk for adverse effects and eliminate potential boosting, when testing is repeated. Nevertheless, IGRAs also have important disadvantages, including higher material cost, need for an equipped laboratory, and a requirement to draw blood with subsequent careful handling to maintain viability of lymphocytes. The variability of these tests, when repeated after months or years, such as in serial testing of exposed populations, has not been well studied. The greatest disadvantage is the paucity of prospective data regarding the future risk for active tuberculosis in persons with positive results on IGRAs (positive predictive value - PPV) (92). In clinical practice, IGRAs, especially QFT-G-IT, and TST are predominately used to diagnose LTBI. However, there is no gold standard for the diagnosis of LTBI. Most investigators agree that positive IGRAs would occur almost exclusively in subjects who have encountered *M. tuberculosis*, i.e. subjects who actually have either LTBI, active tuberculosis or who had tuberculosis in the past (94). The consensus is, therefore, that positive IGRAs should not be considered false-positive, although the cut-offs for positivity of the commercial tests may need to be adjusted to optimise their accuracy (95).

1.5 Rationale for the study

Epidemiology and interactions of tuberculosis and HIV have been well researched and the increased morbidity and mortality in dually infected subjects have become obvious. Thus, rapid identification and preventive treatment of HIV-infected individuals with LTBI, who are at risk of developing tuberculosis, is a priority to fight tuberculosis in Africa, where tuberculosis and HIV are leading public health problems (2, 96).

In the past, the TST has been widely used as the only method to detect LTBI, although it contains some major disadvantages, and new immunological tests for tuberculosis have recently been developed. Yet, the number of studies comparing the different performances of the two commercially available interferon- γ release assays under the circumstances of immunodeficiency is very limited, although these tests might prove to be a possible alternative to the TST and a reliable means to detect LTBI in immunosuppressed persons, even in countries with high HIV-

prevalence and endemic for tuberculosis. In addition, the different conditions and mechanisms on the cellular levels of the two IGRAs are only poorly understood and increased knowledge in this field will add an important step forward towards revealing the cause of different test performances and results.

Since HIV, the model-disease for immunodeficiency in this study, affects especially CD4⁺ T-lymphocytes, the current gold standard for measuring the stage of HIV-disease is the absolute CD4⁺ cell count. With falling T-cell count the infection progresses and the likelihood of reactivation of LTBI increases. In such immunocompromised circumstances, the possibility of a false-negative response in the TST in persons with presumptive LTBI is high (75, 96-98), and standardised IGRAs stand a great chance to be more reliable for the diagnosis, as they seem to be unimpaired or at least less impaired by further stages of CD4⁺ T-cell depletion (99-101). Nevertheless, specificity and sensitivity between the tests and among different study groups seem to vary (88). In IGRAs on the other hand, CD4⁺ T-lymphocytes sensitized to *M. tuberculosis*-specific antigens ESAT-6 and CFP-10 produce IFN- γ which can be detected by two enzyme-linked methods.

To evaluate the different current methods for the immunodiagnosis of LTBI and understand their relationship to the level of immunodeficiency, this study compares the performance of two IGRAs and the TST among HIV-infected and non-infected patient groups in the high-tuberculosis-incidence country of Uganda, and a control group from the low-incidence country of Italy.

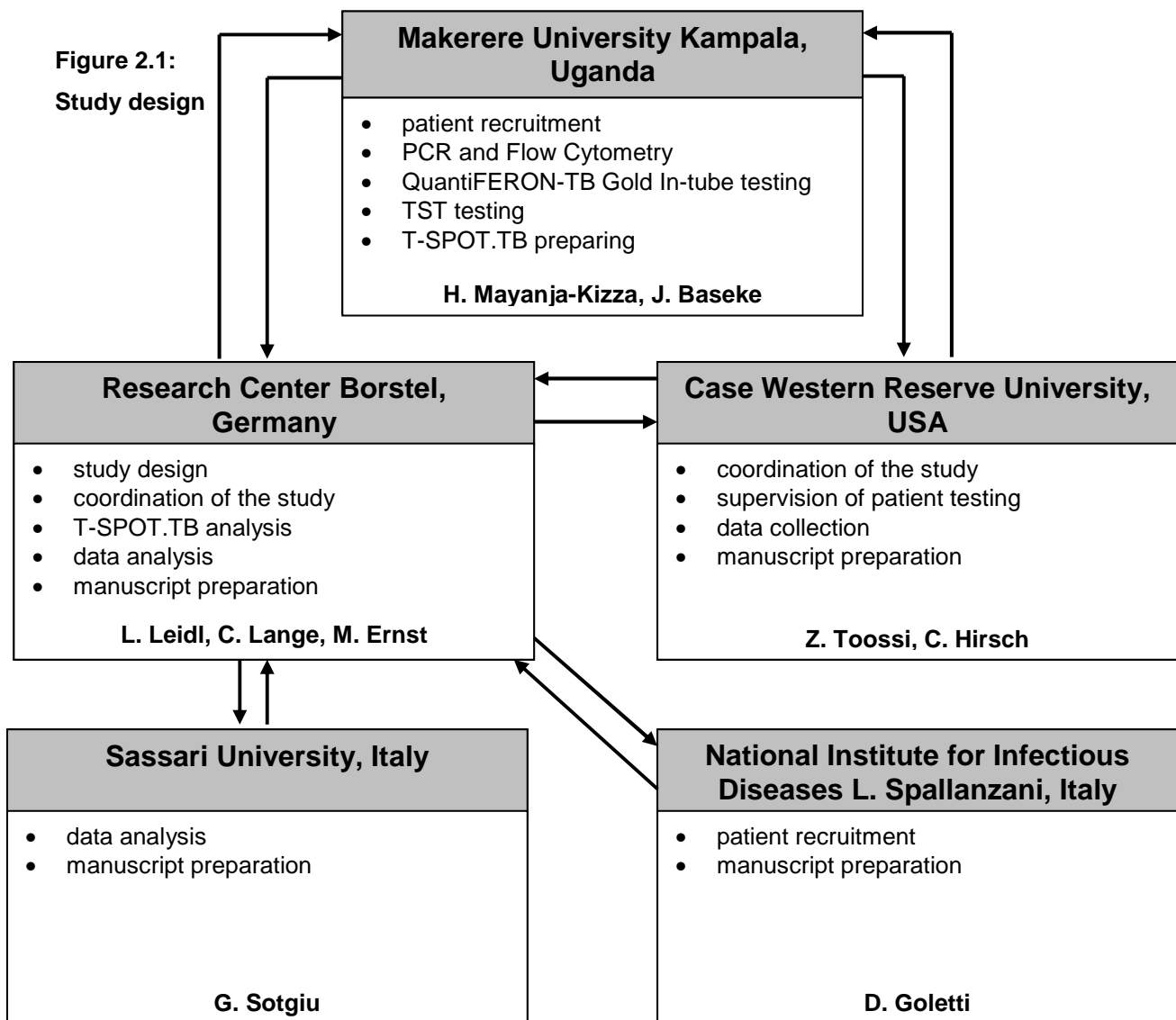
2. Material and Methods

2.1 Study design

The study was designed as collaboration of the Divisions of Clinical Infectious Diseases and Immune Cell-Analytics at the Research Center Borstel, Borstel, Germany, the Institute of Infectious Diseases at Makerere University in Kampala, Uganda, the Hygiene and Preventive Medicine Institute at Sassari University in Sassari, Sardinia, Italy, the Tuberculosis Research Unit at Case Western Reserve University in Cleveland, Ohio, USA, and the Translational Research Unit at the National Institute for Infectious Diseases L. Spallanzani in Rome, Italy. The respective contributions of the different institutions are shown in **figure 2.1**.

The study was supported by a personal research grant to Prof. Dr. C. Lange by the H.W. & J. Hector foundation, Weinheim (Germany), and by NIH grants HL 51636 and AI 70022.

Figure 2.1:
Study design



2.2 Study subjects

The study was conducted in Kampala, Uganda, at the HIV-outpatient clinic of the Infectious Diseases Institute of Makerere University. In 2008, the incidence of tuberculosis in Uganda was 310 per 100.000 (1). In recent years, approximately 71 % of tuberculosis patients tested for HIV were HIV-positive in Uganda (102), however, current HIV-prevalence rates in patients with tuberculosis are estimated to be lower at 59 % (1). Approximately 6.1 % of Ugandan adults and 150.000 children were HIV-positive in 2007, and of the total population of 30.884.000 people 13 % lived in urban areas (9). Kampala is the biggest city and capital of Uganda with 1.35 million inhabitants, several kilometres north of Lake Victoria.



Figure 2.2: Photographs of the outskirts of Kampala

The study was approved by the ethical committees of Makerere University, Uganda, Case Western Reserve University in Cleveland, USA, and Lübeck University, Germany. All subjects provided written informed consent, consistent with good clinical practice and ethical guidelines of the US Department of Health and Human Services.

Following voluntary counselling and testing for HIV-1 infection, adult residents of Kampala who were referred to the Infectious Diseases Institute at Makerere University were invited to participate. All study subjects were antiretroviral therapy naïve. Prior tuberculosis, Isoniazid preventive therapy, steroid therapy, pregnancy, and in HIV-infected people, a Karnofsky score ≤ 60 (score system on status of disease referring to symptoms and ranging from 100 % for no current symptoms to 0 % for death) or current opportunistic infection were exclusion criteria. The presence

of any one of the symptoms cough, chest pain, recent weight loss, night sweats, fever, loss of appetite, swelling of lymph nodes or generalized tiredness triggered referral to the Tuberculosis Research Unit clinic to rule out active tuberculosis, where sputum examinations were performed. All individuals were clinically examined and received a chest X-ray to exclude active pulmonary tuberculosis. Healthy HIV-seronegative controls (staff of the Infectious Diseases Institute at Makerere University) were also enrolled.

Furthermore, HIV-seropositive individuals without risk factors for active tuberculosis were recruited at the National Institute for Infectious Diseases L. Spallanzani, Rome, Italy, as additional controls.

2.3 Performing Tuberculin Skin Test

At the first study visit, blood was taken and a 2 TU/0.1 ml TST (RT23, Statens Serum Institute, Copenhagen, Denmark) was placed on the volar aspect of the forearm, and the site was marked with a circle by felt tip pen.

At 48 hours, the transverse diameter of the visible and palpable TST-induration was determined by the ball-point pen and ruler method (68) (**figure 2.3**). TST-positivity was defined according to ATS-guidelines (54).



Figure 2.3: Photograph of measuring TST-induration

2.4 Performing Interferon- γ Release Assays (IGRA)

T-SPOT.TB (Oxford Immunotec, Abingdon, UK) and QuantiFERON-TB Gold In-tube (QFT-G-IT; Cellestis, Carnegie, Australia) were performed according to the manufacturer's guidelines. **Figure 2.4** shows the basic differences of the two tests.

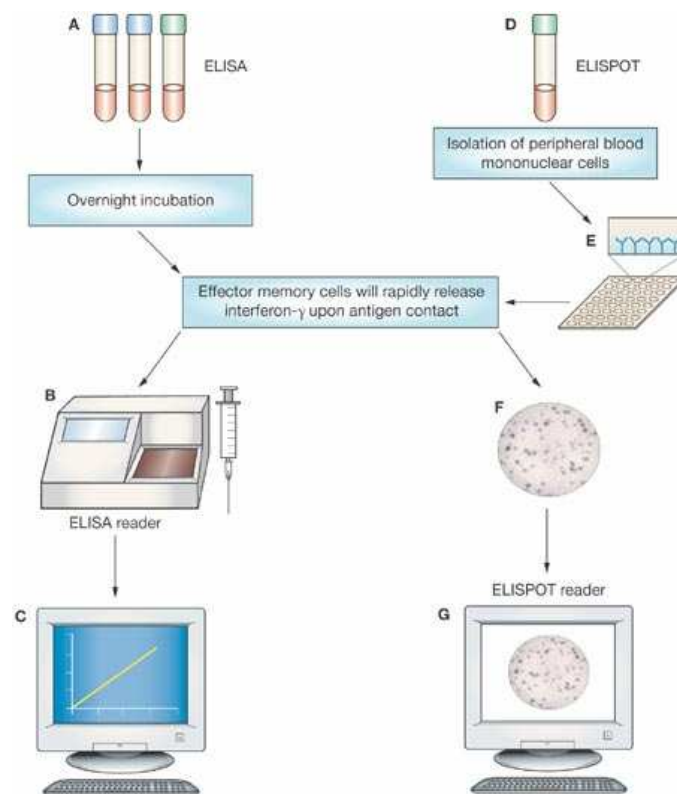


Figure 2.4: QuantiFERON-TB Gold In-tube ("ELISA") and T-SPOT.TB ("ELISPOT") (103)

2.4.1 T-SPOT.TB assay

For the T-SPOT.TB assays, the patient's venous blood was collected in Vacutainer[®] Cell Preparation Tubes[™] and peripheral blood mononuclear cells (PBMCs), which included T-cells, were separated from the heparinized sample by centrifugation, were washed in culture media to remove any background interference, counted to correct for the patient's immune status, and added in even amounts to the wells of the commercially available IFN- γ ELISPOT kit with a pre-coated 96-well microtiter plate. 250.000 cells per well were stimulated with phytohaemagglutinin (positive control),

were left unstimulated (negative control) or contained *M. tuberculosis*-specific peptides of ESAT-6 and CFP-10. After incubation for 18 hours in a CO₂- incubator to allow for the T-cells to encounter the antigen, plates were washed removing both the T-cells and the antigen from the wells and leaving any T-cell secreted cytokine captured by the antibodies lining the wells. If the patient was infected with *M. tuberculosis*, his T-cells had recognized the antigens and secreted IFN- γ . The cytokine was captured in the immediate vicinity of the cytokine-secreting T-cell by monoclonal IFN- γ specific antibodies coated on the bottom of each well. An enzyme-conjugated secondary antibody that binds to another epitope on the captured cytokine was then added, and coloured spots were generated by the conjugated enzyme upon application of a colorimetric substrate ("sandwich capture technique"). The spots were counted on an Immunospot Analyzer (AID, Strassberg, Germany) (see figure 2.4), each one representing the footprint of an individual T-cell and, therefore, giving the number of *M. tuberculosis*-specific T-cells in the blood. A positive result was defined as spot count in the ESAT-6 and/or CFP-10 panel minus spot count in the nil control panel ≥ 5 spots; a negative result was defined as < 5 spots. Results were defined as indeterminate, if more than 10 spot-forming cells (SFC) were present in the nil-control wells (high background) and/or less than 20 SFC in the mitogen-positive control wells (see figure 2.4.1).

