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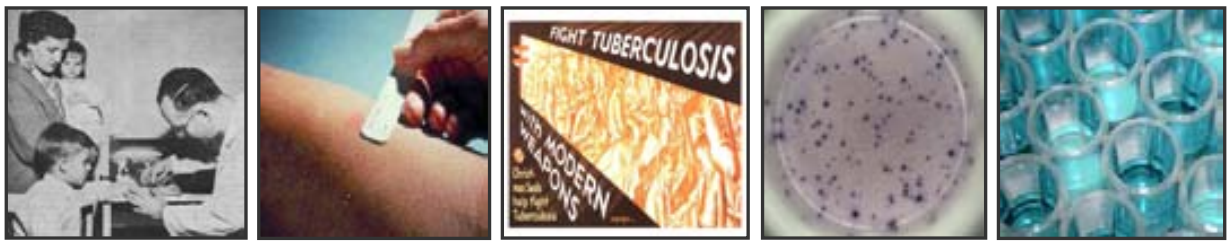
DIAGNÓSTICO DE LA INFECCIÓN POR *Mycobacterium tuberculosis* MEDIANTE ESTIMULACIÓN DE LAS CÉLULAS T SENSIBILIZADAS CON ANTÍGENOS ESPECÍFICOS



Irene Latorre Rueda

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Memoria presentada por Irene Latorre Rueda para optar al grado de Doctor con Mención Europea* por la Universitat Autònoma de Barcelona.

VºBº de los Directores de la Tesis

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* En cumplimiento con la normativa vigente de Mención de Doctor Europeo, los apartados de la Tesis *Summary* y *Conclusions* están redactados en inglés.

A mis padres y a David.

La alegría de ver y entender
es el más perfecto don de la naturaleza.

Albert Einstein

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Índice

SUMMARY

The bases of tuberculosis (TB) control programs consist of a diagnosis and correct treatment of patients with active TB. An essential factor for controlling the spread of this disease is the ability to diagnose infected individuals in their early stages before they become infectious to others through the progression to active TB. Traditional tools for diagnosis of clinical TB remain in clinical examination, combined with direct microscopic examination of sputum samples and culture of bacteria. Patients with pulmonary TB could be smear-negative for acid-fast bacilli, and mycobacterial culture can take several weeks. Therefore, diagnosis is often in an advanced disease stage.

The tuberculin skin test (TST) has been until now the only tool available for the diagnosis of latent tuberculosis infection (LTBI) and is commonly used as complementary test for diagnosis of active TB. Unfortunately, this test has some disadvantages because of its poor specificity, that lead to false positive results by the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine strain and non-tuberculous mycobacteria (NTM) cross-reaction. Moreover, TST has a low sensibility on high risk groups with impaired cellular immunity giving false negative results.

Upon exposure to *Mycobacterium tuberculosis*, antigen-presenting cells (APCs), such as alveolar macrophages and interstitial dendritic cells (DCs) engulf the bacteria in the lung alveoli. These macrophages produce different cytokines that attract other macrophages and lymphocytes in order to phagocyte extracellular bacteria, and also to generate an inflammatory focus. Some APCs stay in the lung parenchyma to form local inflammatory processes that later will form the basis of granuloma, and some of the DCs migrate to the draining lymph nodes where they present *M. tuberculosis*-derived antigenic peptides to circulant T cells.

CD4 T cells that are restricted by gene products of the major histocompatibility complex (MHC) class II represent the major players in the protection against TB. Of greatest relevance are the T helper (Th) 1 cells, that produce interferon-gamma (IFN-γ). The IFN-γ cytokine activates macrophages for the development of a protective immunity against *M. tuberculosis*. Therefore, an immunodiagnostic assay based on the *in vitro* quantification of this cellular immune response could be an alternative to the TST for the diagnosis of LTBI.

In the last years, two *M. tuberculosis* region of difference (RD) 1 antigens have been described. These antigens are early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), absent in BCG strain or in the majority of NTM. Recently, a new third specific *M. tuberculosis* antigen called TB7.7, which is encoded in RD11, has been also studied. *In vitro* assays for the diagnosis of LTBI, based on the detection of IFN-γ secreted by effector T cells stimulated with these specific antigens, have been developed.

Nowadays, there are two commercially available IFN-γ T-cell based assays: QuantiFERON-TB Gold In-Tube (QFN-G-IT, Cellestis Limited, Carnegie, Australia) and T-SPOT.TB. Both assays are approved by the U.S. Food and Drug Administration as an aid for diagnosing LTBI. QFN-G-IT test stimulates whole-blood with ESAT-6, CFP-10 and TB7.7 in the same tube, and measures the concentration of IFN-γ in supernatants with an enzyme-linked-immunosorbent assay. On the other hand, T-SPOT.TB assay stimulates isolated peripheral blood mononuclear cells with ESAT-6 and CFP-10 separately, and detects number of IFN-γ producing T cells by means of an enzyme-linked immunospot assay (ELISPOT).

In this sense, the main objectives of this Thesis are the standardization and clinical evaluation of these new immunologic assays for the diagnosis of LTBI (contact tracing and screening studies) and active TB, their application in clinical practice in adult and pediatric population, and the evaluation of NTM effect in the LTBI diagnosis.

We have assessed the ability of the T-SPOT.TB and QFN-G-IT assays in 492 adults and 134 children to diagnose *M. tuberculosis* infection in clinical practice, comparing the results with the TST. We have observed that T-SPOT.TB gives more positive results than QFN-G-IT in diagnosing active TB and LTBI. Furthermore, the results demonstrate that both IFN-γ tests are unaffected by prior BCG vaccination, and that the three tests have a higher concordance in non-BCG vaccinated patients; however, we have detected a poor concordance between IFN-γ assays and TST in non-BCG vaccinated children. Our results show enough evidence to state that with the utilization of IFN-γ tests, unnecessary LTBI treatment among adult and pediatric populations could be avoided.

In our experience, among non-BCG vaccinated children with a positive TST, the T-SPOT.TB was negative in a high number of cases. Therefore, we have studied the role

of NTM as a discordant factor between TST and T-SPOT.TB by the detection of IFN-γ released by sensitized T cells after being stimulated with *Mycobacterium avium* sensitin. We enrolled 21 non-BCG vaccinated children with suspicion of LTBI that yielded a positive TST and a negative T-SPOT.TB. From these patients, in 10 cases (47.6%) we obtained a positive ELISPOT result after stimulation with *M. avium* sensitin. Our results demonstrate that previous NTM sensitisation induces false-positive results in the TST for diagnosing LTBI, and the use of IFN-γ tests could avoid unnecessary chemoprophylaxis treatment among children.

It has been described that asymptomatic infections with *M. avium* and other NTM are common and probably acquired in childhood. In our area, the estimation of NTM infection in children with a positive TST (5–10 mm) ranged from 20–50%. According to our results, using the *ex vivo* ELISPOT, 8 of the 10 children reactive against *M. avium* sensitin had a positive TST between 5 and 10 mm. In addition, in our study, 8 out of 10 children with a positive *M. avium* sensitin T-cell assay were enrolled from a routine screening of LTBI without any known exposure to an active TB patient. Given that NTM infection affects the TST reading, it is in this group of children that IFN-γ tests could be used to confirm the diagnosis in case of a positive TST result.

On the other hand, the sensitivity of TST is reduced in immunosuppressed patients because of false negative results, as a result of cutaneous anergy. In this sense, another purpose of the Thesis is the study of IFN-γ assays in 75 HIV-infected individuals for the diagnosis of LTBI.

In this study, we observe a low number of positive results with T-SPOT.TB, QFN-G-IT and TST in HIV-infected patients; and a poor concordance between the three tests. Moreover, our results show that IFN-γ assays are more specific than TST in HIV-infected individuals because they are not affected by BCG vaccination. Regarding the number of positive results according to the number of CD4, both *in vitro* assays are influenced by the level of immunosuppression. Notwithstanding, T-SPOT.TB seems to be less affected than QFN-G-IT by severe immunosuppression because we obtained one positive result with T-SPOT.TB, that corresponded to one patient with less than 100 CD4 T cells, and none for QFN-G-IT or TST. This study demonstrates that IFN-γ assays in combination with TST are potential tools for diagnosing LTBI in HIV population severely immunosuppressed.

Nowadays, IFN-γ assays cannot distinguish between active TB and LTBI. However, it has been hypothesized that the magnitude of the response may provide information such as the likelihood of transition to latency *versus* disease. In this regard, we have evaluated the quantitative difference of *M. tuberculosis* specific antigen T cell response in adult and pediatric populations by T-SPOT.TB and QFT-G-IT, to differentiate between subjects with active TB and those with LTBI. Secondly, we have correlated the quantitative response of T cells with TB immunodiagnosis, mycobacterial burden, and disease activity.

In our experience, in adult patients, the number of responder T cells after ESAT-6 and CFP-10 antigens stimulation was higher in active TB than in LTBI patients. However, there is a large amount of overlapping that makes it difficult to distinguish active TB from LTBI. Moreover, our results demonstrate that the specific antigen CFP-10 response could be a specific marker of active TB. Consequently, although there is overlapping, in patients with clinically suspected TB, a T cell count above 116 reactive T cells after RD1 antigens stimulation in T-SPOT.TB, could help in the diagnosis of active TB with a 43% of sensitivity and a 81% of specificity, especially in patients with a high suspicion of having active TB in low LTBI prevalence countries.

The final objectives of the Thesis are the study and evaluation of new antigens and potential biomarkers for the diagnosis of active TB and LTBI. These alternative antigens or markers could increase the sensitivity of LTBI and active TB immunodiagnosis. On the other hand, the monitoring of anti-TB treatment is difficult in patients with TB based solely on clinical findings. There is no evidence, besides clinical examination, of a specific marker for evaluating and/or predicting the correct progress of patients during therapy and the assessment of an adequate treatment. In this regard, the identification of new antigenic targets, may improve the knowledge of the host response against *M. tuberculosis* during active disease, and its kinetics during treatment.

Recently, RD 1 selected peptides have been described. They are a selection of HLA class II-restricted epitopes of ESAT-6 and CFP-10 *M. tuberculosis* proteins. We have evaluated the host IFN-γ T cell response to RD1 selected peptides in 29 and 24 pulmonary TB patients enrolled at the beginning and during anti-TB therapy.

In this study, the number of responder T cells in TB patients at the beginning of therapy with regard to those studied during treatment was significantly different for both RD1

antigens included in T-SPOT.TB and RD1 selected peptides. However, conversion to negative responses for RD1 selected ELISPOT was significantly higher than for T-SPOT.TB. In our experience, specific *M. tuberculosis* T cell responses to RD1-selected peptides decline during anti-TB treatment, and immunological assays based on selected peptides correlate well with bacterial burden and maybe are promising tools for studying host immune response during anti-TB therapy.

Additionally, a wide range of potential TB biomarkers have been studied, of which the cytokine IP-10 has been the most promising one. The aim of our study was to determine the sensitivity and specificity of an IP-10 test for TB diagnosis in 168 active TB patients and 101 healthy controls. We measured IFN-γ by QFN-G-IT and IP-10 by a Luminex assay. In some patients T-SPOT.TB was also developed. According to our results, we have found that the IP-10 test has comparable performance to QFN-G-IT and T-SPOT.TB, and that QFN-G-IT and IP-10 tests could be combined for a significant improvement in sensitivity without a compromise in specificity.

In summary, IFN-γ tests are more specific than TST because they are less affected by BCG vaccination and NTM sensitization. So, the utilization of these assays can help to reduce unnecessary LTBI treatment among adult and pediatric populations. Therefore, detection of IFN-γ produced by T cells after *M. tuberculosis* specific antigen stimulation is an alternative diagnostic tool for LTBI in immunocompetent and immunosuppressed patients. In addition, IFN-γ assays could help in the immunodiagnosis of active TB. On the other hand, the study of new specific antigens and biomarkers is a potential tool for studying host immune response during anti-TB therapy and finding novel diagnostic markers for active TB and *M. tuberculosis* infection.

LISTADO DE ABREVIATURAS

TB	Tuberculosis
OMS	Organización Mundial de la Salud
VIH	Virus Immunodeficiencia Humana
LAM	Lipoarabinomanano
CD	Célula dendrítica
CPA	Célula presentadora de antígeno
MHC	Complejo mayor de histocompatibilidad
Th	Célula T *helper*
IFN-γ	Interferón-gamma
IL	Interleuquina
TNF-α	Factor de Necrosis Tumoral-alpha
IP-10	*IFN-γinducible protein 10*
TBNET	*Tuberculosis Network European Trialsgroup*
PT	Prueba de la tuberculina
PPD	Derivado proteico purificado
MNT	Micobacterias no tuberculosas
ECC	Estudio convencional de contactos
H	Isoniacida
R	Rifampicina
Z	Pirazinamida
RD	*Region of Difference*
ELISA	*Enzyme-Linked Immunosorbent Assay*
E	Etambutol
S	Estreptomicina
ELISPOT	*Enzyme-Linked Immunospot Assay*
ESAT-6	*Early Secretory Antigen Target-6*
CFP-10	*Culture Filtrate Protein 10*
QFN-G-IT	QuantiFERON-TB Gold In-Tube
PBMCs	Células Mononucleares de Sangre Periférica

ARTÍCULOS EN LOS QUE SE BASA ESTA TESIS

Artículo 1

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1. INTRODUCCIÓN

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1.1. La tuberculosis: Epidemiología y situación actual

La tuberculosis (TB) continúa siendo causa de una elevada morbilidad y mortalidad en todo el mundo. A finales del año 1980 hubo un incremento de la incidencia de TB, primero en EEUU y luego en el resto de mundo, de forma que la Organización Mundial de la Salud (OMS) la declaró como una enfermedad reemergente [1]. La OMS ha estimado que el número de nuevos casos de TB en el 2009 fue de 9.4 millones (137 por 100.000 habitantes), entre los cuales, 1.1 millones (12%) fueron nuevos casos de TB en personas infectadas por el virus de la inmunodeficiencia humana (VIH) [2]. India, China, Indonesia, Nigeria y Sudáfrica son los países con casos incidentes de TB más elevados. Prácticamente, el número total de enfermos con TB se concentran en 22 países, donde el 75% de los casos afecta a población activa, provocando un grave problema sanitario y económico. En la **Figura 1** se muestra un mapa que representa la distribución de la incidencia estimada de TB por países.

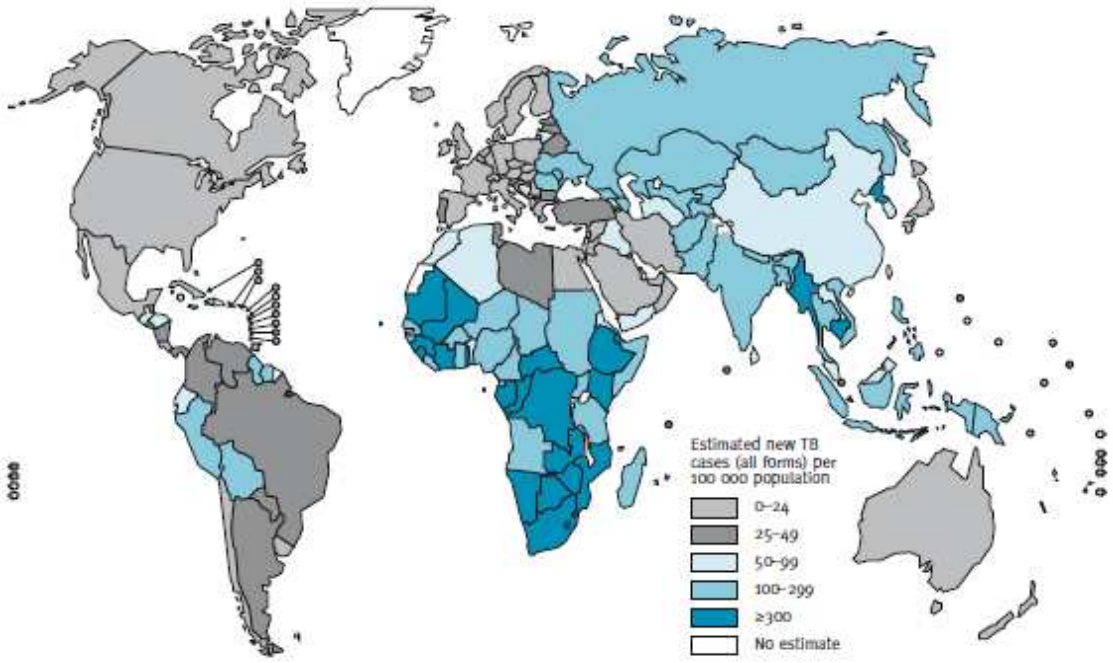

Figura 1. Incidencia estimada de TB por países en 2009. De: Informe OMS, 2010.

En España, en el año 2008, la tasa de incidencia de TB fue de 18.1 casos por 100.000 habitantes, y en Catalunya, de 22.2 casos por 100.000 habitantes [3].

Las personas que están infectadas por *Mycobacterium tuberculosis*, no están clínicamente enfermas ni pueden transmitir la TB, pero albergan microorganismos viables y se enfrentan a un riesgo durante toda su vida de presentar una TB clínica. Datos longitudinales de vigilancia de contactos con pacientes con TB realizados entre 1930 y 1960 sugieren que entre un 5-10% de las personas infectadas por *Mycobacterium tuberculosis* desarrollarán una TB en 1 o 2 años tras adquirir la infección, y en otro 5% aparecerá la enfermedad en algún momento de su vida [4].

Asimismo, las personas coinfectadas por VIH y TB son 20 veces más susceptibles de sufrir TB que los que no están infectados por VIH. En España, la epidemiología de la TB estuvo muy influenciada en los años 80 y 90 por la epidemia del VIH. Recientemente, el gran cambio demográfico debido a la llegada masiva de personas procedentes de países en vías de desarrollo, ha propiciado un patrón epidemiológico, también observado en otros países de Europa Occidental, con un alto porcentaje de casos que corresponden a personas de esos países.

El factor esencial para el control de la expansión de esta enfermedad radica en la capacidad de diagnosticar precozmente y tratar a los individuos enfermos de forma apropiada. Los métodos microbiológicos de referencia en el diagnóstico de la TB continúan siendo el examen microscópico, el cultivo y aislamiento de *M. tuberculosis* y la detección de sus ácidos nucleicos. Sin embargo, como es bien conocido, estas técnicas actualmente disponibles son insuficientes. Por otro lado, las personas infectadas representan un peligro potencial de nuevos casos de TB. Se estima que en el mundo existen unos 2000 millones de infectados. El estudio de las personas infectadas que no han enfermado permite aplicar, según los casos, medidas de prevención y evitar que desarrollen la enfermedad. De este modo se contribuye a romper la cadena de transmisión del microorganismo.

1.2. *Mycobacterium tuberculosis*

La especie patógena más frecuente dentro de *M. tuberculosis* complex es *M. tuberculosis*. El complejo incluye las especies *M. tuberculosis*, *Mycobacterium bovis*, *M. bovis* BCG, *Mycobacterium africanum*, *Mycobacterium microti* y *Mycobacterium canetii*. Estas especies son los agentes causantes de la TB en humanos y animales. A pesar de su estrecha relación genética, los miembros de *M. tuberculosis* complex distan respecto de su epidemiología y su papel como agente etiológico de la enfermedad tuberculosa en humanos. *M. tuberculosis* causa el mayor porcentaje de

TB en humanos. *M. africanum* se aísla de forma predominante en diferentes regiones de África. *M. microti* produce TB principalmente en pequeños roedores. *M. canetii* puede originar TB en humanos, pero su contribución a la enfermedad permanece todavía confusa. Los huéspedes de *M. bovis* son amplios, puede causar TB en varios animales domésticos o salvajes, pero también en humanos. *M. bovis* BCG es usado como vacuna antituberculosa en diferentes partes del mundo, tiene las mismas propiedades que *M. bovis*, pero con una virulencia más atenuada.

M. tuberculosis es un bacilo patógeno intracelular inmóvil y no esporulado. Su tamaño oscila entre 0,5 µm y 3 µm. El requerimento atmosférico de *M. tuberculosis* es aeróbico o microaerófilo y su crecimiento se ve favorecido en una atmósfera de un 5% a 10% de CO_2. La temperatura óptima de crecimiento es de 37 ºC, y su metabolismo de crecimiento es lento. Su tiempo de división es de 18 horas, dando lugar a colonias visibles en medio sólido entre 3 y 5 semanas. Tienen un alto contenido de %C+G (entre el 62 y 72%).

M. tuberculosis presenta una pared celular muy compleja y muy abundante en lípidos (suponen el 40% del peso seco de la pared celular), esto provoca que los bacilos no se tiñan fácilmente mediante la tinción de Gram. La coloración requiere el calentamiento de la célula para que la fucsina atraviese la pared bacteriana, fundamento en el que se basa la tinción de *Ziehl-Neelsen* (**Figura 2**). Una vez teñidas, muestran resistencia a decolorarse incluso al utilizar una mezcla de alcohol y ácido, por ello se denominan **bacilos ácido-alcohol resistentes**. Esta propiedad de ácido-alcohol resistencia depende esencialmente de la presencia de ácidos micólicos y otros lípidos de la membrana celular.

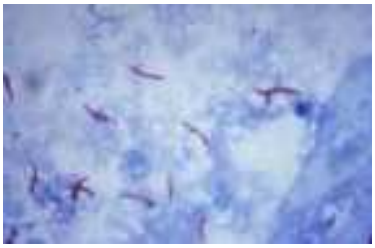

Figura 2. Tinción de *Ziehl-Neelsen* donde se observan bacilos ácido-alcohol resistentes.

La pared celular micobacteriana está compuesta por un esqueleto formado por dos tipos de polímeros, unidos covalentemente entre sí, el **peptidoglicano** y el **arabinogalactano**. Los ácidos micólicos están unidos al esqueleto de la pared celular

de forma uniforme. Aparte de este esqueleto, la pared celular presenta diversos glicolípidos, que actúan como importantes factores de virulencia (**Figura 3**).

El lipoarabinomanano (LAM), otra molécula presente en la pared celular micobacteriana, está relacionada con la interacción del patógeno con la célula huésped y facilita la supervivencia de *M. tuberculosis* dentro de los macrófagos.

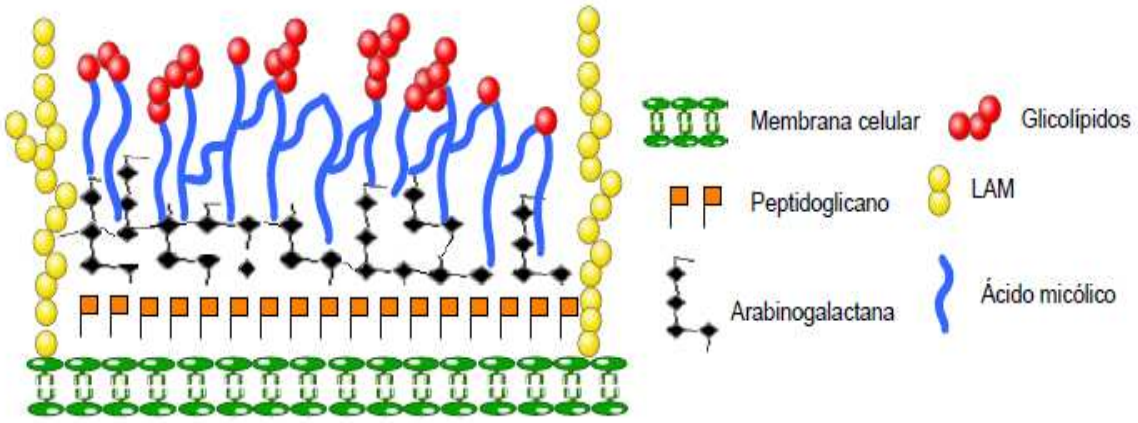

Figura 3. Pared celular de *M. tuberculosis*.

