Rational use of immunodiagnostic tools for tuberculosis infection

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Executive summary

Tuberculosis (TB) still remains a public health challenge with nearly 2 billion people—one third of the world's population—that have been exposed to the agent causing the disease, the *Mycobacterium tuberculosis* (MTB), and 8 million new cases every year together with 2 million deaths.
The rise in HIV infection and the neglect of TB control programs have enabled a resurgence of TB.
The control of TB requires a multifaceted approach integrating efficient public health interventions with the use of efficient and new diagnostic tools, vaccines and drugs. In particular, as the disease is transmitted usually from people with active — not latent— TB, the rapid identification of subjects with active TB together with the identification of the infected subjects represent one of the main measures to control efficiently the disease diffusion.
MTB elicits a strong immune response upon infection by stimulating both CD4+ and CD8+ T-cells as well other cells of the immune system, determining a strong Type 1 response dominated by interferon (IFN)-gamma. The overall response is at the basis of the so called delayed-type hypersensitivity (DTH) caused by MTB antigens. This phenomenon has been used since more than one century for the identification of subjects infected by MTB by using the tuberculin skin test (TST), which attempts to measure cell-mediated immunity in the form of a DTH response to the most commonly used purified protein derivative (PPD) of tuberculin.
However, the test is affected by many limitations not last the fact of high rate of positivity consequently to vaccination with *M. bovis* BCG.
In the last decade, extensive studies on subtractive DNA hybridization of pathogenic *M. bovis* and BCG together with comparative genome-wide DNA microarray analysis of *MTB* H37Rv and BCG, identified several regions of difference between *MTB* and *M. bovis.* In particular, RD1 region was lost early during the process of *M. bovis* BCG attenuation and is therefore missing in all the daughter strains known today. This region has been the subject of detailed studies and a number of antigens have recently been characterized as candidate antigens for diagnostic and vaccine development, these include the early secreted antigen target (ESAT–6) and culture filtrate protein (CFP–10) identified to be immunodominant antigens. Based on these studies, one of the most significant developments in the diagnostic armamentarium for TB in the last hundred years seems to be the assays based on IFN-γ determination (IGRAs). The assays stem from the principle that T cells of sensitized individuals produce IFN-gamma when they re-encounter the antigens of *MTB*. IGRAs' clearest advantage is the increased specificity for detection of *MTB* infection thanks to their utilisation of *MTB*-specific antigens encoded in region of difference (RD)1, a genomic segment absent from the BCG vaccine and most environmental mycobacteria. The evaluation in different clinical settings of the IFN-γ assays using the *MTB* RD1 antigens, have shown many advantages over tuberculin skin testing.
IFN-gamma assays that are now commercially available are: the original QuantiFERON-TB, and its enhanced versions QuantiFERON-TB Gold and QuantiFERON-TB Gold In-Tube assays (Cellestis International, Carnegie, Australia), and the enzyme-linked immunospot (ELISPOT) T-SPOT.TB assay (Oxford Immunotec, Oxford, United Kingdom).

The rapid and large diffusion in using worldwide the IGRA tests, increased a lot the knowledge about the clinical use of these tests in different settings and conditions. Further, as the tests have been designed for the diagnosis of TB infection and do not distinguish among active and inactive or latent disease, their rational use respect (or together with) the TST, has been largely discussed and generated a number of guidelines in many Countries for their best use in different settings of application. In summary, with all the suggestions used by the different countries for the correct use of the IGRA tests, it can be observed that:

- The two-step approach (first use the PPD skin test followed by the IGRA test for confirmation) seems to be the most favored strategy for IGRA use and implementation in the general population;
- The two-step approach is particularly favored in contacts, especially BCG- vaccinated contacts;
- There is a general trend in suggesting the use of IGRAs alone in particular clinical settings and/or patient groups. These include: (i) subjects that undergo anti-TNF-alpha therapy, (ii) other immunodepressed individuals, in particular HIV- infected subjects and (iii) children.

Furthermore, even if these tests are still very costly and their introduction is not suggested for the potentially negative economical impact on the National's TB control programmes, there are a number of cost effective advantages in the "mirate" use of IGRAs vs the TST. These include: (i) the single visit, (ii) the high sensitivity (up to 93%) and specificity (>99%) for detecting active TB and MTB infection, (iii) the IGRAs avoids false positives due to BCG vaccination and most environmental non-tuberculous mycobacteria, (iv) unlike the TST, they are not subject to errors in test placement or reading, (v) it can be possible a reduction in personnel cost (in fact the TST reagents represent less than 1.5% of the total cost of TST screening programs), (vi) it can be possible a reduction in additional costs—such as chest X-rays—associated with investigating false positive cases and (vii) IGRAs avoid boosting and eliminate the need of the two-visit system for testing.

The present work would like to summarise the most recent advances in the immunodiagnosis of TB infection and the best rational use of the new immunodiagnostic tools for TB infection in different clinical settings.

INTRODUCTION

Tuberculosis (TB) is a public health challenge of paramount importance (1). According to the World Health Organization (WHO), nearly 2 billion people—one third of the world's population—have been exposed to the TB pathogen (1). Annually, 8 million people become ill with TB, and 2 million people die from the disease worldwide (1). The rise in HIV infection and the neglect of TB control programs have enabled a resurgence of TB (2). These factors, together with the emergence of drug-resistant strains have also contributed to this new epidemic with, from 2000 to 2004, 20% of TB cases being resistant to standard treatments and 2% resistant to second-line drugs (3).
The rate at which new TB cases occur varies widely, even in neighboring countries, apparently because of differences in health care systems (4). Further, the incidence of TB varies with age. In country with high-medium incidence, TB primarily affects adolescents and young adults (2). However, in countries where TB has gone from high to low incidence, TB is mainly a disease of older people, or of the immunocompromised (2). Consequently, control of TB will require a multifaceted approach integrating efficient public health interventions with the discovery and use of new diagnostic tools, vaccines and drugs.

TRANSMISSION AND LIFETIME RISK

The Mycobacterium tuberculosis (MTB), the causing agent of the disease, is transmitted usually from people with active — not latent — TB (2). The probability of transmission from one person to another depends upon the number of infectious droplets expelled by a carrier, the effectiveness of ventilation, the duration of exposure, and the virulence of the MTB strain (5). People with prolonged, frequent, or intense contact are at particularly high risk of becoming infected, with an estimated 22% infection rate (1, 2). A person with active but untreated TB can infect 10–15 other people per year (1, 2). Therefore, the chain of transmission can be broken by isolating patients with active disease and starting effective anti-tuberculous therapy. After two weeks of such treatment, people with non-resistant active TB generally cease to be contagious (1, 2, 5).
It has been estimated that the lifetime risk of developing active TB following infection with MTB is approximately 10 percent (2, 6, 7). However, among of those persons who develop disease, approximately half do so during the first few years after infection, where the probability to develop active disease is the highest (8). After that, the incidence decline to low than 0.1% yearly to come back at a cumulative risk frequency of about 10% after 30-40 years from the original infection, as established with statistical model on epidemiological data (9).
Therefore, about 90% of those infected with MTB have asymptomatic, LTBI (2).

PATHOGENESIS AND IMMUNITY TO MTB

TB infection begins when the mycobacteria reach the pulmonary alveoli, where they invade and replicate within the endosomes of alveolar macrophages (10)
The primary site of infection in the lungs is called the Ghon focus, and is generally

located in either the upper part of the lower lobe, or the lower part of the upper lobe. Bacteria are picked up by dendritic cells, which do not allow replication, although these cells can transport the bacilli to local (mediastinal) lymph nodes. Further spread is through the bloodstream to other tissues and organs where secondary TB lesions can develop in other parts of the lung (particularly the apex of the upper lobes), peripheral lymph nodes, kidneys, brain, and bone (11). All parts of the body can be affected by the disease, though it rarely affects the heart, skeletal muscles, pancreas and thyroid (12).
When MTB enters the lung after being expelled from a patient with active TB, its genetic program reflects the high metabolic and replicative activity characteristic for the pathogen, which flourishes in caseous lesions. MTB ends up in a macrophage that participates in the contained lesion. These lesions are surrounded by a fibrotic wall and are, therefore, likely to be hypoxic causing a stage of dormancy in the pathogen (13, 14).
In vitro studies revealed upregulation of the DosR regulon comprising 48 genes including heat shock proteins (Hsp), notably HspX or a-crystallin (15-19). The bacterium switches to reduced respiration and metabolism and the total absence of growth. Evidence exists that dormant bacteria become non-stainable by conventional methods (20). Once the lesion is disrupted, oxygen levels rise, MTB starts to synthesize RNA and DNA and to divide – the bacteria become resuscitated. Recent experiments suggest that a few microorganisms, which regain metabolic and replicative activity, produce resuscitation promoting factors (Rpfs), which act as pheromones for dormant microbes in their vicinity (21-23). These Rpfs are muralytic enzymes that enzymatically dissolve the cell wall (24). MTB becomes then metabolically active, flourishes in the liquefied lesions and is transmitted in this form through the blood or airways to distant tissue sites or innocent contacts, respectively. The vicious circle of the pathogen is completed.
If this described above is in summary what is happening from the bacteria point of view, the host put together a number of mechanisms to control the MTB infection. The efficacy of these mechanism of control of the mycobacterial replication are at the basis of the balance between LTBI and activation driving to active TB.
After its arrival in the alveolar space, MTB is phagocytosed by alveolar macrophages which in their quiescent state mount little resistance, both in terms of antimicrobial effector functions and in terms of pro-inflammatory responses. Neutrophils accumulate early at the site of infection, but are incapable of killing MTB directly. Dying neutrophils and macrophages shed blebs containing MTB antigens or spill intact MTB; both can be taken up by alveolar dendritic cells which migrate to the regional lymphnodes, where they present MTB antigens to major histocompatibility complex (MHC) class-I restricted CD8+ T-cells and MHC class-II restricted CD4+ T-cells. In the lymph node, stimulated CD4+ and CD8+ T-cells differentiate respectively into (i) interferon (IFN)-gamma secreting T-helper (Th) type 1 or (ii) cytotoxic Tc1 cells accumulating into their granules molecules such as granzymes and granulysin (25). These MTB -specific effector cells enter the blood circulation and get access to sites of inflammation, such as the lung. At this stage, a delayed-type hypersensitivity (DTH) response in the skin or in blood may become positive upon tuberculin skin testing and intragranulomatous necrosis may ensue. It is conceivable that during the initial growth phase within alveoli, MTB may also spread bronchogenically to other parts of the lung. Following transportation to the

regional lymph node, haematogenous dissemination of MTB is thought to be the rule. Whichever body part they reach, the micro-organisms may gain entry to epithelial cells, fibroblasts or even adipocytes (26, 27), or become engulfed by local tissue macrophages. This usually elicits a low-grade inflammatory response which attracts antigen-responding T- and B-cells into granulomas. Scars of TB are most commonly found on autopsy in the lung apices. Post-primary reactivation disease often starts its apicocaudal propagation through the lung tissue from the apices.

The presence of activated Th1 cells at the site of MTB implantation alters the infiltrate in a striking fashion: mononuclear cells are attracted and organised in a highly specific way to form the granuloma, in which mycobacteria-containing macrophages that morphologically differentiate into ''epithelioid'' cells are surrounded by lymphocytes (28). This juxtaposition affords effective activation of macrophages, mostly via IFN-gamma, and antimycobacterial effector molecules. These include reactive oxygen and nitrogen species that are delivered to the fusing phagolysosome. The host Tc1 cells deliver granulysin and other mycobactericidal molecules in a perforin-dependent fashion to the macrophage. This T-cell response leads to a reduction of mycobacterial growth and even, to a certain extent, to the killing of MTB although this is difficult to demonstrate in vitro using, for example, human macrophages.

The evidence that B-cells and MTB-specific antibodies can mediate protection against extracellular MTB is highly controversial as their contribution is probably of minor importance. Depending on the number of mycobacteria present in the lesion and the level of cellular immune responses generated, the antibacterial protective response can also lead to the destruction of macrophages, resulting in necrosis of the central areas of the granuloma. A concomitant, overwhelming Th2 response with IL-4/IL-13 secreting T-cells may also counteract the mycobactericidal mechanisms in macrophages and may contribute to central granuloma caseation (29). This solid necrotic mass is thought to be devoid of oxygen and may, over time, become sclerotic and even calcified, commonly esulting in containment or death of MTB (30).