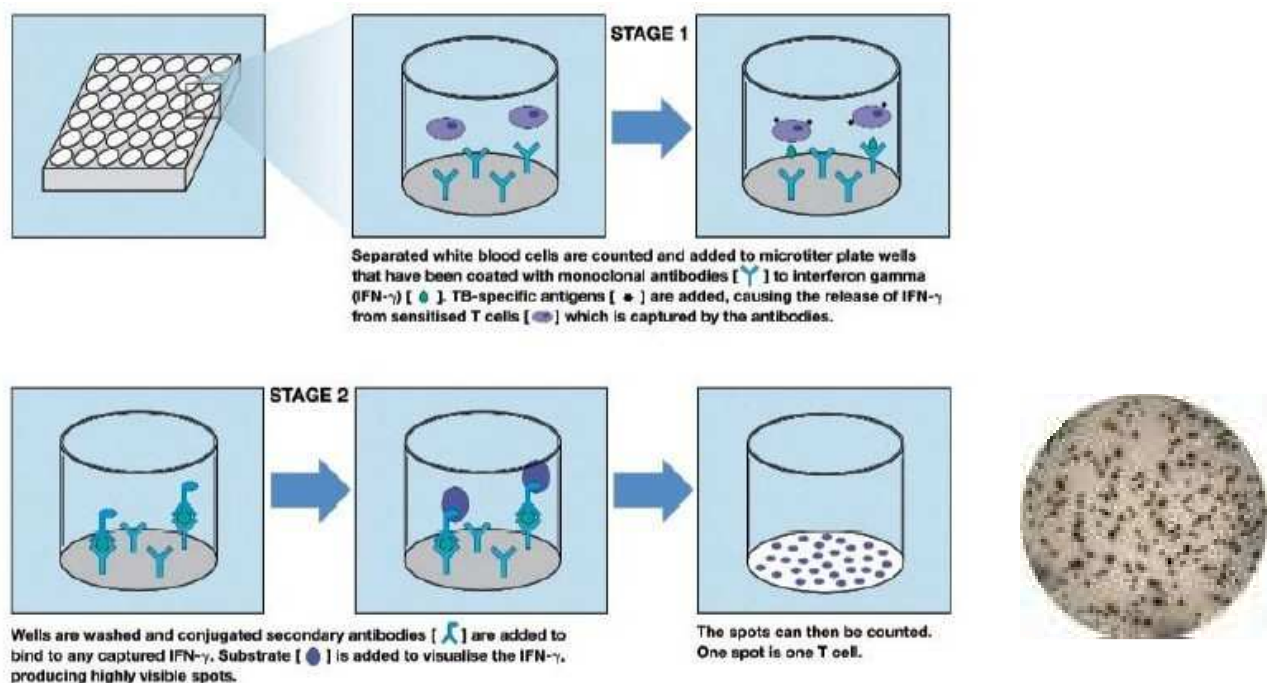


Figure 2.4.1: Procedure of T-SPOT.TB testing (104) and photograph of positive result

2.4.2 QuantiFERON-TB Gold In-tube assay

For the Whole Blood Assay (WBA) QFT-G-IT, three 1 ml venous whole blood samples were collected in three evacuated tubes (grey, red, pink) which were pre-coated with the antigens ESAT-6, CFP-10, and TB7.7 for the test, phytohaemagglutinin for the positive control, or no antigen for the negative control. After shaking the tubes carefully for about 5 seconds to ensure sufficient mixing of the components, the tubes were placed in a 37°C-incubator for 24 hours. On the next day, the tubes were centrifuged and plasma was harvested to add to ELISA plates together with conjugate and standards. After incubating for another 2 hours at room temperature, samples were washed and the substrate was added. If the patient was infected with *M. tuberculosis*, the T-cells had recognized the specific antigens and had secreted IFN- γ . The amount of IFN- γ was measured by an enzyme-linked immunosorbent assay (ELISA) after 30 minutes (see figure 2.4.2). Results were considered positive, if the level of IFN- γ in the ESAT-6, CFP-10 and TB7.7 antigen-exposed sample minus the level in the negative control was ≥ 0.35 IU/ml and $\geq 25\%$ of the IFN- γ concentration in the negative-control plasma. Indeterminate results were defined as either an unprovoked IFN- γ level of ≥ 8.0 IU/ml in the negative control plasma or an IFN- γ response of ≤ 0.5 IU/ml on phytohaemagglutinin stimulation with a level of IFN- γ in the tuberculosis antigen-exposed sample minus the level in the negative control of either < 0.35 IU/ml or $< 25\%$ of the IFN- γ concentration in the negative control plasma.

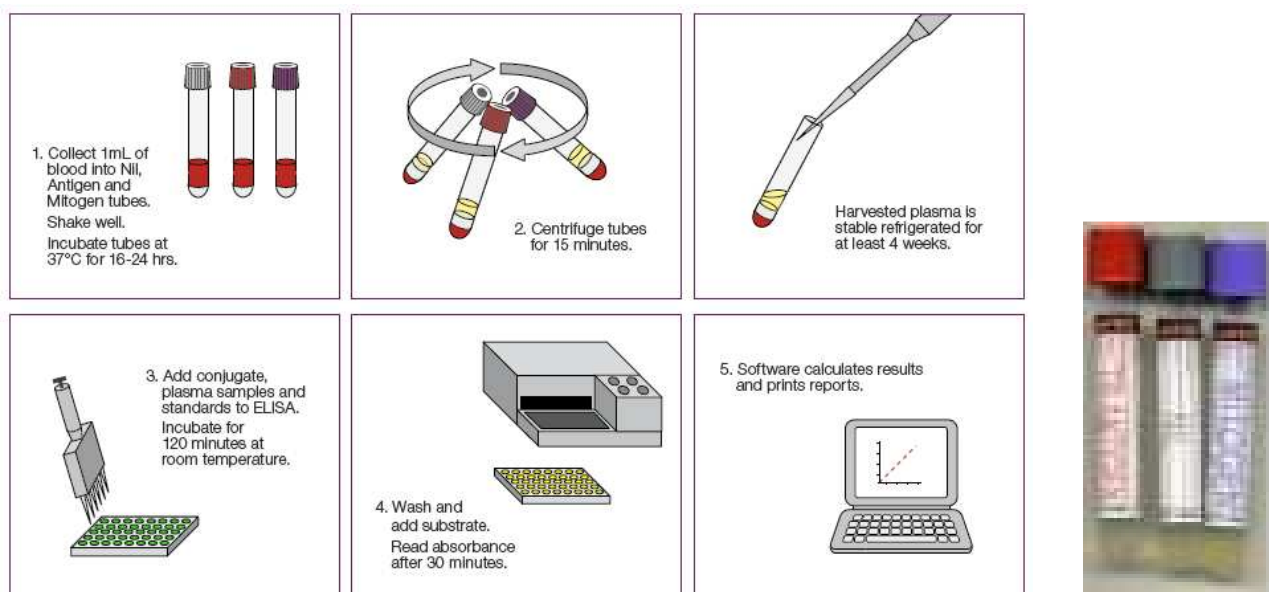


Figure 2.4.2: Procedure of QuantiFERON-TB Gold In-tube (104) and 3 venous blood tubes

2.5 Laboratory evaluation

The patient's plasma HIV viral load was examined by Amplicor (Roche™) and blood CD4+ cell counts were detected by flow cytometry (Becton Dickinson Inc., San Jose, CA, USA).

2.5.1 AMPLICOR HIV-1 MONITOR test (Roche™)

The AMPLICOR HIV-1 MONITOR test (Roche™) is an *in vitro* nucleic acid amplification test to quantitate Human Immunodeficiency-virus type 1 (HIV-1)-RNA in human plasma. The test can be used with either the Standard or UltraSensitive Specimen Processing Procedure. With the Standard Specimen Processing Procedure the test can measure HIV-1-RNA over the range of 400 – 750.000 copies/ml, while the UltraSensitive Specimen Processing Procedure measures HIV-1-RNA over the range of 50 – 75.000 copies/ml. It is intended for the use with clinical presentation and other laboratory markers of disease progress for the clinical management of HIV-1-infected patients. The test can assess patient prognosis by quantitation of the baseline HIV-1-RNA level or monitor the effects of antiretroviral therapy by detecting changes in plasma HIV-1-RNA levels during the course of antiretroviral treatment.

In short, the AMPLICOR HIV-1 MONITOR test (Standard/UltraSensitive) quantifies HIV-1-RNA levels in human plasma, yet, it should not be used as a screening test for HIV or as a diagnostic test to confirm the presence of HIV-infection. In addition, the test provides accurate quantitative results in both symptomatic and asymptomatic patients and is less labour intensive than quantitative culture procedures of plasma or PBMC-associated HIV-1.

To perform the test, human plasma is collected in standard blood collection tubes containing the anticoagulants acid citrate dextrose (ACD) or ethylene-diamine-tetraacetic acid (EDTA). As heparin inhibits the polymerase chain reaction (PCR), specimens anticoagulated with heparin are unsuitable for the use with this test, unless further processed to remove heparin.

The test is based on the five major processes specimen preparation, reverse transcription of HIV-1 and OS-Target-RNA, PCR-amplification of HIV-1 and OS-Target-DNA, hybridization of amplified products to target-specific DNA-probes, and detection of the amplified product.

2.5.2 Technical principles of Flow Cytometry

Flow cytometry is a technology that simultaneously measures and analyzes multiple physical characteristics of single microscopic particles from 0.2 – 150 µm suspended in a fluid stream flowing through a beam of light. The physical and/or chemical properties measured can be the particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic apparatus that records, how the cell or particle scatters incident laser light and emits fluorescence.

The beam of light (usually laser-light) is directed onto the hydro-dynamically focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the laser light, and when the cell or particle deflects the laser light, light scattering occurs. The extent to which this occurs depends on the physical properties of a particle, namely its size and internal complexity. Factors that affect light scattering are the cell's membrane, nucleus, and any granular material inside the cell. Cell shape and surface topography also contribute to the total light scatter. One of the detectors is positioned in line with the light beam and measures forward scattered light (FSC), and several others are placed perpendicular to it detecting side scattered light (SSC). Forward scattered light (FSC) is proportional to cell-surface area or size and provides a suitable method of detecting particles greater than a given size independent of their fluorescence and is therefore often used in immunophenotyping to trigger signal processing. Side scattered light (SSC) is proportional to cell granularity or internal complexity and is collected at approximately 90 degrees to the laser beam by a collection lens and then redirected by a beam splitter to the appropriate detector. Correlated measurements of FSC and SSC can allow differentiation of cell types in a heterogeneous cell population and major

leucocyte subpopulations, such as CD4⁺ T-cells, can be distinguished using FSC and SSC. This process of separating cells is referred to as “sorting”.

Fluorescence activated cell sorting (FACSTM, Becton-Dickinson Inc. San Jose, CA, USA) is a specialised type of flow cytometry. It provides a method for separating a mixture of cells into different tubes, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell.