1.3. Historia natural e inmunopatología

Atendiendo a la historia natural de la enfermedad, la principal vía de infección es la llegada de *M. tuberculosis* a los alvéolos pulmonares, donde es fagocitado por las células dendríticas (CD) y las células presentadoras de antígeno (CPA) como los macrófagos alveolares.

Dado que *M. tuberculosis* es un patógeno intracelular, el bacilo consigue evitar su destrucción impidiendo la unión del fagosoma y el lisosoma, y multiplicarse así en el interior del macrófago hasta posteriormente destruirlo. Los factores de virulencia de *M. tuberculosis*, como los glicolípidos y las lipoproteínas de membrana, son reconocidos por los *toll like receptors* en la superficie de las CPA. Estas interacciones específicas, provocan la activación de las CPA y la inducción de diferentes patrones de expresión de citoquinas y quimioquinas que desencadenan un proceso inflamatorio local, y con ello una respuesta inespecífica de defensa que se caracteriza por el acúmulo inicial de neutrófilos y posteriormente de nuevos macrófagos en el foco de infección [5, 6].

En la **Figura 4** y **Figura 5** se resume el inicio de la inmunidad protectora, que depende de la activación de muchos tipos de células y complejas interacciones entre citoquinas y receptores. *M. tuberculosis* reside en el fagosoma, allí los antígenos peptídicos son presentados por las moléculas del complejo mayor de histocompatibilidad (MHC) de clase II a las células T CD4. Por otro lado, los antígenos citosólicos son presentados por las moléculas MHC de clase I a las células T CD8. Existen otros tipos de células T no tan comunes, las células T γδ y las células T CD1, que también son activadas durante la infección por *M. tuberculosis*. Las células T γδ reconocen pequeñas moléculas que contienen residuos de pirofosfato, y las células T CD1 glicolípidos [5, 7-9].

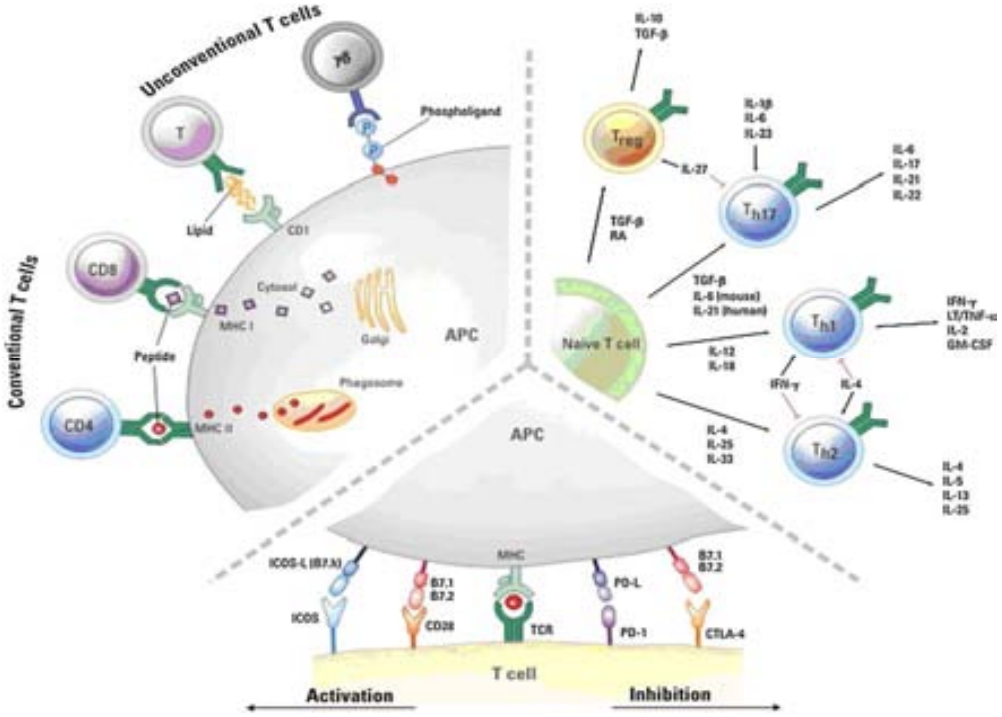

Figura 4. Activación de las células T en respuesta a la infección por *M. tuberculosis*. De: Stefan H.E. Kaufmann and Shreemanta K. Parida. Tuberculosis in Africa: Learning from pathogenesis for biomarker identification. *Cell Host and Microbe*. 2008;4:219-28.

Las células T CD4 se dividen en diferentes subpoblaciones de células T *helper* (Th), en función del patrón de citoquinas que expresan. La inmunidad protectora contra *M. tuberculosis* está mediada mayoritariamente por linfocitos T CD4 específicos, que se transforman en linfocitos Th1. Las células Th1 se caracterizan por producir principalmente interferón-gamma (IFN-γ), interleuquina (IL)-2 y el factor de necrosis

tumoral-alpha (TNF-α). Sin embargo, la respuesta inmunitaria humoral contra *M. tuberculosis* también está mediada por células T CD4 del tipo Th2. Las citoquinas de tipo Th2 más relevantes son la IL-4, IL-5 y IL-13. Las células T reguladoras producen TGF-β y IL-10, las cuales inhiben la respuesta inmunitaria [7, 9].

Los linfocitos Th1 tienen un particular interés, puesto que migran hacia el foco de infección liberando citoquinas que activan a otros macrófagos. Hay dos citoquinas claves que son las integrantes mayoritarias de los granulomas: el IFN-γ, que activa a los macrófafos [10], y el TNF-α, que está implicado en el desarrollo y mantenimiento del granuloma. La quimioquina *IFN-γ inducible protein* (IP)-10 también juega un papel muy importante en la migración de las células Th1 activadas al foco de infección cuando se une a su receptor CXCR3 [11].

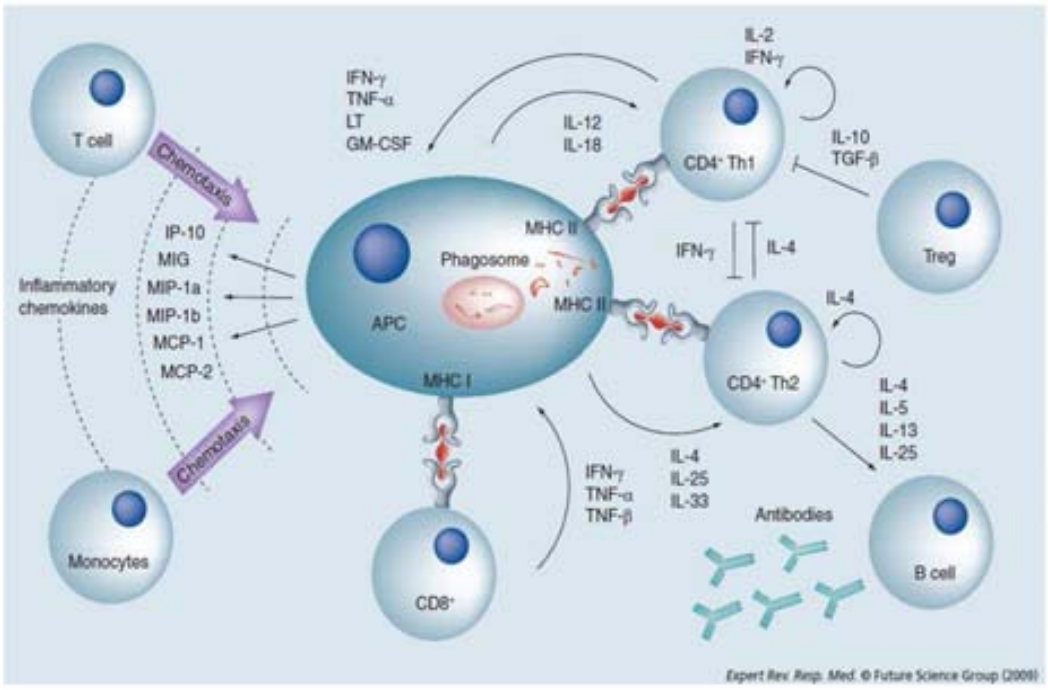

Figura 5. Interacción entre las células T y las CPA en respuesta a la infección por *M. tuberculosis*. De: M. Ruhwald and P. Ravn. Biomarkers of latent TB infection. *Expert Rev Resp Med*. 2009;3:387-401.

Los integrantes mayoritarios de los granulomas son los linfocitos Th1. Además, éstos son los responsables de la hipersensibilidad retardada, al desplazarse y proliferar ante la presencia de pequeñas concentraciones de proteínas de la pared celular de *M. tuberculosis*. Permiten focalizar macrófagos y linfocitos específicos alrededor del bacilo, evitando su crecimiento y su diseminación (**Figura 6**). La activación de los

macrófagos mediante IFN-γ desencadena una acidificación y un incremento de los radicales de oxígeno y de nitrógeno en el interior del fagosoma que permite destruir un alto porcentaje de población bacilar. El porcentaje restante es capaz de adaptar su metabolismo para responder a esta situación de estrés y consigue sobrevivir en estado de latencia.

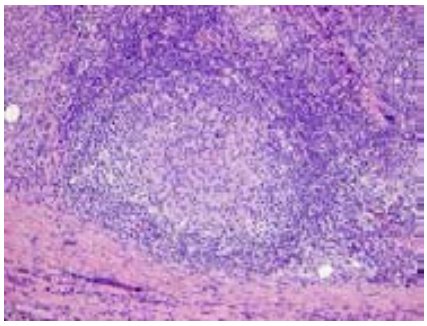

Figura 6. Imagen histológica del granuloma tuberculoso.

1.4. La infección tuberculosa

Tal y como se ha definido en el documento de consenso del *Tuberculosis Network European Trialsgroup* (TBNET) [12], el término de infección tuberculosa se utiliza para describir a aquellas personas que son portadoras de bacilos de *M. tuberculosis* vivos y no presentan signos ni síntomas clínicos de enfermedad, en este caso, *M. tuberculosis* es capaz de sobrevivir en estado de latencia. Contrariamente, cuando *M. tuberculosis* se manifiesta clínicamente como una enfermedad activa se le llama simplemente TB.

Para conocer si un individuo ha sido infectado por *M. tuberculosis*, clásicamente se ha estudiado su respuesta de hipersensibilidad retardada frente a determinados compuestos antigénicos específicos del bacilo. Este sería el principio en que se basa la tuberculina. Con la prueba de la tuberculina (PT) se pone de manifiesto una respuesta inmunológica medida por las células, que da lugar a una reacción inflamatoria en el lugar donde se inyecta la tuberculina. Esta respuesta se detecta mediante una induración visible y palpable.

1.4.1. Diagnóstico de la infección tuberculosa

1.4.1.1. Historia de la tuberculina

En el X Congreso Internacional de Medicina, celebrado en Berlín en 1890, **Robert Koch** anunció el descubrimiento de un posible remedio contra la TB [13]. Estudió la inmunización de cobayas sanas contra la TB y la detención de la enfermedad en

cobayas tuberculosas al inocular un extracto de bacilos tuberculosos, que fue denominado tuberculina.

La terapia con tuberculina fue aceptada con grandes esperanzas en aquellos tiempos, lo que provocó que se enviaran médicos procedentes de toda Europa a Berlín con el fin de aprender a utilizar la que se conoce como la Tuberculina Antigua de Koch. Se ensayaron diversas pautas y técnicas de administración, así como diversos tipos de tuberculinas. Uno de los principales problemas que se presentaron fue determinar la dosis de tuberculina a utilizar, ya que una dosis alta producía reacciones tóxicas, y una dosis baja podía ser insuficiente [14].

Robert Koch realizó otra comunicación en el año 1891 para comentar las reacciones observadas por otros médicos que utilizaban la tuberculina con fines terapéuticos y que llevaban a cabo sus primeras recomendaciones de uso. Las prácticas de Robert Koch rápidamente generaron muchas críticas, la mayoría negativas, ya que se notificaban muchas reacciones adversas, de manera que los riesgos superaban los beneficios del tratamiento.

No obstante, con los estudios de las reacciones cutáneas provocadas por la tuberculina en personas con TB, rápidamente se observó que la tuberculina podía ser utilizada para el diagnóstico de la infección tuberculosa. Incluso, hasta el mismo Robert Koch fue el primero que pensó en utilizar la reacción producida por la inyección de la tuberculina con fines diagnósticos, tal y como comunicó en el congreso de Berlín en 1890 [13].

La búsqueda de una vía más adecuada para la utilización de la tuberculina como herramienta diagnóstica tuvo su fruto en 1908. **Charles Mantoux** propuso un método que fue adaptado por los clínicos, y que consistía en inyectar por vía intradérmica una cantidad dosificada de tuberculina [15], de forma que si la reacción era positiva, se observaba la aparición de una infiltración de color rosado al cabo de las 48 horas. Esta reacción de Mantoux era valorada como positiva o negativa, pero no era medida.

Los amplios estudios que se realizaron sobre la TB en todo el mundo evidenciaron la necesidad de disponer de una tuberculina estándar para poder comparar los resultados de la PT entre diferentes individuos y también las PT realizadas en una misma persona, ya que la Tuberculina Antigua de Koch y las posteriores tuberculinas proporcionaban diferentes reacciones según su procedencia.

En 1932, **Florence Seibert** obtuvo un derivado proteico purificado (PPD) por precipitación del principio activo de la tuberculina. El PPD de Seibert (PPD-S) fue admitido como tuberculina estándar. Se estableció que la dosis óptima a utilizar del PPD-S no debía exceder a 0.0001 mg, lo que significaba 5UT por 0.1 ml de dilución (1UT=0.00002 mg).

Entre 1943 y 1949 se realizó un estudio [16] en el que se realizaron 21.313 PT a estudiantes de enfermería, utilizando el PPD-S. Hasta el momento, se había considerado que la sensibilidad de la tuberculina era un signo inequívoco de infección tuberculosa, pero con este estudio se observaron sensibilidades inespecíficas relacionadas con el país y el lugar de residencia. Se sugirió que el agente responsable de esta sensibilidad inespecífica debía estar relacionado con *M. tuberculosis*, aunque debía ser investigado testando a la población con tuberculinas preparadas a partir de otros géneros micobacterianos (sensitinas).

La sensibilización a micobacterias no tuberculosas (MNT) provoca reacciones cruzadas en la PT. Este tipo de reacciones fueron estudiadas por el *dual testing*, procedimiento que consiste en inyectar en un brazo PPD de *M. tuberculosis* y el otro PPD de sensitinas procedentes de MNT. Los resultados se estudiaban comparando las induraciones provocadas por cada antígeno.

1.4.1.2. Fundamento de la prueba de la tuberculina

La tuberculina se obtiene del filtrado del cultivo de *M. tuberculosis* esterilizado y concentrado. Actualmente está constituida por PPD que consiste en una mezcla de más de 200 proteínas de *M. tuberculosis*. En España se recomienda emplear la tuberculina PPD RT23 con Tween 80, preparada por el *Statens Serum Institute* de Copenhague, a dosis de 2 UT por 0,1 ml, que es la bioequivalente a la dosis recomendada (5 UT) de la tuberculina patrón internacional, la PPD-S [17].

La PT se realiza según la técnica de Mantoux, mediante la inyección intradérmica en la cara anterior del antebrazo de una cantidad constante de líquido diluyente (0,1 mL) con la dosis correspondiente de tuberculina. Si la administración es correcta, aparecerá en el lugar de la inyección una pápula que desaparece en pocos minutos. La sensibilización del individuo se manifiesta por una reacción de hipersensibilidad retardada, que produce una induración en el sitio de la inyección, que ha de comprobarse a las 48-72 horas (**Figura 7**). Esta respuesta está mediada por células

Th1 que migran al punto de inyección del antígeno y producen la liberación de diversas citoquinas al reconocer los antígenos presentados por las moléculas MHC de clase II, y activando posteriormente a los macrófagos provocando una reacción caracterizada por la aparición de eritema e induración.

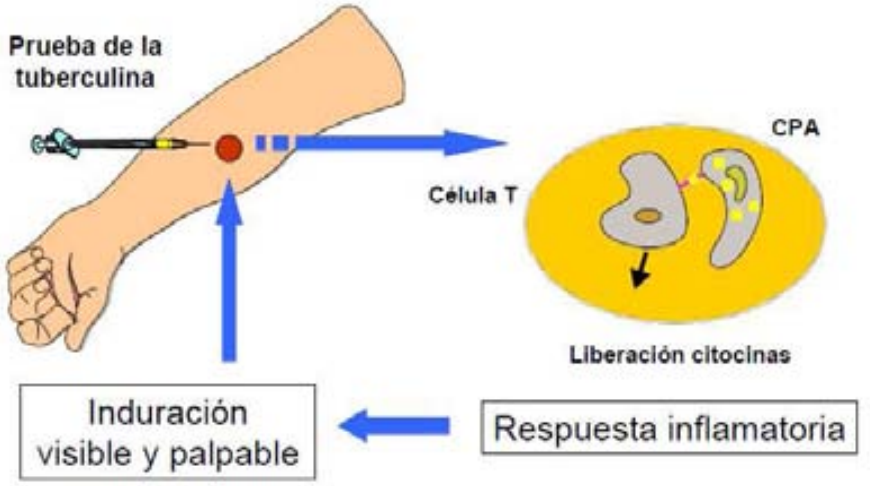

Figura 7. Esquema de la reacción de hipersensibilidad retardada al PPD. *Figura cedida por: Cellestis Limited (Australia).*

1.4.1.3. Interpretación del resultado

La lectura de la PT se realiza midiendo los milímetros de la induración que se obtiene en el sitio de la inyección, haciendo la medición del diámetro transversal al eje longitudinal del antebrazo. Solamente se deben medir los límites de la induración, si sólo hay eritema el resultado se debe registrar como negativo (**Figura 8**).

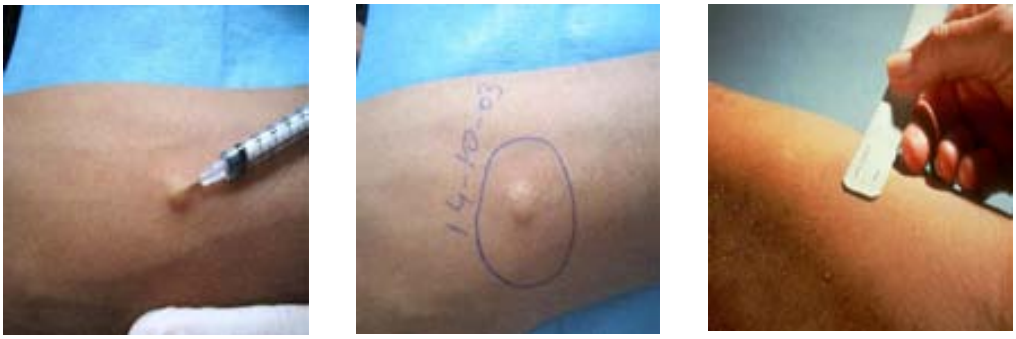

Figura 8. Administración del PPD y lectura de la PT.

Se considera que una PT es positiva cuando hay una induración superior a 5 mm, exceptuando los pacientes que hayan sido vacunados con BCG, en este caso no se puede diferenciar si la reacción de la PT es debida a la infección por *M. tuberculosis* o a la vacuna de la BCG. En los pacientes vacunados con BCG, se evaluará individualmente cada caso teniendo en cuenta que a mayor diámetro de la induración obtenida, más probabilidad hay de que la causa de la respuesta a la PT sea por infección tuberculosa, en especial si la induración supera los 15 mm. Sin embargo, en los colectivos con alto riesgo de infección, la vacuna de la BCG no debe tenerse en cuenta. En los vacunados se acepta que una respuesta a la PT de 5 mm indica infección por *M. tuberculosis*, cuando son convivientes o contactos frecuentes de una persona con TB bacilífera o infectados por el VIH [17]. En estos casos, la probabilidad de que una PT con induraciones de 5 mm signifique infección por *M. tuberculosis* es de hasta 70% [18] (**Tabla 1**).

Tabla 1. Criterios de infección tuberculosa según la positividad de la PT.

Situación	Positividad de la PT
No vacunados con la BCG	≥ 5 mm
Vacunados con la BCG, contactos íntimos o frecuentes de enfermos con baciloscopia positiva	≥ 5 mm
Vacunados con la BCG, contactos esporádicos de enfermos con baciloscopia positiva	≥ 15 mm (entre 5 y 15 mm: a más induración, más posibilidad de infección)
Vacunados con la BCG, contactos íntimos o frecuentes de enfermos con baciloscopia negativa	≥ 15 mm (entre 5 y 15 mm: a más induración, más probabilidad de infección)
Infectados por el VIH u otros inmunodeprimidos	Cualquier induración
Virajes tuberculínicos durante el estudio de contactos	≥ 5 mm

Tabla modificada de: Grupo de Estudio de Contactos de la UITB. Documento de Consenso sobre el estudio de contactos en los pacientes tuberculosos. *Med Clin (Barc)*. 1999;112:151-6.

Tras la infección por *M. tuberculosis*, han de transcurrir entre 2-12 semanas para que las células T sensibilizadas pasen a la sangre y puedan reconocer la tuberculina que se ha inoculado en el antebrazo. La capacidad de respuesta a la tuberculina no permanece toda la vida, ya que, aunque no llega a desaparecer, puede debilitarse con

el tiempo. Para poder descartar esta respuesta debilitada tras una PT negativa, se realiza una segunda PT entre 7-10 días después de la primera, produciéndose un efecto "empuje" o *booster,* para aumentar la respuesta inmune debilitada y poder evidenciar la sensibilización a la tuberculina.

1.4.1.4. Inconvenientes de la prueba de la tuberculina

La PT ha sido utilizada desde hace más de 100 años para el diagnóstico de la infección tuberculosa. El principal inconveniente de la tuberculina es su limitada especificidad. El PPD contiene más de 200 antígenos que no son específicos de *M. tuberculosis*, y que se comparten con la vacuna de la BCG y otras MNT. Esto provoca una disminución de la especificidad de la prueba, ya que individuos sensibilizados por MNT o vacunados con BCG también responden inmunológicamente al PPD [19]. Además, la PT presenta una sensibilidad reducida en pacientes con alteraciones en la inmunidad celular, dando como resultado falsos negativos.

Otros inconvenientes de la PT son: la subjetividad con la que los resultados pueden ser interpretados; los errores a la hora de su inoculación; y que requiere de una segunda visita para la lectura del resultado, con la consecuente pérdida de pacientes que no acuden a esta visita.