In summary, well-organised granulomas are entirely dependent on an effector Th1 response and mediate restriction of MTB growth either within IFN-gamma-activated macrophages or within the adverse conditions of the necrotic caseum. In contrast, if no or only low-level T-cell immunity is generated under conditions of T-cell deficiencies, MTB growth is not contained. Nevertheless, this is associated with comparatively little tissue damage.

Granulomas are dynamic lesions with cells continuously dying, debris being removed, and new cells entering. When cells are prevented from entering, the granulomatous structure disintegrates leading to the dissemination of its content. Within a granuloma, it is assumed that there is equilibrium of actively dividing MTB and MTB adapted to the stress generated within activated or foamy macrophages that do not completely destroy MTB but prevent their growth. Therefore, MTB is thought to enter a state of non-replicating persistence within the necrotic part of the lesion (31).

It is likely that small foci of resuscitation occur from time to time, resulting in longterm persistence of MTB progeny, but it is also possible that individual mycobacteria may remain in a truly quiescent state over decades. It is not clear

which antigens are expressed and secreted during the stage of stress-adapted metabolism in granulomatous lesions or extracellularly, and how these antigens are processed for T-cell recognition (32). ESAT-6 and CFP-10, antigens secreted during active replication, are likely to play a much less dominant role than proteins from the DosR operon which is highly upregulated in latency. Given that all anti-TB drugs are metabolic blockers, and specifically that isoniazid is effective only against multiplying MTB and that isoniazid treatment of latent infection with MTB is effective in 60–90% of cases, latent infection with MTB must to some extent be accompanied by some replication of MTB (33). The larger the viable mycobacterial load and the remaining lesions are, the more likely constant antigen-specific re-stimulation of the memory/effector T-cell pool becomes. Conversely, the more encapsulated and quiescent the lesions and the smaller in size they are, the more likely it is that Th1 cell immunity will decline (25).
T-cell immunity will fade or become impaired for various reasons, particularly in acquired or drug-induced immunodeficiency. This may lead to recrudescence of mycobacterial growth, which is often localised in, and often seemingly limited to, the upper lobes where oxygen supply is thought to favour replication and to where MTB has earlier been lodged after haematogenous dissemination. Consequently, T-cells encounter an increased antigenic load that precipitates a hyperinflammatory immune response with necrotic and cavitating lesions. Overall, however, reactivation disease is a rare event in immunocompetent individuals (34-37).
Recent advances have shed new light on the MTB specific T-cell responses occurring in vaccinated, MTB-exposed, and TB patients. The most dramatic improvement in data generation has been in analyzing antigen-specific responses and identifying several parameters of responding cells using flow cytometry. Analysis of cellular responses has shown that infected and diseased individuals express a high frequency of multifunctional cells (38). Recent studies of a European population reported a limited MTB-specific IL-17-specific response but a strong IL-17 response to fungal antigens (39). In contrast, when South African populations are examined, MTB-specific IL-17- and IL-22-producing T-cells can clearly be identified both in exposed and diseased individuals, although the frequency of such cells in the periphery is lower in the diseased patients (40). Despite the low frequency of these T-cells peripherally, IL-22 can be detected in the bronchoalveolar lavage of patients, suggesting that IL-22-producing cells are active in the lung of patients (40). This compartmentalization of the response has also been seen in a recent study using an Ag85/HLA-A*0201 pentamer to assess frequency of antigen-specific cells in children with active TB before and after drug treatment. These patients had a low frequency of antigens-specific CD8+ T-cells in the blood, but this frequency increased after treatment (41), possibly because the treatment reduced inhibition of T-cell activation by the bacteria or because lung lesions were resolving and thus recruitment of antigen-specific cells from the blood to the lesional site was reduced. Interestingly, the circulating antigen-specific cells had low cytokine and cytolytic activity before treatment, whereas more activated cells were present in the lung before treatment (41). A separate study found that at the beginning of treatment the frequency of antigen-specific IFN-gamma-producing cells is higher than that for IL-2-producing antigen-specific cells but that as treatment progressed over 28 months the dominance of the IFN-gamma response is lost and most

responding cells are of the IL-2-producing phenotype (42). Further detailed studies will allow for greater understanding of the kinetics and nature of the antigen-specific cellular response in humans. The high level of BCG vaccination occurring in newborns in South Africa has allowed for an unprecedented screen of the ability of BCG to induce specific cell types in this population. In a recent study, cells from BCG-vaccinated newborns were restimulated in vitro with BCG, and the cellular response was analyzed by flow cytometry. Importantly, whereas IL-4- and IL-10-producing CD4+ T-cells occurred at low frequency, IFN-gamma, IL-2, and TNF-alpha were all produced either singly or in combination by activated CD4+ T cells. Activated CD8+ T-cells were less frequent and when present were predominantly IL-2 and/or IFN-gamma positive. In a telling detail, many of the responding T cells were not positive for IFN-γ; thus, the effector phenotype of many of the responding cells is still to be determined. The majority of the responding IFN-γ-producing cells had an effector cell surface phenotype, whereas those expressing IL-2 alone were of the central memory phenotype (43).
One aspect of the human immune response that has received increased attention recently
is the identification of antigens recognized by exposed individuals. In a study using synthetic
peptide arrays of known immunodominant antigens, the antigen specificity of a large number of CD8 T-cells clones was obtained and new epitopes identified (44). In a separate study, the regions of difference (RD) that have been identified between MTB and BCG were used to compare the peptide-specific responses of patients with TB and healthy individuals vaccinated with BCG. In this study, both groups responded to peptides from the RD1 region (perhaps reflecting a high degree of exposure to Mtb). However, RD12, RD13, and RD15 peptides elicited an IL-10 response from peripheral blood mononuclear cells, suggesting that antigens within the different RD are associated with distinct cellular responses (45). These kinds of studies are being used to fill the gaps in our understanding of the specificity of the T-cell response to MTB in humans (46).

CLASSIFICATION OF TB

The most widely used clinical classification system for TB is the one last review in 1999 by the American Thoracic Society (47). It is based on the combination of the pathogenesis, exposure evidences and supported by microbiological for MTB identification and immunological test for TB diagnosis. It is based on 6 groups (see table), of which people falling in the class 3 to 5 should be reported promptly to the local health department.

Class	Type
0	No TB exposure, Not infected
1	TB exposure, No evidence of infection
2	TB infection, No disease
3	TB clinically active
4	TB Not clinically active
5	TB suspect

Another, important classification made by the Center for Diseases Control (5), is considering to target subject with clinically active TB (ATS class 3) in order to contain the diseases diffusion based on the evidence that only subjects with an high burden of bacteria have to be isolated.

Class	*Chest X-ray*	*AFB smear*
A - TB, clinically active, infectious	*Active TB*	*Positive*
B1 - TB, clinically active, not infectious	*Active TB*	*Negative*
B2 - TB, not clinically active	*Inactive TB*	*Not required unless symptomatic*
No class (Normal)	Normal	Not required

As stated in the "Transmission and life time risk of infection" section, the largest group of persons infected by MTB is represented by the ones with asymptomatic infection. On the contrary of most other infectious where the asymptomatic subjects are capable to eliminate, hence transmit, the infectious agent, subjects infected by MTB but not presenting the active sign of the disease are not capable to transmit and spread the infection. Thus, these are defined as subjects with LTBI and fall in the category 2 of the ATS. LTBI subjects keep a cumulative risk frequency of about 10% of developing an active diseases longlife. Thus, although it is not possible to demonstrate a replication of MTB, it has been postulated that or (i) MTB is surviving in its dormant phase or (ii), more likely, that MTB is surviving in an equilibrium among the dormant phase and the replication activities, in an existing equilibrium tightly controlled by the immune response. As a consequence, the immune reaction against MTB is the only possibility to demonstrate the presence of a LTBI infection.

DIAGNOSIS OF TB

Due to the extreme advances on the microbiological diagnosis of active TB as well as in the immunodiagnosis of TB infection in the past years, new tools have been made available to the clinicians and laboratorists for the best classification of subjects with TB suspect or exposed to TB patients. In any case, these new tools need to be properly used and the results carefully interpreted in the context of what the different tests are developed for and not overinterpreted by given a different meaning.

MICRIBIOLOGICAL DIAGNOSIS

The gold standard for diagnosis of active TB is the demonstration of mycobacteria from various body fluids. However, in the overall the sensitivity and the specificity of the two standard microbiological tests for the diagnosis of TB, i.e. the Acid Fast Bacilli (AFB) stain and the cultures techniques, are not satisfactory raging from 20-80% and 60-99% respectively (47). The significant improvement in understanding of

molecular biology of MTB till the availability of its whole genome, has led to development of newer diagnostic techniques for TB. Because of their ability to detect even a single copy of MTB, nucleic acid amplification tests (NAAT) and their modifications have been welcomed as a revolutionary diagnostic tool, capable of reducing the time to diagnosis of patients with suspected TB from weeks to hours and are taking hold in clinical practice (47, 48). The NAAT have been used to:

(a) diagnose TB rapidly by identifying DNA or RNA from *MTB* in clinical samples, in particular in the samples negative by microscopic examination.
(b) determine rapidly whether acid-fast micro-organisms identified by microscopic examination in clinical specimens are *MTB* or atypical mycobacteria.
(c) identify the presence of genetic modifications known to be associated with resistance to some anti-mycobacterial agents.

During the 1990s, however, it became apparent that NAAT are not sufficiently reliable to replace conventional diagnostic methods. Both inherent test characteristics and errors in testing procedures may account for NAAT inaccuracy (48). As for microscopy and culture, the key factor in determining NAAT false negatives is the density of mycobacteria in the specimen, since it can result in the absence of nucleic acids in the small volumes used for the test. The presence of enzymes in respiratory secretions able to inhibit amplification reactions may account for a percentage varying from 3% to 25% of false negative NAAT results. On the other hand, false positives mainly arise from contamination of negative samples with target DNA from samples containing large MTB numbers or from target sequences of previous amplifications accumulated in the laboratory room (48).
In the attempt to minimize these defects, the industry developed automated commercial systems which were made more robust by means of the use of standardized procedures and reagents for sample processing, amplification and detection. These tests, allowing different step of the process to take place in a single sealed tube, should have rendered commercial NAAT less prone to contamination. At the same time, measures such as the choice of target sequences present in multiple copies, the use of larger sample volumes or the introduction of internal amplification controls to detect inhibitors, have been adopted for cutting down on false negatives. However, notwithstanding these precautionary measures, a considerable variability of diagnostic accuracy of commercial NAAT is still apparent from published studies (49). A statement from the U.S. Centers for Disease Control (CDC) indicates that commercial NAAT should be used besides to microscopy as in parallel tests to improve diagnostic certainty, pending culture results and/or patient's response to therapy. According to CDC, a diagnosis of pulmonary TB can be presumed in AFB-positive patients with a positive NAAT result and in AFB-negative patients with two subsequent positive NAAT results. In the case of at least two negative, inhibitor-free NAAT results, an AFB-positive smear can be presumed diagnostic of non tuberculous mycobacterial disease, while an AFB-negative can be considered indicative of non contagiousness (50). The CDC recommendations are limited to the two FDA-approved commercial NAAT (50). Furthemore, the CDC strongly advise physicians

to rely upon clinical judgement in the interpretation of the NAAT laboratory data (49, 50).

IMMUNOLOGICAL DIAGNOSIS OF TB. MECHANISMS OF READOUT MEASURES OF AN ADAPTIVE MTB -SPECIFIC *IMMUNE RESPONSE*

Due to the limitations of the microbiological and molecular biology tests for the diagnosis of TB, particularly in children and extrapulmonary for of TB, and in the search for rapid and cost-effective diagnostic methods for TB, immunodiagnosis has been considered an attractive option. Basically, it uses the specific humoral and cellular immune responses of the host to infer the presence of infection or disease. The tuberculin skin test (TST) (51) and, more recently, the set up of antigen-specific *ex vivo* release of IFN-gamma (IGRAs) have been used to detect infection with MTB (52).

As largely discussed in the previous section, strong evidences support the notion that both TB patients and those infected with MTB present a strong cellular immune response to MTB antigens both in *in vivo* and *ex vivo. In vivo*, this reaction can be measured by DTH response to the tuberculin purified protein derivative (PPD); *ex vivo*, by the proliferation of lymphocytes (or the release of their immunomediators) to different compounds of the bacteria (52, 53).