2.6 Statistical analysis

Data were analysed using Stata 9.0 (StataCorp, Stata Statistical Software Release 9, College Station, TX, USA, 2005). Assay responses were evaluated as continuous variables and as categorical variables (positive or negative responses). Comparisons between proportions were performed using Fisher's exact test; the Student's t-test was used for continuous measurements to test relationships in unpaired analysis, while the Wilcoxon-Mann-Whitney test was used, when assumed that the dependent variable is a normally distributed interval variable. Group means (> 2) were compared using analysis of variance; its non-parametric version (Kruskal-Wallis test) was used, when appropriate. Test concordance was assessed by κ -statistics with agreement considered ‘slight’ for $\kappa \leq 0.20$, ‘fair’ for $0.20 < \kappa \leq 0.40$, ‘moderate’ for $0.40 < \kappa \leq 0.60$, ‘substantial’ for $0.60 < \kappa \leq 0.80$ and ‘optimal’ for $0.80 < \kappa \leq 1.00$. All analyses were two-sided, with a p-value of 0.05 considered significant (105, 106).

3. Results

3.1 Baseline characteristics of participants

Of 190 persons initially evaluated, 135 met the inclusion/exclusion criteria and were included in the analysis, and 55 were excluded due to unknown HIV-status and/or missing data of IGRA-tests. Of the included persons, 109 were newly diagnosed HIV-1-positive patients with no signs or symptoms of active tuberculosis, 19 were newly diagnosed HIV-1-positive patients with active tuberculosis, and 7 were HIV-uninfected healthy controls. As IGRA-tests detect IFN- γ released by CD4+ T-cells, they might depend on the absolute amount of these cells in the patient's blood. Considering this, we stratified our results by CD4+ cell count in groups of individuals with < 100/ μ l, 100 – 250/ μ l and > 250/ μ l. The study design, the demographic and clinical characteristics of the study participants and results of the immunodiagnostic assays are shown in **table 2.1** and **figure 3.1**.

Median numbers of circulating CD4+ T-cells were 182/ μ l (interquartile range-IQR 118) and 283/ μ l (IQR 226) in HIV-infected patients with and without active tuberculosis ($p = 0.0008$). HIV viral load in HIV-infected persons with tuberculosis was significantly higher than in persons without tuberculosis (71.331 (IQR 245.202) vs. 26.805 (IQR 95.003); $p = 0.01$). In HIV-infected patients without active tuberculosis ($n = 109$), median numbers of circulating CD4+ T-cells were 64/ μ l (IQR 43), 179/ μ l (IQR 68) and 368/ μ l (IQR 206), in those with CD4+ T-cell counts of < 100 ($n = 10$), 100 – 250 ($n = 33$) and > 250 ($n = 66$) cells/ μ l. HIV viral load was inversely related to CD4+ T-cell counts as expected with 104.004 (IQR 150.718), 55.234 (IQR 124.166), and 22.058 (IQR 42.692), respectively ($p < 0.01$). Gender and age were equally distributed among patients from the three different CD4+ T-cell count strata with generally more women than men being enrolled (males: 4 (40.0 %); 4 (12.1 %); 17 (25.8 %); $p = 0.12$; age: 34.3 ± 6.6 ; 35.6 ± 9.8 ; 33.4 ± 7.7 ; $p = 0.48$), altogether 25 with the median age of 34.1 ± 8.3 years. While there were 11 men in the group of HIV+/active tuberculosis patients ($p = 0.004$), only two of the seven members of the healthy control group were male ($p = 0.008$). For the healthy control group, CD4+ T-cell count and HIV viral load had not been registered.

Between HIV+ patients and HIV+/tuberculosis patients, the median age was more or less the same (mean = 34.1 and 33.4 years; $p = 0.7$), while HIV-uninfected controls were younger (mean = 25.7 years; $p = 0.03$) than the two other groups.

For all healthy persons, TST, T-SPOT.TB and QFT-G-IT could be performed, and for the 109 HIV-infected patients, T-SPOT.TB, QFT-G-IT and TST were done, but 20/109 (18.3 %) did not return for TST-reading. In the 19 HIV+ patients with active tuberculosis, T-SPOT.TB and QFT-G-IT were run, but no TST was performed.

In the 10 HIV-positive only male individuals from Italy who were included as additional negative controls to document test specificity, the median patient age was 41.4 (IQR 34 – 51) years and median number of circulating CD4+ T-cell counts was 480 (IQR 303.5 – 778) cells/ μ l.

Table 2.1: Baseline characteristics in 109 HIV+ persons, 19 HIV+/tuberculosis co-infected patients and 7 healthy individuals in Kampala, Uganda.

Variables	HIV+ patients			p-value [^]	HIV+ patients n = 109	HIV+/TB patients n = 19	p-value ^{^^}	Healthy controls n = 7	p-value ^{^^^}
	CD4+ cell counts < 100/ μ l n = 10	CD4+ cell counts 100 – 250/ μ l n = 33	CD4+ cell counts > 250/ μ l n = 66						
Males (%)	4 (40)	4 (12.1)	17 (25.8)	0.12*	25 (22.9)	11 (57.9)	0.004*	2 (28.6)	0.008*
Age (years, mean \pm SD)	34.3 \pm 6.6	35.6 \pm 9.8	33.4 \pm 7.7	0.48***	34.1 \pm 8.3	33.4 \pm 6	0.7**	25.7 \pm 4.9	0.03***
CD4+ cell count / μ l (median; IQR)	64; 43	179; 68	368.5; 206	0.0001*****	283; 226	182; 118	0.0008*****	-	-
HIV viral load (copies/ml, median; IQR)	104,004; 150,718	55,234; 124,166	22,058; 42,692	0.01*****	26,805; 95,003	71,330.5; 245,202	0.01****	-	-
HIV viral load (log copies/ml, mean \pm SD)	5 \pm 0.6	4.5 \pm 0.8	4.3 \pm 0.7	0.01***	4.4 \pm 0.8	4.9 \pm 0.7	0.01**	-	-

*Fisher's exact test

**Two independent samples t-test

***Analysis of Variance

****Wilcoxon-Mann-Whitney test

*****Kruskal-Wallis test

IQR: Inter-Quartile Range

TST: tuberculin Skin Testing

[^] Comparison of the demographic and clinical characteristics among HIV+ patients (n = 109) with CD4+ T-cell counts < 100/ μ l, those with 100 – 250/ μ l and those with > 250/ μ l

^{^^} Comparison of the demographic and clinical characteristics among HIV+ patients (n = 109) and HIV+/TB co-infected patients (n = 19)

^{^^^} Comparison of the demographic and clinical characteristics among HIV+ patients (n = 109), HIV+/tuberculosis co-infected patients (n = 19) and healthy controls (n = 7)

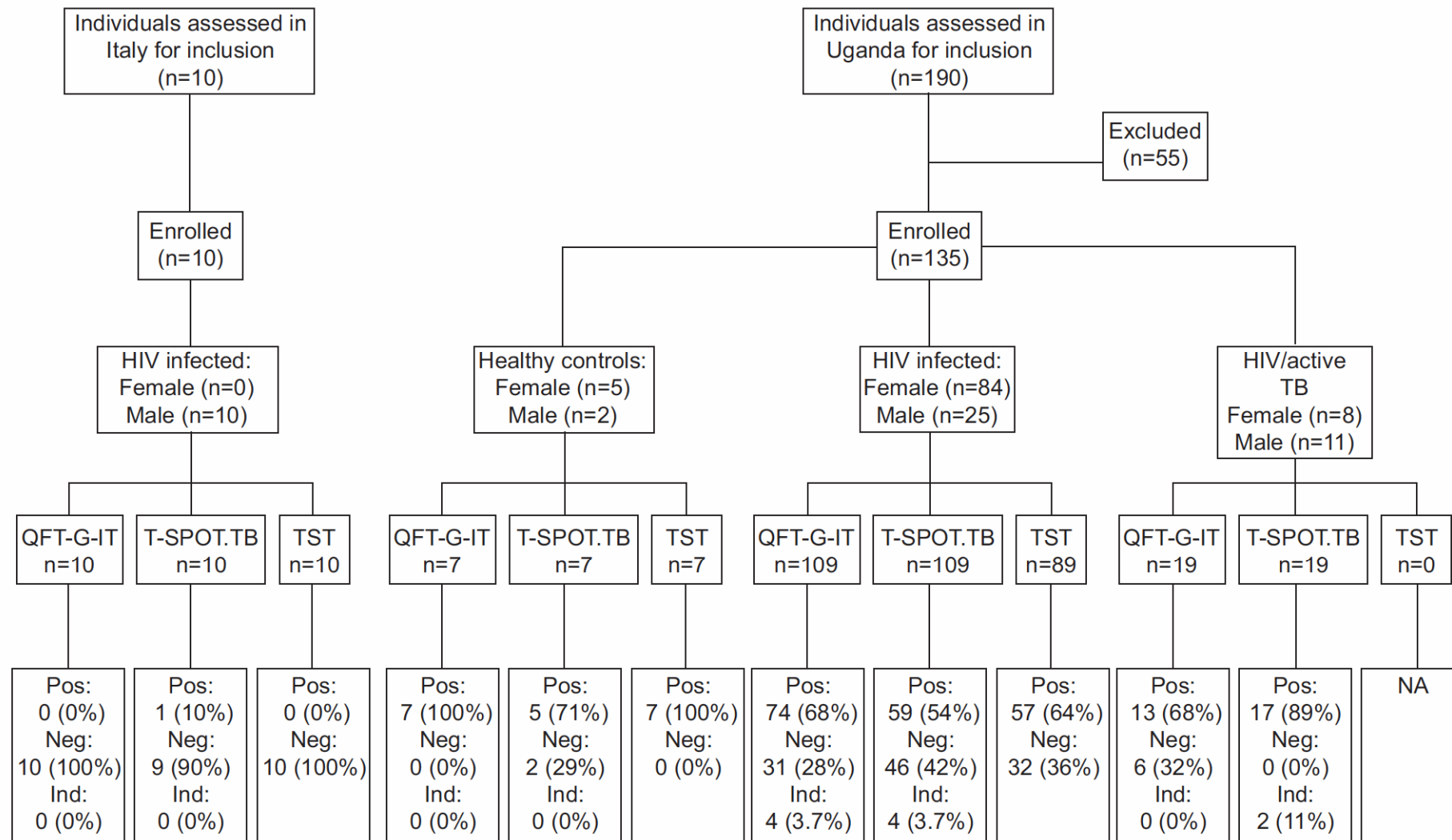


Figure 3.1: Flowchart of the study design and the main outcomes. TB: tuberculosis; QFT-G-IT: QuantiFERON-TB-Gold-In-Tube; TST: tuberculin skin test; Pos: positive; Neg: negative; Ind: indeterminate; NA: not applicable.

3.2 Test results according to HIV- and tuberculosis-status

Stratified according to numbers of circulating CD4+ T- cells with < 100 cells/ μ l, 100 – 250/ μ l and > 250/ μ l, in the individuals with HIV-infection and without tuberculosis frequencies of positive test results for T-SPOT.TB were 70.0 %, 57.6 % and 50.0 % ($p = 0.63$), respectively, and were not significantly different from the frequency of positive T-SPOT.TB results in the group of HIV-uninfected controls ($p = 0.76$) (**table 2.2; figure 3.2**). In contrast, frequencies of positive test results for QFT-G-IT tests declined significantly from 77.3 % to 66.7 % and 10.0 % ($p < 0.0001$), respectively, while QFT-G-IT and T-SPOT.TB results were positive in all (7/7; 100 %) and 5/7 (70.0 %) of healthy controls, respectively. In addition, all HIV-uninfected controls had a TST-reaction of ≥ 5 mm induration and in 6/7 (85.7 %) and 4/7 (57.2 %) of HIV-negative individuals, the TST-induration was ≥ 10 mm and ≥ 15 mm, respectively. On the contrary, HIV-infected patients without active tuberculosis were mostly TST-negative or only had small TST-diameters ranging from 5 to 10 mm (47.2 %), and 30 of 89 (33.7 %) of these patients had a TST-induration of 0 mm (**see chapter 3.5**).

Indeterminate test results in QFT-G-IT test and T-SPOT.TB test in HIV-infected patients without active tuberculosis were observed in 4/109 (3.7 %) each ($p = 0.48$; $p = 0.46$). Baseline characteristics of all persons with indeterminate test results were not different from the patients with tests that could be interpreted.

In the HIV+ patients with active tuberculosis, 17/19 (89.5 %) had a positive and 2/19 (10.5 %) had an indeterminate T-SPOT.TB test result, respectively ($p = 0.2$). In the same group, results of the QFT-G-IT were positive in 13/19 (68.4 %; $p = 0.97$) and negative in 6/19 (31.6 %).