1.4.2. Estudio de contactos

El estudio convencional de contactos (ECC) se realiza sistemáticamente a los convivientes y a las personas que han estado en contacto con un enfermo de TB. Es una de las actividades sanitarias más eficaces desde el punto de vista del control de la TB, ya que permite la detección de todos los posibles nuevos casos de enfermos e infectados originados como consecuencia del contacto con el nuevo foco infeccioso, intentando romper de esta forma la cadena de transmisión de la enfermedad. También se considera ECC, el estudio de todos los contactos de un niño enfermo de TB y/o con PT positiva para poder descubrir el caso índice.

El método tradicional para realizar un ECC se basa en la aplicación del sistema de círculos concéntricos (**Figura 9**), en cuyo centro se encuentra el caso índice o fuente; en el primer circulo se encuentran las personas de mayor riesgo, es decir, los convivientes con un contacto íntimo diario mayor de 6 horas, por los que se iniciará el estudio, ampliando éste a los siguientes círculos, hasta llegar a los contactos

esporádicos. Si en algún círculo se detecta la presencia de un nuevo caso, entre sus contactos debe iniciarse un nuevo estudio en círculos concéntricos.

Los estudios publicados muestran que los ECC presentan una rentabilidad diagnóstica de nuevos casos de TB detectados entre el 3-6% [20-22] y más del 50% de los que conviven con pacientes con TB bacilífera están infectados [23].

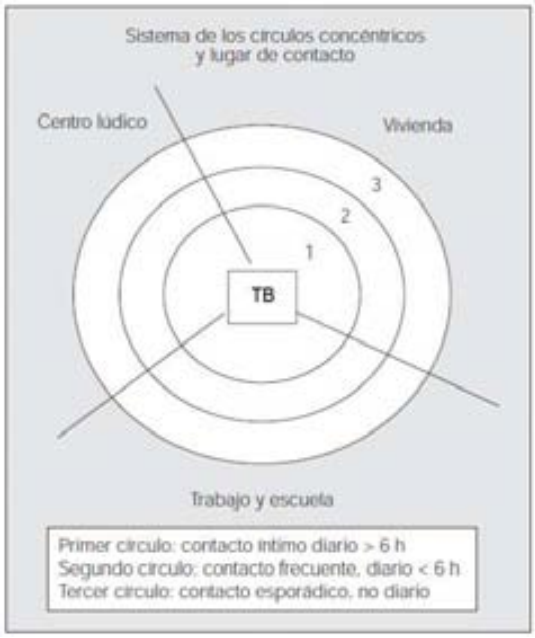

Figura 9. Sistema de círculos concéntricos. De: Grupo de Trabajo del área TIR de SEPAR. Normativa sobre la prevención de la TB. *Arch Bronconeumol*. 2002;38:441-51.

Los objetivos que se pretenden con esta forma de seguimiento son: descubrir casos ocultos de TB bacilífera; diagnosticar y tratar nuevos casos de TB e infección tuberculosa; prevenir infección en grupos de riesgo; prevenir TB en contactos con viraje tuberculínico; y reconstruir la cadena de transmisión para identificar el caso índice y cortar la cadena epidemiológica.

En la primera visita del contacto se realiza la PT, y en la segunda (48-72 horas) se efectúa la lectura de dicha prueba. Se recomienda hacer una radiografía de tórax para descartar enfermedad en los positivos para la PT y contactos íntimos de bacilíferos [23].

1.4.3. Tratamiento de la infección tuberculosa: Quimioprofilaxis

El tratamiento de la infección tuberculosa tiene como objetivo evitar la progresión de la infección a enfermedad. Para realizarla se requiere un medicamento seguro, bactericida y fácil de tomar; estas condiciones sólo las cumple la isoniacida (H), que, además, tiene un bajo coste [24]. Se ha demostrado su efectividad cuando se utiliza durante 6 meses o más. La protección de la pauta de 9 meses es superior a la de 6 meses en personas inmunocompetentes, pero el beneficio adicional es mínimo si se prolonga a 12 meses [25].

La quimioprofilaxis primaria tiene como objetivo evitar la infección tuberculosa en contactos íntimos de enfermos tuberculosos con baciloscopia positiva que no son reactores a la tuberculina (PT negativa). La quimioprofilaxis se debe mantener entre 2-3 meses y repetir la PT transcurrido este tiempo. Si la PT resulta negativa, el tratamiento se suspende, en cambio, si es positiva se debe continuar el tratamiento como si se tratara de una quimioprofilaxis secundaria durante 6 meses. La quimioprofilaxis secundaria pretende evitar el desarrollo de la enfermedad tuberculosa en pacientes ya infectados (PT positiva), sobretodo en aquellas personas que se han infectado recientemente.

En los últimos años se están impulsando pautas más cortas con la finalidad de favorecer el cumplimiento. Entre éstas cabe citar la rifampicina (R) más H durante tres meses [17, 26] y R sola durante cuatro meses [27]. Por el contrario, otras pautas como la combinación de R y pirazinamida (Z) durante 2 meses se desaconseja por sus efectos secundarios [28]. De acuerdo con nuestras guías nacionales [17], se recomienda la pauta de quimioprofilaxis en los casos indicados en la **Tabla 2** y **Tabla 3**.

Tabla 2. Indicaciones quimioprofilaxis primaria.

Quimioprofilaxis primaria (negativos para la PT)
Niños menores de 5 años
Pacientes con infección por el VIH
Adolescentes o adultos jóvenes (valoración individual)
Contactos con inmunodepresión

Tabla modificada de: J. Ruiz-Manzano, et al. Normativa SEPAR: Diagnóstico y tratamiento de la TB. *Arch Bronconeumol*. 2008;44:551-66.

Tabla 3. Indicaciones quimioprofilaxis secundaria.

Quimioprofilaxis secundaria (positivos para la PT)
Contactos de un enfermo con TB bacilífera
Conversores de la PT en los dos últimos años
Pacientes con infección por el VIH
Pacientes con lesiones residuales en la radiografía de tórax que no hayan recibido tratamiento previo
Pacientes infectados que vayan a iniciar tratamiento con inhibidores del TNF-α
Pacientes infectados candidatos a transplante

Tabla modificada de: J. Ruiz-Manzano, et al. Normativa SEPAR: Diagnóstico y tratamiento de la TB. *Arch Bronconeumol*. 2008;44:551-66 y Grupo de Trabajo del área TIR de SEPAR. Normativa sobre la prevención de la TB. *Arch Bronconeumol*. 2002;38:441-51.

1.4.4. Vacunación con BCG

1.4.4.1. Origen de la BCG

Desde que en el año 1882 Robert Koch demostró que la TB era una enfermedad infecciosa, rápidamente se empezó a trabajar en la elaboración de una vacuna.

Hasta el momento, la única vacuna utilizada en el mundo para el control de la TB ha sido la que obtuvieron **Albert Calmette** y **Camille Guérin** en el Instituto Pasteur. Se necesitaron 13 años y 230 cultivos sucesivos de una cepa virulenta de *M. bovis*, de origen bovino, sobre patata impregnada de bilis de buey, para conseguir un bacilo avirulento [29]. El bacilo vacunal recibió el nombre de BCG (Bacilo de Calmette y Guérin).

Las experimentaciones comenzaron a partir de 1903, pero durante la ocupación alemana en Francia, los experimentos realizados con la vacuna fueron interrumpidos, y no se volvieron a reanudar hasta que Calmette regresó a París en 1919. Se realizaron un gran número de investigaciones con la vacuna en bueyes, monos y cobayas, mostrándose eficaz al preservar a éstos animales de las infecciones por TB.
En 1921, un médico del Hospital de la Caridad, el Dr. Weill-Hallé, comunicó al Instituto Pasteur acerca de un niño nacido de una madre con TB, que acababa de fallecer. El

niño debía ser cuidado por su abuela, también con TB. Este hecho tan preocupante, bajo las coincidencias experimentadoras, llevó a que el niño fuera vacunado y que éste no presentara ningún incidente patológico. A partir de entonces se empezó a vacunar a niños.

En 1924, Calmette y su equipo hicieron una comunicación de sus resultados a la Academia de Medicina de París [30], fue a partir de este momento cuando el Instituto Pasteur inició la producción de la vacuna de la BCG para su uso por los profesionales de la Medicina.

El 26 de Abril de 1930 falleció en Lübeck (Alemania) un niño que había sido vacunado con la BCG y algunos niños más enfermaron de TB. Aunque rápidamente se suspendió la vacunación, fallecieron un total de 71 niños. Después de numerosas investigaciones para poder esclarecer el asunto, y tras estudiar las causas del incidente, todo apuntó a que en el laboratorio del Hospital de Lübeck se tomó una cepa patógena por una de BCG, ya que la cepa vacunal que envió el Instituto Pasteur a Lübeck también fue expedida en otras regiones sin ningún incidente. Calmette fue exculpado y se confirmó la inocuidad del BCG, aunque todo este proceso le afectó gravemente.

1.4.4.2. Respuesta inmunológica frente a BCG

Actualmente, existen cuatro variedades de cepas de la BCG. La cepa del Instituto Pasteur es la que está considerada como cepa de referencia de la vacuna, ya que los distintos laboratorios en todo el mundo han introducido variaciones genéticas que han afectado la inmunogenicidad y posiblemente la protección frente a la enfermedad.

La BCG es un inmunógeno altamente complejo que induce una respuesta básicamente de tipo celular, y se considera que la protección no dura más de 10-20 años después de su administración [31]. La vacuna no evita la infección por *M. tuberculosis*, pero ayuda a evitar la multiplicación incontrolada del bacilo en el foco de infección primario, y previene su diseminación evitando las formas clínicas graves como la TB miliar y meníngea en niños [32]. En general, no consigue evitar el desarrollo de TB pulmonar, por tanto, no puede descartarse el diagnóstico de TB en una persona vacunada que presente clínica sugestiva de dicha enfermedad.

Con la secuenciación completa del genoma de *M. tuberculosis* y de *M. bovis BCG* se ha evidenciado que ambos microorganismos tienen una composición genética diferente. *M. bovis BCG* ha perdido una región genómica, llamada *Region of Difference* (RD) 1, implicada en la existencia de antígenos específicos de *M. tuberculosis* complex, por lo tanto, no puede proporcionar una protección completa frente al desarrollo de TB [31, 33].

La vía de administración es intradérmica mediante escarificación en la zona deltoidal del brazo. Al ser una vacuna atenuada está contraindicada en personas inmunodeprimidas, ni tampoco se puede administrar durante el embarazo.

1.4.4.3. Controversia de la efectividad de la BCG

Al finalizar la segunda Guerra Mundial se implantó el uso masivo de la BCG como medida preventiva de la TB. Ya en 1948 se habían vacunado unos 5 millones de personas en 35 países y se consideró que era el único método que conseguía una inmunidad eficaz frente a la TB. En la década de los años 70 la vacunación se practicaba en 169 países y se estimaba en 2.000 millones el número de personas vacunadas. En 1988 la OMS incluyó la vacuna BCG en el Programa Ampliado de Vacunación de modo que en el año 2002, aproximadamente el 80% de todos los niños menores de 1 año habían sido vacunados.

En España, la vacunación comenzó en Barcelona en el año 1924. El profesor **Luis Sayé** la inició en el Dispensario Central Antituberculoso de Barcelona con una cepa vacunal proporcionada por Calmette. En el resto de España se introdujo en 1927 por el Instituto de Higiene Alfonso XIII. En 1965 se puso en marcha el Plan Nacional de Erradicación de la TB. Entre las estrategias que formaban parte de dicho plan, figuraba una campaña de vacunación masiva en recién nacidos, escolares y adolescentes negativos a la PT. El Plan de Erradicación finalizó en noviembre de 1973, con la recomendación de mantener la vacunación en los recién nacidos. Dicha vacunación sistemática con BCG en los recién nacidos se abandonó en 1980 (1974 en Cataluña), manteniéndose solamente en algunas provincias [29]. En 1992, la Conferencia de Consenso para el Control de la TB en España recomendó la suspensión de la vacunación con BCG sistemática; no obstante, durante algún tiempo se siguió utilizando en algunas comunidades autónomas de España y actualmente se sigue vacunando con BCG a los recién nacidos en el País Vasco [34].

Actualmente, la OMS recomienda la vacunación con BCG en niños de países con elevada incidencia de TB. Las indicaciones de la vacuna en países con incidencia de TB media o baja son en neonatos o niños que han tenido un contacto íntimo y prolongado con un caso de TB pulmonar bacilífera, y que presentan un elevado riesgo de desarrollar la enfermedad. La vacunación con BCG no está recomendada en adultos, aunque se puede indicar en algunos casos, como por ejemplo, contactos con casos de TB multiresistente. Sin embargo, la OMS acepta que hay países de baja incidencia de TB que han sustituido la vacunación con BCG por medidas más activas, como son la búsqueda de casos y el tratamiento supervisado. España ha integrado las estrategias de control y eliminación de la TB dentro de los programas autonómicos, y recientemente se ha desarrollado el "Plan para la prevención y control de la TB en España" [35]. Evitar la infección es el principio básico del control de la TB, para lo cual las estrategias son el diagnóstico precoz y el tratamiento de los enfermos hasta su curación. La vacuna de la BCG no tiene ningún papel en la prevención de la transmisión de la infección [36]. Evitar su desarrollo es la estrategia básica para la eliminación de la TB, para lo cual el estudio de contactos y el tratamiento de los infectados son los mecanismos indicados.

La vacuna está contraindicada en personas con inmunodeficiencias primarias o secundarias, así como personas infectadas por el VIH, pacientes en tratamiento inmunosupresor y mujeres embarazadas [31]. La revacunación no está recomendada.

A pesar de que cada año son vacunados aproximadamente 100 millones de niños, se ha debatido acerca de la eficacia de la vacuna durante décadas. Tanto los ensayos clínicos como los estudios de casos y controles han mostrado que la vacuna protege eficazmente contra la TB miliar y la meningitis tuberculosa en los niños entre 0-4 años, pero su eficacia es muy variable frente a la TB pulmonar de los adolescentes y adultos [31].

La eficacia en diversos estudios y poblaciones es muy variable (del 0 al 80%), siendo las razones para esta variabilidad múltiples. Influyen en esta variabilidad los factores debidos a la vacunación (técnica, dosis, vía y edad de administración), factores de la propia vacuna (fabricación conservación, viabilidad, tipo de vacuna), factores del huésped (inmunodepresión, infección por el VIH, desnutrición), prevalencia de infección por MNT o por cepas altamente virulentas de *M. tuberculosis* o diferencias a nivel poblacional [31, 37].

1.5. La enfermedad tuberculosa

1.5.1. Manifestaciones clínicas

Existen factores que favorecen el riesgo de desarrollar enfermedad tuberculosa una vez el paciente se ha infectado. Los más importantes son: el tiempo transcurrido desde la infección, sobretodo en los dos primeros años; la edad, siendo los niños hasta 4 años, los adolescentes y los adultos jóvenes particularmente más susceptibles; factores genéticos; el sexo, los hombres tienen el doble de riesgo que las mujeres; o factores medioambientales de la comunidad o de la virulencia de la cepa.

No existe una clínica exclusiva de la TB. La clínica es inespecífica, los síntomas y signos dependen de la localización apareciendo de forma tardía, en ocasiones cuando la persona enferma ya es contagiosa. Los síntomas pueden ser sistémicos (fiebre, pérdida de apetito y peso, astenia, sudoración nocturna profusa y malestar general) o específicos de órganos. Éstos últimos varían según la localización de la enfermedad, pero la forma más frecuente es la pulmonar (tos y expectoración hemoptoica).

1.5.2. Técnicas diagnósticas de la enfermedad tuberculosa

Los métodos microbiológicos de referencia en el diagnóstico de la TB continúan siendo el examen microscópico, el cultivo y el aislamiento de *M. tuberculosis*. Sin embargo, como es bien conocido, estas técnicas son insuficientes para las necesidades de la clínica. Las técnicas de examen microscópico presentan una baja sensibilidad y las técnicas de cultivo en medio sólido son lentas requiriendo períodos de incubación de hasta dos meses. Además, los métodos de identificación tradicionales son laboriosos y lentos. La incorporación de nuevos medios líquidos adaptados a equipos automatizados ha permitido reducir el tiempo de aislamiento. Además, la utilización de estos sistemas para el estudio de la sensibilidad a los antituberculosos permite disponer de los resultados en 15-20 días desde el aislamiento de la cepa de *M. tuberculosis* [38].

Las técnicas basadas en la biología molecular han sido consideradas como una alternativa a los métodos convencionales. Por sus características intrínsecas, estas técnicas moleculares nos deberían ayudar a reducir el tiempo de diagnóstico, a realizar la identificación y a detectar las resistencias más frecuentes de forma rápida y, además, a realizar estudios de epidemiología molecular.

1.5.2.1. Examen microscópico

La tinción y observación del bacilo de la TB permite realizar un diagnóstico presuntivo rápido. A pesar de los múltiples avances efectuados en los últimos años en el diagnóstico de la enfermedad tuberculosa, el examen directo continúa siendo la base del diagnóstico y seguimiento de la TB por su sencillez, rapidez, reproducibilidad y bajo coste.

Las técnicas utilizadas para la tinción de bacilos ácido-alcohol-resistentes son la clásica de Ziehl-Neelsen con fucsina fenicada y la tinción con fluorocromos, como la tinción de auramina.

La tinción de Ziehl-Neelsen está ligada a los ácidos micólicos de la pared micobacteriana. Éstos están presentes en el resto de especies micobacterianas y no se pierden con la muerte del bacilo. Por lo tanto, una baciloscopia positiva puede dificultar su interpretación en el seguimiento de los enfermos en tratamiento o infectados con otras micobacterias. Su principal inconveniente es su moderada sensibilidad, que está condicionada por la localización, el grado de afectación de la enfermedad y la calidad de la muestra. Sin embargo, su especificidad es muy elevada, superior al 95%. Por consiguiente, una baciloscopia negativa no descarta TB, pero una baciloscopia positiva prácticamente la confirma en el 95% de los casos y es indicación de iniciar tratamiento [17].

La tinción de auramina es más sensible y facilita el examen directo al poder efectuarse con menores aumentos, abarcando mayor superficie de campo observado y ser, por lo tanto, suficiente un menor tiempo de observación. Sin embargo, es menos específica y, en caso de positividad, debe confirmarse por tinción de Ziehl-Neelsen.

1.5.2.2. Técnicas tradicionales y nuevas de cultivo

El cultivo es el único método que puede asegurar con certeza la presencia de TB si se acompaña de identificación, y el único que es completamente válido para evaluar el seguimiento del paciente y garantizar su curación. Sin embargo, es necesaria una larga incubación para obtener un resultado, que es superior a 2-4 semanas.

Las muestras clínicas para el cultivo de micobacterias pueden proceder de lugares con flora comensal (respiratorias, heces, etc.) o de territorios estériles en condiciones de normalidad (orina, líquido pleural, articular, cefalorraquídeo, etc.). El rápido crecimiento de la flora comensal impediría el crecimiento de las micobacterias en los medios de cultivo, por lo que es necesario aplicar técnicas de descontaminación de la flora comensal y de homogeneización de las muestras clínicas. Con la homogeneización se consigue que en las muestras respiratorias se elimine la capa de moco que envuelve los bacilos, facilitando el acceso de éstos a los nutrientes del medio.

Los medios de uso habitual para el cultivo de micobacterias pueden ser sólidos o líquidos. El cultivo sólido más utilizado es el clásico de Löwenstein-Jensen, preparado a base de huevo. Sin embargo, la utilización combinada de un medio sólido y un medio líquido se considera actualmente idónea para tener sensibilidad óptima y rapidez en la detección.

1.5.2.2.1. Medios de cultivo sólidos

Los medios de cultivo sólidos pueden tener como base huevo coagulado, como el medio de Löwenstein-Jensen (**Figura 10**), o el de Coletsos, o bien, el agar, como en los medios 7H10 y 7H11 de Middlebrook. Los medios sólidos, a diferencia de los líquidos, permiten la visualización de las colonias y su morfología. La mayor desventaja de los cultivos sólidos es la larga incubación que se necesita para obtener un resultado positivo. Para hacer frente a esta limitación, se han comercializado medios de cultivo líquido.

Figura 10. Colonias de *M. tuberculosis* en medio de cultivo Löwenstein-Jensen.

1.5.2.2.2. Medios de cultivo líquido

Los medios de cultivo líquido presentan como mayor ventaja el acortamiento en 2-3 semanas de la detección del crecimiento de las micobacterias.

El sistema BACTEC 460TB (Becton Dickinson Microbiology Systems, EE.UU) incorpora una sustancia, generalmente un ácido graso como el ácido palmítico, marcado con carbono radioactivo (^{14}C). El crecimiento de las micobacterias se comprueba al detectar la aparición de CO_2 radiactivo en el frasco de cultivo. El principal inconveniente de este sistema es que es radiométrico, por lo que requiere instalaciones autorizadas para la manipulación y eliminación de los residuos radioactivos [39].

En los últimos años, los medios líquidos de cultivo no radiométricos han ido reemplazando a los radiométricos. En estos sistemas, la positividad del cultivo se basa en la detección del consumo de oxígeno [BD BACTEC MGIT 960 (Becton Dickinson Microbiology Systems, EE.UU)], en la disminución de la presión en la atmósfera del vial [ESP II (AccuMed International Inc. EE.UU)], o en la liberación de CO_2 del medio de cultivo [MB-BacT (BioMérieux, Francia)] como resultado del crecimiento micobacteriano [40-45].

1.5.2.3. Métodos de identificación

La identificación de especie confirma el diagnóstico de TB al permitir diferenciar el complejo *M. tuberculosis* del resto de micobacterias. La identificación puede efectuarse por técnicas bioquímicas, por técnicas cromatográficas y por técnicas de biología molecular.

La complejidad de las técnicas bioquímicas y cromatográficas, y la demora en la obtención de los resultados, han hecho que en la actualidad se realice la identificación mediante técnicas génicas, tanto sobre aislados clínicos como directamente de muestra clínica.

1.5.2.3.1. Técnicas bioquímicas

Durante muchos años, las micobacterias aisladas en cultivo se han identificado mediante sus características de cultivo y metabólicas. Las pruebas como la

acumulación de niacina, reducción de nitratos, catalasa, sensibilidad a la hidracida de tiofeno-2-carboxílico, sensibilidad a la cicloserina y Z, y test de NAP [46] permiten la identificación de las especies que integran el complejo de *M. tuberculosis*.

1.5.2.3.2. Técnicas cromatográficas

El contenido lipídico de la pared celular de cada especie micobacteriana presenta un carácter estable y específico. Las técnicas de cromatografía en capa fina que estudian el perfil de ácidos micólicos, o la cromatografía gas-líquido que estudia el perfil de los ácidos grasos constitutivos, alcoholes secundarios y productos de pirólisis de los ácidos micólicos, o la cromatografía líquida, permiten la identificación de las micobacterias [47-49].

1.5.2.3.3. Técnicas inmunocromatográficas

Se ha desarrollado recientemente una técnica inmunocromatográfica rápida para detectar el antígeno específico MPT64 de *M. tuberculosis* complex. Actualmente existen dos métodos comerciales que detectan este antígeno mediante inmunocromatografía en cepas aisladas en cultivo sólido o medios líquidos: TB Ag MPT64 Rapid (Standard Diagnostics, Seoul, South Korea) y BD MGIT TBc Identification Test (BD Biosciencies, Sparks, Md. USA). Se ha descrito que estas dos técnicas presentan una sensibilidad alrededor del 98% y una especificidad del 100% [50, 51].