THE CENTURY-OLD SKIN TEST FOR DETECTION OF LATENT TB

In 1882, about eight years after the discovery of the tubercle bacillus, Robert Koch announced a cure for TB. He obtained a heat-inactivated filtrate from cultures of *MTB*, and found that this material would protect guinea pigs from experimental TB. This product, known as "Koch´s Old Tuberculin", was then administered to patients with TB, and Koch claimed that this treatment resulted in the cure of the disease (54-56). However, TB patients who received tuberculin had generalized systemic reactions, including fever, muscle aches, and abdominal discomfort with nausea and vomiting, in contrast to people without TB, who did not develop this violent reaction. These observations were the basis for the proposal of the use of tuberculin as a diagnostic test, despite its failure as a therapeutic substance. The intradermal injection of tuberculin was described by Mantoux, and his method became widespread because of the reproducibility of the results.

The reaction to intracutaneously injected tuberculin is the classic example of a delayed (cellular) hypersensitivity reaction. T-cells sensitized by prior infection are recruited to the skin site where they release lymphokines (57). These lymphokines induce induration through local vasodilatation, edema, fibrin deposition, and recruitment of other inflammatory cells to the area (58). Typically, the reaction to tuberculin begins 5 to 6 h after injection, causes maximal induration at 48 to 72 h, and subsides over a period of days. In a few individuals (the elderly and those who are being tested for the first time), the reaction may not peak until after 72 h (59). Such delayed reactions do not alter the intrepretation of the test. Immediate hypersensitivityreactions to tuberculin or constituents of the diluent can also occur. These reactions disappear by 24 h, and should not be confused with delayed hypersensitivity reactions.

The reaction is strongly supported by infiltrating cells in the site of tuberculin injection. At very early time points (after 4–6 h) the majority of infiltrating cells are neutrophils (60). Approximately 12 h after challenge, T-cells begin to appear

around dermal blood vessels (61). Maximal numbers of infiltrating activated macrophages are present at 24 h and, by 48 h, the majority of infiltrating cells are T-cells that accumulate perivascularly (62, 63); however, some T-cells diffuse into the epidermis and the interstitium. CD4 T-cells always exceed the number of CD8 T-cells (64, 65). The mechanism of this cellular infiltration is not entirely clear but it appears that very early after the injection, pro-inflammatory cytokines, such as IFN-gamma, TNF-alpha and TNF-beta stimulate expression of adhesion molecules on the endothelium (E-selectin) and increase permeability of the local blood vessels. It has been observed that the frequency of circulating CD4+CD25+FoxP3+ regulatory T-cells might influence the size of the induration of the TST (66). Cutaneous CD4 T-cells accumulating after tuberculin stimulation in the skin are predominantly of a CD45 RO memory phenotype (66).

The first Koch's tuberculin was an impure extract of boiled cultured tubercle bacilli. In 1934, Siebert (67) made a simple protein precipitate of the old tuberculin and named it purified protein derivative (PPD).

Standardization should guarantee equivalent potency between PPD preparations isolated by different precipitation methods and between successive lots isolated by a single method. Most of the constituents of PPD are small proteins with molecular masses of approximately 10,000 Da, but there are also polysaccharides and some lipids present (68). The relatively small size of the protein constituents in PPD is the reason that PPD does not sensitize individuals who have not been exposed to mycobacteria (68). A batch of PPD (lot 49608) called PPD-S, which was produced by Seibert and Glenn in 1939, has continued to serve as the international standard (69). All PPD lots must be bioassayed to demonstrate equal potency to PPD-S (70).

The standard 5-tuberculin unit (TU) dose of PPD-S is defined as the delayed skin test activity contained in a PPD-S dose of 0.1 mg/0.1 ml. The standard test dose of a commercial PPD preparation is defined as the dose of the product that is biologically equivalent to that contained in 5 TU of PPD-S.

The tuberculin test, like all medical tests, is subject to variability, but many of the inherent variations in administration and reading of tests can be avoided by careful attention to details. The test is administered by injecting 0.1 ml of 5-TU PPD intradermally (Mantoux method) into the volar or dorsal surface of the forearm. Other areas may be used, but the forearm is preferred. The use of a skin area free of lesions and away from veins is recommended. The injection is made using a onequarter- to one-half-inch, 27-gauge needle and a tuberculin syringe. The tuberculin should be injected just beneath the surface of the skin, with the needle bevel upward or downward (71). A discrete, pale elevation of the skin (a wheal) 6 to 10 mm in diameter should be produced when the injection is done correctly.

Tests should be read between 48 and 72 h after injection, when the induration is maximum. Tests read after 72 h tend to underestimate the true size of induration. Reading should be performed in a good light, with the forearm slightly flexed at the elbow. The basis of reading is the presence or absence of induration, which may be determined by inspection (from a side view against the light as well as by direct light) and by palpation. For standardization, the diameter of induration should be measured transversely to the long axis of the forearm and recorded in millimeters (71). The absence of induration should be recorded as “0 mm,” not

"negative." Interobserver variability may be decreased by using the ball-point pen method of Sokal to measure induration (72, 73).

To interpret the TST appropriately, one must understand the sensitivity and specificity of the test as well as the positive and negative predictive value of the test. The sensitivity of a test is the percentage of people with the condition who have a positive test. If false-negative results are uncommon, the sensitivity is high. The PPD skin test has a reported false-negative rate of 25% during the initial evaluation of persons with active TB (74). This high false-negative rate appears to be due to poor nutrition and general health, overwhelming acute illness, or immunosuppression. Immunosuppression can be either specific, which may be seen early during disease, or nonspecific, which can be the result of medications, malignancy, or HIV infection (75). Because of the low sensitivity of the test, especially in acutely ill patients and those who are infected with HIV, the tuberculin test cannot be used to eliminate the possibility of active TB (76).

Vaccination with live-attenuated virus can cause suppression of the PPD response in patients known to be infected with *MTB.* Live-attenuated vaccines that may cause false-negative PPD results are measles, mumps, rubella, oral polio, varicella, yellow fever, BCG, and oral typhoid (TY21a). False-positive results decrease the specificity of a test. False-positive tuberculin tests occur in individuals who have been infected with other mycobacteria, including vaccination with BCG. Some antigens in PPD are shared with the other mycobacteria (77) and thus can elicit a skin test response. These cross-reactions tend to result in smaller amounts of induration than reactions due to *MTB*, but the overlap may be considerable in areas of the world where the other mycobacteria are common (76). In these populations, the specificity of the test is highly dependent on the criterion used to define a "positive" test. Thus, the specificity of the test can be improved by progressively increasing the cut point for positivity. In any population, the likelihood that a positive test represents a true infection is influenced by the prevalence of infection with *MTB.* The TST has a specificity of approximately 99% in populations that have no other mycobacterial exposures or BCG vaccination, but the specificity decreases to 95% in populations where cross-reactivity with other mycobacteria is common (78).

On the basis of the sensitivity, specificity, and the prevalence of TB in different groups, three cut points have been recommended for defining a positive tuberculin reaction. For individuals who are at great risk of developing TB disease if they become infected with *MTB* (78), a cut point of > 5 mm is recommended. Reactions in persons who have had recent close contact with TB and in persons with abnormal chest radiographs consistent with TB are more likely to represent infection with *MTB* than cross-reactions. Persons who are immunosuppressed because of disease (e.g., HIV infection) or drugs (e.g., corticosteroids) are more likely to progress to TB disease if they are infected with *MTB.* Therefore, using a lower cut point (e.g., 5 mm) for separating positive from negative reactions is appropriate in these groups. This will ensure that few persons infected with *MTB* will be classified as having negative reactions, although a few persons not infected with tubercle bacilli will be classified as having positive reactions. A cut point of > 10 mm is suggested for individuals who have normal or mildly impaired immunity and a high likelihood of being infected with *MTB* but are without other risk factors that would increase their likelihood of developing active disease. In addition to

those groups listed, other high-prevalence populations may be identified locally. Persons who are not likely to be infected with *MTB* should generally not be tuberculin tested since the predictive value of a positive test in low-prevalence populations is poor. However, if a skin test is done, e.g., at entry into a work site where some risk of exposure to TB is anticipated and a longitudinal tuberculin testing program is in place, a higher cut point of > 15 mm is suggested in order to improve the specificity of the test (76) These internationally accepted standard guidelines for the use of the TST are summarized in Table.

GUIDELINES FOR DETERMINING A POSITIVE TUBERCULIN SKIN TEST REACTION

Induration ≥ 5 mm	Induration ≥ 10 mm	Induration ≥ 15 mm
HIV-positive persons	Recent arrivals (< 5 yr) from high-prevalence countries	Persons with no risk factors for TB
Recent contacts of TB case	Injection drug users	
	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	
	Mycobacteriology laboratory personnel	
Fibrotic changes on chest radiograph consistent with old TB	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 (e.g., carcinoma of the head or neck and lung), weight loss of > 10% of ideal body weight, gastrectomy, jejunoileal bypass	
Patients with organ transplants and other immunosuppressed patients (receiving the equivalent of > 15 mg/d Prednisone for > 1 mo)	Children < 4 yr of age or infants, children, and adolescents exposed to adults in high-risk categories	

* For persons who are otherwise at low risk and are tested at entry into employment, a reaction of > 15 mm induration is considered positive.

INTERFERON-GAMMA BASED DETERMINATIONS

In the last decade, extensive studies have shown that immunodominant antigens, such as the 6-kDa early secretory antigenic target (ESAT-6) and its homologues, are highly suitable for detecting infection. There is no cross-reaction with the BCG vaccine, since these antigens are absent in the BCG vaccine strains. By screening eluted fractions of antigens from *MTB* and *M. bovis* culture filtrates for recognition by T cells from infected humans and cattle, respectively, Andersen and co-workers identified several low-molecular mass antigens that are major targets of cellular mediated immune responses (79). Subtractive DNA hybridization of pathogenic *M. bovis* and BCG (80) and comparative genome-wide DNA microarray analysis of *MTB* H37Rv and BCG (81) identified several regions of difference, designated RD1 to RD16, between *MTB* and *M. bovis.* All represent segments that have been deleted from the *M. bovis* genome. RD1 was lost early during the process of *M. bovis* BCG attenuation and is therefore missing in all the daughter strains known today (80). This region has been the subject of detailed studies and a number of antigens have recently been characterized as candidate antigens for diagnostic and vaccine development (82-84). Antigens, such as early secreted antigen target (ESAT–6) and culture filtrate protein (CFP–10), are located in this region and have already shown great potential for TB diagnosis (85-88).

Based on these studies, one of the most significant developments in the diagnostic armamentarium for TB in the last hundred years seems to be the assays based on IFN-γ determination (IGRAs). The assays stem from the principle that T-cells of sensitized individuals produce IFN-gamma when they re-encounter the antigens of *MTB* (89). IGRAs' clearest advantage is increased specificity for detection of *MTB*

infection thanks to their utilisation of *MTB*-specific antigens encoded in region of difference (RD)1, a genomic segment absent from the Bacille Calmette-Guérin (BCG) vaccine and most environmental mycobacteria. Recent evaluations showed that IFN-γ assays that use *MTB* RD1 antigens, such as ESAT6 and CFP10, may have advantages over tuberculin skin testing (87, 88, 90).
IFN-gamma assays that are now commercially available are: the original QuantiFERON-TB, and its enhanced versions QuantiFERON-TB Gold and QuantiFERON-TB Gold In-Tube assays (Cellestis International, Carnegie, Australia), and the enzyme-linked immunospot (ELISPOT) T SPOT-TB assay (Oxford Immunotec, Oxford, United Kingdom).

QuantiFERON-TB TEST
QuantiFERON-TB® and Bovigam® are two registered products which measure the release of interferon-gamma in whole blood from human subjects and cattle infected with *MTB* and *M. bovis* respectively, in response to stimulation by PPD. The IFN-gamma secreted by T-cells into the plasma is measured by ELISA to indicate the likelihood of TB infection. Different studies demonstrated that the QuantiFERON-TB test was comparable to TST in its ability to detect LTBI. These studies also showed that the QuantiFERON-TB test was less affected by BCG vaccination, discriminated responses due to non-tuberculous mycobacteria, and also avoided the variability and subjectivity associated with administering and reading the skin test (91).
QuantiFERON-TB® was approved by the Food and Drug Administration (FDA) of the United States (US) in 2001. In 2003, the US Centers for Disease Control and Prevention released guidelines for using the QuantiFERON®-TB Test in the diagnosis of latent *MTB* infection, which can be found on the internet at http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5202a2.htm.
More recently, an evaluation of the whole blood IFN-γ test for TB diagnosis, based on the specific antigens ESAT-6 and CFP-10, showed that the recombinant antigens could increase the specificity of the whole blood test and enhance the discriminative power of the test between TB infection, atypical mycobacterial reactivity, and reactivity due to BCG vaccination (92).
QuantiFERON®-TB Gold is an *in vitro* diagnostic test to aid in the detection of *Mycobacterium TB* infection. It combines the simplicity of the QuantiFERON® technology with the diagnostic power of synthetic TB-specific peptides (ESAT-6 and CFP-10) to provide the best available method of diagnosing TB infection. The antigens used in the Gold version are provided by the Statens Serum Institute in Denmark (http://www.ssi.dk/sw162.asp). Because QuantiFERON®-TB Gold uses whole blood, with no need for cell isolation and fractionation, it is inexpensive and fast. The assay measures IFN-γ secreted from stimulated T-cells previously exposed to *MTB* to assess TB infection status. The QuantiFERON®-TB Gold test is approved by the US FDA for *in vitro* diagnostic use (December, 2004), is approved for sale in Japan (April, 2005), and has CE Marking for use in Europe.
In addition to all the benefits of QuantiFERON-TB Gold, in the new In-Tube method a third antigen is included (TB7.7) and the assay has the following advantages:

- Minimises blood handling and simplifies the blood stimulation step of QuantiFERON®-TB Gold.
- Is highly compatible with automated ELISA platforms, allowing large volume screening.