In the Italian control group, results of the TST, the QFT-G-IT and the T-SPOT.TB were positive in 0/10, 0/10 and 1/10 patients, respectively. Indeterminate results in the QFT-G-IT and T-SPOT.TB assays were observed in 0/10 in each test (**figure 3.3**).

Table 2.2: Immune responses in 109 HIV+ persons, 19 HIV+/tuberculosis co-infected patients and 7 healthy individuals in Kampala, Uganda.

Variables	HIV+ patients			p-value [^]	HIV+ patients n = 109	HIV+/TB patients n = 19	p-value ^{^^}	Healthy controls n = 7	p-value ^{^^^}
	CD4+ cell counts < 100/ μ l n = 10	CD4+ cell counts 100 – 250/ μ l n = 33	CD4+ cell counts > 250/ μ l n = 66						
TST (%)	8 (80)	27 (81.8)	54 (81.8)	-	89 (81.7)	-	-	7 (100)	0.21*
TST-induration (median; IQR)	0; 2.5	5; 15	8; 13	0.01****	5; 18	-	-	18; 7	0.01****
TST-induration > 5 mm (%)	1/8 (12.5)	9/27 (33.3)	32/54 (59.3)	0.01*	42/89 (47.2)	-	-	7/7 (100)	0.01*
QFT-G-IT positive result (%)	1 (10)	22 (66.7)	51 (77.3)	< 0.0001*	74 (67.9)	13 (68.4)	0.97*	7/7 (100)	0.5*
QFT-G-IT indeterminate result (%)	1 (10)	-	3 (4.6)	0.48*	3 (3.7)	-	-	-	-
T-SPOT. TB positive result (%)	7 (70)	19 (57.6)	33 (50)	0.63*	59 (54)	17 (89.5)	0.004*	5 (71.4)	0.001*
T-SPOT .TB indeterminate result (%)	-	2 (6.1)	2 (3)	0.46*	4 (3.7)	2 (10.5)	0.2*	-	-

*Fisher's exact test

**Two independent samples t-test

***Analysis of Variance

****Wilcoxon-Mann-Whitney test

*****Kruskal-Wallis test

IQR: Inter-Quartile Range

TST: Tuberculin Skin Testing

[^] Comparison of the demographic and clinical characteristics among HIV+ patients (n = 109) with CD4+ T- cell counts < 100/ μ l, those with 100 – 250/ μ l and those with > 250/ μ l

^{^^} Comparison of the demographic and clinical characteristics among HIV+ patients (n = 109) and HIV+/tuberculosis co-infected patients (n = 19)

^{^^^} Comparison of the demographic and clinical characteristics among HIV+ patients (n = 109), HIV+/tuberculosis co-infected patients (n = 19) and healthy controls (n = 7)

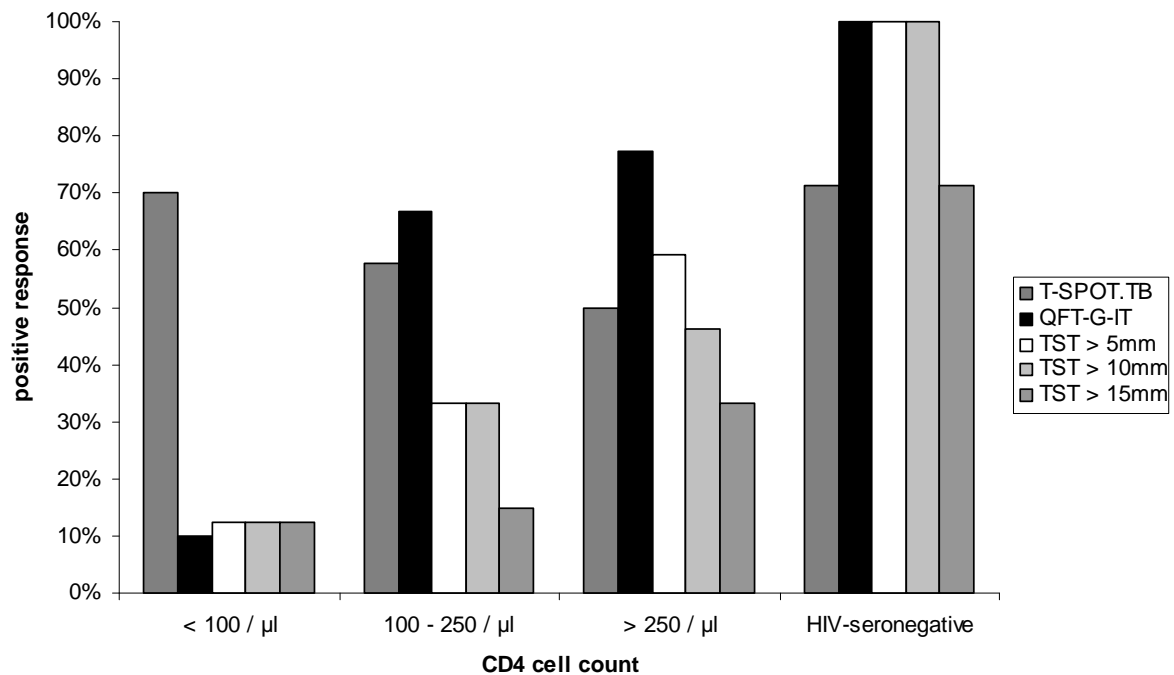


Figure 3.2: Frequency of positive immune responses in HIV-positive and -negative individuals

3.3 Agreement between tests

Overall concordance between the T-SPOT.TB and the QFT-G-IT was only 60.8 % ($\kappa = 0.17$; SE = 0.09) in the HIV-infected persons without active tuberculosis, shown in **table 2.3**. Between T-SPOT.TB and TST concordance was even worse with 40.4 % ($\kappa = 0.37$; SE = 0.11), while QFT-G-IT and TST showed the best result in agreement with 66.3 % ($\kappa = 0.34$; SE = 0.1). Considering patients with CD4+ cells < 100/ μ l, concordant test results between the T-SPOT.TB and the QFT-G-IT, between the T-SPOT.TB and the TST and between the QFT-G-IT and the TST were observed in 44.4 % ($\kappa = 0.12$; p = 0.16), 37.5 % ($\kappa = 0.09$; p = 0.15) and 71.4 % ($\kappa = -0.17$; p = 0.38), respectively, representing agreement less than chance, slight, or fair agreement. In the second group with 100 – 250 CD4+ cells/ μ l, slight agreement with 61.3 % ($\kappa = 0.16$; p = 0.18) could be found for concordance between T-SPOT.TB and QFT-G-IT, whereas concordance between IGRAs and TST was slightly better with 68.0 % ($\kappa = 0.39$; p = 0.18) and 55.6 % ($\kappa = 0.22$; p = 0.15), respectively. In HIV-infected individuals, overall test result concordance was best in the group of patients with > 250 circulating CD4+ T-cells/ μ l. In this group, highest concordance among test

results was observed between the TST and the T-SPOT.TB test with 73.1 % ($\kappa = 0.46$; $SE = 0.14$), QFT-G-IT and TST only counted 71.2 % ($\kappa = 0.35$; $p = 0.12$), and T-SPOT.TB and QFT-G-IT only 62.9 % ($\kappa = 0.24$; $p = 0.1$). On the contrary, for the healthy control group, the distribution of concordant tests was different and for the agreement between T-SPOT.TB and QFT-G-IT (71.4 %) and between QFT-G-IT and TST (57.1 %) Cohen's κ could not be calculated due to the small number of persons. For T-SPOT.TB and TST, agreement was 57.1 % ($\kappa = 0.09$; $p = 0.36$). The IGRA-tests in the HIV-infected patients with active tuberculosis were concordant in 68.4 %. **Figure 3.4** summarizes the percentages of concordance between the different LTBI assays in the Ugandan individuals.

Table 2.3: Concordance between immunoassays in HIV-infected persons and healthy controls from Kampala, Uganda

Concordance (%)	HIV+ patients			HIV+ patients	Healthy controls
	CD4+ cell counts < 100/ μ l	CD4+ cell counts 100 - 250/ μ l	CD4+ cell counts > 250/ μ l		
T-SPOT.TB/QuantiFERON (k ; SE)	44.4 (0.12; 0.16)	61,3 (0.16; 0.18)	62,9 (0.24; 0.1)	60.8 (0.17; 0.09)	71,4 (0; 0)*
TST/T-SPOT.TB (k ; SE)	37.5 (0.09; 0.15)	68 (0.39; 0.18)	73.1 (0.46; 0.14)	40.4 (0.37; 0.11)	57.1 (0.09; 0.36)
TST/QuantiFERON (k ; SE)	71.4 (-0.17; 0.38)	55.6 (0.22; 0.15)	71.2 (0.35; 0.12)	66.3 (0.34; 0.1)	57.1 (0; 0)*

* Cohen's kappa index, in order to evaluate agreement inter assay, could not be calculated because of the small number of persons

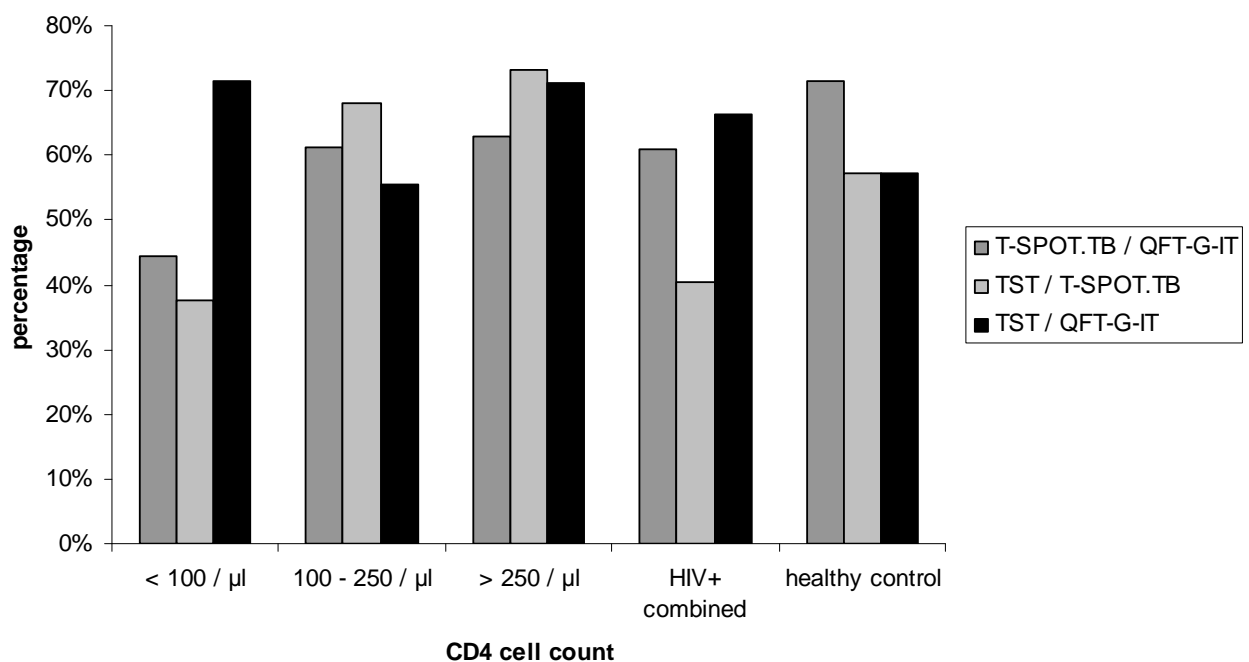


Figure 3.4: Concordance of test results in HIV-positive individuals stratified by CD4+ T-cell count, in all HIV-positive individuals without tuberculosis, and in healthy controls

3.4 Comparison of test results between the different assays

When comparing the results of the three different assays in the HIV-infected patients without tuberculosis, QFT-G-IT showed the highest percentage of positive results, followed by T-SPOT.TB and TST > 5 mm (67.9 %; 54.0 %; 47.2 %). Yet, after stratification by CD4+ T-cell count, T-SPOT.TB reached a significantly higher proportion of positive results in patients with CD4+ cells < 100/μl than QFT-G-IT (70.0 % vs. 10.0 %), whereas both of the IGRAs had similar sensitivity in the group of people with 100 – 250 cells/μl (57.6 % vs. 66.7 %), with QFT-G-IT being only slightly better. On the other hand, QFT-G-IT performed a significantly better outcome in patients with > 250 CD4+ T-cells/μl than T-SPOT.TB (50.0 % vs. 77.3 %).

T-SPOT.TB scored 71.4 % positive in the healthy control group, QFT-G-IT even 100 % indicating higher usefulness for people with little or no immunodeficiency.

For each of the three stratified groups of HIV+ individuals, TST had lower positive proportions than T-SPOT.TB and than QFT-G-IT except for the very immunocompromised with < 100 CD4+ cells/μl (10.0 % vs. 12.5 %). TST-results at higher cut-off levels showed equal, but nevertheless poorer results than the other tests. In the healthy control group, TST > 5 mm and > 10 mm detected latent tuberculosis infection in 100 % of cases, TST > 15 mm only in 71.4 %.