1.5.2.3.4. Técnicas moleculares

1.5.2.3.4.1. Sondas de ácidos nucleicos

El desarrollo de la biología molecular ha permitido identificar secuencias de ADN o ARN específicas de cada especie micobacteriana. Se dispone de sondas marcadas con sustancias quimioluminiscentes que permiten la identificación de *M. tuberculosis*, del complejo *Mycobacterium avium-intracellulare*¸ *M. kansasii* y *Mycobacterium gordonae* a partir de cultivo sólido y líquido, sin necesidad de amplificación genómica (AccuProbe. Gen-Probe Incorporated, San Diego, USA) [52]. Alternativamente, también se han diseñado sondas de ácidos nucléicos peptídicos marcadas con sustancias fluorescentes (Dako Probe *M. tuberculosis* Culture Confirmation Test. Dako, Denmark) que permiten diferenciar entre micobacterias del complejo *M. tuberculosis* del resto de micobacterias [53].

1.5.2.3.4.2. Técnicas de amplificación genómica

Las técnicas de amplificación de ácidos nucleicos han resultado de gran utilidad para la identificación de micobacterias, permitiendo la mayoría de ellas realizar la identificación en unas pocas horas. Se pueden aplicar tanto a cepas aisladas en cultivo sólido como a partir de medios líquidos. Éstas técnicas de amplificación genómica varían en función del método de revelado utilizado.

Hibridación en fase sólida

Estas técnicas se basan en la amplificación de una secuencia diana y su posterior hibridación en tiras de nitrocelulosa marcadas con sondas específicas para las diferentes especies que se quieran identificar. Estas técnicas son rápidas y de fácil interpretación. En estos momentos se dispone de dos sistemas comercializados: INNO-LiPA Mycobacteria v2 (espacio intergénico 16S-23S, Innogenetics, Ghent, Belgium) [54, 55] y GenoType (23S rRNA, Hain Lifescience GmbH, Nehren, Germany). INNO-LiPA Mycobacteria v2 incluye 16 sondas diferentes y Genotype se presenta en diferentes formatos: GenoType Mycobacterium CM para la identificación de 14 de las especies de micobacterias de mayor interés clínico, GenoType Mycobacterium AS para la identificación de 16 especies micobacterianas adicionales, y GenoType MTBC para identificar especies dentro del complejo *M. tuberculosis* [56, 57].

Polymorphism Restriction Amplification (PRA)

Consiste en la amplificación mediante PCR del gen *hsp65*, seguida de una restricción del producto de amplificación mediante dos enzimas de restricción (*Bst*EII y *HaeIII*) [58, 59]. El análisis del polimorfismo de los fragmentos de restricción (RFLP) permite diferenciar entre las diferentes especies micobacterianas. Los resultados se basan en el análisis de los patrones de restricción obtenidos en la electroforesis.

PCR en tiempo real

Estas técnicas se basan en la amplificación y detección del amplificado de forma simultánea. Se utilizan sondas complementarias a las secuencias de interés. Al ir marcadas con fluorocromos, a medida que se amplifica el fragmento seleccionado se detecta la emisión de fluorescencia. Las sondas más empleadas son las TaqMan, Beacon y FRET. En estas técnicas, al no ser necesaria la manipulación del producto

amplificado se minimizan los problemas de contaminación. Se pueden emplear para la identificación a partir de cultivo sólido o líquido [60].

Secuenciación

Las técnicas de secuenciación se pueden emplear para la identificación a partir de cultivo líquido o sólido. Las secuencias más empleadas para la identificación son 16S rDNA y *hsp65*. Las secuencias obtenidas pueden analizarse en diversas bases de datos internacionales. Estas técnicas son laboriosas y requieren de un equipamiento que no siempre se encuentra disponible en los laboratorios de microbiología.

1.5.2.4. Técnicas serológicas

Hace más de 100 años que se intenta conseguir un test diagnóstico de TB utilizando el suero de pacientes enfermos. Aunque a lo largo del tiempo se han ido introduciendo mejoras significativas en esta tecnología (purificación, antígenos recombinantes, tests de detección más sensibles, etc), las técnicas serológicas no se han mostrado útiles para introducirlas rutinariamente en los programas de control de la TB.

El principal papel que tienen las técnicas serológicas es en el diagnóstico de la TB activa. Estas técnicas tienen que ser especialmente importantes allí donde los métodos diagnósticos convencionales fallan, como por ejemplo, en los pacientes con TB pulmonar y extrapulmonar con baciloscopia negativa.

La historia de las técnicas serológicas empieza alrededor de 1898, cuando se intentó desarrollar un test de aglutinación utilizando el bacilo entero. Desde entonces se han desarrollado numerosas técnicas utilizando antígenos no específicos de *M. tuberculosis* como por ejemplo, técnicas de aglutinación, fijación del complemento, precipitación e inmunoensayo [61]. En general los datos son muy variables, con sensibilidades muy elevadas pero con especificidades bajas.

Actualmente existen en el mercado diferentes técnicas, basadas en diferentes antígenos, que detectan IgG, IgM o IgA con las técnicas de *Enzyme-Linked Immunosorbent Assay* (ELISA), DOT-ELISA e inmunocromatografía. En general, estas técnicas detectan desde el 30 al 75% de enfermos con tinción positiva, y una proporción más pequeña con tinción negativa [62-70].

En la actualidad se está tratando de mejorar estas técnicas mediante la selección de antígenos proteicos a través de la síntesis recombinante de antígenos lipídicos. Todavía se requieren ajustes de la técnica que proporcionen la combinación óptima de antígeno y anticuerpo y que permita alcanzar niveles de sensibilidad aceptables.

1.5.2.5. Técnicas de detección de antígeno

Recientemente, se ha desarrollado un ELISA para detectar el antígeno LAM en orina en pacientes con TB activa que están infectados por el VIH (Clearview TB ELISA, Inverness Medical Innovations, USA). Debido a que este tipo de pacientes tienen un sistema inmunitario debilitado, presentan elevados niveles de *M. tuberculosis* en la sangre, que posteriormente se metabolizan en los riñones filtrándose el antígeno LAM en la orina [71].

Estudios recientes han demostrado que la detección del antígeno LAM en orina posiblemente tiene un valor diagnóstico en pacientes infectados por el VIH [72-75], pero no en pacientes con sospecha de TB que no están infectados por el VIH [76, 77]. No obstante, son necesarios más estudios para poder determinar la utilidad de este ELISA en orina o otros fluidos biológicos, como por ejemplo líquido pleural [78] y líquido cefaloraquídeo [79].

1.5.2.6. Diagnóstico mediante técnicas de amplificación genética

En los últimos años se han desarrollado y evaluado un gran número de técnicas para la detección de micobacterias, fundamentalmente *M. tuberculosis,* directamente de muestras clínicas. En estas técnicas se amplifican diferentes dianas genéticas y se utilizan diferentes métodos de amplificación y de revelado del producto amplificado. Es necesario señalar que la sensibilidad de estas técnicas es dependiente en gran medida de la utilización de métodos de calidad y muy optimizados para la extracción del material genético y que es recomendable emplear técnicas que incluyan controles internos de amplificación para detectar las posibles inhibiciones.

Se han descrito diferentes protocolos de técnicas no comerciales basadas en la amplificación de diferentes secuencias diana (IS6110, *hsp*65 o *rpoB*) mediante PCR o *nested-PCR*. El revelado del producto amplificado se realiza mayoritariamente mediante electroforesis en gel de agarosa.

Las técnicas de amplificación estandarizadas y disponibles comercialmente varían según la técnica de revelado utilizada.

Revelado automatizado o semi-automatizado

Las técnicas más ampliamente utilizadas son, por un lado, el Amplified *Mycobacterium tuberculosis* Direct Assays–AMTD2 (Gen-Probe Incorporated, San Diego, USA) [80-82] y LCx *Mycobacterium tuberculosis* Assay (Abbott Laboratories, Illinois, USA) [83, 84] que son técnicas semiautomatizadas; y por otro lado, las técnicas Amplicor *Mycobacterium tuberculosis* Test y COBAS Amplicor *Mycobacterium tuberculosis* Test, (Roche Molecular Diagnostics, California, USA) [85, 86] y BD ProbeTec^{TM} ET assay (BD Biosciencies, Sparks, Md. USA) [86-88], que son técnicas totalmente automatizadas. La mayoría de estudios demuestran que la especificidad de estas técnicas es aceptable tanto en muestras respiratorias como extrarespiratorias, mientras que la sensibilidad es variable, encontrando siempre la mayor sensibilidad en muestras respiratorias con baciloscopia positiva. En este sentido existe aún gran controversia sobre su utilidad real en muestras con baciloscopia negativa.

Revelado por hibridación en fase sólida

En estas técnicas la detección del producto amplificado se realiza mediante sondas de DNA específicas fijadas en tiras de nitrocelulosa. Fundamentalmente existen técnicas comercializadas, la primera de ellas es el INNO-LiPA Mycobacteria v2 que, a pesar de que se desarrolló para la identificación de *M. tuberculosis* y otras especies micobacterianas a partir de aislamientos, también ha demostrado ser de utilidad en la detección directa de muestra clinica, especialmente en muestras respiratorias con baciloscopia positiva [89]. La segunda técnica es el GenoType Mycobacteria Direct que detecta la presencia de *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium kansasii, Mycobacterium malmoense* y *M. tuberculosis* complex con una buena sensibilidad [90]. Estas técnicas a pesar de que son laboriosas y no están automatizadas, presentan la ventaja de que son capaces de detectar en el mismo ensayo otras especies micobacterianas además de *M. tuberculosis.*

PCR en tiempo real

Estas técnicas ya comentadas en el apartado de identificación, se pueden emplear también para la detección de *M. tuberculosis* directamente de muestra clínica. Se puede disminuir el tiempo de detección e identificación a unas 3 horas en total y es más sencilla de realizar que las técnicas anteriormente citadas.

Por el momento los datos disponibles con técnicas de PCR a tiempo real parecen indicar que son técnicas sensibles y con una buena especificidad.

Actualmente existe la técnica comercial GeneXpert MTB/RIF (Cepheid, USA) que detecta simultáneamente a partir de cepa y también de muestra directa la presencia de *M. tuberculosis* complex y resistencia a R [91-93]. En un estudio multicéntrico que se ha llevado a cabo recientemente [93], se han evaluado 1730 muestras de pacientes con sospecha de TB por la técnica de GeneXpert MTB/RIF. Esta técnica detectó el 98.2% de los pacientes con cultivo positivo para *M. tuberculosis* y el 72.5% de los pacientes con TB y baciloscopia negativa, con una especificidad del 99.2%.

Situación actual. Ventajas e inconvenientes.

La mayoría de evaluaciones llevadas a cabo con sistemas de amplificación ofrecen resultados satisfactorios, aunque todavía quedan por resolver algunas limitaciones de estas técnicas y su aplicación en el diagnóstico de la TB.

En muestras Ziehl-Neelsen positivas, las técnicas han demostrado una excelente sensibilidad y especificidad. Sin embargo, en muestras de pacientes con diagnóstico de TB y tinción negativa, las técnicas reflejan una gran variabilidad de los resultados, dependiendo del sistema de amplificación utilizado y del origen de las muestras. El grado de sospecha clínica es fundamental para la interpretación de los resultados.

Dada la óptima sensibilidad y especificidad de las técnicas de amplificación cuando se aplican en muestras con baciloscopia positiva, un valor positivo de la amplificación establecería el diagnóstico de la TB. En cambio, un valor negativo de amplificación obtenido de una muestra con baciloscopia negativa, no descartaría en ningún caso el diagnóstico de la TB.

Finalmente, aunque las posibilidades de aplicación de esta tecnología pueden ser de gran utilidad, es importante conocer sus limitaciones y sus ventajas para poder aplicarlas en el diagnóstico de la TB.

1.5.2.7. Micobacteriófagos

Desde la década de los sesenta hasta el presente, los bacteriófagos han sido un sistema ideal para la investigación de los mecanismos genéticos a nivel molecular. Además, los micobacteriófagos, han sido también el material de elección para diseñar experimentos en virología, debido a las significativas diferencias de costes y tiempo frente a la metodología cara y compleja de los virus de eucariotas.

En este sentido, los micobacteriófagos se han estudiado desde 1954 cómo herramienta en el diagnóstico de la TB y como método alternativo para la obtención rápida de resultados de resistencia antibiótica. Se han descrito dos métodos que tienen cierta utilidad clínica: el *Luciferase Reporter Phage Assay* y el *Mycobacteriophage-based assay* [94]. Las principales diferencias entre estas técnicas residen en el método de detección de las células micobacterianas infectadas y en el tipo de fago que se utiliza.

Luciferase Reporter Phage Assay es una técnica bioluminiscente en la que mediante micobacteriófagos específicos modificados se introduce en el genoma de la micobacteria el gen que codifica la enzima de la luciferasa. La expresión de la luciferasa se puede utilizar como un sistema sensible de cuantificar ATP en la micobacteria y constituye un sistema rápido de estudiar la viabilidad de *M. tuberculosis* tras su exposición a diferentes agentes antimicobacterianos.

Mycobacteriophage-based assay se basa en la capacidad de los micobacteriófagos de infectar micobacterias y lisarlas, de forma que si una muestra clínica contiene *M. tuberculosis* y se le añade un micobacteriófago, éste penetra en el interior de la célula procariota y realiza un ciclo lítico. La presencia de fagos libres, que pueden detectarse por la formación de calvas en la superficie de un cultivo confluente de otra micobacteria huésped (*Mycobacterium smegmatis*), es indicativa de la presencia de *M. tuberculosis* en la muestra clínica estudiada (**Figura 11**).

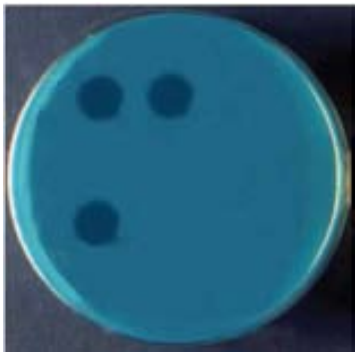

Figura 11. Detección de calvas en un cultivo confluente de *Mycobacterium smegmatis* mediante *Mycobacteriophage-based assay*. De: N. Galí, et al. Use of a Mycobacteriophage-Based Assay for Rapid Assessment of Susceptibilities of *Mycobacterium tuberculosis* Isolates to Isoniazid and Influence of Resistance Level on Assay Performance. *J Clin Microbiol.*2006;44:201-205.

Esta tecnología tiene posibilidades de ser aplicable también en la determinación de la susceptibilidad de las micobacterias a los fármacos antituberculosos, de forma que si la micobacteria es resistente al antibiótico continuará siendo viable y permitirá el desarrollo del ciclo lítico del fago con su consecuente detección posterior [95-97].

No obstante, aunque para la R han demostrado una buena correlación con los métodos fenotípicos, se ha observado que la resistencia de bajo nivel a la H puede no ser detectada [98]. Además, para otros fármacos, como etambutol (E), estreptomicina (S) y Z, han mostrado peor sensibilidad y especificidad [94]. Por estos motivos, aunque tal vez puedan tener cierta utilidad en laboratorios de países con recursos limitados, se encuentran pendientes de evaluación y estandarización.

1.5.3. Tratamiento de la enfermedad tuberculosa

Las micobacterias presentan una resistencia natural a numerosos antibacterianos, ya que poseen una pared muy compleja, hidrófoba y con una permeabilidad reducida para un gran número de compuestos [99]. Debido a esto, el tratamiento de la TB se realiza con antimicrobianos específicos.

El objetivo principal de todo tratamiento antituberculoso es eliminar el bacilo rápidamente y evitar la aparición de resistencias. El tratamiento de la TB es de larga duración y se basa en la asociación de diversos fármacos.

De forma espontánea, en una población de bacilos tuberculosos que nunca ha estado en contacto con fármacos, cuando alcanza un número determinado de bacilos, aparecen mutantes resistentes naturales a dichos fármacos. Esta aparición está relacionada con la densidad de la población bacilar, su velocidad de crecimiento y las características de los fármacos. El número de bacilos necesarios para que aparezca un mutante resistente a la H es de 10^5-10^6, para la R 10^7-10^8, para la S 10^5 -10^6, para la Z 10^2 -10^4 y para el E 10^6, siendo mucho menor para los fármacos de segunda línea. Se comprende pues, que si se administra un solo fármaco a un paciente cuya densidad de población bacilar es elevada, inicialmente se destruye la mayoría de la población bacilar, pero con el paso del tiempo los mutantes resistentes darán origen a una población bacilar totalmente resistente [4].

En base a esto, el tratamiento debe de constar de dos fases: una fase inicial en la que se han de usar al menos tres fármacos para impedir el desarrollo de mutantes resistentes entre los bacilos que existen en el huésped, y posteriormente una vez conseguida la reducción del mayor número de bacilos posible, basta con seguir con dos fármacos, es la llamada fase de consolidación. La pauta terapéutica recomendada en los nuevos casos de TB según nuestras guías nacionales [17] es de 6 meses, con los fármacos H, R, E y Z durante los dos primeros; e H y R durante los cuatro restantes. La indicación de añadir E a todos los pacientes se establece por motivos operativos y para cubrir la posibilidad de una elevada resistencia primaria a la H, que no se conoce con exactitud en todas las comunidades autónomas españolas. El E se podrá eliminar de la pauta terapéutica cuando el antibiograma muestre sensibilidad a todos los fármacos.

Existen dos tipos de resistencias en *M. tuberculosis*: la resistencia primaria y la resistencia adquirida o secundaria. La resistencia primaria, es la que presenta un paciente que nunca ha recibido fármacos antituberculosos, y aunque el mecanismo de adquisición todavía no está del todo definido, se cree que es debido al contagio por bacilos con resistencias. La resistencia adquirida o secundaria se produce cuando el paciente que inicialmente tenía una cepa de *M. tuberculosis* sensible se convierte en resistente. Este tipo de resistencia puede ser debida a una indicación de un tratamiento antituberculoso incorrecto, como por ejemplo, cuando se administra tratamiento con un solo fármaco o una asociación de dos fármacos a los que el paciente ya tiene resistencia a uno de ellos. También se puede producir por un incumplimiento del tratamiento.

Los fármacos antituberculosos se clasifican en dos grupos: los fármacos de primera línea y los de segunda línea. Los de primera línea son H, R, Z y S; se caracterizan por ser bactericidas y son de elección para el tratamiento de nuevos casos de TB. En este grupo también se incluye el E, que solamente es un fármaco bacteriostático, pero se usa con los de primera línea. Los de segunda línea presentan menor actividad antituberculosa y se aconseja que los utilicen personal especializado, ya que su manejo es más complicado. Los más utilizados son: protionamida, etionamida, capreomicina, kanamicina, cicloserina, PAS, tioacetazona y quinolonas. Tras un fracaso terapéutico o recidiva con bacilos resistentes a algún fármaco de primera línea, se recomienda una pauta de fármacos distinta a la estándar (2HREZ/4HR). Esta pauta tiene siempre una duración superior a los 6 meses (entre 9 y 24 meses) y casi siempre incluye fármacos de segunda línea.

En los últimos años, ha habido un incremento mundial de casos de TB multiresistente, es decir, cepas de *M. tuberculosis* con resistencia simultánea a H y R. Asimismo, han aparecido resistencias expandidas o extremadamente resistentes. Este tipo de resistencia se define como la causada por cepas multiresistentes, pero con otra resistencia añadida a alguna fluoroquinolona y, al menos, a un fármaco de segunda línea que se administre de forma inyectable [100, 101].

1.5.4. Métodos de estudio de la sensibilidad a fármacos tuberculosos

Estudios genéticos han demostrado que las resistencias a los fármacos antituberculosos se deben a mutaciones cromosómicas de los genes que codifican la diana del fármaco, o a enzimas implicadas en la activación del fármaco [102-104]. Se han descrito mutaciones puntuales, delecciones, o inserciones responsables de la resistencia a fármacos de primera línea o algunos de segunda línea. La detección precoz de la resistencia a los fármacos antituberculosos es esencial para el control de la TB. Además, el conocimiento de los mecanismos de resistencia permite desarrollar técnicas moleculares para su detección precoz.

Existen varios métodos para el estudio de la sensibilidad a los fármacos antituberculosos. En la actualidad, los métodos clásicos o fenotípicos más utilizados son el método de las proporciones y los automatizados en medio líquido [100]. Las pruebas de sensibilidad fenotípicas se pueden realizar directamente a partir de muestra clínica con baciloscopia positiva o sobre cepa de *M. tuberculosis*.

El método de las proporciones, estudia el número de unidades formadoras de colonias que crecen en el medio con los fármacos estudiados y en un medio control que no lo posee. Con este método se determina la resistencia de, al menos, el 1% de la población bacteriana que crece en el medio en presencia de un fármaco a una determinada concentración. Estos porcentajes de resistencia fueron establecidos para cada fármaco por Canetti, Rist y Grosset hace 45 años.

Existen sistemas automatizados que se basan en el método de las proporciones y que detectan correctamente la multiresistencia a la H y R, aunque existe una cierta variabilidad entre los diferentes laboratorios para la detección de resistencias de otros fármacos de primera línea como la Z, el E o la S. Por otro lado, la detección de resistencia a los fármacos de segunda línea es particularmente difícil ya que no se dispone de criterios estandarizados de evaluación e interpretación, aunque los resultados de diferentes evaluaciones son prometedores [94, 100, 105-107].

Por otro lado, los métodos moleculares desarrollados para la detección de mutaciones en los genes relacionados con la resistencia antibiótica parecen ser la mejor alternativa para la determinación rápida de resistencias, sobretodo para la R, que es un marcador de multiresistencia. La resistencia a la R está relacionada con mutaciones en el gen *rpoB* que codifica para la subunidad β de la RNA polimerasa DNA dependiente [104]. En más del 95% de las cepas resistentes, las mutaciones se encuentran en esta zona concreta, lo cual ha facilitado el desarrollo de métodos para su detección. Sin embargo, las bases moleculares de la resistencia a H son más complicadas, ya que las mutaciones involucradas se encuentran en más de un gen o complejos genómicos como *katG*, *inhA*, *KasA* y la región intergénica *oxyR-aphC* [108]. El gen *KatG* codifica la enzima catalasa-peroxidasa, responsable de la activación de la H. La mutación de este gen es la más frecuente y se asocia a alto nivel de resistencia [108, 109]. El gen *inhA* codifica para la enzima enoil ACP reductasa, que participa en el proceso de síntesis de los ácidos micólicos de la pared bacteriana. Las mutaciones en este gen se traducen en un aumento de la síntesis de la enzima, compensando la acción inhibidora de la H, por eso, suelen estar asociadas a resistencias de bajo nivel [108, 110].

Actualmente existen distintas técnicas moleculares para la detección de resistencias de *M. tuberculosis*, que se basan en la amplificación de la región genómica de interés y el posterior análisis del producto amplificado. En función del sistema de revelado los métodos moleculares se dividen en cuatro categorías: electroforesis, hibridación, PCR a tiempo real y secuenciación.