- The simplified blood stimulation step allows the test to be used in any location. The Blood Collection Tubes are easily transported for ELISA testing. A convenient and cost effective option for blood stimulation, regardless of location.
- High sensitivity (~90%) has been shown in culture confirmed TB patients, exceeding that of the Mantoux test. Specificity is also very high (>98%) even in BCG vaccinated individuals. The In-Tube method has equivalent or higher sensitivity and specificity than that seen with QuantiFERON®-TB Gold.

The QuantiFERON®-TB Gold In-Tube test has CE Marking and is available for use in Europe, Australia, Canada (approved by Health Canada August 2006), Africa, Middle East and Asia. QuantiFERON®-TB Gold In-Tube test is approved by the FDA on 12 October 2007 for use in the United States. On April 14th 2009 it was approved for use in Japan.

ENZYME-LINKED IMMUNOSPOT FOR INTERFERON-GAMMA (T-SPOT.TB**)**

The ELISPOT assay for diagnosis of *MTB* infection is based on the rapid detection of T-cells specific for *MTB* antigens. IFN-γ released *ex vivo* from these cells can be detected by the extremely sensitive ELISPOT (93). Each such T-cell gives rise to a dark spot and the readout is the number of spots. The T cells enumerated by the ELISPOT assay are effector cells that have recently encountered antigen in vivo and can rapidly release IFN-γ when reexposed to the antigen (94). In contrast, the long-life memory T-cells, which persist long after clearance of the pathogen, are relatively quiescent and less likely to release IFN-γ during the short period of exposure to antigen in the *ex vivo* ELISPOT assay (93).

Lalvani *et al*. developed the first generation of new ELISPOT tests for latent TB by using the ESAT-6 peptide to stimulate single blood samples. This test detects as few as one in 60,000 IFN-γ producing cells. In a preliminary trial, this test was positive in 96 % of 47 TB patients and in 85 % of 26 persons presumed to have latent TB. The ELISPOT test was negative in 26 BCG-vaccinated control subjects, and this specificity implies a major advantage over TST (95). The assay has been evaluated by different groups (96) and the results have shown that ELISPOT offers a more accurate approach than TST for the identification of individuals who have LTBI. These tests could improve TB control by more precise targeting of the preventive treatment. A commercial ELISPOT test, T SPOT-TB® (Oxford Immunotec, Oxford, United Kingdom) is now available. Related information can be found on the internet at http://www.finddiagnostics.org.

As the T SPOT-TB is based on the same antigens of the QuantiFERON-TB Gold in tube, with the exception of the TB7.7, it present the same general benefits of QuantiFERON-TB Gold. However, some limitation for its general use in clinical laboratories have to be considered:

- Although the blood handling is minimized, PBMC purification is required before the T SPOT-TB test
- Being based on ELISpot technology the T SPOT TB is not compatible with automated ELISA platforms.
- The two previous points underlines the need of specialized/specifically trained personnel.
- The test have to be performed as short as possible after the blood drawn

- Also for T SPOT TB high sensitivity (~90%) has been shown in culture confirmed TB patients, exceeding that of the Mantoux test, with also a very high specificity (>98%) even in BCG vaccinated individuals.
- In theory, the T SPOT TB working with a defined number of PBMCs should have less problem in immunocompromised subjects. However, head to head comparison on T SPOT TB and Quantiferon TB Gold in Tube did not reveal any specific advantage.

RECENT ADVANCES AND CONSIDERATIONS ON *IN VITRO* TESTS FOR THE DIAGNOSIS OF TB INFECTION

While the initial results of IFN-γ determination for the detection of latent infected individuals appear promising, it remains to be seen whether this will translate into practically useful results in the field (97). Indeed, IFNγ assays are expensive tests and their higher cost appears to limit their wider applicability, especially in resource-limited settings and developing countries, where TB is highly rampant. The ELISPOT test is not yet suitable for widespread use, because it is costly and requires isolation of mononuclear cells, a procedure that is not performed in clinical laboratories.

One approach that has been used for both ELISpot and ELISA to increase diagnostic sensitivity is incorporation of additional antigens of established high specificity (98). In a recent large prospective study of patients with suspected TB, incorporation of a novel RD-1-encoded antigen, RV3879c, alongside ESAT-6 and CFP-10 (ELISpot^{PLUS}), significantly improved diagnostic sensitivity over the standard ELISpot test (99). The addition of Rv2645 in QuantiFERON-TB Gold In-Tube significantly improved sensitivity of this assay in diagnosing active TB over QuantiFERON-TB Gold (which, like T-SPOT.*TB*, contains only ESAT-6 and CFP-10) without compromising specificity (100), in agreement with the observed higher positive results using QuantiFERON-TB Gold In-Tube than QuantiFERON-TB Gold in a cross-sectional study of healthy adults in South Africa (101).

Alternative readouts to measure IFN-γ release by *MTB*-specific T-cells have also been explored. For example, using flow cytometry, ESAT-6-specific IFN-γ responses in peripheral blood mononuclear cells were detected in 13 (87%) out of 15 TB patients (102) and measuring IFN-γ mRNA from ESAT-6-stimulated peripheral blood mononuclear cells using quantitative PCR had a sensitivity of 65% in 54 active TB cases and LTBI (103).

Although the most recent studies are intending to increase the sensitivity and the specificity of the IGRAs, it is important to keep in consideration that (mycobacterial) epitopes are recognized in the context of specific HLA antigens (104). Thus, the IFN-γ based assays should be evaluated at multiple geographic locations, among patients of different ethnicities. Further, although BCG vaccination does not yield false-positive results in IFN-γ assays using selected antigens, the specificity of the test should be studied in persons exposed to environmental mycobacteria, as ESAT6-like proteins are largely distributed among the genome of mycobacteria, leading for potential cross-reactivity. Studies with larger numbers of TB patients are needed to address this issue. The diagnosis of latent TB represents a major advance in the quest for better tests. The explosion of microbial genomics, proteomics, and transcriptomics will yield more *MTB* specific

genes and antigens; and IFN-γ assays, using peptides from multiple antigens, should be more sensitive than the ones using only ESAT-6 or/and CFP10 (104).

GUIDELINES ON THE USE OF IGRAS

There is growing interest in the use of IGRAs, although most countries continue to recommend and use TST. In the past 5 years, several guidelines and position statements have been published on the role and use of IGRAs, and even some of the countries have more than one guideline or statement (105).
At the present moment guidelines or statements on IGRAs have been identified from 16 countries: USA, Canada, UK, Japan, France, Spain, Italy, Germany, Switzerland, Australia, Netherlands, Denmark, Czech Republic, Slovak Republic, Korea and Norway (105).
Among the countries that do not yet have an official guideline or statement on IGRAs are: China, India, Russia, Ukraine, Sweden, Brazil, Belgium S Africa, Mexico, New Zealand, Finland, Ireland, Bulgaria, Turkey, Viet Nam, Singapore, Portugal, and Saudi Arabia. At the same time IFN-□ release assays are used in some of these countries like Bulgaria, Turkey, Portugal, Singapore, Finland. In several countries guidelines have been developing: Finland, Saudi Arabia, Portugal (105).
Three main approaches are outlined in the countries that have guidelines:
-TST must be replaced by IGRA (i.e. only IGRA) Germany (anti-TNF-alpha therapy), Switzerland (anti-TNF-alpha), Denmark (anti-TNF-alpha, BCG-vaccinated contacts/adults);
-Either TST or IGRA can be used USA, France, Australia (refugees), Japan (QFT preferred in all groups except in children <5 years), Denmark (child contacts);
-Two-step approach of TST first, followed by IGRA either to improve specificity or sensitivity, Canada, UK, Italy, Spain, Australia, Germany (contacts), Switzerland (contacts), Netherlands (contacts, immigrants), Norway, South Korea (contacts).

Some guidelines recommend more than one approach, depending on the risk group tested (e.g. contacts, immunocompromised, children, etc).

The first published IGRA Guidance was the **Centers for Disease Control and Prevention (CDC) Guidelines 2003**: **QFT** (106)**.** The CDC has issued recommendations on using the QuantiFERON-TB (QFT) test for diagnosing LTBI. This document provided guidance for public health officials, health-care providers and laboratorians with responsibility for TB control activities in the USA in their efforts to incorporate QFT testing for detecting and treating LTBI. Regardless of the test used to identify LTBI, testing should be primarily targeted at diagnosing infected patients who will benefit from treatment. These interim recommendations are intended to achieve a high rate of acceptance and completion of testing for LTBI among groups who have been identified for targeted testing. Testing programs using TST or QFT should only be implemented if plans are also in place for the necessary follow-up medical evaluation and treatment (e.g., chest radiograph or LTBI treatment) of persons who are diagnosed with LTBI and quality laboratory services are ensured.
On May 2nd, 2005 was published the **CDC Guidelines 2005: QFT-G [2G])** (107). CDC recommended that QFT- G may be used in all circumstances in which the TST is currently used, including contact investigations, evaluation of recent immigrants,

and sequential testing surveillance programs for infection control [e.g., those for health-care workers (HCW)].
In December 2005, was published the **Guidelines for Preventing the Transmission of *Mycobacterium tuberculosis* in Health-Care Settings** (108). It is stated that the whole-blood interferon gamma release assay (IGRA), QuantiFERON®-TB Gold test (QFT-G) (Cellestis Limited, Carnegie, Victoria, Australia), is a Food and Drug Administration (FDA)– approved in vitro cytokine-based assay for cell-mediated immune reactivity to *MTB* and might be used instead of TST in TB screening programs for HCWs. This IGRA is an example of a blood assay for *MTB*, does not require two-step testing and is more specific than skin testing and is not expected to result in false-positive results in persons vaccinated with BCG.

Canada

The first Canadian CTC Guidelines on IGRAs, published in 2007, stated that:

- Two-step approach in contacts, immunocompromised is recommended.
- Not recommended in children.
- Not recommended for active TB and serial testing.

An updated version of the CTC guideline was published in 2008 (109):

- Not recommended for active TB in adults.
- Can be used as a supplementary aid in children with suspected TB.
- IGRAs may be used as a confirmatory test for a positive TST in contacts (adult or child).
- IGRA may be performed in TST- positive, immunocompetent adults and children who are at relatively low risk of being infected and of progressing to active disease.
- In an immunocompromisedperson (adult or child), the TST should be the initial test; however, a clinician still concerned about the possibility of LTBI may perform an IGRA.
- Not recommended for serial testing of HCWs; IGRAs may be used as a confirmatory test if a false-positive TST is suspected in a low-risk HCW or prison staff /employee or inmate.

Australia

The Australian NTAC Guidelines was published in 2007 (110) and the main points are:

- Currently TST remains the preferred method of screening for LTBI pending further evaluation.
- TST and IGRAs have almost no place in the diagnosis of active TB disease.
- State-based TB services should be encouraged to participate in the evaluation of the role of IGRAs for the investigation of LTBI.
- IGRAs may be used as a supplementary test in individualized clinical assessment for LTBI where increased specificity is valuable in reducing the confounding effect from prior BCG vaccination or prior exposure to non-tuberculous mycobacteria.