In the HIV-infected persons, four indeterminate results occurred in either IGRA: For the T-SPOT.TB, two (6.1 %) in the group with 100 – 250/μl and two (3.0 %) in the group with > 250/μl ($p = 0.46$), but none in the first group with < 100/μl. In contrast, for the QFT-G-IT, one (10.0 %) in the group with < 100/μl, and three (4.6 %) in the group with > 250/μl, but none in the group with 100 – 250/μl ($p = 0.48$).

In HIV-infected persons without active tuberculosis, stratified by CD4+ cell count, numbers of positive T-SPOT.TB results were 7 (70.0 %), 19 (57.6 %), and 33 (50.0 %), respectively ($p = 0.63$), while for the QFT-G-IT they were 1 (10.0 %), 22 (66.7 %), and 51 (77.3 %), respectively ($p < 0.0001$), and for the TST 1/8 (12.5 %), 9/27 (33.3 %), and 32/54 (59.3 %), respectively ($p = 0.01$). Comparing HIV+ patients and HIV+/active tuberculosis patients, T-SPOT.TB showed 59 (54.0 %) and 17 (89.5 %) positive results ($p = 0.004$), and QFT-G-IT 74 (67.9 %) and 13 (68.4 %) positive results ($p = 0.97$). Taking the healthy controls into account as well, T-SPOT.TB had 5 (71.4 %), QFT-G-IT had 7 (100 %) and TST had 7 (100 %) positive results ($p = 0.001$; $p = 0.5$; $p = 0.01$).

Obviously, low CD4+ T-cell counts did not lead to increased numbers of indeterminate results in either IGRA, the amount of positive results of the QFT-G-IT, on the other hand, seemed to be affected by decreasing CD4+ levels, a phenomenon that cannot be experienced in the T-SPOT.TB.

3.5 Distribution of TST-diameters

In the HIV-infected patients without tuberculosis, the percentage of TST-results was evenly distributed among the three groups with 8 (80.0 %), 27 (81.8 %), and 54 (81.8 %), respectively (**table 2.4; figure 3.5**). Altogether 89 TST-results corresponding to 81.7 % of HIV-positive individuals could be included into the calculations, as well as 7/7 TST-results (100 %) of the healthy control group ($p = 0.21$).

The median TST-diameter in the HIV-infected persons without tuberculosis was 5 mm (IQR 18), while in the healthy controls it was 18 mm (IQR 7; $p = 0.01$). Stratified by CD4+ T-cell number, median TST-induration was 0 mm (2.5 %), 5 mm (15 %), and 8 mm (13 %), respectively ($p = 0.01$).

HIV-uninfected patients had a significantly higher proportion of strongly positive TST-results compared to the HIV-infected persons (42.9 % vs. 19.6 % for 16 – 20 mm), and their TST-results corresponded to the Gaussian distribution, in contrast to the HIV-infected patients who were mostly TST-negative or only had small TST-diameters (range 5 – 10 mm). All HIV-uninfected controls had a TST-reaction of ≥ 5 mm induration and in 85.7 % and 57.2 % of HIV-uninfected individuals the TST-induration was ≥ 10 mm and ≥ 15 mm, respectively. 33.7 % of HIV-infected patients without active tuberculosis had a TST-induration of 0 mm.

Table 2.4: Distribution of TST-results (HIV+/not active tuberculosis, healthy control)

	0 mm	1 - 4 mm	5 - 10 mm	11 - 15 mm	16 - 20 mm	21 - 25 mm	> 26 mm
Patients, HIV+/not active TB (%)	33,7	2,2	25,0	14,1	19,6	3,3	2,2
Patients, healthy control (%)	0,0	0,0	14,3	28,6	42,9	14,3	0,0

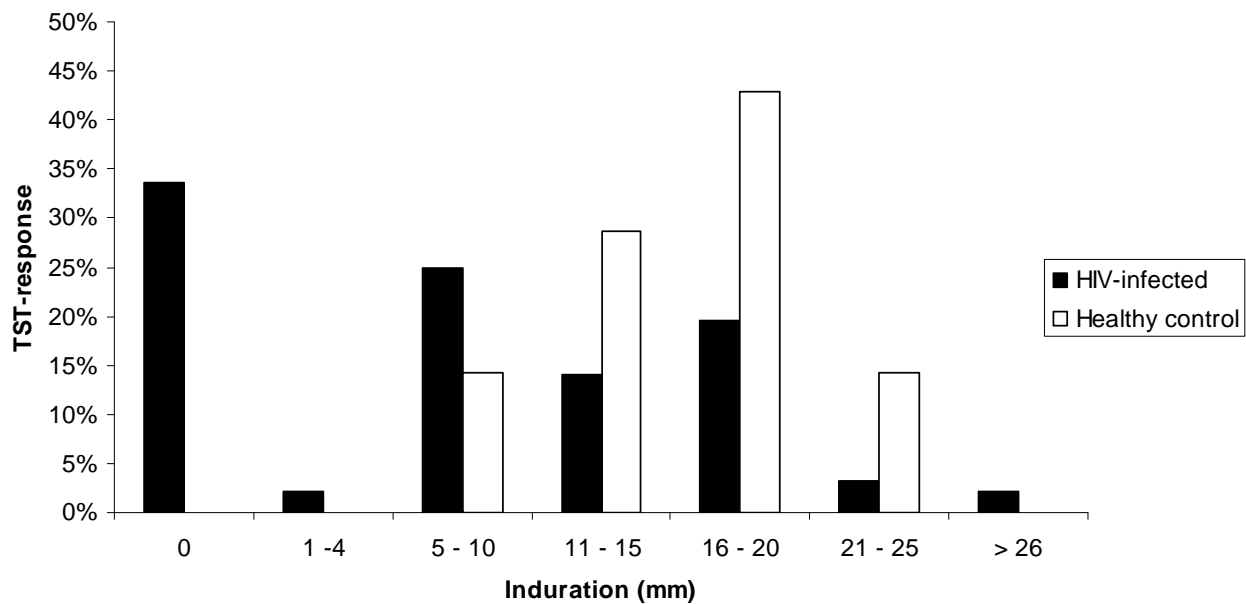


Figure 3.5: Distribution of TST-results in HIV+/not active tuberculosis patients and healthy controls

3.6 Correlation of numbers of circulating CD4+ T-cells

In HIV-infected patients, induration in the TST (mm) was directly correlated to numbers of circulating CD4+ T-cells/ μ l (Spearman's $\rho = 0.41$; $p < 0.0001$) (**table 2.5**).

Indeterminate test results in QFT-G-IT test and T-SPOT.TB test of HIV-infected patients without active tuberculosis were not related to levels of circulating CD4+ T-cells ($p > 0.45$). In HIV-infected patients, the concentration of IFN- γ in the tube with the specific antigens of QFT-G-IT was directly correlated to numbers of circulating CD4+ T-cells/ μ l (Spearman's $\rho = 0.38$; $p = 0.0001$). In contrast, numbers of spots in the ESAT-6 or CFP-10 antigen wells in the T-SPOT.TB test were not correlated to numbers of circulating CD4+ T-cells/ μ l (Spearman's $\rho = 0.03$; $p = 0.77$ and Spearman's $\rho = 0.13$; $p = 0.21$, respectively).

Table 2.5: Correlation (*significant values) of numbers of circulating CD4+ T-cells with skin induration in the TST (mm), IFN- γ concentration in the ESAT-6, CFP-10 and TB 7.7 containing tube in the QuantiFERON-Gold-in tube test and with numbers of spot forming cells (sfc) in response to incubation with ESAT-6 and CFP-10 in the T-SPOT.TB test

Correlation of CD4+ T-cells (cells / μ l) with	Spearman's rho	p-value
TST (mm)	0.41	< 0.0001*
QuantiFERON-Gold-in tube (IFN-γ)	0.38	0.0001*
T-SPOT.TB ESAT-6 (sfc)	0.03	0.77
T-SPOT.TB CFP-10 (sfc)	0.13	0.21
T-SPOT.TB highest value among ESAT-6/CFP-10 (sfc)	0.01	0.31

4. Discussion

Combating co-infection of HIV with *M. tuberculosis* is a major goal of health care interventions in Africa. Tuberculosis is still the leading cause of HIV-related deaths in the developing world (11). Early identification and treatment of latent infection with *M. tuberculosis* in individuals with HIV-infection is efficient (107) and important to reduce the spread of tuberculosis (108). Detection of latent infection with *M. tuberculosis* relies on immunodiagnostic tests, the TST and, more recently, IGRAs, but comparative data on their performance in individuals from high-incidence countries for tuberculosis are still limited (109, 110).

To date, this study is the largest to compare the commercially available IGRAs with the performance of the Tuberculin Skin Test in HIV-infected individuals in a country of high tuberculosis endemicity. Not only do the findings in this study support the statement of higher specificity and sensitivity of the two IGRAs over the conventional TST to detect latent infection with *M. tuberculosis*, but they actually also uncover significant differences between the single blood tests themselves:

HIV-infected individuals were enrolled to directly compare the diagnostic accuracy of the TST, the T-SPOT.TB test and the QFT-G-IT test for the diagnosis of latent infection with *M. tuberculosis*. Results from this comparison add substantially to the knowledge on the performance of these tests in individuals with different levels of immunodeficiency in HIV-infection. In a cohort of African individuals from a community in Uganda, one of the countries with a high tuberculosis incidence, in patients without active tuberculosis, 57.2 % of the HIV-uninfected individuals had a positive TST-reaction of an induration of > 15 mm, the recommended cut-off according to the ATS-criteria (111) (the TST-induration in all HIV-uninfected controls was > 5 mm, which is the recommended cut-off for HIV-infected individuals). Only 12.5 % of HIV-infected persons with < 100 circulating CD4⁺ T-cells exhibited a positive TST-reaction, and the diameter of TST-induration was significantly correlated to numbers of circulating CD4⁺ T-cells in the HIV-infected individuals, as previously demonstrated (112). So, without the additionally performed IGRA-tests and by relying on TST only, in this cohort, just a small percentage of the actually *M. tuberculosis* infected individuals would have been diagnosed with latent infection.

4.1 Sensitivity of IGRA-tests

The results strongly support previous observations that show robustness of antigen specific immune responses in the ELISPOT assays, irrespective of T-cell stratification in patients with HIV-infection (109, 113-115). QFT-G-IT, on the other hand, scored higher percentages of positive results with increasing CD4+ cell numbers, showing a significantly better response than the T-SPOT.TB in individuals with > 250 cells/ μ l or healthy controls.

In the largest comparable trial by Rangaka et al (110). who enrolled 74 HIV-infected individuals and 86 HIV-uninfected individuals in a township in the Cape region of South Africa, frequencies of T-SPOT.TB results in HIV-infected individuals with > 250 CD4+ T-cells/ μ l were comparable to those of individuals with < 250 CD4+ T-cells/ μ l, while frequencies of QFT-G test results were lower in patients with more advanced HIV-infection. However, results did not reach statistical significance in that study.

In addition, immunodiagnostic tests for latent infection with *M. tuberculosis* were compared among HIV-infected individuals in Germany, a country of low tuberculosis incidence (115). In agreement with the results obtained in the current study, positive results of the T-SPOT.TB were independent of the numbers of circulating CD4+ T-cells.

In contrast to these observations, the proportion of patients with a positive response to an *in-house M. tuberculosis*-specific ELISPOT decreased with the numbers of circulating CD4+ cells in HIV-infected individuals from Dakar, Senegal (116). This effect was particularly observed in individuals with circulating CD4+ T-cell counts of less than 50/ μ l, indicating that indeterminate or negative ELISPOT test results for the immunodiagnosis of latent infection with *M. tuberculosis* in severely immunosuppressed individuals need to be interpreted with caution.

In contrast to the findings of the present study, Mandalakas et al. (117) had more positive test results for the T-SPOT.TB than for the QFT-G, and even TST was more often positive than QFT-G, that is in adults. They could not find a significant difference between the proportion of positive T-SPOT.TB and TST-results. For adults, the positive results were 72.2 % for T-SPOT.TB, 62.5 % for TST and only 35.5 % for QFT-G. So in this case, T-SPOT.TB detected a higher proportion of latently infected subjects than QFT-G.

4.2 Concordance of test results

Consequently, there is a high level of discordant results among immunodiagnostic tests for the detection of latent infection with *M. tuberculosis* in HIV-infected individuals (110, 115, 117-119):

In the present study, concordance between T-SPOT.TB and QFT-G-IT was only slight with 62.7 % ($\kappa = 0.15$) in HIV-infected patients, which was, nevertheless, better than concordance between IGRAs and TST at 5 mm cut-off (40.4 % for T-SPOT.TB vs. 49.4 % for QFT-G-IT).