Actualmente, existen dos métodos comerciales que detectan resistencia mediante una hibridación inversa en tiras de nitrocelulosa: INNO-Lipa Rif TB (Innogenetics, Ghent, Belgium), que detecta resistencia a R y GenoType MTBDR*plus* (Hain LifeScience GmbH, Nehren, Germany), que detecta resistencia a R e H. El test INNO-Lipa Rif TB puede identificar *M. tuberculosis* y presencia de resistencia a R por mutaciones en el gen *rpoB* de forma simultánea [111]. GenoType MTBDR*plus* identifica mutaciones en los genes *rpoB*, *katG* y *inhA*, para la detección de resistencias a R y H. Estos métodos son sencillos, rápidos y con buena sensibilidad y especificidad, sobretodo cuando se utilizan a partir de cepas aisladas. También se han usado para la detección de resistencia directamente en muestras clínicas, obteniendo elevados valores de sensibilidad para las muestras con baciloscopia positiva [112-114].

Recientemente, se ha desarrollado un nuevo método comercial, GenoType MTBDR*sl*, para detectar resistencias a E y fármacos de segunda línea. Esta nueva técnica detecta de forma eficiente las mutaciones más comunes involucradas en las resistencias a los fármacos de segunda línea y E. Sin embargo, existen algunas mutaciones que no están incluidas en el test, dando lugar a que los resultados deban ser confirmados por otros tests de susceptibilidad, como ocurre por ejemplo para el E [115, 116].

Tal y como se ha comentado anteriormente, la técnica comercial GeneXpert MTB/RIF (Cepheid, USA) detecta simultáneamente la presencia de *M. tuberculosis* complex y resistencia a R asociada al gen *rpoB*. Recientemente, se ha evaluado esta técnica comparándose con métodos de sensibilidad fenotípica [93]. En este estudio se observó que la concordancia de la técnica GeneXpert MTB/RIF con los métodos de sensibilidad fenotípicos fue del 97.6% en los pacientes con resistencia a R y del 98.1% en los que eran sensibles a éste fármaco.

1.5.5. Pirosecuenciación

La pirosecuenciación [117] es una técnica semiautomatizada de secuenciación que se basa en la detección cuantitativa del pirofosfato que se libera al incorporarse un nucleótido durante la síntesis de DNA. El pirofosfato producido entra en una cascada enzimática, donde una sulfurilasa lo utiliza para sintetizar ATP, que le permite a la luciferasa producir luz al metabolizar la luciferina a oxiluciferina. La luz emitida, que es proporcional al número de nucleótidos que se incorporan se representa en forma de pirogramas (**Figura 12**).

Esta tecnología ha sido utilizada para la identificación, la detección de resistencias a los antibióticos y la tipificación de numerosos microorganismos. Existen algunos estudios que demuestran la utilidad de la pirosecuenciación para la identificación de diferentes especies de micobacterias y la detección de resistencia a R, H, E y fluoroquinolonas tanto a partir de cepas de *M. tuberculosis* como de muestras clínicas directas [118-120].

La pirosecuenciación puede ser una alternativa real a las técnicas actuales, que nos permita detectar la presencia de micobacterias, identificarlas y también detectar mutaciones más asociadas a la resistencia a los fármacos antituberculosos a partir de muestra directa. Además, puede ayudarnos a reducir los retrasos diagnósticos y a mejorar el manejo y tratamiento de la TB multiresistente.

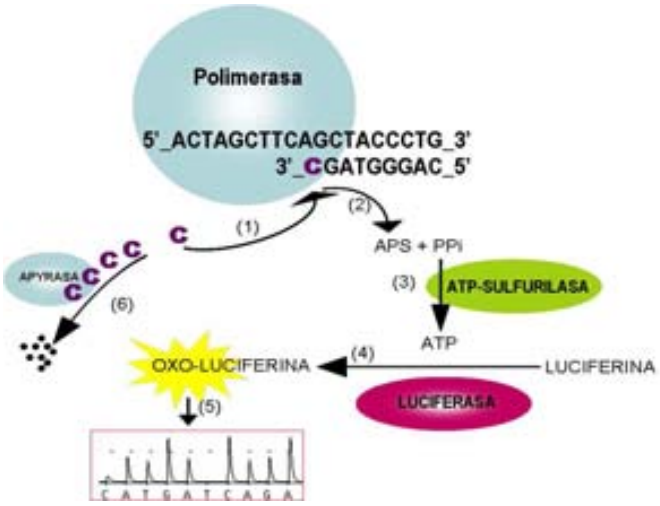

Figura 12. Esquema del fundamento de la técnica de pirosecuenciación. Adaptada de: J. Domínguez, et al. Utilidad de la biología molecular en el diagnóstico microbiológico de las infecciones por micobacterias. *Enferm Infecc Microbiol Clin*. 2008;26:33-41.

1.6. Técnicas basadas en la producción de IFN-γ

1.6.1. Fundamento de las técnicas *in vitro*

Un método de inmunodiagnóstico basado en la cuantificación *in vitro* de la respuesta inmune celular puede ser una alternativa a la PT para identificar la infección tuberculosa y diagnosticar la TB activa. En este sentido, han sido desarrollados diferentes métodos de cuantificación de esta respuesta inmune celular utilizando diferentes antígenos micobacterianos para la estimulación de las células T sensibilizadas y para la detección *in vitro* de la liberación de IFN-γ. Fundamentalmente

se han basado en técnicas de ELISA y en técnicas de *Enzyme-Linked Immunospot Assay* (ELISPOT). La tecnología consiste en una estimulación *in vitro* de los linfocitos con antígenos micobacterianos, seguido de una detección del IFN-γ producido mediante técnica inmunológica [121] (**Figura 13**).

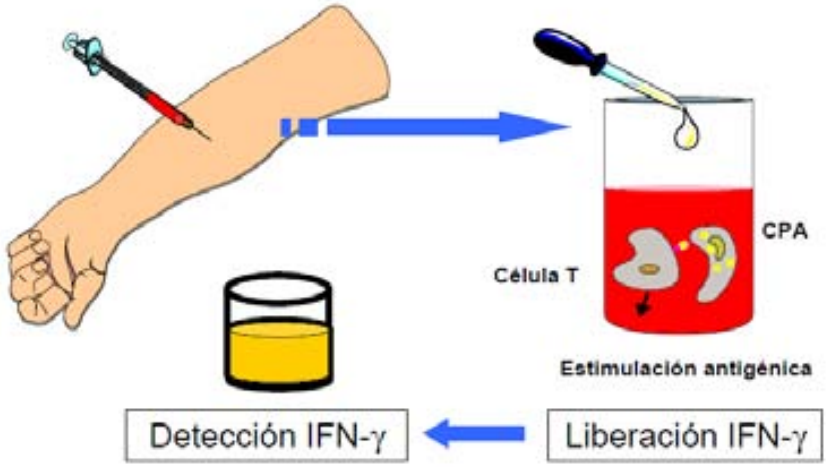

Figura 13. Esquema del fundamento del inmunodiagnóstico basado en la cuantificación *in vitro* de la respuesta inmune celular. *Figura cedida por: Cellestis Limited (Australia).*

1.6.2. Antígenos empleados en la estimulación de las células T

El éxito de esta tecnología depende, entre otros factores, de los antígenos que se emplean en la estimulación. Las técnicas preliminares basadas en la detección de IFN-γ estimulaban los linfocitos con PPD. Sin embargo, los resultados que se obtuvieron eran similares a los que se obtenían con la PT, mostrándose reacciones cruzadas con la vacuna de la BCG. Estas técnicas han sido reemplazadas por nuevas versiones que utilizan antígenos específicos de *M. tuberculosis*.

La secuenciación del genoma de *M. tuberculosis* ha permitido identificar regiones de diferencia (RD) presentes en *M. tuberculosis* pero ausentes en la vacuna BCG y en otras MNT. Estas RD son: *Early Secretory Antigen Target-6* (ESAT-6) y *Culture Filtrate Protein 10* (CFP-10) en la RD1 (**Figura 14**) y la proteína recombinante Rv2654 (TB7.7) en la RD11.

Los genes de la región RD1 están implicados en la secreción de los antígenos ESAT-6 y CFP-10 (**Figura 15**). Las proteínas ESAT-6 y CFP-10 juegan un papel muy importante en la virulencia e inmunidad protectora de *M. tuberculosis*. Estas proteínas son secretadas y son esenciales en la unión específica a la superficie de las células.

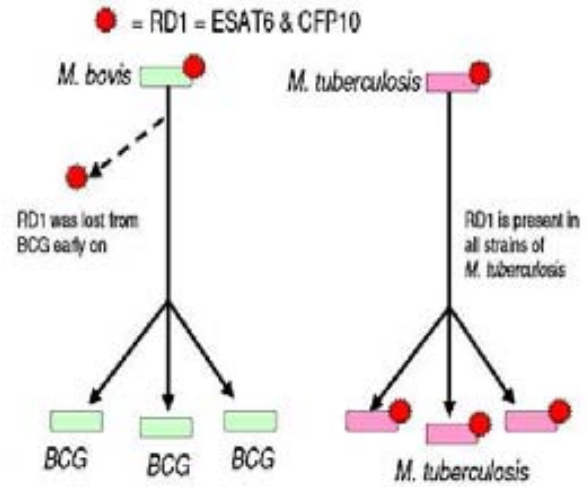

Figura 14. Las proteínas ESAT-6 y CFP-10 están ausentes en la cepa vacunal de la BCG y la mayoría de MNT. Imagen cedida por: A. Lalvani.

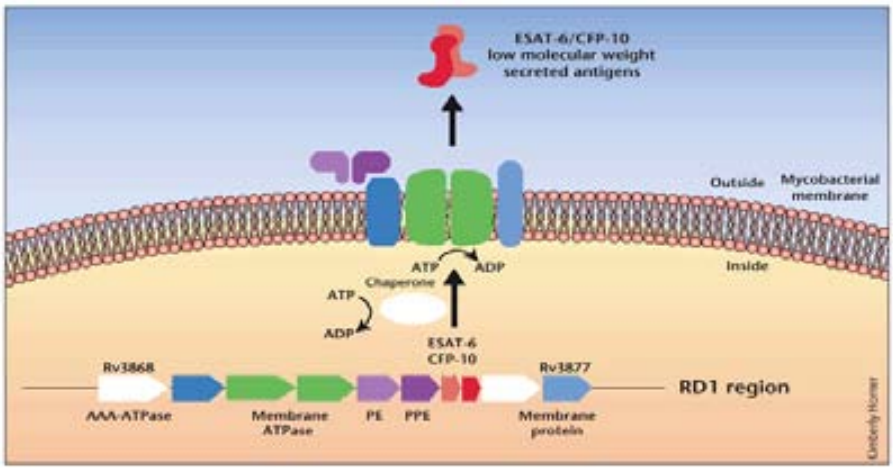

Figura 15. Las proteínas de bajo peso molecular ESAT-6 y CFP-10 son secretadas por *M. tuberculosis*. De: www.medscape.com.

La selección de estos antígenos específicos codificados en estas RD ha permitido desarrollar técnicas con una mejor especificidad que la tuberculina [122-124].

1.6.3. Técnicas *in vitro* estandarizadas

En base a esta tecnología se han estandarizado dos técnicas disponibles comercialmente: QuantiFERON-TB Gold In-Tube (QFN-G-IT) (Cellestis. Australia) y T-SPOT.TB (Oxford Immunotec. Reino Unido). Ambas técnicas, aprobadas por la FDA, se basan en la estimulación de los linfocitos específicos mediante ESAT-6, CFP-10 y/o

TB7.7, con la posterior detección de la producción de IFN-γ. QFN-G-IT estimula los linfocitos presentes en muestras de sangre total con los antígenos ESAT-6, CFP-10 y TB7.7 de forma conjunta y determina la producción de IFN-γ mediante técnica de ELISA, mientras que T-SPOT.TB requiere una separación previa de las células mononucleares de sangre periférica (PBMCs) para su estimulación con los antígenos ESAT-6 y CFP-10 de forma individual, y determina la presencia de IFN-γ mediante ELISPOT (**Figura 16**). Ambas técnicas incluyen un control positivo (fitohemaglutinina) que permite detectar los casos de anergia, lo cual es especialmente útil en pacientes inmunodeprimidos cuya respuesta está disminuida.

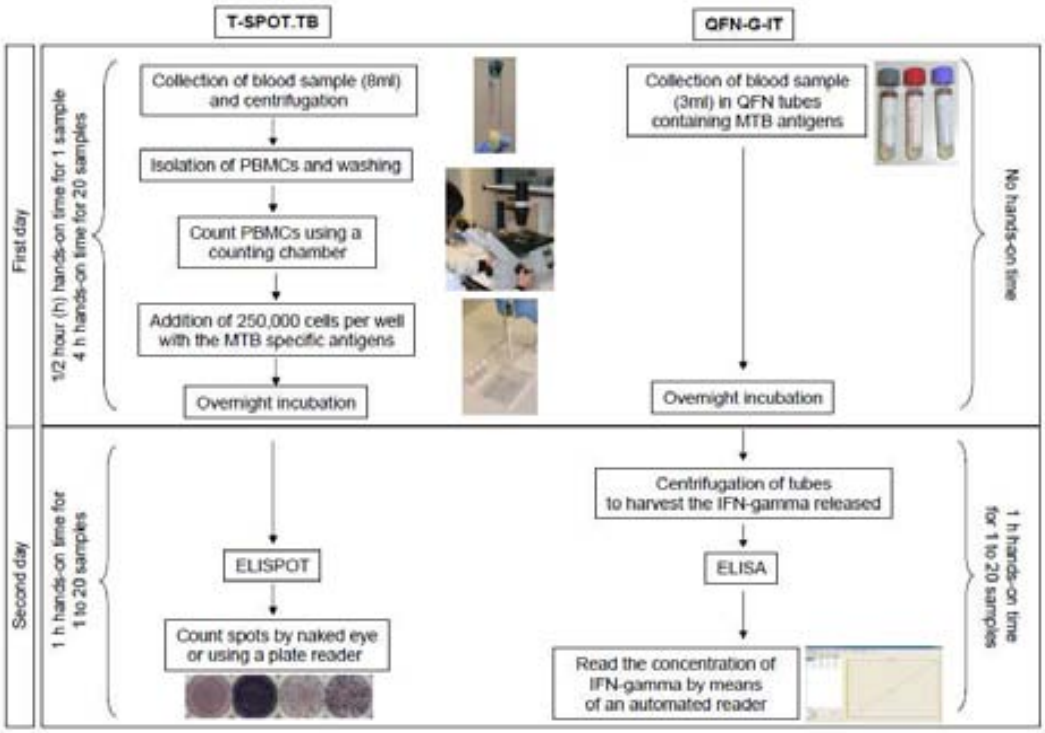

Figura 16. Esquema comparativo de las técnicas T-SPOT.TB y QFN-G-IT. De: J. Domínguez, et al. IFN-γ release assays to diagnose TB infection in the immunocompromised individual. *Expert Rev Resp Med.* 2009;3:309-327.

Varias guías internacionales recomiendan el uso de las técnicas basadas en la detección de IFN-γ. El *Center for Disease Control* (USA) recomienda su uso en las mismas circunstancias en las que se usaría la PT [125]. Por el contrario, el *National Institute for Health and Clinical Excellence* (United Kingdom) propone realizar las técnicas *in vitro* como primer paso diagnóstico en pacientes con riesgo de ser falsos negativos en la PT, o para confirmar los resultados de pacientes con PT positiva [126].

En España se recomienda un algoritmo de utilización de las técnicas de IFN-γ en combinación con la PT (**Figura 17** y **Figura 18**) [17, 127].

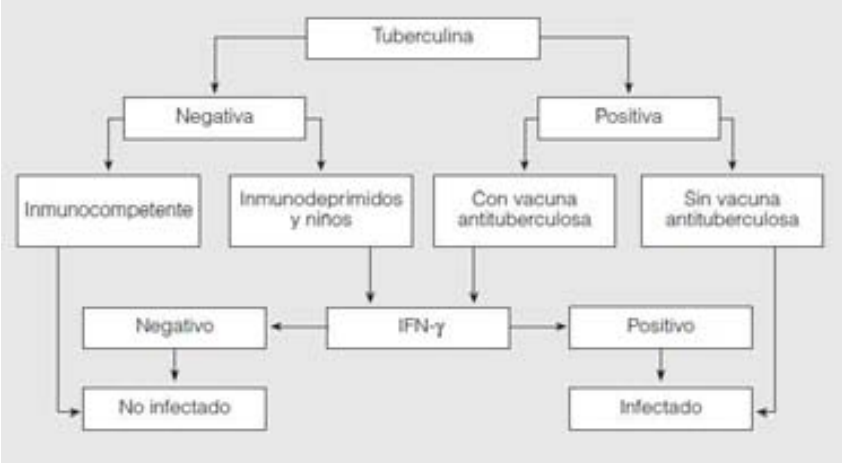

Figura 17. Algoritmo de utilización conjunta de la PT y las técnicas *in vitro* de IFN-γ en el diagnóstico de la infección tuberculosa. De: J. Ruiz-Manzano, et al. Normativa SEPAR: Diagnóstico y tratamiento de la TB. *Arch Bronconeumol*. 2008;44:551-66.

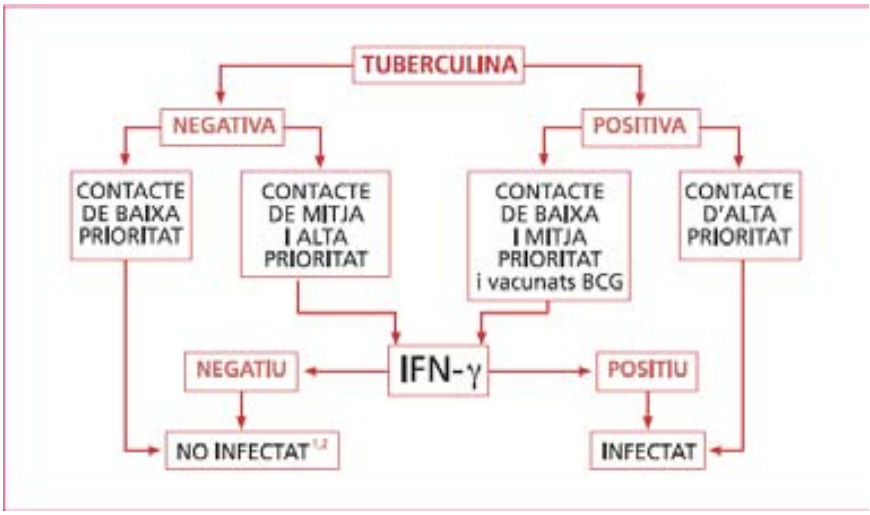

Figura 18. Algoritmo de utilización de la PT conjuntamente con las técnicas de IFN-γ. De: 2008. Guia i proposta d'organització per a la prevenció i control de la tuberculosi a la Regió Sanitària Barcelona. Generalitat de Catalunya.

En los últimos años se han realizado diversos estudios que han comparado las técnicas basadas en la detección de IFN-γ, sugiriendo que son más sensibles y específicas que la PT, y que correlacionan mejor el riesgo de infección con el grado de contacto con un enfermo de TB [128, 129].

El principal inconveniente de las técnicas *in vitro* es su elevado coste, superior al de la PT. De todos modos, estas técnicas son más coste-efectivas, y su implementación puede suponer una reducción en recursos sanitarios, como por ejemplo, en quimioprofilaxis innecesarias, visitas médicas y radiografías.

2. JUSTIFICACIÓN

2. JUSTIFICACIÓN

El factor esencial para el control de la expansión de la TB radica en la capacidad de diagnosticar precozmente y tratar a los individuos enfermos de forma apropiada. Los métodos microbiológicos de referencia en el diagnóstico de la TB continúan siendo el examen microscópico, el cultivo y aislamiento de *M. tuberculosis* y la detección de sus ácidos nucleicos. Sin embargo, como es bien conocido, estas técnicas actualmente disponibles son insuficientes. Por otro lado, las personas infectadas representan un peligro potencial de nuevos casos de TB. El estudio de las personas infectadas que no han enfermado permite aplicar, según los casos, medidas de prevención y evitar que estas personas desarrollen la enfermedad. Con esta medida se contribuye a romper la cadena de transmisión del microorganismo.

Para conocer si un individuo ha sido infectado por *M. tuberculosis* se estudia su respuesta de hipersensibilidad retardada frente a determinados compuestos antigénicos específicos del bacilo. Este sería el principio en que se basa la prueba de la tuberculina (PT). La tuberculina, que se obtiene del filtrado del cultivo de *M. tuberculosis* esterilizado y concentrado, actualmente está constituida por PPD. La tuberculina ha sido utilizada durante los últimos 100 años como herramienta de ayuda en el diagnóstico de la TB. Su principal inconveniente radica en que la mayoría de proteínas presentes en el PPD no son específicas de *M. tuberculosis* sino que las comparte con otras MNT. Esto provoca una disminución en la especificidad de la prueba, ya que individuos sensibilizados por exposición previa a otras micobacterias o vacunados con la BCG también responden inmunológicamente al PPD. Además, la PT presenta una baja sensibilidad en pacientes con alteraciones en la inmunidad celular.

Atendiendo a la historia natural de la enfermedad, la principal vía de infección es la llegada de *M. tuberculosis* a los alvéolos pulmonares, donde es fagocitado por los macrófagos alveolares. Estos macrófagos liberan citoquinas que atraen neutrófilos, linfocitos y más macrófagos para que fagociten a los bacilos extracelulares, así como para que generen un foco inflamatorio. Los linfocitos T CD4 específicos se transforman en linfocitos Th1 bajo la influencia de la IL-12 secretada por los macrófagos. La respuesta Th1 segrega el TNF-α, que permite la llegada de más macrófagos, y de IFN-γ que activa a los que están infectados.

La citoquina efectora clave en el control de la infección micobacteriana para la activación de los macrófagos y el desarrollo de la inmunidad protectora contra *M. tuberculosis* es el IFN-γ. Por lo tanto, un método de inmunodiagnóstico basado en la cuantificación *in vitro* de la respuesta inmune celular puede ser una alternativa a la PT para identificar la infección tuberculosa.

En este sentido se han desarrollado diferentes métodos de cuantificación de esta respuesta inmune celular utilizando diferentes antígenos micobacterianos para la estimulación de las células T sensibilizadas y para la detección *in vitro* de la liberación de IFN-γ. Fundamentalmente se han basado en técnicas de ELISA y ELISPOT, que respectivamente han dado lugar a dos técnicas disponibles comercialmente: QFN-G-IT y T-SPOT.TB. La tecnología consiste en una estimulación *in vitro* de los linfocitos con antígenos específicos micobacterianos, seguido de una detección del IFN-γ producido mediante técnica inmunológica. El éxito de esta tecnología depende entre otros factores de los antígenos que se emplean durante la estimulación. La utilización de antígenos codificados en la región RD1, como por ejemplo ESAT-6 y CFP-10, que son antígenos secretados por el complejo *M. tuberculosis* y que están ausentes en la vacuna BCG y en otras MNT, parecen tener una gran capacidad en la detección de individuos infectados por *M. tuberculosis*.