In 2009 the Australian Society for Infectious Diseases published its guidelines (111) and it states that: "With the exception of those with documented past TB disease, all newly arrived refugees, including children, should be assessed for LTBI , with the following plan:

- Testing is performed with the intention to treat.
- Either a Mantoux test or IGRA may be used for screening.
- Refer those with a positive Mantoux test or a positive IGRA test to the local TB services, for exclusion of active TB infection and consideration of treatment of LTBI.
- A Mantoux greater than 10mm in adults and children of ≥ 5 years of age and a Mantoux of ≥5mm in those younger than 5 years or those who are HIV-infected are considered positive.
- Refugees known to be HIV-infected should have a two-step Mantoux test. In the event that the second test remains < 5mm, specialist advice should be sought from TB/HIV services.
- TB (active disease or latent infection) should be managed by clinicians experienced in doing so as part of a centralized, coordinated TB service.

United States of America

The WHO Guidelines (112) recommend that all people living with HIV be regularly screened for TB but avoid the question of LTBI testing, despite suggesting use of INH prophylaxis.

The American Thoracic Society (AST)/CDC/IDSA Guidelines require symptom screening, chest x-ray, and a TST upon HIV diagnosis and repetition of TST at least yearly.

The more recent US NIH Guidelines state that either a TST or IGRA should be used. Despite the best intentions of these guidelines and recommendations, 2006 statistics show that less than 1% of the world's HIV-infected population is screened for TB, let alone LTBI.

In 2008, came out the Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents (113). It is stated there that immune restoration as a result of antiretroviral therapy may be associated with conversion from a negative to a positive TST or IFN-γ release assay (IGRA) in response to *MTB*- specific proteins. It is recommended that TST or IGRA should be repeated in previously TST-negative or IGRA-negative individuals after initiation of antiretroviral therapy when the CD4 cell count exceeds 200 cells/mm^3. HIV-infected individuals found to have LTBI, defined as >5 mm skin test induration or positive IGRA with no prior treatment for LTBI and after appropriate evaluation to rule out active TB disease and no prior treatment of LTBI, should commence treatment with isoniazid for 6 to 9 months.

On April 10th, 2009 was published the CDC/NIH/IDSA Guidelines 2009 for prevention and treatment of Opportunistic infections in HIV-infected adults and adolescents (114). Evidence suggests that the IGRAs have more consistent and higher specificity (92%–97%) compared with TST (56%–95%), better correlation with surrogate measures of exposure to *MTB*, and less cross reactivity because of Bacillus Calmette-Guérin (BCG) vaccination or other nontuberculous mycobacteria exposure than the TST. Three IGRAs are FDA- approved and available in the United States: the QuantiFERON®-TB Gold, the QuantiFERON®-TB Gold In-Tube (Cellestis Limited), and the T-SPOT™.TB test (Oxford Immunotec). For both the TST and IGRAs, however, HIV- related immunosuppression might be associated with false-negative results. The frequency of false-negative and indeterminate IGRA results increases with advancing immunodeficiency. Results from comparative studies of TST and IGRAs in HIV-infected patients indicate that

concordance between the tests is not complete. The TST remains useful for diagnosing LTBI, particularly for patients who have not been vaccinated for BCG and in settings with cost constraints. The optimal application of IGRAs in HIV-infected persons will be better defined when the results of ongoing studies become available. IGRAs might be used in combination with TST to improve sensitivity and specificity for detection of LTBI. Given the high risk for progression to active disease in HIV-infected persons, any HIV- infected person with reactivity on any of the current LTBI diagnostic tests should be considered infected with MTB.
In the USA AAP Red Book 2009 (115) it is stated that at this time neither IGRA not the TST can be considered "gold standard" for diagnosis of LTBI. Current recommendations for the use of IGRAs in children are as follows:

- For immune competent children 5 years of age and older, IGRA can be used in place of a TST to confirm cases of TB or cases of LTBI and likely will yield fewer false-positive results.
- Children with a positive result from IGRA should be considered infected with *MTB* complex. A negative IGRA result cannot universally be interpreted as absence of infection.
- Because of their higher specificity and lack of cross-reactions with BCG, IGRAs may be useful in children who have received BCG vaccine. IGRAs may be useful to determine whether a BCG-immunized child with a reactive TST more likely has a false-positive TST reaction caused by the BCG.
- IGRAs cannot be recommended routinely for use in children younger than 5 years of age or for immune-compromised children of any age because of a lack of published data about their utility with these groups.
- Indeterminate IGRA results do not exclude TB infection and should not be used to make clinical decisions.

Soon will be released the CDC Guidelines 2009: QFT-GIT/TSPOT.TB, comparing the two ex vivo immunodiagnostic tests for TB. At the 2nd Global Symposium on IGRAs, Dr Ken Castro, Director of the CDC's Division of Tuberculosis Elimination, presented IGRAs as the 'preferred' test in BCG-vaccinated individuals and some other populations. The approach is a significant change from the previous guideline and now considers the wide prevalence of BCG-vaccinated populations within the U.S.

Japan

In 2006, in Japan was published the JST Guidelines, regarding the use of QFT-2G (131). These are the main recommendations:

Children < 5 years:

- QFT not recommended [TST preferred] for LTBI
- QFT can be used as adjunct for active TB

Contacts:

- Less than 5 years old: TST is preferred over QFT.
- From 5 to 12 years old: Use QFT with considering use of TST together. The QFT results should be interpreted carefully.
- From 12 to 18 years: QFT is preferred over TST where QFT test is available. Use TST if necessary.
- From 18 to 49 years old: QFT is preferred over TST.
- Over 50 years old: Limited use of QFT or TST

HCWs:

- QFT preferred over TST

Active TB:

- Adjunct (supporting) evidence

High risk groups (diabetes, steroids, TNF-alpha blockers):

- QFT is preferred and can be used for deciding on LTBI treatment

Even when QFT is preferred

- TST may be done first, followed by QFT in TST+, in order to be cost effective (especially in case of mass screening)

In 2009 the QuantiFERON-TB Gold In - Tube assay was approved in Japan.

South Corea

The KCDC Guidelines in South Korea came out in 2009. It states that:

- IGRA is required to confirm TB infection in contacts over 6 year old with a positive TST (5mm in BCG unvaccinated and 10mm in BCG vaccinated.
- In contacts with negative TST results, IGRA can be performed based on the attending doctor's clinical decision.
- Not for active TB or serial testing.

In Europe there are several countries that released Guidelines on LTBI diagnosis during the last 3 to 4 years.

United Kingdom

On 22 March 2006, The National Institute for Health and Clinical Excellence (NICE) published the Guidelines for TB control in England and Wales (116).
The NICE Guidelines recommend:

- Use IGRAs as the front line test for LTBI in preference to the TST where the skin test may be "less reliable" including all immunocompromised patients
- Use IGRAs as a secondary, confirmatory test in all cases when the TST is positive. The IGRA is used as a means of screening out TST false positives.
- IGRAs also have a role to play in the diagnosis of TB disease especially in non-pulmonary TB and as a rule-out test in TB suspects.

In October 2007, The Health Protection Agency published a position statement on the use of IGRA tests for TB, providing an update on the NICE Guidelines (117). The main points of this statement are:
Active disease:

- IGRAs may be used when it has not been possible to confirm a diagnosis by culture and when radiological and histopathological evidence is lacking.

Latent Infection:

- In LTBI IGRAs are at least as sensitive at the TST and in BCG vaccinated populations are more specific.
- TST should be carried out first and those that are positive should be considered for IGRA testing if available.
- This would also apply to new entrant screening.

IGRAs should be the only test used in the following situations:

- Where TST may be falsely negative due to immunosuppression
- When screening a large number of people as part of a public health investigations since repeated visits would be impractical

Health care worker screening:

- New health care workers should be tested with IGRAs as they may come into contact with immunosuppressed patients and because of the logistical simplicity of the tests.

Pre-TNF- alpha screening

- IGRAs may be a suitable alternative in BCG vaccinated subjects.

In July 2009, Guidelines for the Diagnosis and treatment of tuberculosis of the central nervous system in adults and children was published (118). The recommendation made are that CSF adenosine deaminase activity is not recommended as a routine diagnostic test for CNS TB. The TST and IGRAs may provide indication of previous TB infection and are probably most useful in young children, but results need to be interpreted cautiously as neither is sufficiently sensitive nor specific to diagnose active disease. Currently, IGRAs are only licensed for the diagnosis of latent TB and cannot be recommended for the diagnosis of active CNS disease.

Switzerland

In November 2005 the Swiss Lung Association released recommendations for the diagnosis of TB infection in contact investigations using blood tests in Bulletin 45/10 of the Office Federal de la Sante Publique (119). The main points are:

- Confirm a positive Mantoux tests with an IGRA
- Use only an IGRA in immunocompromised subjects
- Children are excluded from being tested with IGRAs

In December 2007 the following guidelines were released for the use of IGRAs to screen patients prior to administration of anti-TNF alpha therapies (120). All patients should be screened for LTBI before being given anti-TNF alpha therapies

- Screening should be based on history, chest x-ray and IGRA (TST is no longer recommended).
- Preventive treatment should be given where LTBI is suspected as a result of:
    1. Positive IGRA
    2. Abnormal x-ray suggesting TB which was not adequately treated
    3. History of significant prior exposure

Italy

Recommendations for the identification of LTBI were released jointly by the Associazione di Microbiologia Clinica Italiana (AMCLI) and the Federazione Italiana per le Malattie Polmonari Sociali e la Tubercolosi (FIMPST) in May 2006 (121). The main points are:

- Mantoux testing should be carried out and where positive an IGRA should be performed.
- In subjects with an expected TST positivity rate of 40% or more, and in immunosuppressed patients an IGRA should be carried out without a prior TST.
- IGRAs may be used along with other tests in the diagnosis of active disease.

In 2009 came out a draft awaiting approval by the Italian Ministry of Health (122) which states that:

- TST is the standard test for LTBI diagnosis; there is not enough evidence to recommend complete replacement of TST with IGRAs.
- IGRAs are recommended to confirm LTBI diagnosis in TST+ BCG- vaccinated individuals.
- IGRAs are recommended to diagnoseLTBI in TST- HIV+ or other immunosuppressed individuals.

The Netherlands

Dutch Guidelines for contact tracing and screening were released in 2007 (123). The main points are:

- Contact investigations: TST is the initial test, if 5 mm or more followed by IGRA.
- Immigrant screening of children 4 weeks to 12 years old: initially TST, if 5 mm or more, then IGRA.
- Repeated screening of risk groups (e.g. occupational): either TST or IGRA as initial test.
- For detecting LTBI before start of iatrogenic immune suppression (e.g. anti-TNF-alpha therapy): TST and clinical information only
- In subjects where the Mantoux "may be less reliable" perform an IGRA without a prior Mantoux.
- IGRAs can be used in place of a Mantoux in the work up for active disease diagnosis.

France

The Haute Autorite de Sante (HAS) issued preliminary guidelines in December 2006 suggesting that IGRA tests should be used in the following settings (124):

- Contact tracing in subjects older than 15 years.
- Health care workers where a TST may not be reliable.
- To assist in the diagnosis of active disease, particularly non-pulmonary.
- Pre-TNF-alpha therapy screening.

Ireland

Draft guidelines published in July 2008 proposed that IGRAs can be used in the following settings (125).

- Contact tracing (in conjunction with a TST).

In certain circumstances IGRAs, if available can be considered as the sole test for LTBI:

- Where the TST may be falsely negative due to immunosuppression.
- When screening large numbers of individuals as part of a public health investigation.
- Pre-employment screening of healthcare workers.
- For individuals, commencing immunosuppressive therapy e.g. TNF-a antagonists.

Germany

In 2007 Recommendations for contact tracing in TB were published (126) and in July 2009 New TB testing recommendations for autoimmune diseases came out (127). It is stated that due to the increased risk of TB under treatment with TNF-alpha inhibitors for rheumatoid arthritis and other autoimmune diseases, precautionary measures are required before initiating TNF-alpha inhibitor therapy. Patients should have active TB ruled out and screening for LTBI should be performed. The screening should include chest X-ray, complete medical history, and the administration of a highly specific interferon-γ release assay (IGRA). (In the future, the reimbursement of IGRA tests under an analogue procedure code is expected to be formalized by the application of a code specific to the TB IGRA procedure.) As TST results can be expected to be either false-positive or false-negative in these patients, the TST, as commonly performed in the past, is recommended only in exceptional situations.

Spain

The Recommendations of the Spanish Society of Pulmonology and Thoracic Surgery (SEPAR) published in 2008 (128), state that IGRA tests could be performed in immunocompromised individuals and children and prior to TB vaccination. They are not recommended in active TB and serial testing.

Denmark

A Draft Guidelines on the use of IGRAs is proposed for discussion in 2009:
TB screening before anti TNF-alpha therapy:

- IGRA is preferred; TST when IGRA is not available.