In a South African study by Mandalakas et al. (117) on the comparison of IGRAs between adults and children, high levels of discordant IGRA-results were found, too. Concordance between the TST and the IGRAs in adults was only moderate, but at least fair agreement could be reached between T-SPOT.TB and QFT-G with 37.5 % discordant results in adults and agreement ranging from 48.0 % to 79.0 % among all subjects.

Similar results are presented in the study by Rangaka et al. (110): Concordance between the TST at 5 mm and 10 mm cut-offs and the two IGRAs were fair in the HIV-infected group, whereas it was only moderate at the 15 mm cut-off level. The T-SPOT.TB and the QFT-G were moderately concordant in the HIV-infected persons (66.0 %), too, although for the HIV-uninfected group, agreement between these two tests was only poor. In addition, agreement of QFT-G and TST was poor, as well, only T-SPOT.TB and TST showed a slightly better agreement ranging from poor to moderate.

The German study by Stephan and colleagues (115) found only poor concordance of T-SPOT.TB and QFT-G ($\kappa = 0.146$), with the majority of patients having a negative TST-result. Nevertheless, the positive TSTs were fairly concordant with T-SPOT.TB ($\kappa = 0.201$) and QFT-G ($\kappa = 0.335$).

The study by Clark et al. (120) was conducted in London, UK, among patients either with symptoms, signs and X-ray findings suggestive of active tuberculosis or asymptomatic infection with certain tuberculosis risk factors. Here, discordance between the results of TST and T-SPOT.TB occurred in 11.0 % of patients tested. In this low prevalence setting, history of previous bacille Calmette–Guérin vaccination, exposure to environmental mycobacteria, recent exposure to tuberculosis or previous

tuberculosis treatment may have accounted for some TST-positive/IGRA-negative results. Reduced sensitivity of IGRA compared with TST has been described in healthy children (121), and may be a feature of individuals with a history of remote tuberculosis exposure (95, 122).

Another study in a low-prevalence-country by Talati and colleagues (119) compared the concordance between TST, T-SPOT.TB and QFT-G-IT in HIV-positive possible LTBI-patients. Of the 27 patients with at least one positive test result, only three people tested positive with more than one test and only one person tested positive for all three tests. They then evaluated, whether lowering the interferon- γ cut-off would improve concordance between tests since the current cut-off values are based on receiver operator characteristics in immunocompetent individuals, but lowering the interferon- γ cut-off did not improve concordance.

Also, only 5/24 (21.0 %) individuals with either a positive TST or QFT-G-IT were positive by both tests in a study conducted in New York, USA (123). This rate and those for discordant results of TST-/QFT-G-IT+ (3.0 %) and TST+/QFT-G-IT- (4.0 %) are similar to the ones found by the previously named study by Luetkemeyer et al. (100), where 167 of 196 (85.2 %) were concordant in their negative results and 8 (4.1 %) were concordant in their positive results, for an overall concordance of 89.3 %. Of subjects without valid TST-results, 6.7 % (6/89) had positive QFT-G-IT-results. TST+/QFT-G-IT- discordant results were found in 5.1 % (10/196) of subjects and TST-/QFT-G-IT+ results in 5.6 % (11/196) of subjects.

In the present study, due to the different distribution of positive results, overall concordance among the results of all three tests was not more than fair in HIV-infected individuals without active tuberculosis. Similar to the results of the above cited study from Germany, the lowest agreement among test results was observed between the two IGRAs ($\kappa = 0.17$). Moderate test agreement was only observed among the TST and T-SPOT.TB in patients with more than 250 circulating CD4+ T-cells ($\kappa = 0.46$).

4.3 Indeterminate results

Another issue that arises in using IGRAs in immunocompromised persons is the rate of indeterminate test results. In the present study, these indeterminate results of the two IGRAs were equally distributed in the HIV-infected group in this setting with four for the T-SPOT.TB and also four for the QFT-G-IT. Although it has often been reported in similar studies, in our setting, an increasing number of indeterminate results with decreasing CD4⁺ T-cells could not be examined, actually the opposite was the fact, as T-SPOT.TB counted none indeterminate in the group with the highest immunosuppression and QFT-G-IT only one. One blood sample was tested indeterminate by both assays.

Yet, according to an Italian study by Ferrara and colleagues (124), indeterminate results of IFN- γ release assays are more frequent in immunosuppressed individuals than in immunocompetent persons. Especially for the QFT-G test, indeterminate results are supposed to be more common than for the T-SPOT.TB.

Similarly, in additional studies on the performance of IFN- γ release assays in HIV-positive patients, various amounts of indeterminate results could be found, in particular for people with low CD4⁺ cell counts. On the contrary, T-SPOT.TB seemed to be less influenced by higher immunodeficiency and, therefore, counted less indeterminate results (125).

For the adults in their study-cohort, Mandalakas et al. (117) found 15.0 % indeterminate results for QFT-G and 10 % for T-SPOT.TB, which concurs with findings of similar studies mentioned earlier. All indeterminate results occurred in persons with low CD4⁺ cell number (mean count < 209/ μ l) and 0 mm for TST.

In the study by Aichelburg et al. (126) from Austria, higher rates of indeterminate results of QFT-G-IT (53.2 %) among patients with advanced immunodepletion of < 200 cells/ μ l were found. In the groups with 200 – 350 and > 350 cells/ μ l, indeterminate results occurred in 17 % and 29.8 %, respectively.

As well, in patients with a CD4⁺ cell count < 100 cells/ μ l, Brock et al. (127) from Denmark found a higher proportion of indeterminate QFT-G-IT test results (24.0 % vs. 2.8 %) due to low PHA-response, and there was a significant correlation between CD4⁺ cell count and PHA induced IFN- γ release. These findings again support the

assumption that the performance of the IFN- γ based tests is impaired in patients with advanced immunosuppression.

In the German cohort of Stephan et al. (115), indeterminate results are spread vice versa: QFT-G showed only 0.4 % indeterminate, whereas T-SPOT.TB counted 3.0 % in all patients with valid results in both blood tests. Seven of the eight indeterminate samples in T-SPOT.TB were tested negative by QFT-G, the remaining one was indeterminate by QFT-G as well.

Of the proportion of indeterminate QFT-G-IT results (14.0 %) in a Tanzanian study (128), the majority was found in HIV-positive patients and could be explained by a low CD4+ cell count, which demonstrates the trend of decreasing sensitivity with decreasing CD4+ number.

In the American study by Talati et al. (119), an indeterminate test result occurred in 14.0 % of patients with T-SPOT.TB and 1.8 % of patients with QFT-G-IT. An indeterminate result was associated with a CD4+ count of < 200 cells/ μ l. In indeterminate QFT-G-IT tests, patients might require retesting or testing with a larger volume of blood, in order to obtain more PBMCs for T-SPOT.TB.

In the study by Haustein et al. (129), 35.0 % of 237 children tested with the whole blood assay QFT-G-IT had an indeterminate result. These indeterminate results were highest for children < 5 years (44.0 %) and for those who were immunocompromised (66.0 %). Similar data were reported in 315 children in Italy tested with QFT-G-IT, where overall indeterminate rates were 16.4 %, but there was a significant difference in indeterminate rates in children < 4 years compared with those \geq 4 years (35.9 % vs. 5.6 %) (130). In both of these studies, the indeterminate results were primarily a result of poor response to the positive control mitogen, suggesting that particularly in young children (< 5 years old) more work needs to be done to define the appropriate response of T-cells to mitogens, before relying on whole blood assays to define infection with *M. tuberculosis*.

Jones and colleagues (123) found that all subjects who had an indeterminate QFT-G-IT result had CD4+ cell counts < 200 cells/mm³, and the majority (7/10) had CD4+ counts < 100 cells/mm³. The overall rate of indeterminate results was 4.9 %, while Luetkemeyer et al. (100) discovered indeterminate results in 5.1 % (15/294) of subjects. All their indeterminate results were due to a failure to respond adequately to the positive control.

Rangaka and colleagues (110) found 1.0 % of T-SPOT.TB tests indeterminate and 7.0 % of QFT-G tests in the HIV-infected cohort. For the HIV-uninfected, only 2.0 % of QFT-G tests and none of T-SPOT.TB tests were indeterminate, also underlining the assumption that QFT-G produces more indeterminate results.

Therefore, compared to studies with similar settings the distribution of indeterminate results in the present study seems to be an exception which at the moment cannot be explained. The fact that only one blood sample with a CD4+ count of 62 was tested indeterminate by QFT-G-IT and that the other indeterminate results occurred in samples with CD4+ T-cells ranging from 143 to 608 (T-SPOT.TB at 143 and 155 CD4+ cells) contradicts the assumption that especially the QFT-G-IT scores indeterminate more often with low CD4+ cells.

4.4 Distribution of positive test results and dependency on CD4+ T-cell count

All HIV-uninfected individuals without active tuberculosis had a positive reaction to one of the *ex vivo* IGRAs to test for memory against *M. tuberculosis*-specific antigens, namely the QFT-G-IT or the T-SPOT.TB. Detection of high frequencies of LTBI in healthy individuals provide a basis to study *M. tuberculosis*-specific immune responses in relation to the degree of immunosuppression in HIV-infected individuals from the same community (131).

A striking difference could be found in the frequencies of positive immune responses assayed by the different tests in HIV-infected individuals without active tuberculosis at different levels of circulating CD4+ T-cell counts. When we compared the size of TST-induration and the concentration of IFN- γ (in IU/ml) from the tubes containing the *M. tuberculosis*-specific antigens ESAT-6, CFP-10 and TB7.7 in the QFT-G-IT, they correlated negatively to numbers of circulating CD4+ T-cells in individuals with HIV-infection. This correlation was not found for numbers of antigen-specific spots in the T-SPOT.TB test. When the frequencies of positive immune responses in groups of patients with different levels of immunodeficiency were evaluated, again TST and

QFT-G-IT were dependent upon the level of circulating CD4⁺ T-cell counts, while this relationship could not be observed for the T-SPOT.TB test. The highest frequency of positive TST and QFT-G-IT test results was observed in HIV-infected individuals with > 250 CD4⁺ cells/μl, indicating a dependency of these tests on the absolute number of circulating CD4⁺ T-cells. In contrast, frequencies of positive immune responses in the T-SPOT.TB test were independent of the level of immunodeficiency. Rates of positive T-SPOT.TB results were comparable in HIV-infected patients with < 100, 100 – 250 and > 250 CD4⁺ T-cells/μl and HIV-uninfected controls.

Although QFT-G-IT had the highest percentage of positive results (67.9 %), stratified by CD4⁺ T-cell number the T-SPOT.TB showed significantly more positive results in persons with < 100 cells/μl, as well as in persons with 100 – 250/μl than QFT-G-IT and TST. For the patients with > 250 CD4⁺ cells/μl, however, QFT-G-IT scored the best results, which indicates a dependency of this test on the absolute number of CD4⁺ T-cells in the patients' blood sample unlike the T-SPOT.TB. Nevertheless, the extraordinary result of the T-SPOT.TB in persons with very low CD4⁺ levels (70.0 % positive) might partly be due to the small number of participants in this group (n = 10).

Similarly, in a study by Day et al. (113) on the performance of the T-SPOT.TB assay, no correlations between the responses of IFN- γ releasing CD4⁺ T-cells and the absolute CD4⁺ cell count were observed.

In a Gambian study, carried out by Hammond and colleagues (114), with decreasing amounts of CD4⁺ cells the number of positive TST-tests in HIV-infected persons fell, as well. In contrast, the response of patients to ESAT-6 and CFP-10 in the ELISPOT-assays did not show a difference, when stratified by CD4⁺ cell count. Of the healthy, HIV-uninfected control group, the proportion of positive responses was vice versa with more results for the TST and less for CFP-10.

Surprisingly, the study conducted by Lawn et al. (109) found no association between CD4⁺ cell count in HIV-infected persons and responses to *M. tuberculosis*-specific antigens in either IGRA. So, they could not emphasize the finding that Whole Blood Assays, such as the QFT-G-IT, showed a significantly lower percentage of positive results in patients with CD4⁺ cells < 100/μl, actually only a non-significant trend towards an association in the ELISPOT assay could be found. This is quite the

opposite of the previously named studies. For them, the history of tuberculosis treatment was the more important issue with lower responses in treated patients compared to tuberculosis therapy naïve persons. Dheda et al. (101) performing a small study with 29 HIV-positive patients also concluded that there was no impact of a low CD4+ cell count on the performance of the IFN- γ response in an ELISPOT assay.

Similar to the results of the present study, in a South African study the T-SPOT.TB received a higher proportion identified as tuberculosis-infected in the group with patients < 250 cells/mm³ than the QFT-G test, as well as in two more groups with patients < 350 cells/mm³ and < 200 cells/mm³, yet, they could not reach significance. On the other hand, for the HIV-uninfected control persons, the TST at 5 mm and 10 mm cut-off reached the highest positive responses, whereas the interferon- γ release assays remained constant in their proportion (110).