De esta forma, la Tesis se ha centrado en la estandarización y evaluación clínica de estas nuevas técnicas inmunológicas para el diagnóstico de la infección tuberculosa (estudios de contactos y cribados) y TB activa, así como su aplicabilidad en la práctica clínica en pacientes adultos y pediátricos, y el estudio del efecto de las MNT sobre la positividad de la PT. Las implicaciones del uso de estas técnicas basadas en la detección de la citoquina IFN-γ en la práctica clínica supondrían principalmente una reducción en quimioprofilaxis innecesarias, ya que podrían ser más específicas que la PT, al utilizar antígenos específicos de *M. tuberculosis* en la estimulación.

A pesar de que no existe una técnica de referencia para el diagnóstico de la infección tuberculosa, es bien conocido que en pacientes inmunodeprimidos la PT tiene una sensibilidad bastante reducida en este tipo de pacientes. La PT puede dar resultados falsos negativos en pacientes con deficiencias en la inmunidad celular, como por ejemplo pacientes infectados por el VIH, debido a anergia. De este modo, otra aportación de esta Tesis es el estudio de estas técnicas *in vitro* en pacientes VIH. Esto

supondría una elevada utilidad clínica, ya que este tipo de pacientes presentan mayor riesgo de progresar hacia TB en el caso de estar infectados.

Aunque las técnicas basadas en la detección de IFN-γ liberado han sido diseñadas para el diagnóstico de la infección tuberculosa, también podrían ser de utilidad en el diagnóstico de la TB activa. De este modo, se ha estudiado la correlación de la respuesta cuantitativa de IFN-γ con el inmunodiagnóstico de la TB, carga micobacteriana y enfermedad tuberculosa.

Finalmente, la Tesis se completa con la evaluación de nuevos potenciales marcadores y el estudio de nuevos antígenos alternativos en el diagnóstico *in vitro* de la TB, y que podrían representar una mayor sensibilidad en el inmunodiagnóstico de la infección tuberculosa y la TB activa. Por otro lado, la monitorización de la eficacia del tratamiento antituberculoso es complicada en pacientes con TB, ya que no existe un marcador específico para poder valorar precozmente si el tratamiento es adecuado y si el paciente progresa correctamente. De este modo, el estudio de nuevos biomarcadores nos podría ayudar a conocer mejor la respuesta del huésped contra *M. tuberculosis* durante el tratamiento.

3. OBJETIVOS

3. OBJETIVOS

1. Evaluar la utilidad de las nuevas técnicas *in vitro* basadas en la detección del IFN-γ producido por las células T al ser estimuladas con los antígenos específicos RD1 y RD11 en la práctica clínica:

   1.1. Estudiar la sensibilidad y la especificidad de las técnicas *in vitro* en el diagnóstico de la TB activa e infección tuberculosa en comparación con la PT en población adulta y pediátrica.

   1.2. Estudiar la interferencia de la vacuna de la BCG con los resultados de las nuevas técnicas de detección de IFN-γ.

2. Evaluar si la sensibilización por MNT en población pediátrica puede ser responsable de algunas discordancias entre la positividad de la PT y la negatividad de los test basados en la detección de IFN-γ.

3. Estudiar la posible relación entre la carga micobacteriana y la respuesta inmune frente a los antígenos específicos de *M. tuberculosis*, para poder diferenciar entre infección tuberculosa y TB activa.

4. Evaluar el rendimiento de las técnicas basadas en la detección de IFN-γ en el diagnóstico de la infección tuberculosa en población infectada con el VIH, y estudiar el impacto del grado de inmunosupresión en la respuesta de las células T frente a los antígenos específicos de *M. tuberculosis*.

5. Estudiar nuevos antígenos RD1 seleccionados de las proteínas ESAT-6 y CFP-10 en pacientes con TB activa, en el momento del diagnóstico y durante la terapia tuberculosa.

6. Estudiar nuevas citoquinas liberadas por las células T, tras estimular con antígenos específicos de *M. tuberculosis,* para el diagnóstico de la infección tuberculosa y de la TB activa.

4. RESULTADOS

4. RESULTADOS

4.1. Artículo 1

Comparison of two commercially available gamma interferon blood tests for immunodiagnosis of tuberculosis.

Domínguez J, Ruiz J, De Souza-Galvão M, Latorre I, Milà C, Blanco S, Jiménez MA, Prat C, Lacoma A, Altet N, Ausina V.

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El principal inconveniente de la PT es que presenta reacción cruzada con otras MNT y también con la vacuna de la BCG. No obstante, los antígenos específicos de *M. tuberculosis* recientemente descritos, ESAT-6, CFP-10 y TB7.7, no presentan reacciones cruzadas con el bacilo vacunal de la BCG ni tampoco con la mayoría de MNT [121]. Recientemente, se han desarrollado técnicas *in vitro* que detectan el IFN-γ liberado tras estimular las células T de un paciente con estos antígenos específicos. En base a esta tecnología, se han comercializado dos técnicas llamadas T-SPOT.TB y QFN-G-IT.

De este modo, en este apartado de la Tesis se exponen los resultados obtenidos al estudiar estas dos nuevas técnicas *in vitro* basadas en la detección de IFN-γ en 492 adultos y 134 niños para el diagnóstico de la infección tuberculosa y la TB activa en la práctica clínica, comparando los resultados con los de la PT.

En nuestra experiencia, observamos que el porcentaje de resultados positivos por la técnica del T-SPOT.TB es mayor que la del QFN-G-IT, tanto en el diagnóstico de la TB activa como en el de la infección tuberculosa. Nuestros resultados también muestran que ambas técnicas *in vitro* están menos afectadas que la PT por la vacuna de la BCG, y que las concordancias entre las tres técnicas son más elevadas en población no vacunada. No obstante, en población pediátrica no vacunada con la BCG hemos obtenido una concordancia bastante baja entre la PT y las técnicas *in vitro*. El porcentaje de niños no vacunados con BCG con T-SPOT.TB y QFN-G-IT negativos y PT positiva fue del 50% y 53.3%, respectivamente.

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Comparison of Two Commercially Available Gamma Interferon Blood Tests for Immunodiagnosis of Tuberculosis[▽]

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We evaluated the T-SPOT.TB and Quantiferon-TB Gold *In tube* (QFN-G-IT) tests for diagnosing *Mycobacterium tuberculosis* infection. T-SPOT.TB was more sensitive than QFN-G-IT in diagnosing both active and latent infection. Both gamma interferon tests were unaffected by prior *Mycobacterium bovis* BCG vaccination. Among children who were not BCG vaccinated but had a positive tuberculin skin test, QFN-G-IT was negative in 53.3% of cases, and T-SPOT.TB was negative in 50% of cases.

The tuberculin skin test (TST) is used for diagnosing latent *Mycobacterium tuberculosis* infection (LTBI) (11). The biggest drawback of TST is the cross-reaction with nontuberculous mycobacteria (NTM) or with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine strains (10). The 6-kDa *Mycobacterium tuberculosis* protein early secreted antigenic target 6 (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10), encoded in the region of deletion 1 (RD1), have been described as being present in *M. tuberculosis* but not in any BCG strain or the majority of NTM strains (1).

In vitro assays for measuring gamma interferon (IFN-γ) released by T cells after RD1 antigen stimulation have been developed (7, 14, 18, 19). On the basis of this technology, the following three commercial IFN-γ tests are available: Quantiferon-TB Gold assay (QFN-Gold), Quantiferon-TB Gold *In tube* assay (QFN-G-IT; Cellestis Limited, Carnegie, Victoria, Australia), and T-SPOT.TB assay (Oxford Immunotec, Abingdon, United Kingdom). The main differences between QFN-Gold and QFN-G-IT are that, in the latter, antigens are included together in the same blood sample collection tube and, in addition, a third stimulating antigen, TB7.7 (Rv2654), is included (4). This new antigen is encoded in RD11 and is lacking in the BCG strains as well as in most common NTM strains (4).

The aim of this study was to assess the ability of the new QFN-G-IT and T-SPOT.TB tests to diagnose *M. tuberculosis* infection in clinical practice, comparing the results with those of TST.

Study population. We prospectively recruited 626 individuals between September 2004 and November 2006 who attended the Hospital Universitari Germans Trias i Pujol or the TB Control and Prevention Unit of Barcelona for ongoing studies of active TB or LTBI. We classified the adults and children enrolled in the study into the following three groups of patients: patients with etiological diagnosis of active TB at the beginning of the treatment, individuals enrolled during a contact tracing study as close contacts of patients with active pulmonary TB, and individuals studied for screening of latent TB. The main demographic characteristics of the study population are shown in Table 1. Ethics approval for this study was provided by the corresponding ethics committees.

After obtaining written informed consent from all enrolled persons, a detailed questionnaire about the possible risk factors of exposure to *M. tuberculosis* was completed by each patient. Subjects were also asked to indicate the results of any previous TST, whether they had received BCG vaccination, details of any contact with a person who had TB, any risk factors associated with human immunodeficiency virus infection, and whether they had any other medical conditions. Data were also collected from medical records of chest radiography, along with the results and dates of culture. In our study, only participants with BCG scars were considered BCG vaccinated.

TST. Two tuberculin units of purified protein derivative RT23 (Statens Serum Institut, Copenhagen, Denmark) was administered by the Mantoux method. Induration was measured after 72 h. Indurations of 5 mm or greater were considered positive (20). All purified protein derivative stimuli were placed and read by certified members of the staff who regularly perform these duties.

T-SPOT.TB. T-SPOT.TB assays were performed as described previously, using 35 overlapping peptides spanning the lengths of ESAT-6 and CFP-10 (15). The test and the interpretation of the results were performed following the manufacturer's instructions. The presence of reactive antigen-specific T cells was revealed as a spot on the well. Spots were scored manually in all cases, and in some borderline cases, scores were also obtained with the aid of an automated AID enzyme-linked immunospot assay plate reader (AID Systems, Strassberg, Germany).

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TABLE 1. Demographic characteristics and clinical details for patients in this study[b]

Variable	No. (%) of adults in group			No. (%) of children in group		
	Patients with active TB (n = 33)	Contact tracing study (n = 206)	Screening for LTBI[a] (n = 253)	Patients with active TB (n = 9)	Contact tracing study (n = 64)	Screening for LTBI[a] (n = 61)
Gender						
Female	11 (33.3)	121 (58.7)	101 (39.9)	2 (22.2)	37 (57.8)	32 (52.5)
Male	22 (66.7)	85 (41.3)	152 (66.1)	7 (77.8)	27 (42.2)	29 (47.5)
Age						
<5 yr				3 (33.3)	9 (14.1)	3 (4.9)
5–18 yr				6 (66.7)	55 (85.9)	58 (95.1)
18–35 yr	23 (69.7)	136 (66)	138 (54.5)			
35–75 yr	8 (24.2)	59 (28.6)	115 (45.4)			
>75 yr	2 (6.1)	11 (5.4)	0 (0)			
BCG vaccination						
Yes	9 (27.3)	82 (39.8)	97 (38.3)	1 (11.1)	46 (71.9)	39 (63.9)
No	24 (72.7)	124 (60.2)	156 (61.7)	8 (88.9)	18 (28.1)	22 (36.1)
Immunosuppression status						
AIDS patients	0 (0)	0 (0)	19 (7.5)	0 (0)	0 (0)	0 (0)
Treatment with systemic steroids	0 (0)	0 (0)	2 (0.8)	0 (0)	0 (0)	0 (0)
Cancer chemotherapy	0 (0)	1 (0.5)	3 (1.2)	0 (0)	0 (0)	0 (0)
None	33 (100)	205 (99.5)	229 (90.5)	9 (100)	64 (100)	61 (100)
Country of birth						
Immigrants from countries with high prevalence of TB	17 (51.5)	97 (47.1)	82 (32.4)	2 (12.5)	45 (69.8)	42 (68.9)
Autochthon Spanish population	16 (48.5)	109 (52.9)	171 (67.6)	7 (87.5)	19 (30.2)	19 (31.1)

[a] Conducted on recent immigrants from countries with a high prevalence of TB, the homeless, or school teachers enrolled during preemployment examinations.
[b] There were a total of 626 participants in the study.

QFN-G-IT. Pools of overlapping peptides representing ESAT-6, CFP-10, and TB7.7 were used as TB-specific antigens in the whole-blood IFN-γ assay. The test and the interpretation results were performed according to the manufacturer's instructions.

Statistical methods. Concordance between the tests was assessed using Cohen's kappa coefficient. We used the McNemar test to compare the proportions of indeterminate, negative, and positive results among the QFN-G-IT, T-SPOT.TB, and TST assays. The differences in function of vaccination and immunosuppression status were calculated using the nonparametric Mann-Whitney U test. Differences were considered significant when the P value was <0.05. All analyses were done with SPSS statistical software for Windows (SPSS, version 14.0; SPSS Inc., Chicago, IL).

Positive T-SPOT.TB results were obtained for 44.7% (280/

TABLE 2. T-SPOT.TB, QFN-G-IT, and TST results for different groups in adult and child populations

Test and result	Overall (n = 492)	No. (%) of adults in group			Overall (n = 134)	No. (%) of children in group		
		Patients with active TB (n = 33)	Contact tracing study (n = 206)	Screening for LTBI (n = 253)		Patients with active TB (n = 9)	Contact tracing study (n = 64)	Screening for LTBI (n = 61)
T-SPOT.TB								
Positive	229 (46.6)	30 (90.1)	90 (43.7)	109 (43.1)	51 (38.1)	6 (66.7)	32 (62.7)	13 (21.3)
Negative	259 (52.6)	2 (6.1)	114 (55.3)	143 (56.5)	80 (59.7)	1 (11.1)	31 (48.4)	48 (78.7)
Indeterminate	4 (0.8)	1 (3)	2 (1)	1 (0.4)	3 (2.2)	2 (22.2)	1 (0.7)	0 (0)
QFN-G-IT								
Positive	192 (39)	27 (81.8)	70 (33.9)	95 (37.5)	50 (37.3)	6 (66.7)	28 (43.8)	16 (26.2)
Negative	299 (60.8)	6 (18.2)	135 (65.6)	158 (62.5)	84 (62.7)	3 (33.3)	36 (56.3)	45 (73.8)
Indeterminate	1 (0.2)	0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
TST								
Positive	366 (74.4)	31 (93.9)	150 (72.8)	185 (73.1)	115 (85.8)	9 (100)	53 (82.8)	53 (86.9)
Negative	126 (25.6)	2 (6.1)	56 (27.2)	68 (26.9)	19 (14.2)	0 (0)	11 (17.2)	8 (13.1)
Indeterminate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

TABLE 3. T-SPOT.TB, QFN-G-IT, and TST positive results in diagnosing LTBI regarding BCG vaccination status

Diagnostic test	No. (%) of positive results			
	Contact tracing studies		Screening for LTBI	
	Non-BCG-vaccinated subjects (n = 142)	BCG-vaccinated subjects (n = 128)	Non-BCG-vaccinated subjects (n = 178)	BCG-vaccinated subjects (n = 136)
T-SPOT.TB	67 (47.2)	55 (43)	73 (41)	49 (36)
QFN-G-IT	57 (40.1)	41 (32)	65 (36.5)	46 (33.8)
TST	89 (62.7)	114 (89.1)	121 (68)	117 (86)

626 patients) of all individuals studied, compared with 38.7% for QFN-G-IT (242/626 patients). T-SPOT.TB produced significantly more positive results than did QFN-G-IT ($P < 0.001$). TST was positive in 76.8% of cases. The number of positive results obtained by TST was significantly higher than those obtained by both IFN-γ tests ($P < 0.001$). The agreement between QFN-G-IT and T-SPOT.TB was 83.2% (521/626 samples) (κ = 0.66; standard error, 0.029). The overall agreement between T-SPOT.TB and TST was 64.5% (404/626 samples) (κ = 0.34; standard error, 0.029), and that between QFN-G-IT and TST was 58.1% (κ = 0.26; standard error, 0.026). In Table 2, we show the results of the tests for the different groups of patients (divided between adults and children).

Regarding BCG vaccination status, the overall differences between the results for vaccinated and nonvaccinated subjects were significant for TST ($P < 0.001$) and not significant for QFN-G-IT ($P = 0.174$) and T-SPOT.TB ($P = 0.332$). The number of positive results for each group of patients and the agreement between the tests are shown in Tables 3 and 4.

For pediatric patients, the overall agreement between both IFN-γ tests for patients diagnosed with active TB was 77.8% (7/9 patients) (κ = 0.71; standard error, 0.256) (Table 2). The overall rate of positive results for patients studied for LTBI was 36% (45/125 patients) for T-SPOT.TB, 35.2% (44/125 patients) for QFN-G-IT, and 84.8% (105/125 patients) for TST. However, the agreement rates between T-SPOT.TB and TST for nonvaccinated and BCG-vaccinated patients enrolled for LTBI diagnosis were 62.5% (25/40 patients) (κ = 0.33; standard error, 0.101) and 46.4% (39/85 patients) (κ = 0.12; standard error, 0.043), respectively; those between QFN-G-IT and TST were 57.5% (23/40 patients) (κ = 0.24; standard error, 0.101) and 42.3% (36/85 patients) (κ = 0.08; standard error, 0.044), respectively; and those between T-SPOT.TB and QFN-G-IT were 90% (36/40 patients) (κ = 0.79; standard error, 0.106) and 84.7% (72/85 patients) (κ = 0.68; standard error, 0.84), respectively. The differences in results regarding BCG vaccination were significant for TST ($P = 0.037$) and nonsignificant for T-SPOT.TB ($P = 0.752$) and QFN-G-IT ($P = 0.713$).

In our study, we found few indeterminate results; in seven cases (1.1%), the T-SPOT.TB result was indeterminate, and in another one (0.2%), the QFN-G-IT result was indeterminate (Tables 2 and 3). In our study, the indeterminate results were not obtained for immunosuppressed patients. Among the 25 immunosuppressed patients, the T-SPOT.TB assay was positive in 6 cases, the QFN-G-IT assay was positive in 8 cases, and TST was positive in 12 cases. The agreement between T-SPOT.TB and QFN-G-IT for immunosuppressed patients was 76% (19/25 patients) (κ = 0.41; standard error, 0.198).

In the last year, few studies have been published comparing T-SPOT.TB and QFN-Gold (2, 8, 12, 16). Lee et al. (16) compared T-SPOT.TB and QFN-Gold for 218 subjects (87 people with active TB and 131 people at low risk of TB). They found that T-SPOT.TB was the more sensitive test (95.4%). Kang et al. (12) found that the sensitivities for diagnosing active TB of QFN-Gold and T-SPOT.TB were 89% and 92%, respectively. In our experience, T-SPOT.TB was also a more sensitive test than QFN-G-IT.

Ferrara et al. (8) evaluated the T-SPOT.TB and QFN-Gold tests in a prospective study that enrolled 393 patients who were studied for suspected latent or active TB. They detected more indeterminate results with QFN-Gold than with T-SPOT.TB, and the indeterminate results were associated with immunosuppressive treatments for both tests. In contrast, we did not find indeterminate results to be associated with immunosuppression status. The population studied by Ferrara et al. in-

TABLE 4. Concordance and agreement (Cohen's κ coefficient) between TST, T-SPOT.TB, and QFN-G-IT results for different groups of patients

Patient group	TST vs T-SPOT.TB		TST vs QFN-G-IT		T-SPOT.TB vs QFN-G-IT	
	Concordance[a] (%)	κ (SE)	Concordance[a] (%)	κ (SE)	Concordance[a] (%)	κ (SE)
Patients with active TB	36/42 (85.7)	0.30 (0.237)	34/42 (81)	0.28 (0.159)	34/40 (85)	0.49 (0.173)
Contact tracing study						
Non-BCG-vaccinated subjects	110/142 (77.5)	0.58 (0.066)	100/142 (70.4)	0.44 (0.066)	120/142 (84.5)	0.71 (0.050)
BCG-vaccinated subjects	67/128 (52.3)	0.14 (0.046)	51/128 (39.8)	0.06 (0.036)	98/128 (76.6)	0.52 (0.073)
Overall	177/270 (65.6)	0.35 (0.046)	151/270 (55.9)	0.29 (0.040)	218/270 (80.7)	0.61 (0.047)
Screening for LTBI						
Non-BCG-vaccinated subjects	127/178 (71.3)	0.47 (0.055)	118/178 (66.3)	0.39 (0.053)	153/178 (86)	0.72 (0.054)
BCG-vaccinated subjects	64/136 (47.1)	0.12 (0.042)	61/136 (44.9)	0.11 (0.040)	117/136 (86)	0.69 (0.065)
Overall	191/314 (60.8)	0.30 (0.037)	179/314 (57)	0.25 (0.035)	270/314 (86)	0.71 (0.041)

[a] No. of patients with concordant results/total no. of patients.

cluded many immunosuppressed patients (38%), as opposed to our study, where the immunosuppressed population reached only 3.9%.

Finally, Arend et al. (2) compared the T-SPOT.TB and QFN-G-IT tests for 785 non-BCG-vaccinated adult subjects in a contact tracing study. They obtained an interassay agreement of 89.6% ($\kappa = 0.59$). In our experience, the agreement between both IFN-γ tests for non-BCG-vaccinated adults involved in contact tracing studies was also very high (84.5%; $\kappa = 0.71$).

Very few studies have been conducted on the pediatric population (5–7). Connell et al. (5) compared QFN-Gold and TST for detecting LTBI and found a low agreement between both techniques ($\kappa = 0.3$), with the IFN-γ test being negative for 70% of the 37 children with a positive TST. In our study, the agreement between the IFN-γ tests and the TST was also very low. In our experience, among children not vaccinated with BCG, QFN-G-IT was negative for 53.3% of children with a positive TST, and T-SPOT.TB was negative in 50% of cases. The percentage of positive TST results among pediatric patients as a consequence of NTM infection, as described previously, is not negligible (3). The utilization of IFN-γ tests could reduce the false diagnosis of *M. tuberculosis* infection in children with NTM infection (6).

Although further research in certain areas is required to fully elucidate the real role of IFN-γ tests in the management of *M. tuberculosis* infection (9, 13, 17), our results show enough evidence to state that IFN-γ tests are less affected by BCG vaccination than is TST and could avoid unnecessary latent tuberculosis treatment among adult and child populations.

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4.2. Artículo 2

Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis.

Latorre I, De Souza-Galvão M, Ruiz-Manzano J, Lacoma A, Prat C, Altet N, Ausina V, Domínguez J.

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Hasta el momento, se han publicado resultados prometedores con las técnicas basadas en la detección de IFN-γ en adultos y niños [130-135]. Sin embargo, se han observado algunas discordancias entre la PT y las técnicas *in vitro* [129], sobretodo en niños no vacunados con la BCG que tienen PT positiva y técnicas de IFN-γ negativas. En nuestra experiencia [131], hemos observado que un elevado porcentaje de niños que no estaban vacunados con la BCG y que presentaban una PT positiva, el T-SPOT.TB fue negativo.

En este apartado del trabajo, hemos determinado el papel que juega la sensibilización por MNT en las discordancias entre la PT y la técnica de T-SPOT.TB. Se estudiaron retrospectivamente 21 niños con una PT positiva y un T-SPOT.TB negativo, que no estaban vacunados con la BCG, y se detectó la presencia de células sensibilizadas frente a MNT estimulando con sensitinas de *M. avium*.