Exposure/contact screening:

- IGRA if BCG vaccinated/adult. •
- Either TST or IGRA in children and unvaccinated young people.
- HIV: no specific guidelines.

Norway

The Norwegian Guidelines came out in 2007. The recommendation are as follow:

- TST as a first test and then IGRA as a supplementary test for all TST positives > 6mm.
- Among IGRAs,QFT is recommended as the primary test. If the mitogen control fails or the patient is severely immunosuppressedT-SPOT-TB is recommended as a secondary test.
- Referral to an infectious disease specialist or lung physician is recommended for all IGRA positives and also for TST >15mm/IGRA negative. Result of chest X-ray, symptoms, previous history of TB, immune status is also assessed and part of the decision of referral and prescription of preventive treatment.

Czech Republic

The Recommendation of the Czech Thoracic Society for QuantiFERON-TB Gold test came out in December 2005. According to it the QuantiFERON-TB Gold test should be used in following situations:

- Differential diagnosis of active TB.

- Detection of TB infection in close contacts.
- Screening for LTBI in high risk groups.
- Before and during biological (anti- TNF-alpha) treatment.

Slovak Republic

A Draft of the Slovak Guidelines from 2008 on the use of IGRAs is awaiting approval (129):

Two-step model (TST positive followed by IGRA) is indicated in:

- Before starting anti-TNF-alpha therapy; regularly, once a year, for patients subjected to long-term anti-TNFatherapy;
- Healthcare workers exposed to open forms of TB
- Contacts
- Military personnel after servingin TB endemic countries;
- As a part of medical examination of risk groups in the population –refugees, minorities;
- As a part of differential diagnosis of pulmonary and extrapulmonaryTB.

KNCV/EuroTB

The use of IGRAs in low- and medium- prevalence countries in Europe is stated in a Consensus Statement of a Wolfheze Workshop organized by KNCV/EuroTB, Vilnius, September 2006 (130). According to this statement, IGRAs offer an alternative to tuberculin skin testing for the diagnosis of LTBI or as an additional diagnostic method for active TB. Public health specialists involved in TB control, mainly in European countries with low and intermediate incidence of TB, met in Vilnius, Lithuania, in September 2006, to consider the use of IGRA assays because of the increasing demand for the use of these assays. There was consensus on the value of the use of IGRAs for the diagnosis of LTBI based on the following agreed points:

- Although there is no clear gold standard for LTBI, IGRAs, in published contact tracing incidents, reflect the degree of exposure to infectious cases more accurately than does TST. This suggests that IGRAs are more specific than TST. Discordant results between IGRAs and TST, however, cannot be completely explained by the notion that IGRAs are more specific with regard to cross-reaction with non-tuberculous mycobacterial (NTM) infections or with the BCG vaccine.
- Both commercial systems (QFT and TSPOT) probably perform well for LTBI detection in immunocompetent individuals.
- Studies of IGRA sensitivity suggest they are at least as sensitive as TST in TB patients but may be less sensitive than TST for detecting LTBI in immunocompetent individuals.
- Theoretically, a combination of TST (with its high sensitivity) followed by IGRAs (with their greatest specificity) should be an optimal approach for contact tracing in incidents where there is a known index or source case. Clearly this advantage is negated where the patient does not return for reading of the TST. In those cases, the single-visit IGRA would be more advantageous.
- Although it is reasonable to assume that a positive IGRA is as predictive of later active TB as a positive TST, there is no evidence so far to suggest a higher or lower degree of predictability.

- IGRAs are of value for diagnosing/excluding LTBI in children or HIV- positive (or other immuncompromised) individuals, including those about to receive anti-tumour necrosis factor or other immunomodulating therapy.
- IGRAs are of value in any situation requiring serial TST testing, e.g. occupational health-related screening/exposure.

Summary of the present guidelines on IGRAs

In summary, with all the different suggestions used by the different countries for the correct use of the IGRA tests, it can be observed that:

- Two-step approach seems to be the most favored strategy for IGRA use;
- Two-step approach is particularly favored in contacts, especially BCG-vaccinated contacts;
- Trend towards using IGRAs alone prior to anti-TNF-alpha therapy;
- Some guidelines are still cautious about IGRA use in young children;
- Few guidelines recommend IGRAs for active TB, but some recommend as an adjunct, especially in children;
- Most guidelines do not mention use for serial testing of HCWs;
- While some guidelines have been updated to keep up with the evidence base (e.g. USA, Canada, Germany), others are yet to be updated (e.g. UK, Australia, France, Czech);
- Few of the guidelines used evidence summaries and systematic reviews. Most based on narrative literature reviews and expert opinion.
- Most guidelines did not include a clear description of potential conflicts of interests and industry involvement in guideline development.

RISK FACTORS FOR DEVELOPING ACTIVE TB

The nature of the variability in the clinical and epidemiological consequences of MTB infection remains poorly understood. However, it is clear that different factors are contributing to the development of active TB after establishment of MTB infection. These can be summarized in three main categories: factors of the MTB (strain diversity, infectiousness, virulence factors ect), nutritional factors and concomitant status determining TB reactivation, and host genetic factors.

The rapid increase in the understanding of the molecular basis of MTB over the past decades has revived research into its pathogenesis. DNA fingerprinting techniques have been used to distinguish between strains of MTB, and efforts to characterise the strains present within populations have led to increased understanding of their global distribution. This research has shown that in certain areas a small number of strains are causing a disproportionate number of cases of the disease. In particular some strain, classified as Bejin strain, at the moment seems to overwhelm previous strain in term of infectiousness in TB endemic areas (132).

There are a number of known environmental factors that make people more susceptible to TB infection. A part some co-infections such as HIV (1), Smoking more than 20 cigarettes a day also increases the risk of TB by two to four times (133, 134). Diabetes mellitus is also an important risk factor that is growing in importance in developing countries (135). Other disease states that increase the

risk of developing TB are Hodgkin lymphoma, end-stage renal disease, chronic lung disease, malnutrition, and alcoholism (1).
Diet may also modulate risk. For example, among immigrants in London from the Indian subcontinent, vegetarian Hindu Asians were found to have an 8.5 fold increased risk of TB, compared to Muslims who ate meat and fish daily (136). Although a causal link is not proved by this data, this increased risk could be caused by micronutrient deficiencies: possibly iron, vitamin B12 or vitamin D (137). Further studies have provided more evidence of a link between vitamin D deficiency and an increased risk of contracting TB (138, 139). Globally, the severe malnutrition common in parts of the developing world causes a large increase in the risk of developing active TB, due to its damaging effects on the immune system (138, 139). Along with overcrowding, poor nutrition may contribute to the strong link observed between TB and poverty (140-142).
Other conditions that increase risk include (1) drug abuse; silicosis; prolonged corticosteroid therapy and other immunosuppressive therapy; head and neck cancers; hematologic and reticuloendothelial diseases, such as leukemia and Hodgkin's disease; end-stage kidney disease; intestinal bypass or gastrectomy; chronic malabsorption syndromes; vitamin D deficiency; and low body weight.
Some drugs, including rheumatoid arthritis drugs that work by blocking tumor necrosis factor-alpha (an inflammation-causing cytokine), raise the risk of activating a latent infection due to the importance of this cytokine in the immune defense against TB (143).
The role of the genetic background in susceptibility to TB was all too apparent to physicians at the time of the 18th and 19th century TB epidemic, when mortality rates were close to 2,000 per 100,000 population per year. The sight of entire families wiped out by the white plague while their neighbours were left unscathed led physicians to think of phtysis as an hereditary disease. E.R.N. Grigg (144), using reports and chronicles of the 17th, 18th and 19th centuries and contemporary epidemiological data, described the TB epidemic as a rapidly raising wave reaching its peak over 50 to 100 years. During this period the infection hit and eliminated the more susceptible among the population only to decline slowly thereafter over the ensuing 200-250 years, thus suggesting that genetic susceptibility of large population segments may play an important role in the spread of TB, in the absence of medical intervention (144).
This information is further corroborated by the twin studies in the 1940s showed that susceptibility to TB was heritable (145). If one of a pair of twins got TB, then and the other was more likely to get TB if he was identical than if he was not (145). Since then, Several genes have been implicated in susceptibility and resistance to MTB infection including (i) the natural resistance-associated macrophage protein-1, some rare dysfunctional mutations of the (ii) IFN-γ receptor 1 and (iii) the IL-12 receptor β1 present in homozygous form (145-147).
However, it is unlikely that rare gene mutations play a major role in the TB epidemic wave (144-147). Allelic variants of the HLA locus have also been associated with TB susceptibility in population studies indicating many alleles in HLA class II loci in particular as susceptibility genes with risk ratios ranging from 3.7 to 7.2 (145-147). From these studies, it can also be appreciated that susceptibility-associated HLA alleles are present in wide segments of the population, suggesting that these

genes might have had a profound impact upon the clusterization of disease in families and kindred seen at the peak of the TB epidemic.

MTB-SPECIFIC IMMUNODIAGNOSTIC ASSAYS AS A TOOLS FOR MEASURING THE RISK OF DEVELOPING ACTIVE TB IN IMMUNOCOMPETENT AND IMMUNOCOMPROMISED HOSTS.

Screening for latent infection with MTB aims to identify individuals at risk of developing TB. As indicated in the above paragraph, in immunocompetent hosts it is has been estimated that 50% of cases of active TB will occur within the initial 2 yrs after initial infection (i.e. from the TST conversion) (148). However, the time of TST conversion is only known in selected groups. Furthermore, TST results may be positive because of prior BCG vaccination or infection with environmental mycobacteria. Unfortunately, BCG vaccination does not always leave an identifiable scar and, furthermore, BCG vaccination mediates only partial protection against development of TB. Until recently, it has been exceedingly difficult, if not impossible, to identify latent infection with MTB among BCG-vaccinated subjects and to exclude them from being at risk of TB. Past mycobacterial infections can result in persistently positive TST results. The use of different cut-off values, as indicated in various national guidelines, or depending on the risk of infection and BCG status further complicates the interpretation of TST results (149).

In immunocompromised hosts, all available data should be used to demonstrate or exclude latent infection with MTB. The risk of developing active TB is different between various immunocompromising conditions. However, screening for LTBI in immunocompromised patients is carried out irrespective of the type of immunosuppression, because the risk of developing active TB is probably higher compared with that of immunocompetent individuals. Careful history taking and physical examination in combination with chest radiography and tuberculin skin testing have been the cornerstones for the detection of latent infection with MTB. This approach has resulted in a decrease in the number of cases of TB in patients treated with certain medications, such as anti-TNF-alpha targeting drugs (150). However, the TST has a reduced sensitivity in subjects who are already using immunosuppressive drugs (151) or in those suffering from chronic illnesses such as rheumatoid arthritis (152-154), chronic renal insufficiency (155) or HIV infection (156-160). Reduced sensitivity in patients on immunosuppressive drug therapy may be a direct result of inhibitory drug action (e.g. corticosteroids, calcineurin inhibitors and methotrexate) on cytokine signalling, antigen-presenting cells or T-cell proliferation (161, 162). Moreover, immunosuppressive drugs may favour pathogen reactivation and progressive consumption of antigen-specific T-cells over time. In patients with renal insufficiency, skin testing may be adversely affected by an altered expression of costimulatory molecules on antigen presenting cells (163). Finally, in HIV infected patients, low numbers of circulating CD4 T-cells and high frequencies of circulating regulatory T-cells directly correlate with skin test anergy (66).

While it is reasonable to assume that these factors may also adversely affect in vitro testing, accumulating evidence suggests that IGRAs are of superior sensitivity compared with skin testing as experimental conditions in vitro may be optimised with respect to incubation time and/or adjustment of cell numbers. In the setting of TB, studies are hindered by the lack of a gold standard for establishing absence or

presence of latent infection with MTB. In order to estimate the specificity and sensitivity of the test formats, the type and extent of immunodeficiency has to be taken into account and surrogate conditions such as TB or patients with defined risk of exposure are needed.

With the early acceptance of IGRAs as a tool to detect MTB infection (164) it became impossible to conduct adequate research into their predictive value for the development of TB. It is considered to be ethically unacceptable to disregard a positive IGRA test result as general consensus dictates that treatment of latent infection with MTB should be considered. However, the predictive role of IGRAs in detecting LTBI in immunosuppressed individuals has not been investigated in detail so far. Retrieval of data on these patients will, however, not be easy and is dependent on an adequate national or regional TB registry. The ability of IGRA to predict development of TB remains an elusive goal. In an ideal setting, large cohorts of subjects with both known TST and IGRA results should be followed for the development of TB. Preferably this follow-up should be done in a setting with a low risk of reinfection during follow-up. To allow the study of both the positive and negative predictive value of IGRA, a sufficient proportion of subjects with positive or negative results should have deferral of treatment of latent infection with MTB, that make the study ethically unfeasable.