In a study conducted in the low prevalence country of Germany by Stephan and colleagues (115) comparing the two IGRAs with the TST in HIV-infected people, a significant difference in median CD4+ cell count between patients with a positive QFT-test result and a negative one was found. For the T-SPOT.TB and the TST on the contrary, this association could not be proven. This may not be the exact same differentiation as in the above named studies, but it still underlines the dependency of QFT-results on the CD4+ cell count.

Luetkemeyer et al. (100) stratified their QFT-G-IT results by CD4+ cell counts of < 100 , $100 - 350$, and > 350 cells/mm³. Higher CD4+ cell count was correlated with higher IFN- γ release in response to tuberculosis antigens and with higher IFN- γ response to the positive control. The tuberculosis antigen induced IFN- γ release was also correlated with the TST-induration.

Converse and colleagues (132) using the 1st generation Quantiferon test found a reduced rate of responders and in average a lower IFN- γ response in HIV-positive individuals with a CD4+ cell count of less than 200 cells/ μ l. Another study used the ELISPOT test and found that the number of responders, as well as the mean IFN- γ response was reduced in HIV-positive individuals (133). However, the CD4+ T-cell count was unfortunately not available in this study.

4.5 Possible explanation for the performance of immunodiagnostic tests in HIV-infection

Our findings do suggest that both the T-SPOT.TB and the QFT-G-IT have a higher specificity and sensitivity than the TST for the detection of latent tuberculosis infection, especially in immunocompromised patients. Nevertheless, IGRAs cannot distinguish between latent and active tuberculosis and due to the lack of a gold standard the comparability of IGRAs with TST - and with each other - is diminished.

Taking a closer look at the patients with HIV/known active tuberculosis regarding IGRA results, in the present study T-SPOT.TB showed a significantly better performance than QFT-G-IT. In contrast, QFT-G-IT reached 100 % positive test results in healthy, not immunocompromised people, where T-SPOT.TB only found 71.4 %.

The cause for different results is the fact that for the T-SPOT.TB the same amount of IFN- γ producing PBMCs is added to the microtiter wells (250.000 PBMC are plated into each well), thus normalizing the individual impairment of the immunosystem, whereas the QFT-G-IT as a whole blood assay is more affected by an increasing amount of immunodeficiency. This leads to the assumption that the implementation of special cut-off points for the QFT-G-IT, like for the TST (**see chapter 1.4.1**), is necessary. By using such cut-off points, comparability of QFT-G-IT, T-SPOT.TB, and TST, respectively, would be improved.

4.6 Limitations of the study

The limitations of our study need to be addressed. While we performed the largest comparison of the three currently available immunodiagnostic tests for latent tuberculosis infection in HIV-infected individuals in a country of high tuberculosis incidence to date, numbers of HIV-infected individuals with less than 100 circulating CD4⁺ T-cells per μ l and numbers of HIV-uninfected controls in our study were still relatively small. However, the robustness of the data is supported by different statistical methods, including a very high correlation between the magnitude of

immune-responses in the TST and QFT-G-IT and the numbers of CD4+ T-cells in the blood. HIV-uninfected controls were younger than HIV-infected individuals, leading to a possible bias in frequencies of positive immune responses in this group. However, HIV-infected individuals were on average younger than 35 years and it is not expected that age influenced the test results substantially.

There are now growing numbers of studies reporting cross sectional data on frequencies of immune responses by IGRA in immunocompromised individuals without active tuberculosis. While these data provide important information, longitudinal studies on the predictive values of IGRA for the development of active tuberculosis are now urgently needed (134).

4.7 Conclusion

In the present study, it could be demonstrated that T-SPOT.TB positive results were independent from numbers of circulating CD4+ T-cells, unlike QFT-G-IT and TST results. In addition, indeterminate results occurred within a broad range of CD4+ numbers. On the other hand, QFT-G-IT diagnosed more individuals LTBI-positive in the group of HIV-infected patients with > 250 cells/ μ l than any other test. These findings add substantial knowledge to the understanding of the mechanism and especially the use of the two latest available IFN- γ release assays.

The observed findings can be explained by the fact that the detrimental effects of lymphopenia associated with the late stages of HIV-disease in reducing the efficiency of IGRA-tests can be overcome or reduced by the ELISPOT procedure. In fact, ELISPOT involves a normalization of the input of PBMC used in the assays. Therefore, although QFT-G-IT produces easier logistic calculation in which the cell input is not normalized, it could suffer from a high rate of negative results among those HIV-infected patients with advanced immunodeficiency (100, 123, 127, 135), as also shown in this study. Since the definition of a positive test results depends on the cut-off point, re-defining the cut-off points for the QFT-G-IT test may be warranted to the possibility to better identify individuals who are latently infected with *M. tuberculosis* and also have underlying immunodeficiencies.

The low frequency of positive results in the QFT-G-IT test in persons with advanced CD4+ T-cell depletion in our cohort was not explained by a high number of indeterminate results in this group. Frequencies of indeterminate results were only observed in 3.6 % of HIV-infected individuals by either IGRA, independently of the circulating CD4+ T-cell count. These data differ from other studies, where high numbers of indeterminate results were found by the QFT-G-IT assay (128, 135) or by “in house” tests of ELISPOT assays (119, 136). This is probably due to the fact that in the present study, the group of patients with CD4+ T-cells < 100/μl was relatively small to have a profound effect on the number of indeterminate results compared to those previous studies, and the majority of the HIV-positive individuals with circulating CD4+ T-cells above 100/μl did not have active disease. These individuals were, therefore, less prone to immune deficiency induced by tuberculosis disease per se (137-139).

In summary, in individuals with HIV-infection in an area of high tuberculosis incidence, results in TST and QFT-G-IT are strongly related to the degree of immunodeficiency, thus demanding new cut-offs, while T-SPOT.TB works independently from CD4+ T-cell depletion.

5. Summary

Tuberculosis and infection with Human Immunodeficiency virus (HIV) are two of the major public health problems in the world, especially in sub-Saharan Africa. Epidemiology and interactions of tuberculosis and HIV have been well researched in the past, but the number of studies evaluating immunodiagnostic tests to identify HIV-infected individuals who are at risk to develop tuberculosis are limited. Interferon- γ release assays (IGRA), novel *ex vivo* immunodiagnostic tests, may prove to be superior to the TST for the diagnosis of latent infection with *M. tuberculosis*, as they seem to be less impaired by further stages of CD4+ T-cell depletion. Interferon- γ release assays have operational advantages over the TST, as they include a positive and negative test-control and they exhibit superior test specificity, as the antigens that are used to elicit immune responses are absent in *M. bovis* BCG and most non-tuberculous mycobacteria. Current generations of IGRAs include two different enzyme-linked methods of the T-SPOT.TB assay and the QuantiFERON-Gold-In tube (QFT-G-IT) test, respectively.

An evaluation of the two IGRAs and the TST among individuals from a high-tuberculosis-incidence country offers the opportunity to compare these methods for the immunodiagnosis of latent infection with *M. tuberculosis* (LTBI) directly.

Following informed consent, 190 individuals were enrolled at the HIV outpatient clinic of the Infectious Diseases Institute of Makerere University in Kampala, Uganda. Following inclusion and exclusion criteria, 135 individuals were included in the final analysis. Of those, 109 were newly diagnosed HIV-1 positive patients with no signs or symptoms of active TB, 19 were newly diagnosed HIV-1 positive patients with active tuberculosis, and seven were HIV-uninfected healthy controls. In addition, a control group of ten individuals from the low-incidence country of Italy was included as additional negative controls to document test specificity.

HIV-infected patients without active tuberculosis ($n = 109$) were stratified by CD4+ T-cell counts in < 100 ($n = 10$), $100 - 250$ ($n = 33$) and > 250 ($n = 66$) cells/ μ l. All HIV-uninfected controls had a TST-reaction of ≥ 5 mm induration, and in 6/7 (85.7 %) and 4/7 (57.2 %) HIV-negative individuals the TST-induration was ≥ 10 mm and ≥ 15 mm, respectively. QFT-G-IT test and T-SPOT.TB results were positive in all (7/7) and 5/7 (70 %) of healthy controls, respectively. In HIV-infected patients, induration in the

TST (mm) was directly correlated to the numbers of circulating CD4+ T-cells (cells/ μ l) (Spearman's $\rho = 0.41$; p -value < 0.0001) and the concentration of IFN- γ in the tube with the specific antigens QFT-G-IT was directly correlated to numbers of circulating CD4+ T-cells (cells/ μ l) (Spearman's $\rho = 0.38$; p -value: 0.0001). In contrast, numbers of spots in the ESAT-6 or CFP-10 antigen wells in the T-SPOT.TB test were not correlated to numbers of circulating CD4+ T-cells (cells/ μ l) (Spearman's $\rho = 0.03$; p -value: 0.77 and Spearman's $\rho = 0.13$; p -value: 0.21, respectively). 30/89 (33.7 %) HIV-infected patients without active tuberculosis had a TST-induration of 0 mm.

In HIV-infected individuals without tuberculosis, frequencies of positive results for the TST were 59.3 %, 33.3 % and 12.5 % ($p = 0.01$) at the > 5 mm cut off for the group of patients with > 250 , $100 - 250$, and < 100 circulating CD4+ t-cells. For QFT-G-IT test, positive test results declined significantly from 77.3 % to 66.7 % and 10.0 % ($p < 0.0001$) respectively, while frequencies of positive test results for T-SPOT.TB test were 50.0 %, 57.6 % and 70.0 %, respectively, and were not significantly different from the frequency of positive T-SPOT.TB test results in the group of HIV-uninfected controls ($p = 0.76$). In HIV-infected individuals, overall test result concordance was best in the group of patients with > 250 circulating CD4+ T-cells. In this group, highest concordance among test results was observed between the TST and the T-SPOT.TB test with 73.1 % ($\kappa = 0.46$; $SE = 0.14$). Indeterminate test results in QFT-G-IT test and T-SPOT.TB test were observed in a total of 3/109 (2.8 %) and 4/109 (3.7 %) of HIV-infected patients without active tuberculosis. Indeterminate test results were not related to levels of circulating CD4+ T-cells ($p > 0.45$).

In 10 HIV-seropositive individuals (10 male) from Italy, results of the TST (> 5 mm), the QFT-G-IT and the T-SPOT.TB were positive in 0/10, 0/10 and 1/10 patients respectively. Indeterminate results in the QFT-G-IT and T-SPOT.TB assays were observed in 0/10 in both tests.

In conclusion, in HIV-infection, immune responses in the TST and the QFT-G-IT assay are both strongly related to the degree of immunodeficiency, while the T-SPOT.TB assay seems to function independently from the level of CD4+ T-cell depletion. These findings suggest that T-SPOT.TB is superior to TST and QFT-G-IT in HIV-infected individuals with advanced levels of immunodeficiency.

6. Ausführliche deutsche Zusammenfassung

Tuberkulose und die Infektion mit dem Humanen Immundefizienzvirus (HIV) zählen zu den größten gesundheitlichen Herausforderungen, vor allem in den afrikanischen Ländern südlich der Sahara. Im Jahr 2008 traten 9,4 Millionen neue Fälle einer Tuberkulose auf. Ca. 1,8 Millionen Menschen verstarben an einer Tuberkulose. Die Weltgesundheitsorganisation (WHO) schätzt, dass etwa ein Drittel der Weltbevölkerung mit *Mycobacterium tuberculosis* infiziert ist, obwohl nur ein kleiner Teil davon tatsächlich eine aktive Tuberkulose entwickelt. Die Mehrheit bleibt, dank einer erfolgreichen Immunantwort, nur latent infiziert. Diese latente Infektion mit *M. tuberculosis* (LTBI) ist durch eine positive Reaktion im Tuberkulin-Hauttest (THT) und/oder ein positives Ergebnis im Interferon- γ release assay (IGRA) bei gleichzeitigem Fehlen einer akuten Tuberkulose-Erkrankung definiert.

Die Ausbreitung des HI-Virus ist die Hauptursache für den Anstieg der Fallzahlen der Tuberkulose in den vergangenen zwei Jahrzehnten in Afrika, da die Interaktion zwischen beiden Krankheiten zu einem drastischen Anstieg von Morbidität und Mortalität bei koinfizierten Patienten führt. Schätzungen zufolge lebten im Jahr 2008 weltweit etwa 33,4 Millionen Menschen mit einer HIV-Infektion. Etwa 2,0 Millionen Menschen verstarben an dem Erworbenen Immundefizienz-Syndrom (engl.: Acquired Immunodeficiency Syndrome - AIDS). Mehr als zwei Drittel aller HIV-infizierten Menschen weltweit, ca. 22,4 Mio. Menschen, leben in afrikanischen Ländern südlich der Sahara. Die Prävalenz von HIV bei Tuberkulose-Patienten in Afrika liegt bei ca. 40 %. Das jährliche Risiko für eine aktive Tuberkulose bei HIV-Infizierten mit einer LTBI liegt bei 5 – 10 %. Das relative Risiko für den Erwerb der Tuberkulose ist bei HIV-seropositiven Personen gegenüber HIV-seronegativen Personen 6 – 50 mal erhöht.