De los 21 niños, en 10 (47.6%) casos se obtuvo un resultado positivo tras estimular con sensitinas de *M. avium*. De los 10 niños en que se obtuvo un resultado positivo tras estimular con sensitinas de *M. avium*, 8 fueron incluidos durante estudios de cribado de infección tuberculosa, mientras que solamente 2 de ellos pertenecían a estudios de contactos. Respecto a las induraciones de la PT de estos 10 niños, en 8 (80%) casos ésta resultó estar entre 5 y 10 mm.

Nuestros resultados evidencian que una sensibilización previa en niños por MNT puede inducir un resultado falso positivo en la PT en el diagnóstico de la infección tuberculosa.

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Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis

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ABSTRACT: The aim of the present study was to determine the role of previous non-tuberculous mycobacteria sensitisation in children as a factor of discordant results between tuberculin skin test (TST) and an *in vitro* T-cell based assay (T-SPOT.TB; Oxford Immunotec, Oxford, UK).

We enrolled 21 non-bacille Calmette-Guérin-vaccinated paediatric patients for suspicious of latent tuberculosis infection (LTBI). These patients yielded a positive TST and a negative T-SPOT.TB. Cells were stimulated with *Mycobacterium avium* sensitin (having cross-reaction with *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*) and the presence of reactive T-cells was determined by an *ex vivo* ELISPOT.

From the 21 patients, in 10 cases (47.6%), we obtained a positive ELISPOT result after stimulation with *M. avium* sensitin, in six (28.6%) cases, the result was negative and in the remaining five (23.8%) cases, the result was indeterminate.

In conclusion, previous non-tuberculous mycobacteria sensitisation induces false-positive results in the TST for diagnosing LTBI and the use of γ-interferon tests could avoid unnecessary chemoprophylaxis treatment among a child population.

KEYWORDS: Childhood, ELISPOT, interferon-γ release assays, latent tuberculosis infection, *Mycobacterium avium* sensitin, non-tuberculous mycobacteria

The detection and treatment of active TB is a key strategy in the control of childhood tuberculosis (TB) [1]. Children have a high risk of progression to active TB [2]. Therefore, a rapid and specific diagnosis of latent TB infection (LTBI) is essential in preventing the progression to disease. The tuberculin skin test (TST) attempts to measure cell-mediated immunity in the form of a delayed-type hypersensitivity response to the purified protein derivative (PPD) [3]. The biggest drawback of TST is that individuals sensitised by previous exposure to non-tuberculous mycobacteria (NTM) or vaccinated with *Mycobacterium bovis* bacilli Calmette-Guérin (BCG) respond immunologically to PPD. Consequently, unnecessary latent tuberculosis treatments are prescribed.

In vitro assays for measuring T-cell-mediated immune responses have been developed. In these assays, infected individuals are identified by the detection of γ-interferon (IFN-γ) released by the T-cells that are sensitised after being stimulated with the specific *Mycobacterium tuberculosis* (MTB) antigens of region of deletion (RD) 1 (early-secreted antigenic target protein (ESAT)-6 and 10-kD culture filtrate protein (CFP)-10) [4, 5]. Promising results from these diagnostic tests in both adults and children have been published [6–11].

However, there are several discordant results between the IFN-γ tests and the TST [12]. One of the more challenging correct interpretations remains in the instance of positive TST and negative IFN-γ results in non-BCG vaccinated children. In our experience [7], among unvaccinated children with a positive TST, the T-SPOT.TB result was negative in 56.6% of the cases.

The aim of the present study was to determine the role of previous NTM sensitisation in children as a factor of discordant results between TST and the T-SPOT.TB test.

MATERIAL AND METHODS

Patients and inclusion criteria

We retrospectively enrolled a total of 21 paediatric patients, who attended Hospital Universitari Germans Trias i Pujol (Badalona, Spain) or TB Control and Prevention Unit of Barcelona (CAP

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Drassanes, Barcalona, Spain) for suspicion of LTBI. These patients were enrolled for contact tracing studies or for screening of LTBI. Inclusion criteria for this selected population were a positive TST, a negative T-SPOT.TB, non-BCG vaccination and no more than 2 weeks of chemoprophylaxis when blood sampling. None of the children presented lymphadenitis at the time of inclusion. We have also included control groups to validate the methodology and the results: 11 children with both TST and T-SPOT.TB negative results, and six individuals with microbiologically confirmed *M. avium* infection (four lymphadenitis and two respiratory infections). Another additional group of 10 children with both TST- and T-SPOT.TB-positive results was included in order to know the background of *M. tuberculosis* and *M. avium* sensitisation in the population. The main demographic characteristics of the groups included in the study are shown in table 1.

Ethics approval for this study was provided by the corresponding Ethics Committees. We obtained written informed consent from all parents before blood sampling. A detailed questionnaire from all patients was completed to indicate the results of any previous TST, BCG vaccination status, details of any contact with a person diagnosed of active TB, history of prior active TB, LTBI and HIV infection, chest radiography and other medical conditions.

TST

Two intradermal tuberculin units of PPD RT23 Tween 80 (Statens Serum Institut, Copenhagen, Denmark) were used to perform TST. The tuberculin was administered using Mantoux method, and the size of the induration was interpreted after 48–72 h by trained personnel. In this study, TST indurations $\geqslant$5 mm were classified as positive [13].

Detection of T-cell sensitised against MTB specific antigens

Peripheral blood mononuclear cells (PBMCs) were stimulated with ESAT-6 and CFP-10 antigens individually. The presence of reactive antigen-specific T-cells was revealed by ELISPOT (T-SPOT.TB; Oxford Immunotec, Oxford, UK). The test was performed in accordance with the manufacturer's instructions. Unstimulated cells were washed with RPMI medium (Invitrogen, Auckland, New Zealand) and resuspended in freeze medium (80% RPMI and 20% free bovine serum (PAA Laboratories GmbH, Pasching, Austria)), adding dropwise 10% DMSO (Merck, Darmstadt, Germany) and frozen at -80°C.

TABLE 1 Demographic characteristics of patients studied

Variable	Study group	Control groups		
		TST and T-SPOT.TB negatives	TST and T-SPOT.TB positives	Microbiologically confirmed *M. avium* infection
Subjects	21	11	10	6
Sex				
Male	10 (47.6)	3 (27.3)	6 (60)	4 (66.7)
Female	11 (52.4)	8 (72.7)	4 (40)	2 (33.3)
Age yrs	8.81±4.03	11.55±4.52	10±3.02	17.5±20.92
BCG vaccinated				
Yes	0 (0)	5 (45.5)	3 (30)	0 (0)
No	21 (100)	6 (54.5)	7 (70)	6 (100)
Immunosupression				
Yes	0 (0)	0 (0)	0 (0)	0 (0)
No	21 (100)	11 (100)	10 (100)	6 (100)
Birth country				
Immigrants from countries with high prevalence of TB infection	5 (23.8)	6 (54.5)	3(30)	0 (0)
Residents in a non-epidemic TB country	16 (76.2)	5 (45.5)	7 (70)	6 (100)
Origin				
Contact tracing studies	6 (28.6)	8 (72.7)	10 (100)	
Screening of LTBI at school	15 (71.4)	3 (27.3)	0 (0)	
T-SPOT.TB				
Positive	0	0	10	2[#]
Negative	21	21	0	3
Indeterminate	0	0	0	1
***Ex vivo* ELISPOT *M. avium* sensitin stimulation**				
Positive	10	0	5	4
Negative	6	11	3	1[¶]
Indeterminate	5	0	2	1

Data are presented as n, n (%) or mean±SD. TST: tuberculin skin test; *M. avium*: *Mycobacterium avium*; BCG: bacilli Calmette-Guérin; TB: tuberculosis; LTBI: latent TB infection. [#]: in one case, active TB was documented 8 yrs before; [¶]: *M. avium* infection was reported 2 yrs before.

Detection of T-cell sensitised against NTM sensitin

The stimulation of the T-cells was performed using *M. avium* sensitin (Statens Serum Institute, Copenhagen, Denmark). The manufacturer informed that this sensitin has cross reaction with *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*. In order to perform *ex vivo* ELISPOT, stimulating with *M. avium* sensitin, cells were thawed and re-suspended in 10 mL of RPMI medium. Finally, cells were washed, re-suspended in AIM-V medium (Invitrogen, Auckland, New Zealand) and stimulated with medium alone (as nil control), phytohaemagglutinin (as positive control) and *M. avium* sensitin at a concentration of 10 $\mu g \cdot mL^{-1}$. Plates were incubated for 16–20 h at 37°C with 5% CO_2. Following incubation, wells were washed with PBS and incubated for 1 h at 2°C with a monoclonal antibody to IFN-γ conjugated to alkaline phosphatase. The presence of reactive antigen-specific T-cells was revealed as a spot in the well.

Interpretation of the results

Spots were scored using an automated ELISPOT plate reader (Lector AID Elispots; Autoimmun Diagnostiks GmbH, Germany). All readings were also manually verified. The results of the assays were expressed as ESAT-6, CFP-10 and *M. avium* sensitin specific responder cells per million PBMCs. Test wells were scored as positive if the number of responder cells per million PBMCs minus their number in the control negative was >24. The result of the assay was considered indeterminate if the number of positive control cells per million PBMCs was <80, and the response to both of the antigen panels was negative.

RESULTS

From the 21 children with positive TST and negative T-SPOT.TB, a positive ELISPOT result after stimulation with *M. avium* sensitin was obtained in 10 (47.6%) cases. In six (28.6%) cases the result was negative and in the remaining five (23.8%) cases the result was indeterminate. The number of responder T-cells after *M. avium* sensitin stimulation was significantly higher than the number of responder T-cells after specific MTB antigens (ESAT-6 and CFP-10) stimulation: p=0.001 and p<0.001, respectively.

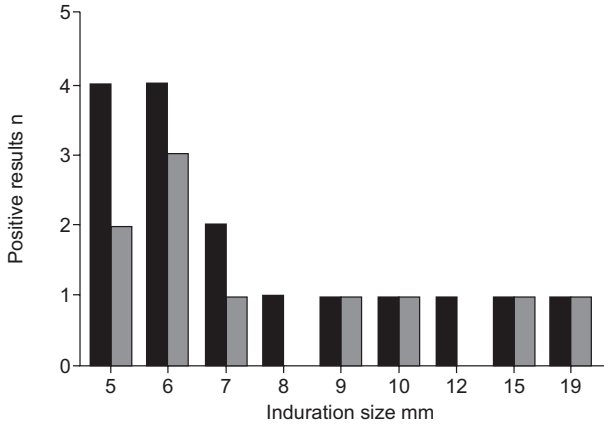

FIGURE 1. Induration size distribution of positive results of the tuberculin skin test (■) and *ex vivo Mycobacterium avium* sensitin ELISPOT (■) among the children with a valid result.

Among the 10 children that obtained a positive result after stimulation with *M. avium* sensitin, five children were aged 6–7 yrs and the other five children were aged 11–16 yrs. Additionally, eight were enrolled during LTBI screening at school and the remaining two, during a contact tracing study. Regarding the induration of the TST, eight of these children were in the range of 5–10 mm, one case was 15 mm and the other case was 19 mm (fig. 1).

In all children with both TST- and T-SPOT.TB-negative results included as controls, negative ELISPOT results after stimulation with *M. avium* sensitin were obtained. There were no significant differences between the number of responder T-cells after stimulation with ESAT-6, CFP-10 and *M. avium* sensitin. The differences in the number of responder T-cells to *M. avium* sensitin between the patients study group and this control group were significant (p=0.004) (fig. 2). In the group of individuals with microbiologically confirmed *M. avium* infection, four out of five cases with valid results, cells

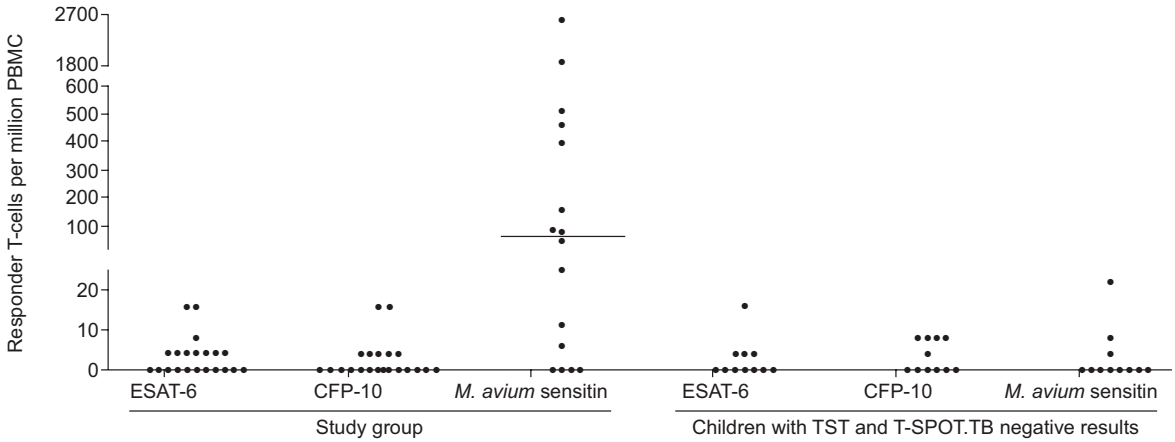

FIGURE 2. Number of responder T-cells enumerated by *ex vivo* ELISPOT after stimulation with the specific *Mycobacterium tuberculosis* antigens (early secretary antigen target (ESAT)-6 and culture filtrate protein (CFP)-10) and *Mycobacterium avium* (*M. avium*) sensitin in the study group and children with tuberculin skin test (TST)- and T-SPOT.TB-negative results. PBMC: peripheral blood mononuclear cells.

sensitised against *M. avium* were detected. The results obtained by the study and all control groups are presented in table 1. The indeterminate results were due to the low number of cells recovered after thawing.

DISCUSSION

Although specificity of IFN-γ tests is excellent because the assay is not affected by BCG vaccination [6–8], frequent discordant results with TST have been described [6, 7, 14]. In fact, it has been recommended for priority research to obtain data to understand discordant TST and IFN-γ tests results, including the role of NTM [12]. To date, the effect of NTM on IFN-γ tests results has been poorly studied. In this sense, we have studied the effect of previous NTM sensitisation to try to give an explanation for the discordant results of positive TST and negative IFN-γ results in non-BCG vaccinated children. Among the 16 children with a valid result, 10 (62.5%) children had a specific response of T-cells after stimulation with *M. avium* sensitin.

It has been described that asymptomatic infections with *M. avium* and other NTM are common [15] and probably acquired in childhood [16–19]. In our area, the estimation of NTM infection in children with a positive TST (5–10 mm) ranged 20–50% [16]. According with our results, using the *ex vivo* ELISPOT, eight (80%) of the 10 children reactive against *M. avium* sensitin had a positive TST between 5 and 10 mm, and nine (90%) of them between 5 and 15 mm. Indeed, in the children control group with TST- and T-SPOT.TB-positive results, the presence of T-cell sensitised against *M. avium* was detected in five out of the eight cases with valid result.

In our study, eight out of 10 children with a positive *M. avium* sensitin T-cell assay from our study group were enrolled from a routine screening of LTBI without known exposure to any active TB patient. Given that NTM infection affects the TST reading, it is in this group of children where IFN-γ tests could be used to confirm the diagnosis in case of a positive TST result.

Regarding the six remaining discordant results without T-cell response after *M. avium* sensitin stimulation, there are three possible explanations. First, a real LTBI not detected by the IFN-γ test. Nevertheless, the sensitivity of the IFN-γ tests is considered to be higher than the TST, or at least at the same level. Secondly, the IFN-γ test enumerates effectors T-cells that have recently been in contact with the antigen, in contrast, TST remains positive a long period after past *M. tuberculosis* infection [20]. However, in children the infection is usually recent. The third explanation is that the positive TST was due to a previous infection by a NTM without *M. avium* sensitin cross-reaction. It was impossible to test more NTM sensitins given that we didn't have more PBMCs stored from these patients.

One limitation of our study is that the skin test reactions to *M. avium* sensitin were not performed at the moment of inclusion of the children; therefore, it was not possible to correlate with the *ex vivo* result. Another limitation is that we have tested a reduced number of children. Nevertheless, despite these limitations, the results obtained are sufficiently consistent to draw some conclusions.

In summary, our results show enough evidence to state that previous NTM sensitisation in children induces false-positive results in the TST for diagnosing LTBI and that the IFN-γ tests could avoid both unnecessary chemoprophylaxis treatment among child populations and consuming resources searching the index case.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

A statement of interest for J. Domínguez can be found at www.erj.ersjournals.com/misc/statements.dtl

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Discordance between TSTs and IFN-γ release assays: the role of NTM and the relevance of mycobacterial sensitins

To the Editors:

We read with interest the recent study by LATORRE *et al.* [1] entitled ''Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis''. We agree that discordance between tuberculin skin tests (TSTs) and interferon (IFN)-γ release assays (IGRAs) presents physicians with a considerable management dilemma when evaluating children for latent tuberculosis (TB) infection (LTBI) in routine clinical practice. We have previously urged caution in the interpretation of discordant results and have highlighted this area as a research priority [2, 3]. We therefore commend the authors for investigating a potential underlying cause of discordance. However, we believe that the interpretation of the data presented by LATORRE *et al.* [1] is based on erroneous assumptions, and that as a result the conclusions are overstated. We suggest that a more cautious and contextualised interpretation of the study findings is warranted.

As indicated by LATORRE *et al.* [1], previous bacille Calmette–Guérin immunisation and exposure to non-tuberculous mycobacteria (NTM) are frequently cited as the primary factors underlying discordance between TSTs and IGRAs, although convincing data to support these concepts are currently lacking. In the study by LATORRE *et al.* [1], children with suspected LTBI were assessed with a TST, a commercial IFN-γ ELISpot assay (the T.SPOT.*TB* assay, incorporating early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10)) and an in-house IFN-γ ELISpot assay using *Mycobacterium avium* sensitin (MAS) as the stimulating antigen. In the subgroup of children that had TST+/T.SPOT.*TB*- discordance, 47.6% showed a ''positive'' response in the IFN-γ ELISpot using MAS as the stimulant (contrasting with the absence of response to ESAT-6 and CFP-10). The authors interpret this observation as evidence that previous NTM sensitisation in these children resulted in a false-positive TST result and thereby discordance.

While we agree that this is one possible explanation, there is an alternative explanation that would equally account for these observations. Importantly, significant cross-reactivity between different mycobacterial sensitins has been previously consistently shown in animal models [4]. Furthermore, more than a decade ago, LEIN *et al.* [5] convincingly demonstrated that T cell assays incorporating MAS cannot reliably distinguish between *M. tuberculosis* and *M. avium* complex (MAC) infection in humans. In that study, the authors used the same MAS preparation as LATORRE *et al.* [1] to assess T-cell responses in adults with culture-confirmed TB (n=27) or MAC (n=10) infection. Somewhat unexpectedly, higher mean IFN-γ concentrations were observed in supernatants from peripheral blood mononuclear cells stimulated with MAS in patients with TB than those with MAC infection. In addition, MAS-sensitised T cells were detected in the majority of patients with TB. These data strongly suggest that there is considerable cross-reactivity between antigens encountered by the human immune system during *M. tuberculosis* infection and antigens contained in MAS. We also note that in the study by LATORRE *et al.* [1], in the subgroup of children that were TST+/T.SPOT.*TB*+ (and therefore highly likely to have LTBI), 50% showed a ''positive'' response to MAS in the in-house IFN-γ ELISpot assay, which further questions the ability of MAS-based assays to discriminate between TB and NTM infection, or alternatively exposure.

The limited ability of MAS to distinguish between different mycobacterial infections is not surprising. Unlike the well-defined peptides ESAT-6 and CFP-10, which are thought to be relatively *M. tuberculosis*-specific (despite orthologues of these proteins being present in several other mycobacterial species including *M. kansasii*, *M. marinum* and *M. szulgai*), MAS is a mixture of heterogenous mycobacterial antigens, analogous to the purified protein derivative used in the TST [6]. Cross-reactivity with other mycobacterial species is therefore likely to occur, as indicated by the manufacturer's warning mentioned by LATORRE *et al.* [1], that is particularly likely to be the case with *M. intracellulare* and *M. scrofulaceum*.

Taken together, these facts make it questionable whether the observations by LATORRE *et al.* [1] in the subgroup of children with TST+/T.SPOT.*TB*- discordance truly reflect previous NTM exposure. An alternative explanation is that the assays using MAS detected T-cell sensitisation resulting from previous *M. tuberculosis* exposure and/or LTBI (*i.e.* confirming the positive TST), while the T.SPOT.*TB* produced a false-negative result. Published data show that up to one-third of children with culture-proven active TB have negative or indeterminate T.SPOT.*TB* assay results [7], which highlights the limitations of these assays and lends support to the latter explanation. In the absence of a gold standard for LTBI, neither hypothesis can be tested with certainty. However, given these uncertainties we believe it is premature of the authors to suggest that chemoprophylaxis could be safely withheld in these patients. Contrary to the authors' assertions, we believe their study does not provide ''enough evidence'' to justify changes in clinical practice.

We concur with LATORRE *et al.* [1] that there remains an urgent need to explore the immunology of underlying discordance between TSTs and IGRAs in greater detail. However, in view of the comparatively poor performance of IGRAs in children and the uncertainties surrounding their interpretation we, and other researchers in this field, firmly believe that research to identify better biomarkers and immunological correlates of TB infection remains crucial [3].

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Statement of Interest: None declared.

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From the authors:

We do appreciate the comments by M. Tebruegge and co-workers about our recently published manuscript in the *European Respiratory Journal* [1]. The aim of this reply is to clarify some points in order to interpret better the results of the study, given that we think there were some misunderstandings.

In vitro assays for measuring interferon (IFN)-γ released by the T-cells after specific *Mycobacterium tuberculosis* stimulation have demonstrated promising results in adults and also in children for diagnosing tuberculosis (TB) infection [2, 3]. However, there are discordant results between IFN-γ based assays and the tuberculin skin test (TST) that require clarification in order to assess the real utility of the *in vitro* tests in the management of patients [2, 4].

In our study we determined the potential role of non-tuberculous mycobacteria (NTM) sensitisation in children as a factor of discordant results between TST and an *in vitro* T-cell based assay (T.SPOT.*TB*; Oxford Immunotec, Oxford, UK). We enrolled 21 non-bacille Calmette–Guérin vaccinated paediatric patients for suspicion of latent TB infection (LTBI). These patients yielded a positive TST and a negative T.SPOT.*TB*. Cells were stimulated with *Mycobacterium avium* sensitin (MAS) and the presence of reactive T-cells was determined by an *ex vivo* enzyme-linked immunospot assay. From the 16 patients with a valid result, in 10 cases we obtained a positive ELISPOT result after stimulation with MAS.

Our main disagreement with the argumentation by M. Tebruegge and co-workers resides in the fact that we are not using MAS for distinguishing *M. tuberculosis* from NTM infection. For this objective, we used the specific *M. tuberculosis* RD1 antigens included in the T.SPOT.*TB* test, and, as no response against RD1 antigens was obtained, we assessed T-cell sensitisation against MAS antigens to investigate if NTM sensitisation could be responsible for TST positivity. Indeed, LEIN *et al*. [5], also referred to in the letter by M. Tebruegge and co-workers, obtained significant immune responses to ESAT-6 from 59% of pulmonary *M. tuberculosis* disease patients diagnosed, but no response was obtained from patients with *M. avium* complex pulmonary disease.