Recently, a large study described the rate of progression to TB within 2 yrs of contact screening in subjects being close contacts (defined as 40 h of cumulative contact time) of patients with pulmonary TB (165). In this study the total number of positive ELISA-based IGRA results was four-fold lower compared with the TST (using a cut-off of 5 mm or more to denote test positivity). Of 41 IGRA positive individuals who refused preventive chemotherapy, six developed active pulmonary TB. Because of the lower number of initial positive results for the IGRA, its positive predictive value for development of TB was significantly better than that of the TST. Among those diagnosed as having TB, MTB could not be isolated from sputum culture in four out of six cases, a definite study limitation. None of the subjects reported immunocompromising conditions.

Thus, it has to be established whether the positive and negative predictive values in immunocompromised patients will be the same as those in immunocompetent individuals. In general, a considerable proportion of indeterminate IGRA results due to impaired mitogen reactivity could be a problem when analysing immunocompromised subjects (166).

A single large prospective study has been published describing the risk of progression to TB after conversion to a positive ELISPOT assay in Gambian case contacts (167). In this study, ELISPOT positive contacts had a similar rate of progression to those who were skin test positive, whereas those negative on either or both tests had the lowest rate of progression. Since initial ELISPOT test and skin tests were positive in only about half of cases, whereas 71% were positive by one or the other test, positivity by either skin testing or IGRA was suggested as the best indication for preventive treatment. As more such studies, especially in low prevalence countries, are lacking, the use of surrogate studies is required. A review article has described a sensitivity for active TB of 88%, 76% and 70% for T-SPOT TB, Quantiferon-TB and TST, respectively (168). The sensitivity of the TST could be improved to 73% and 80% after lowering the cut-off values to 10 and 5 mm, respectively, yet at the cost of compromised specificity.

In a number of contact investigations, substantially fewer BCG unvaccinated subjects had positive IGRA results compared with positive TST results (153, 165, 169-179). Comparability between the two IGRAs was generally good, whereas the agreement between TST and IGRA was poor, owing to false-positive TST results in BCG-vaccinated subjects or, based on studies among BCG unvaccinated subjects, to lower sensitivity of IGRA for detection of infections acquired in the past. Although future studies are needed to establish the implications for the clinical use of the IGRA in both immunocompetent and immunocompromised patients, some studies clearly indicates their superior performance respect to the TST. In this context, in contacts from an outbreak at a supermarket (169), conversion to positive TST results 1 yr after exposure has been demonstrated in a small subgroup of patients with an initially negative TST in association with a positive IGRA result (180). As the TST has been an adequate, albeit nonspecific, tool for the detection of infected persons at risk of TB reactivation, and that low sensitivity has not been a problem in immunocompetent individuals, it is highly unlikely that those late TST converters would have an increased risk of progression to TB.

Depending on the selection of the cut-off value for the TST the sensitivity and specificity for detection of MTB latent infection will differ. Thus, for cut-off values as low as 5 mm, individuals with positive TST results will reflect a mix of subjects with latent infection with MTB, environmental mycobacteria infection or BCG vaccination. The proportion of subjects with true latent infection with MTB results will increase with increasing cut-off values to designate a positive TST result. However, in immunocompromised hosts with similar risk profile the median indurations will be lower due to an altered ability to produce an adequate immune response, although this notion has been challenged in HIVinfected patients with sputum smear-positive pulmonary TB from high-prevalence countries (158). The clinical relevance or the predictive value of a positive TST (which patients will develop TB in the near future) is generally poor, while the negative predictive value (those subjects with a negative TST who will not develop TB in the future) is high.

The positive predictive value for the development of TB will most likely be higher with an IGRA than with the TST because of the higher test specificity and similar, or probably higher, sensitivity. In immunocompetent individuals, the negative predictive value of IGRA for active TB is very high, if combined with a negative result of the TST (181). However, in immunocompromised individuals the negative predictive value of IGRA needs to be established.

In conclusion, the TST has been used for several decades now. Despite its limitations, clinicians feel comfortable with this test format in immunocompetent hosts who are not vaccinated with BCG. In this condition, owing to the high negative predictive value in immunocompetent hosts, few patients will be incorrectly withheld adequate treatment. However, this profile is not applicable in the countries were BCG vaccination is mandatory or, more in general, in BCG-vaccinated subjects where the negative predictive value for developing active TB is much lower.

Using IGRAs, the number of patients treated for latent MTB infection will most probably be reduced. Clinicians have to carefully weigh the benefit from not overtreating patients against the still unclear negative predictive value from IGRA tests. This problem might be easely overcome by the use of a two-step approach

(TST and confirmation of positive TST results by IGRA) proposed in many guidelines for IGRAs particularly in Europe.

MTB-SPECIFIC IMMUNODIAGNOSTIC ASSAYS AS A TOOLS FOR MEASURING THE RISK OF DEVELOPING ACTIVE TB IN CHILDREN.

Special considerations apply to the management of latent infection with MTB in children. As a public health intervention, a sensitive and specific diagnosis of MTB latent infection in children coupled with appropriate preventive chemotherapy might reduce the future burden of disease.
If a source of infection emerges in a household, small children are at particularly high risk of becoming infected with MTB because exposure time is frequently long. Subsequent to infection, the risk of progression to TB differs greatly with maturation. It is largest in the youngest children and drops to one of the lowest in life during primary school to increase again with onset of adolescence to reach a second peak among young adults (182).
The main effect of BCG vaccination is the reduction in the risk for the most severe forms of TB in infants such as disseminated and meningeal TB; it reduces the risk of pulmonary TB to a lesser extent, and may have a limited role in the prevention of acquisition of infection (183, 184). If vaccinated at birth, BCG coverage was between 83% and 99.8%, and children originating from high-burden countries showed 60–90% BCG coverage (185).
The diagnosis of TB infection in children relies on history of exposure, clinical symptoms and radiological findings consistent with TB and positivity to specific immunodiagnostic assays, of which the largely used so far has been, of course, the TST.
The diagnosis is difficult due to deficiencies in the specificity of the TST, which is compounded if there is prior BCG vaccination. In children vaccinated at birth, positive TST results may be observed for up to 10 yrs, depending on the vaccine strain and may last even longer in the case of revaccination or repeated tuberculin skin testing (186, 187). Conversely, high-risk groups, such as immunocompromised children, often have a false-negative skin test. Cut-off points for the interpretation of skin test results in children have been defined to facilitate decision making for when an intervention is required, integrating vaccination history, epidemiologic and other risk factors, in an attempt to balance errors resulting from incomplete sensitivity and lack of specificity (188). There are only few data available on the incidence of environmental mycobacterial infection or mycobacterioses in Europe, but the reported increase of the latter, especially since the discontinuation of BCG vaccination, suggests the potential for causing false-positive TST results (189-191).
As with adults, IGRAs have been shown to better discriminate between MTB and common environmental mycobacteria in children, and they are most notably not confounded by prior BCG vaccination (192). Moreover, IGRAs show a better correlation with the extent of TB exposure than the TST (184).
Data on the performance of IGRAs in very young and immunocompromised children are still scarce but, unlike the TST, diagnostic sensitivity the tests in active TB appears to be independent of HIV co-infection and malnutrition (193).
Thus, although the IGRAs are better performing in paediatric subjects in particular for their specificity, both tuberculin skin and IGRA test results have to be interpreted

with caution and the individual risk factors and clinical signs have to be taken into account.
There are few data on the predictive value of IGRAs for the development of TB and recommendations for their use in children remain highly heterogeneous. According to a recent prospective cohort study from Turkey, the risk of progression to TB in household contact children with a positive T SPOT TB result was 3–4-fold increased compared with children with negative T SPOT TB results (194), which was, however, not significantly superior to the TST in predicting the progression to TB in these children.
Thus, in summary, at present TST should perhaps best remain the basic diagnostic screening tool for latent infection with *MTB* in children. Taking into account existing paediatric data, an IGRA should be considered if the following conditions are met. First, in children with a high risk of infection (especially young children aged below 5 yrs and immunocompromised children) an IGRA should be performed in addition to the TST to increase both diagnostic specificity and sensitivity. If either test gives a positive result, this may be regarded as supportive evidence of infection (167), and the children should be offered preventive chemotherapy. Secondly, in children with a low risk of latent infection with MTB (e.g. a positive TST during indiscriminate screening without an identified putative source), an IGRA can be used to confirm a positive TST result to increase diagnostic specificity and reduce the risk of a false diagnosis of latent infection with MTB.
The following recommendations can be summarised for the management of LTBI in children. First, TB needs to be excluded in any child prior to prescribing preventive therapy. Secondly, children (particularly those aged below 5 yrs) with exposure to sputum smear-positive TB patients who are TST negative at the time of screening should be offered preventive chemotherapy with isoniazid and should be re-evaluated with a TST and/or IGRA after 3 months. If the test remains negative, the probability of infection is very low and treatment may be stopped. If either test converts to positive, preventive therapy should be continued, unless 3 months of preventive therapy with both isoniazid and rifampicin have already been completed. Children aged 5 yrs with exposure to sputum smear-positive TB should also be screened and a positive TST be confirmed by IGRA, where available. In case in which the treating paediatrician opts not to provide preventive therapy to TST-positive but IGRA-negative children, surveillance for a minimum of 12–24 months is indicated for observation and to collect outcome data, until the positive and negative predictive value of IGRA are better established in the setting of paediatric TB.

COST EFFECTIVENESS OF IGRAS VS PPD SKIN TEST. INDICATIONS FOR A COUNTRY WITH AN INTERMEDIATE INCIDENCE OF TB AS BULGARIA.

There are several major IGRA clinical advantages that provide economic benefits for TB infection control programs:

- Single visit.

- High sensitivity (up to 93%) and specificity (>99%) for detecting active TB (195). Avoids false positives due to BCG vaccination (196) and most environmental non-tuberculous mycobacteria (197).
- Unlike the TST is not subject to errors in test placement or reading.
- Reduction in personnel cost—which is the major cost component of a TST program. TST reagents represent less than 1.5% of the total cost of TST screening program (198).
- Reduction in additional costs—such as chest X-rays—associated with investigating false positive cases (199).
- Avoids Boosting (200). Eliminates need for two-step testing.

"In situations with serial testing for *MTB* infection, initial two-step testing - which is necessary with the TST - is unnecessary with the QuantiFERON-TB Gold and is not recommended" (201*).*

The use of both IGRA tests is a cost effective solution for TB screening when compared to the significant costs associated with the TST. Total costs for the TST are often believed to be lower than the actual cost. According to a published study by Lambert et al., the total cost of a TST per healthcare worker tested ranged from $41.00USD to $362.00USD (202).

The source of many of the costs for the TST include:

- **Procedural**:
    - Two visits are required, one for administration of the PPD and the second for interpretation of the test result.
    - Subjectivity of test administration and interpretation of results.
- **Operational**:
    - Training and retraining in proper test inoculation and interpretation.
    - Getting the individual to return for the second visit.
    - Following up on those who do not return for the second visit.
    - Two-step testing for new employees requires a total of four visits.
- **Performance:**
    - The TST cross-reacts with the BCG vaccine resulting in false-positive results.
    - TST results are affected by immunosuppression resulting in false-negative results (203).
    - The IGRA tests address the sources of these costs associated with the TST:

- Single blood test, only one visit required. IGRAs results are not affected by immunosuppression or BCG vaccine.

There are several economic assessments coming from different countries.

Japan economic assessments

A cost-effectiveness analysis of QFT in a TB contact investigation in Japan was performed by Mori T. and Harada N. (204). A model was built assuming that a group of young people was exposed to an infection source with different degrees of intensity. The strategies for investigating this group included using QFT to test subjects with erythema size exceeding 30 mm, 20 mm and 10 mm, as compared with the strategy of only using TST or QFT. The analysis confirmed that the additional use of QFT would greatly reduce the number of indications for chemoprophylaxis of uninfected cases and that the use of QFT is cost effective.