In Uganda, wo diese Studie durchgeführt wurde, traten im Jahr 2008 98.000 neue Tuberkulose-Fälle auf mit einer Inzidenz von 310 pro 100.000 Menschen. Im Jahr 2007, dem Jahr der letztmalig für Uganda erhobenen epidemiologischen Daten für die Prävalenz der HIV-Infektion, lag diese bei einer Zahl von 1 Million Menschen bzw. 6,1% der Erwachsenen. 89000 Menschen starben an AIDS oder opportunistischen Krankheiten.

Der Tuberkulin-Hauttest (THT) war seit Beginn des letzten Jahrhunderts die einzige Methode und lange Zeit der Goldstandard zur Diagnose einer latenten Infektion mit *M. tuberculosis*. In den meisten Ländern wird zur Durchführung des THT das Tuberkulin RT-23 des dänischen Statens Serum Institut (SSI) in der Standarddosis von 2TU verwendet, von dem 0,1 ml intrakutan an der Innenseite des Unterarms appliziert werden. Das Tuberkulin wird von Antigen-präsentierenden Zellen (Makrophagen, dendritischen Zellen, Langerhans-Zellen) in der Haut aufgenommen und in regionalen Lymphknoten T-Lymphozyten präsentiert. Antigenspezifische T-Zellen expandieren klonal und gelangen in den Blutkreislauf. Am Ort der Antigeninokulation treten die T-Zellen aus dem Blutstrom aus und verursachen eine lokale Entzündungsreaktion, die als tastbare Induration und Rötung registriert werden kann. Nach 48 bis 72 Stunden wird das Ausmaß der Induration (in mm) gemessen. Der Grenzwert des THT für HIV-positive Patienten liegt bei ≥ 5 mm. Positive Ergebnisse des THT können aber auch bei einer vorangegangenen Tuberkulose-Impfung mit BCG (Bacille Calmette-Guérin) oder einer Infektion mit nicht-tuberkulösen Mykobakterien (NTM) auftreten. Menschen mit einer Immunsuppression, wie HIV-Infizierte, besonders in fortgeschrittenen Stadien der Erkrankung, können häufig keine adäquate Reaktion auf das Tuberkulin mehr ausbilden, obwohl eventuell eine Infektion mit *M. tuberculosis* besteht.

Inzwischen konnte im Genom von *M. tuberculosis* eine Region identifiziert werden, die sowohl in Mycobacterium Bacille Calmette-Guérin als auch in den meisten nicht-tuberkulösen Mykobakterien, außer *M. kansasii*, *M. marinum* und *M. szulgai*, nicht vorhanden ist. Zwei Proteine, die in dieser genomischen Region kodiert werden, nämlich das early secretory antigenic target-6 (ESAT-6) und das culture filtrate protein-10 (CFP-10), sind mit einer Virulenz von *M. tuberculosis* assoziiert. Auf der Basis dieser Erkenntnis sind in den vergangenen Jahren *ex vivo* Interferon- γ release assays (IGRA) als diagnostische Alternative zum THT entstanden. Aktuell sind als enzyme-linked immunosorbent assays (ELISA) der QuantiFERON-TB Gold Test und seine neuere Version QuantiFERON-TB Gold In-tube (QFT-G-IT, Cellestis, Carnegie, Australien) und der T-SPOT.TB Test (Oxford Immunotec, Oxford, England), der auf der Basis der enzyme-linked immunospot (ELISPOT) Technik arbeitet, erhältlich. Beim T-SPOT.TB werden Antigen-spezifische T-Zellen identifiziert, die in Gegenwart von ESAT-6 und CFP-10 IFN- γ sezernieren. Der QFT-G-IT dagegen misst die Konzentration an IFN- γ nach 24-stündiger Inkubation von T-Zellen mit den *M.*

tuberculosis-spezifischen Antigenen. Sensitivität und Spezifität der beiden IGRA-Tests für die Diagnose einer latenten Tuberkulose liegen höher als beim THT.

Das größere Risiko für eine Tuberkulose-Erkrankung bei HIV-infizierten Personen und die damit verbundene erhöhte Morbidität und Mortalität sind seit langem bekannt, der THT jedoch stellt nur eine unzureichende Diagnosemöglichkeit für koinfizierte Patienten dar. Da sich die beiden IGRAs als mögliche Alternative zum Tuberkulin-Hauttest erweisen könnten, wurde diese Studie in dem für Tuberkulose bzw. die HIV-Infektion Hochprävalenzland Uganda durchgeführt.

Nach positiven Voten der Ethikkommissionen der Universität zu Lübeck, der Makerere University, Kampala, Uganda, und der Case Western Reserve University in Cleveland, USA, wurden 190 Personen an der HIV-Ambulanz der Makerere University in Kampala rekrutiert. Nach Evaluation der Ein- und Ausschlusskriterien wurden 135 Patienten in die abschließende Analyse aufgenommen. Zusätzlich wurden zehn HIV-infizierte Kontrollpersonen aus Italien eingeschlossen. Von den 135 ugandischen Teilnehmern waren 109 neu HIV-positiv diagnostiziert ohne Zeichen einer aktiven Tuberkulose, 19 waren ebenfalls neu diagnostiziert HIV-infiziert mit aktiver Tuberkulose und sieben waren HIV-seronegative gesunde Probanden. An allen Personen wurden die beiden IGRAs durchgeführt, der THT aber nur bei denjenigen ohne aktive Tuberkulose.

Bei allen 7/7 bzw. 5/7 HIV-seronegativen Personen waren der QFT-G-IT und der T-SPOT.TB positiv. Im THT hatten alle eine Induration von > 5 mm, wobei bei 6/7 bzw. 4/7 das Ergebnis bei ≥ 10 mm bzw. ≥ 15 mm lag. Bei den HIV-seropositiven Personen insgesamt lag die mittlere Zahl der zirkulierenden CD4⁺ Zellen bei 182/ μ l (Interquartile Range-IQR 118) bzw. 283/ μ l (IQR 226). Die Patientengruppe der HIV-infizierten Personen ohne Tuberkulose ($n = 109$) wurde entsprechend ihrer CD4⁺ Zellzahl zusätzlich unterteilt in die drei Gruppen < 100 / μ l, $100 - 250$ / μ l und > 250 / μ l, die mittleren Werte der CD4⁺ Zellen lagen bei 64/ μ l (IQR 43), 179/ μ l (IQR 68) und 368/ μ l (IQR 206). Die HI-Viruslast war umgekehrt proportional zur Anzahl der CD4⁺ Zellen mit 104.004, 55.234 bzw. 22.058 (IQR 150.718, 124.166, 42.692; $p < 0.01$). Der THT konnte bei 89/109 Patienten abgelesen werden, die Induration betrug 0 mm oder lag zwischen 5 und 10 mm. Das Ergebnis des THT korrelierte mit der Anzahl der CD4⁺ Zellen im Blut (Zellen/ μ l). In den drei stratifizierten Gruppen nahmen die positiven Testergebnisse des QFT-G-IT signifikant ab mit 77,3 %, 66,7 % und 10,0 %

($p < 0.0001$), wohingegen sie beim T-SPOT.TB mit 50,0 %, 57,6 % und 70,0 % im Wesentlichen mit den Ergebnissen der HIV-seronegativen Kontrollgruppe übereinstimmten. Die Gesamtübereinstimmungen zwischen T-SPOT.TB und QFT-G-IT, zwischen T-SPOT.TB und THT und zwischen QFT-G-IT und THT lagen bei 60,8 % ($\kappa = 0,17$; $SE = 0,09$), 40,4 % ($\kappa = 0,37$; $SE = 0,11$) und 66,3 % ($\kappa = 0,34$; $SE = 0,1$). Die höchste Übereinstimmung erreichten die Patienten mit > 250 CD4+ Zellen pro μl , und zwar zwischen THT und T-SPOT.TB mit 73,1 % ($\kappa = 0,46$; $SE = 0,14$). Bei den Patienten mit aktiver Tuberkulose hatten 17/19 (89,0 %) ein positives T-SPOT.TB Ergebnis und 2/19 (11,0 %) ein unbestimmbares. Für den QFT-G-IT hingegen waren 13/19 (68,0 %) positiv und 6/19 (32,0 %) negativ.

Unbestimmbare Ergebnisse im QFT-G-IT und T-SPOT.TB traten auf bei 3/109 (2,8 %) und 4/109 (3,7 %) der HIV-Infizierten ohne Tuberkulose, wobei diese unbestimmbaren Testergebnisse nicht in Bezug standen zur Anzahl der zirkulierenden CD4+ Zellen ($p > 0,45$). In dieser Personengruppe korrelierte die Konzentration an IFN- γ im QFT-G-IT direkt mit der Zahl der CD4+ Zellen im Blut der Patienten (Spearman-rho = 0,38; p-Wert: 0,0001), im Gegensatz zur Anzahl der abgelesenen Punkte im T-SPOT.TB (Spearman-rho = 0,03; p-Wert: 0,77 bzw. Spearman-rho = 0,13; p-Wert: 0,21).

Bei den zehn HIV-seropositiven italienischen Kontrollteilnehmern lag die mittlere CD4+ Zellzahl bei 480/ μl (IQR 303,5 – 778). Die Ergebnisse für den THT (> 5 mm), den QFT-G-IT und den T-SPOT.TB waren positiv bei 0/10, 0/10 und 1/10. In keinem der beiden IGRA-Tests wurde ein unbestimmbares Ergebnis erzielt.

Sowohl beim THT als auch beim QFT-G-IT Test hängt die Stärke der gemessenen Immunantwort vom Ausmaß der Immunsuppression der Patienten ab. Da beim T-SPOT.TB Test eine konstante Anzahl von mononukleären Zellen im Testsystem verwendet wird, ist der T-SPOT.TB Test unabhängiger vom Ausmaß der Immundefizienz bei der HIV-Infektion. Zur Immundiagnose der latenten Infektion mit *M. tuberculosis* bei HIV-infizierten Patienten mit fortgeschrittenem Immundefekt sollte der T-SPOT.TB Test aufgrund der höheren Sensitivität anstelle des THT oder des QFT-G-IT Tests verwendet werden.

Konsequente Identifikation von HIV-infizierten Personen mit latenter Infektion mit *M. tuberculosis* und consequente präventive Chemotherapie im Falle eines positiven Testergebnisses der *M. tuberculosis*-spezifischen Immundiagnostik können die Inzidenz der Tuberkulose bei HIV-infizierten Personen deutlich senken.

7. References

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Leidl L, Mayanja-Kizza H, Sotgiu G, Baseke J, Ernst M, Hirsch C, Goletti D, Toossi Z, Lange C. "Relationship of immunodiagnostic assays for tuberculosis and numbers of circulating CD4+ T-cells in HIV-infection". *Eur Respir J* 2010; 35: 619–626

Oral presentations:

Leidl L, Mayanja-Kizza H, Sotgiu G, Baseke J, Ernst M, Hirsch C, Goletti D, Toossi Z, Lange C. "Comparison of T-cell interferon gamma release assays for the diagnosis of latent tuberculosis infection in HIV-seropositive individuals from a country of high tuberculosis prevalence - an interim analysis". 49. *Congress of the German Society for Pneumology and Respiratory Medicine, Lübeck, April 09th - 12th, 2008*

Leidl L, Mayanja-Kizza H, Sotgiu G, Baseke J, Ernst M, Hirsch C, Goletti D, Toossi Z, Lange C. "Comparison of T-cell interferon-gamma release assays for the diagnosis of latent tuberculosis infection in HIV-seropositive individuals – an interim analysis". *Congress of the European Respiratory Society, Berlin, October 04th - 08th, 2008*

Leidl L, Mayanja-Kizza H, Sotgiu G, Baseke J, Ernst M, Hirsch C, Goletti D, Toossi Z, Lange C. "Relationship of immunodiagnostic assays for tuberculosis and numbers of circulating CD4+ T-cells during HIV-infection". 50. *Congress of the German Society for Pneumology and Respiratory Medicine, Mannheim, March 18th - 21st, 2009*

Poster presentations:

Leidl L, Lange C: „HIV und Tuberkulose in Afrika: Erprobung neuer Testverfahren zur Diagnose der latenten Infektion mit *Mycobacterium tuberculosis* bei HIV-seropositiven Personen in Uganda“. 2. Lübecker Doktorandentag, Juni 2008