UK economic assesments

UK National Institute for Health and Clinical Excellence (NICE) (205) considered the cost-effectiveness of 4 different strategies—no test, TST alone, IGRA alone and IGRA testing for individuals with a positive TST—for diagnosing LTBI. The assessment showed that:

- At all prevalence levels, an IGRA-only strategy was cheaper than a TST-only strategy.
- Overall, the two-stage TST/IGRA strategy was most cost effective, however the impact of false negative results or logistical issues involved with two step testing was not considered in the assessment.
- One step IGRA testing can be used in individuals "in whom tuberculin skin testing may not be reliable" such as those with immune suppressing diseases (including HIV) or on immune suppressive treatment (e.g. corticosteroids), Hodgkin's disease, infectious mononucleosis and viral infections in general (including those of the upper respiratory tract).
- The NICE assessment showed that above a prevalence level of 40%, one step testing (that is a single IGRA test) is the most cost effective option.

NICE guidelines state that "Interferon-gamma tests showed little evidence of being affected by prior BCG vaccination, and showed stronger correlation with exposure categories than did TST. The specificity of interferon-gamma tests seemed better, and there was less potential for false positive results."

German economic assessments

A Markov model was used to assess the health and economic outcomes of isoniazid treatment of 20 year old TB contacts using two different TST cut-offs (5 mm and 10 mm), QFT alone and QFT as a confirmatory test for TST results. The number treated to prevent one TB case was 22 for the two QFT based procedures, 40 for the TST at a cut-off of 10 mm, and 96 for the TST at a cut-off of 5 mm" which may appear to be a striking argument from an ethical point of view" for using only QFT.

This analysis showed that the two TST-based strategies "when performed alone, in each case [was] more costly and less effective than the Quantiferon®-TB Gold in-Tube assay, the higher cost of implementation of which was outweighed by the averted cost of unnecessarily treating contacts who otherwise would have been wrongly classified as LTBI cases."
Of the four strategies, QFT following the TST screening of close-contacts at a cut-off of 5 mm was the most cost-effective option, followed by the QFT alone strategy. However the cost of combining the two tests was "only marginally lower than the total cost of the program based on QFT-G assay alone per 1000 close-contacts by approximately $ 1,397 (0.61%)."
In another German cost-minimisation analysis (207), the costs of investigating a cohort of adult TB contacts over a period of 2 years was calculated. In this assessment the total cost of simply administering the TST was € 19.24 per person (includes tuberculin material costs, as well as TST administration and reading costs) while the total cost of performing QFT was € 47.68 per person (includes blood sampling, sample transport and all laboratory material and labour costs). These costs do not include follow up costs for those testing positive by either test. Such follow-up comprised three chest X-rays at a cost of € 74.3 per X-ray—which includes all labour and material costs of performing a chest X-ray. This analysis showed that:
When TST was used alone the average costs for every contact followed amounted to € 91.
If instead of TST, QFT alone was performed, the cost per contact was reduced by 33% to € 61.
If both test were combined (validation of a positive TST by QFT) the costs were reduced by 43% to € 52.
A two step approach proved to be marginally cost effective compared to only using QFT. However this sacrifices the operational ease of only using QFT, but also has the risk of missing individuals (eg. Those with immunosuppression) with false negative TST results. As a result the authors do state that "the TST/QFT-G two step strategy should be reassessed in the presence of such specific epidemiological conditions."

USA/Canada economic assessments

In this assessment (208) a Markov model was used to compare the cost effectiveness of 3 strategies (QuantiFERON-TB Gold In-Tube, QuantiFERON-TB Gold and TST) for detecting LTBI in new health care workers (HCW) with or without prior BCG vaccination. It showed that for non-BCG vaccinated HCW, the incremental cost effectiveness of QuantiFERON-TB Gold, compared with QuantiFERONTB Gold In-Tube was $ US14,092/QALY. For BCG vaccinated HCWs, the incremental cost-effectiveness of QuantiFERON-TB Gold was $US 103,020/QALY. Sensitivity analyses show that if the sensitivity of QuantiFERON-TB Gold In-Tube exceeds that of QuantiFERON-TB Gold, QuantiFERON-TB Gold In-Tube is the most effective and least costly strategy.
A costeffectiveness analysis was done by Oxlade O, Schwartzman K and Menzies D (209). The researchers used a Markov model to compare the expected TB cases and costs of various screening methods among immigrants to Canada and TB contacts over a period of 20 years. Sequential screening with TST then QFT was

more cost-effective than QFT alone in all scenarios and more cost-effective than TST alone in selected subgroups. In both immigrants and TB contacts who had received BCG vaccination after infancy, QFT was more cost-effective than TST, because of reduced TST specificity.

M.A. de Perio et al. (210) evaluated the cost-effectiveness of Interferon Gamma Release Assays vs. TSTs in Health Care Workers. The authors state that the QFT is more effective and less costly than the TST for detection of LTBI in both low- and high-prevalence populations, independent of BCG vaccination status.

At the 2nd Global Symposium on IGRAs in Dubrovnik, 2009, L. Masae Kawamura (211) presented the Cost Effectiveness Analysis made in the San Francisco TB Control Section of the Department of Public Health at the Francis J. Curry National TB Center. It was found that the QFT-Gold in tube with investment in automation is the least costly IGRA (If IGRA chosen, investment in automation is easily economically justified). A model predicted IGRA is most cost-effective choice over wide range of assumptions. The testing strategy for different populations should be selected based on local characteristics and experience.

Akiko Kowada from the Department of Health Service, Katsushika City Public Health Center in Tokyo, presented two studies at the 2nd Global Symposium on IGRAs in Dubrovnik, 2009: Cost effectiveness of IGRAs for TB contact screening and Cost effectiveness of IGRAs for annual TB healthcare worker screening (212). The main conclusions of the first study were:

1.The QFT alone strategy is the most cost effective for TB contactscreening in Japan.

2.When the TST specificity is over 0.72, the TST followed by the QFT strategy is more cost effective than the QFT alone strategy at the level of $US 25,000/QALY gained as a willingness to pay.

3.The QFT alone strategywould be more cost effective in individuals at high risk of TB mortality, such as the elderly.

About the cost effectiveness of IGRAs for annual TB healthcare worker screening, it was stated that:

1. The QFT alone strategy is the most cost effective in BCG vaccinated healthcare workers in Japan.

2. When the probability of having LTBIis over 0.463, the QFT/Chest X ray strategy is more cost effective than the QFT alone strategy at the threshold of$US25,000/QALY as a willingness to pay.

THE USE OF IGRAs IN BULGARIA AND HOW THEY SHOULD BE USED

Bulgaria is a country with an intermediate incidence of TB (37.1/100 000 population; Public Health Statistics, 2007, National Center of Health Informatics, Republic of Bulgaria), and with a mandatory BCG-vaccination at childbirth.

In 1999 Bulgaria was the first country in Europe that introduced into clinical practice the use of the first generation kit of the QuantiFERON technology, the QuantiFERON –TB kit (Cellestis, Ltd, Australia). Later on, in 2004 the second generation kit – QuantiFERON –TB Gold was introduced, and in 2006 - the last generation kit QuantiFERON –TB Gold In-Tube. In late 2003 was introduced the CLINISPOT test, which later was named as T-SPOT.TB test (Oxford Immunotec, UK).

The new IGRAs were introduced for the first time in Bulgaria in the Laboratory "Mediators of Inflammation and Immunity" at the Department of Immunology and Allergology of the National Center of Infectious and Parasitic Diseases in Sofia.
During a period of 10 years different settings were tested and these include HIV-negative individuals with LTBI or active TB, HIV/AIDS patients and other immunocompromised individuals, as well as children suspicious of MTB infection.
IGRAs were successfully applied in both active TB and LTBI, pulmonary and extrapulmonary TB, patients with MDR TB, children of any age, and immunocompromised individuals with HIV/AIDS. Also, IGRAs are very useful for diagnosing TB infection in patients with transplantation, immune suppressive therapies, anti-TNF-alpha treatment, cancers, chronic renal failure and diabetes. The use of IGRAs was successful in epidemiological studies (contact tracing, testing of HCWs), for monitoring of anti-TB therapy and discrimination between BCG vaccination and MTB infection. Also, of great significance is the evaluation of the positive and negative predictive value of IGRAs in different settings.
For a period of four years (1999-2003), in the Laboratory "Mediators of Inflammation and Immunity", 1810 persons, both HIV- negative and HIV- positive, most of them with a suspect of a TB infection were tested with the first generation QuantiFERON – TB test (213, 214, 215).
Starting from the beginning of 2004 till late 2006, 490 persons were tested with the QuantiFERON – TB Gold test, which appeared to be more specific and sensitive than the QuantiFERON – TB test.
During the last three years using the QuantiFERON – TB Gold In-Tube test, 860 both HIV- negative and HIV- positive individuals (216), 150 of them children (217, 218), were tested. During that time IGRAs, both QuantiFERON–TB Gold In-Tube (219) and T-SPOT.TB (220), were used successfully for monitoring of anti-TB therapy in adults (HIV- negative and HIV- positive) and in children.
Based on its excellent experience with IGRAs, and as a member of the European TBNET Organization, Bulgaria is participating in a TBNET – priority research project – "TIGRA in Immunocompromised patients" which was started in 2008. This is a comparative study between the two commercially available tests and in immunocompromised patients. The study is designed to carry out a head-to-head comparison of the T.SPOT.TB and the ELISA- based QuantiFERON-TB Gold In-Tube test with the TST in immunosuppressed populations. In a second step this study will be extended to longitudinally assess the predictive value of a positive blood test for progression to active TB disease.
There is a good experience with the QuantiFERON-TB Gold In-Tube test in Bulgaria (221). The test is used in several laboratories (4 in Sofia and 6 in the country). At the same time the T-SPOT.TB test (222) is performed only in two laboratories in Sofia (NCIPD and University Hospital Alexandrovska). During the last 4 years the NCIPD offers in its Educational programme a training course on IGRAs which is regularly visited by medical doctors working in the field of TB. Two symposiums on IGRAs with international participation, in 2005 and 2007, were carried out in Sofia and were very well attended. Also, IGRA tests are successfully used in the development of scientific research projects.

What is the preferred approach for the diagnosis of MTB infection in a country with intermediate incidence of TB, like Bulgaria?

TST sensitivity and specificity is influenced by the cut-off used. A lower cut-off will result in a higher sensitivity and a lower specificity for *MTB* infection. TST and IGRA results in general correlate poorly, mainly because of positive TST results in individuals vaccinated with BCG. The results from IGRAs (ELISA and ELISPOT) in general, have a better correlation.

In immunocompromised hosts, all available data should be used to demonstrate or exclude latent infection with *MTB*. The risk of developing active TB is different between various immunocompromising conditions. However, screening for latent infection with *MTB* in immunocompromised patients is carried out irrespective of the type of immunosuppression, because the risk of developing active TB is probably higher compared with that of immunocompetent individuals. Sensitivity of the TST is limited in immunocompromised individuals and specificity is limited because of cross reactivity due to prior infection with environmental mycobacteria or BCG vaccination. IGRAs have a higher specificity in populations with a high prevalence of BCG vaccination compared with TST.

Contacts potentially benefiting from preventive therapy should be identified hierarchically according to likelihood of having become infected by a putative source and by presence of potentially aggravating risk factors. IGRAs may be superior to the TST in identifying contacts at risk of developing TB.

Children are more likely to develop TB than adults after exposure to an active TB case, hence contact screening and chemoprophylaxis are particularly important.

In children with a high risk of infection (especially young children aged < 5 years and immunocompromised children) an IGRA should be performed in addition to the TST to increase sensitivity. If either tests give a positive result, this may be interpreted as supportive evidence of infection, and the children should be offered preventive chemotherapy.

Children aged ≥ 5 yrs with exposure to sputum smear-positive TB should also be screened and a positive TST be confirmed by IGRA, where available. In cases in which the treating paediatrician opts not to provide preventive therapy to TST-positive but IGRA- negative children, surveillance for a minimum of 12–24 months is indicated for observation and to collect outcome data, until the positive and negative predictive value of IGRA are better established in the setting of paediatric TB.

In summary, the following approach could be recommended in accordance to the setting that should be tested:

- The two-step approach seems to be the most favored strategy for IGRA use;
- The two-step approach for use of IGRAs in young children;
- The two-step approach for active TB in children;
- The two-step approach for use of IGRAs in contacts, especially BCG-vaccinated;
- The use of IGRAs alone in HIV/AIDS patients;
- The use of IGRAs alone prior to anti-TNF-alpha therapy;
- The use of IGRAs alone in hemodialysis patients;

- The use of IGRAs alone in patients receiving immunosuppressive drug therapy;
- The use of IGRAs alone in patients with organ transplantation;
- The two-step approach for use of IGRAs in active TB;
- The two-step approach for use of IGRAs for serial testing of HCWs;
- The use of IGRAs' positive predictive value for the development of active TB; it is likely to be equal or better than that of the TST for immunocompetent individuals.

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