However, we agree with M. Tebruegge and co-workers that in some cases alternative explanation can also be possible. Given that MAS are not totally specific, and cross-reactions with other mycobacteria species have been described, we cannot totally exclude the possibility that we are detecting, in some cases, a response of specific T-cells against some *M. tuberculosis* antigens different from ESAT-6 and CFP-10; or a false-negative result of the T.SPOT.*TB*.

On the one hand, M. Tebruegge and co-workers have shown some concerns about our group of children with positive TST and positive T.SPOT.*TB* where 50% of children responded to the MAS. The results are in concordance with the known cross-reaction between MAS and other mycobacteria. Nevertheless, we cannot totally reject simultaneous infection of *M. tuberculosis* and NTM. Furthermore, these results are in total agreement with those obtained by LEIN *et al*. [5], where they found response against MAS in 24 out of 27 *M. tuberculosis* disease patients.

On the other hand, we want to point out that the main MAS positive results were obtained in children enrolled during LTBI screening at school with TST induration >5 mm and <10 mm. In all these children a complete medical exploration, including clinical and radiographic studies, was performed, and active TB was excluded. In the subsequent contact tracing studies no index case was found. Based on the classical studies performed by NYBOE [6], the main guidelines in this kind of child population consider as a cut-off for *M. tuberculosis* infection a TST induration ⩾10 mm, in order to avoid false-positive TST results induced by NTM immunisation [7]. Nevertheless, indurations >15 mm [8] and 20 mm [9] have been reported in children with NTM infections. Therefore, our results reinforce, in part, the guidelines in that unnecessary chemoprophylaxis treatment in this unexposed population could be avoided, and that IFN-γ based assays could help to confirm a positive TST result.

Children from contact-tracing studies truly exposed to an active TB case merit special consideration as they can develop the disease very quickly after primary infection, with the most severe forms prevailing in younger children [10]. For this child population we did not recommend withholding the chemoprophylaxis;

but we stated that, according to our results, IFN-γ based assays could reduce unnecessary chemoprophylaxis in non-*M. tuberculosis* infected children. In fact, BAKIR *et al.* [11] in a recent study concluded that a positive IFN-γ based assay result predicted the development of active TB as well as the TST, allowing more focused preventive therapy to fewer contacts.

In conclusion, we believe our results provide enough evidence that previous NTM sensitisation induces false-positive results in the TST for diagnosing LTBI; but, we also strongly agree with TEBRUEGGE *et al.* that additional studies are needed in order to clarify different issues related to the discordant IFN-γ based assay results, and to assess the real utility in the management and benefit of a child population.

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Statement of Interest: A statement of interest for J. Domínguez can be found at www.erj.ersjournals.com/misc/statements.dtl

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Complete smoking cessation is beneficial in older and more advanced COPD patients

To the Editors:

We read with great interest the article by TASHKIN *et al.* [1] in a recent issue of the *European Respiratory Journal* evaluating effects of smoking status on long-term responses to maintenance bronchodilator therapy in the Understanding Potential Long-term Impacts on Function with Tiotropium (UPLIFT®) trial. The UPLIFT® trial [2] is a recent investigation in a long series of clinical trials assessing, among other things, the effects of different drugs on long-term forced expiratory volume in 1 s (FEV1) decline in patients with COPD, a ''holy grail'' of the pulmonological community.

As tobacco smoking is the most frequent risk factor for COPD, researchers in the UPLIFT® trial paid the utmost attention to smoking status of the investigated cohort. They registered smoking status at inclusion, offered smoking cessation to every smoking patient before entry and checked smoking status at each follow-up visit during the 4 yrs of study. Study participants were classified into three subgroups: continuing current smokers (CS), continuing ex-smokers (CE) and intermittent smokers (IS).

The authors concentrate on analysis of effects of tiotropium in relation to smoking status on bronchodilation, exacerbation

4.3. Artículo 3

Quantitative evaluation of T-cell response after specific antigen stimulation in active and latent tuberculosis infection in adults and children.

Latorre I, De Souza-Galvão M, Ruiz-Manzano J, Lacoma A, Prat C, Fuenzalida L, Altet N, Ausina V, Domínguez J.

Diagnostic Microbiology and Infectious Diseases. 2009. 65:236-246.

Las técnicas basadas en la detección de IFN-γ no pueden distinguir entre infección tuberculosa y enfermedad. Sin embargo, se ha hipotetizado que la magnitud de la respuesta de IFN-γ podría ayudarnos a discriminar entre TB activa e infección tuberculosa.

El objetivo de este trabajo, fue evaluar la cuantificación de la respuesta de las células T a los antígenos específicos de *M. tuberculosis*, ESAT-6; CFP-10 y TB7.7, para poder diferenciar entre infección tuberculosa y TB activa en población adulta y pediátrica mediante las técnicas basadas en la detección de IFN-γ. Y en segundo lugar, correlacionar esta respuesta cuantitativa de IFN-γ con el inmunodiagnóstico de la TB, carga micobacteriana y enfermedad tuberculosa.

La respuesta de IFN-γ de las células T tras estimular con los antígenos ESAT-6, CFP-10 y RD1 fue mayor en los pacientes con TB activa. Nuestros resultados muestran que la respuesta al antígeno CFP-10 podría ser un marcador específico de TB activa. En nuestro estudio observamos que aunque existe solapamiento, un recuento de células T reactivas (tras estimular con los antígenos RD1 con la técnica de ELISPOT) en pacientes con elevada sospecha clínica de TB por encima del punto de corte descrito, podría sugerir TB activa. El punto de corte propuesto es de 116 células, obteniendo una sensibilidad del 43% y una especificidad del 81%.

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Quantitative evaluation of T-cell response after specific antigen stimulation in active and latent tuberculosis infection in adults and children☆

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Abstract

We have evaluated the quantitative T-cell response after specific *Mycobacterium tuberculosis* antigen stimulation in active tuberculosis (TB) and latent TB infection (LTBI) patients. In adults, the median number of T cells after RD1 antigen stimulation was significantly higher in active TB patients than in LTBI patients. In children, the number of responder T cells against the specific antigens was higher in active TB than in LTBI patients, although the differences were not significant. In summary, in patients with suspected clinical TB, although there is overlapping in the number of responder T cells between both groups, a T-cell count above the described threshold could suggest active TB, especially in patients with a high probability of having active TB and low probability of having LTBI. In addition, the results are consistent with the current evidence that T-cell response may indicate mycobacterial burden and disease activity.

Keywords: IFN-γ tests; Active TB; LTBI; Children; Mycobacterial burden

1. Background

Tuberculosis (TB) remains a serious public health problem. Approximately 9 million people develop active TB, mostly pulmonary TB (pTB), annually (Dye et al., 2006). The basis of TB control programs consists of diagnosing and correcting the treatment of patients with active TB. An essential factor for controlling the spread of this disease is the ability to diagnose it in its early stages, especially in the pediatric population. The traditional tools for diagnosing clinical TB are still clinical and radiologic examination, combined with direct microscopic examination of sputum samples and culture of bacteria. Patients with pTB may be smear-negative for acid-fast bacilli, and mycobacterial culture may take several weeks. Therefore, diagnosis often occurs in an advanced stage of the disease (Dinnes et al., 2007). Although in vitro amplification of mycobacterial targeted DNA via polymerase chain reaction-based methods can provide a rapid answer, the technology is not fully standardized and is often unsuitable for routine clinical practice. Moreover, appropriate specimens are difficult to obtain in young children because of the fact that they rarely produce sputum. In addition, there is a high proportion of extrapulmonary TB that often requires invasive diagnostic methods (Liebeschuetz et al., 2004; Migliori et al., 2006; Valdes et al., 1998).

The tuberculin skin test (TST) has been, until now, the only tool available for the diagnosis of latent TB infection (LTBI) and is commonly used as a complementary test in the diagnosis of active TB (Huebner et al., 1993). Unfortunately, the disadvantage of this test is that its poor specificity can lead to false-positive results by means of a cross-reaction

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with the *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) vaccine strain and non-tuberculous mycobacteria (NTM) (Latorre et al., 2009). Moreover, TST has a low sensitivity in high-risk groups with impaired cellular immunity, such as children younger than 5 years, giving false-negative results (Horsburgh, 2004).

In the last years, 2 *Mycobacterium tuberculosis* (MTB) region of difference (RD) 1 antigens have been explained. These antigens are early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), which are absent in all BCG and in the majority of NTM strains (Andersen et al., 2000). Recently, a third specific MTB antigen, TB7.7 (Rv2654 [only peptide 4]), encoded in RD11 has also been studied (Brock et al., 2004). In vitro assays for the diagnosis of LTBI, based on the detection of interferon-γ (IFN-γ) secreted by effector T cells stimulated with these specific antigens, have been evaluated by several authors in the diagnosis of LTBI and active TB (Domínguez et al., 2009b; Ewer et al., 2003; Lalvani, 2007; Lalvani et al., 2001; Menzies et al., 2007; Mori et al., 2004; Pai et al., 2008; Richeldi, 2006).

Three commercialized in vitro assays based on this technology have been developed: *QuantiFERON-TB GOLD* (QFT-G), *QuantiFERON-TB GOLD In-Tube* (QFT-G-IT) (Cellestis, Carnegie, Australia), and T-SPOT.TB (Oxford Immunotec, Oxford, UK). QFT-G and QFT-G-IT detect IFN-γ from T cells stimulated with the specific antigens in whole blood. On the contrary, T-SPOT.TB stimulates the specific mononuclear cells isolated from peripheral blood (PBMCs). QFT-G-IT is the last version of QFT-G. The main difference between these 2 tests is that QFT-G-IT incorporates the 2 RD1-specific antigens and the TB7.7 antigen inside the same blood collection tube.

Nowadays, the IFN-γ assays for the diagnosis of LTBI cannot distinguish between active TB and LTBI. However, it has been hypothesized that the magnitude of the response may provide information such as the likelihood of transition to latency versus disease (Doherty et al., 2002). In this regard, there are 2 studies that have suggested the variation cutoff values of spot-forming units (SFUs) on T-SPOT.TB and the concentration of IFN-γ (IU/mL) on QFT-G. These studies improve the diagnostic accuracy of LTBI to distinguish active TB from LTBI (Janssens et al., 2007; Soysal et al., 2008).

The aim of this study is to evaluate the quantitative difference of *M. tuberculosis*-specific antigen T-cell response in the adult and pediatric populations by T-SPOT.TB and QFT-G-IT, to differentiate between subjects with active TB and those with LTBI and, secondly, to correlate the quantitative response of T cells with TB immunodiagnosis, mycobacterial burden, and disease activity.

2. Materials and methods

2.1. Patients and inclusion criteria

We prospectively recruited adult and pediatric patients between September 2004 and January 2008 who attended the *Hospital Universitari Germans Trias i Pujol* (Badalona, Spain) and the TB Control and Prevention Unit of Barcelona (CAP Drassanes, Barcelona, Spain) with suspected active TB or LTBI. An ethics approval for this study was provided by the Hospital Universitari *Germans Trias i Pujol* and the Fundació Gol i Gurina (Barcelona, Spain) Ethics Committees. We obtained written informed consent from all patients before taking blood samples, and in the case of the pediatric population, parents signed the written consent. A detailed questionnaire from all patients was collected to indicate the results of any previous TSTs, as well as information on BCG vaccination status, details of any contact with a person that has active TB, history of prior active TB, LTBI, and chest radiography, and other medical conditions (i.e., immunosuppression status). In our study, only participants with a BCG scar were considered BCG vaccinated. Patients diagnosed with active TB and those diagnosed with LTBI were included. Of the LTBI patients, we established 2 groups: individuals with a high risk of recent exposure (contact-tracing studies group) and individuals infected but with unknown exposure to an active TB patient. In this last group, it is not possible to eliminate the possibility that some of them were recently infected. In the case of children, the infection, by definition, should be considered as recent. In LTBI patients, the active TB was excluded by clinical and radiologic examination. None of the patients included in this study were HIV seropositive.

The inclusion criteria for all patients were a positive TST, at least 1 positive result for one of the IFN-γ tests studied, and no more than 2 weeks of therapy or chemoprophylaxis when taking blood samples.

We enrolled a total of 175 adult and 162 pediatric patients with a positive TST. Of these patients, we excluded 44 adults and 79 children from the analysis because of negative and/or indeterminate results for both IFN-γ tests. The patients included in the study were classified into 3 groups:

Group 1. Forty adult and 13 pediatric patients diagnosed with active pulmonary and extrapulmonary TB. The inclusion criteria were a positive culture for MTB for adult patients and, for pediatric population, a positive culture for MTB or suspected active clinical TB related to close contact with a smear-positive pTB patient, radiography compatible with TB, and a positive TST.

Group 2. Fifty-six adults and 52 children enrolled during contact-tracing studies as close contacts of a smear-positive pTB case.

Group 3. Thirty-five adults and 18 children examined for LTBI screening because of recent immigration from countries with a high prevalence of TB infection, homelessness, school teachers enrolled during preemployment examinations, or children enrolled during routine TB screening at school.

2.2. Tuberculin skin test

In all subjects studied for contact-tracing studies or screened for LTBI, a TST was performed. Two

intradermal tuberculin units of PPD RT23 (Statens Serum Institut, Copenhagen, Denmark) were used to perform the TST. The tuberculin was administered using the Mantoux method, and the induration size was interpreted after 48 to 72 h by trained personnel. In this study, according to the Spanish Society of Pneumology and Thoracic Surgery guidelines, TST indurations ≥5 mm were classified as positive in patients diagnosed with active TB and contacts. On the contrary, indurations ≥10 mm were considered positive in patients screened for LTBI (Ruiz-Manzano et al., 2008). All individuals with a positive TST induration were referred for chest radiography, and chemoprophylaxis was indicated in asymptomatic patients with normal chest radiography.

2.3. QuantiFERON-TB GOLD In-Tube

The assay is based on the detection of IFN-γ in whole-blood supernatant by an enzyme-linked immunosorbent assay. The test was performed according to the manufacturer's instructions. A total of 3 tubes of 1 mL each (nil control, positive control [phytohemagglutinin], and TB-specific antigens) were drawn by venipuncture from each patient.

Raw optical densities were interpreted by using specific software provided by the manufacturer. The result obtained by the nil control had to be subtracted from the positive control and the antigen-stimulated samples. The cutoff value for a positive test was at least 0.35 IU/mL of IFN-γ in the sample after stimulation with the specific antigens, regardless of the result of the positive control. The result of the test was considered indeterminate if an antigen-stimulated sample was negative and if the value of the positive control was less than 0.5 IU/mL after subtraction of the value of the nil control.

2.4. T-SPOT.TB

The assay detects IFN-γ produced by PBMCs by means of an enzyme-linked immunospot assay (ELISPOT), stimulated with ESAT-6 and CFP-10 individually. The test was performed according to the manufacturer's instructions. Eight milliliters of blood were drawn for the isolation of PBMC in a vacutainer CPT tube (Becton Dickinson Diagnostics, Franklin Lakes, NJ).

On T-SPOT.TB, spots were scored using an automated AID ELISPOT plate reader (Lector AID Elispots; Autoimmun Diagnostiks, Germany). All readings were also manually verified. Subjects were considered positive if there was a positive response to 1 or both of the antigen panels. Test wells were scored as positive if they contained at least 6 SFU cells more than the nil control well and if this number was at least twice the number of the nil control well. The assay result was considered indeterminate if the number of spots in the positive control was less than 20 and if the response to both of the antigen panels was negative. We also studied the overall RD1 response as the sum of ESAT-6 and CFP-10 T-cell enumeration.

2.5. Statistical methods

The comparison of the number of spots and the IFN-γ released was performed using the Mann–Whitney *U* test analysis. Data were expressed as mean and SD, median, and 5th and 95th percentiles. Differences were considered significant when *P* values were less than 0.05. Receiver operating characteristic (ROC) analysis was performed to determine a threshold level for differentiating between active TB and LTBI. The best threshold of SFU enumeration by T-SPOT.TB and international units per milliliter of IFN-γ by QFT-G-IT was given with an optimal specificity equal to or higher than 80%. All analyses were made with SPSS statistical software for Windows (SPSS version 15.0; SPSS, Chicago, IL). Graphical representation is based on GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

3. Results

The main demographic characteristics of adult and pediatric patients and the positive percentages of T-SPOT.TB and QFT-G-IT of the populations included in the study are shown in Table 1. The percentages of positive results in these patients were higher for T-SPOT.TB than for QFT-G-IT, except for contact-tracing studies in the pediatric population where QFT-G-IT showed a slightly higher percentage of positive results than T-SPOT-TB. Moreover, in contact-tracing studies of adult individuals and active TB pediatric patients, the differences between T-SPOT.TB and QFT-G-IT positive results were significant ($P < 0.001$ and $P = 0.046$, respectively).

3.1. Adult population results

The median numbers of T cells after ESAT-6, CFP-10, and RD1 stimulation in T-SPOT.TB were higher in active TB patients than in contact-tracing studies individuals or those screened for LTBI. In contrast, the median IFN-γ release after antigen stimulation in QFT-G-IT was higher in contacts than in the other group of patients (Table 2 and Fig. 1).

Differences in the number of T cells stimulated with ESAT-6 by T-SPOT.TB in active TB patients, contact-tracing studies, and screening of LTBI individuals were only significant when comparing active TB patients with individuals screened for LTBI ($P = 0.039$). In contrast, the differences in the number of T cells stimulated with CFP-10 were significant between active TB patients and contact-tracing individuals and also those screened for LTBI ($P = 0.018$ and $P = 0.01$, respectively). Finally, when we evaluated both antigens together (RD1 response), the differences for active TB patients compared with contact patients and patients screened for LTBI were also significant ($P = 0.042$ and $P < 0.001$) (Fig. 1).

Table 1
Demographic characteristics of adult and pediatric patients studied

	Adult population			Pediatric population		
	Active TB, *n* = 40 (%)	Contact tracing, *n* = 56 (%)	LTBI screening, *n* = 35 (%)	Active TB, *n* = 13 (%)	Contact tracing, *n* = 52 (%)	LTBI screening, *n* = 18 (%)
Sex						
Male	27 (67.5)	29 (51.8)	21 (60)	10 (76.9)	26 (50)	9 (50)
Female	13 (32.5)	27 (48.2)	14 (40)	3 (23.1)	26 (50)	9 (50)
Age, mean ± SD	37.60 ± 15.93	36.20 ± 17.38	32.39 ± 6.16	10.85 ± 3.69	9.37 ± 3.63	10.67 ± 4.19
BCG vaccinated						
Yes	8 (20)	22 (39.3)	18 (51.4)	4 (30.8)	34 (65.4)	12 (66.7)
No	32 (80)	34 (60.7)	17 (48.6)	9 (69.2)	18 (34.6)	6 (33.3)
Immunosuppression						
Yes	0 (0)	0 (0)	2 (5.7)	0 (0)	0 (0)	0 (0)
No	40 (100)	56 (100)	33 (94.3)	13 (100)	52 (100)	18 (100)
Birth country						
Immigrants from countries with high prevalence of TB infection	20 (50)	23 (41.1)	24 (68.6)	4 (30.8)	35 (67.3)	14 (77.8)
Residents in a non-epidemic TB country	20 (50)	33 (58.9)	11 (31.4)	9 (69.2)	17 (32.7)	4 (22.2)
IFN-γ tests						
Positive T-SPOT.TB	37/39 (94.9)	54/56 (96.4)	31/34 (91.2)	12/13 (92.3)	46/52 (88.5)	15/17 (88.2)
Positive QFT-G-IT	34/40 (85)	34/54 (63)	27/35 (77.1)	9/12 (75)	43/48 (89.6)	13/16 (81.3)

There were no significant differences for the IFN-γ released in QFT-G-IT between the different groups of patients (Fig. 1).

To determine an ESAT-6, CFP-10, and RD1 responder T-cell enumeration threshold in T-SPOT.TB, and an IFN-γ release level threshold in QFT-G-IT to differentiate between active TB and LTBI, we identified the areas under the ROC curve in adult patients as 0.599 (95% confidence interval [CI], 0.489–0.710), 0.677 (95% CI, 0.571–0.783), 0.668 (95% CI, 0.563–0.772), and 0.504 (95% CI, 0.387–0.621), respectively (Fig. 2). Although there was overlapping, we could define a threshold to differentiate between active TB and LTBI (Table 3). The optimal threshold was 69 CFP-10 T cells, with a sensitivity of 45.9% and a specificity of 81.2%. In contrast, the best threshold point was 116 RD1 T cells with a sensitivity of 43.2% and a specificity of 81.2%.

3.2. Pediatric population results

In the pediatric population, the median numbers of ESAT-6, CFP-10, and RD1 T cells in T-SPOT.TB and the median IFN-γ release in QFT-G-IT were higher in active TB individuals than in contact-tracing studies or children screened for LTBI (Table 2 and Fig. 3).

The differences in the number of ESAT-6, CFP-10, and RD1 T cells stimulated in T-SPOT.TB and the IFN-γ released in QFT-G-IT between the 3 groups of pediatric patients were not significant (Fig. 3).

Table 2
T-cell enumeration for *T-SPOT.TB* after antigen stimulation and IFN-γ release determined by *QFT-G-IT* in the different groups of patients

Group of patients	Adult population					Pediatric population				
	n	Mean	SD	Median	5–95 percentile	*n*	Mean	SD	Median	5–95 percentile
T-cell enumeration after ESAT-6 stimulation										
Active TB	37	37.95	35.62	36.00	0.90–17.50	12	50.25	52.81	32.00	1.00–152.40
Contact tracing	54	35.31	47.42	11.50	1.00–149.00	46	37.78	41.04	18.00	1.00–128.05
LTBI screening	31	20.13	23.00	12.00	0.20–79.20	15	27.87	31.52	20.00	5.00–87.40
T-cell enumeration after CFP-10 stimulation										
Active TB	37	65.14	53.00	54.00	1.80–192.80	12	45.33	31.64	40.50	4.00–85.10
Contact tracing	54	42.03	42.02	25.00	1.00–144.75	46	46.09	41.66	29.50	2.35–140.40
LTBI screening	31	27.26	27.26	15.00	2.00–91.80	15	41.00	36.12	22.00	4.00–103.40
T-cell enumeration after RD1 stimulation										
Active TB	37	103.08	70.85	87.00	10.70–247.70	12	95.58	65.26	91.50	17.00–213.10
Contact tracing	54	77.33	76.45	50.50	9.75–269.50	46	83.87	72.05	62.00	8.35–224.65
LTBI screening	31	47.39	44.89	26.00	8.00–154.00	15	68.87	42.08	66.00	13.00–136.80
IFN-γ release after antigen stimulation in QFT-G-IT										
Active TB	34	7.74	11.43	3.99	0.66–2.15	9	13.04	18.68	5.92	0.36–21.34
Contact tracing	34	17.71	48.95	6.12	0.37–105.09	43	10.43	21.11	3.55	0.45–35.86
LTBI screening	27	6.56	13.41	2.11	0.42–49.78	13	7.69	8.11	4.68	0.47–23.71

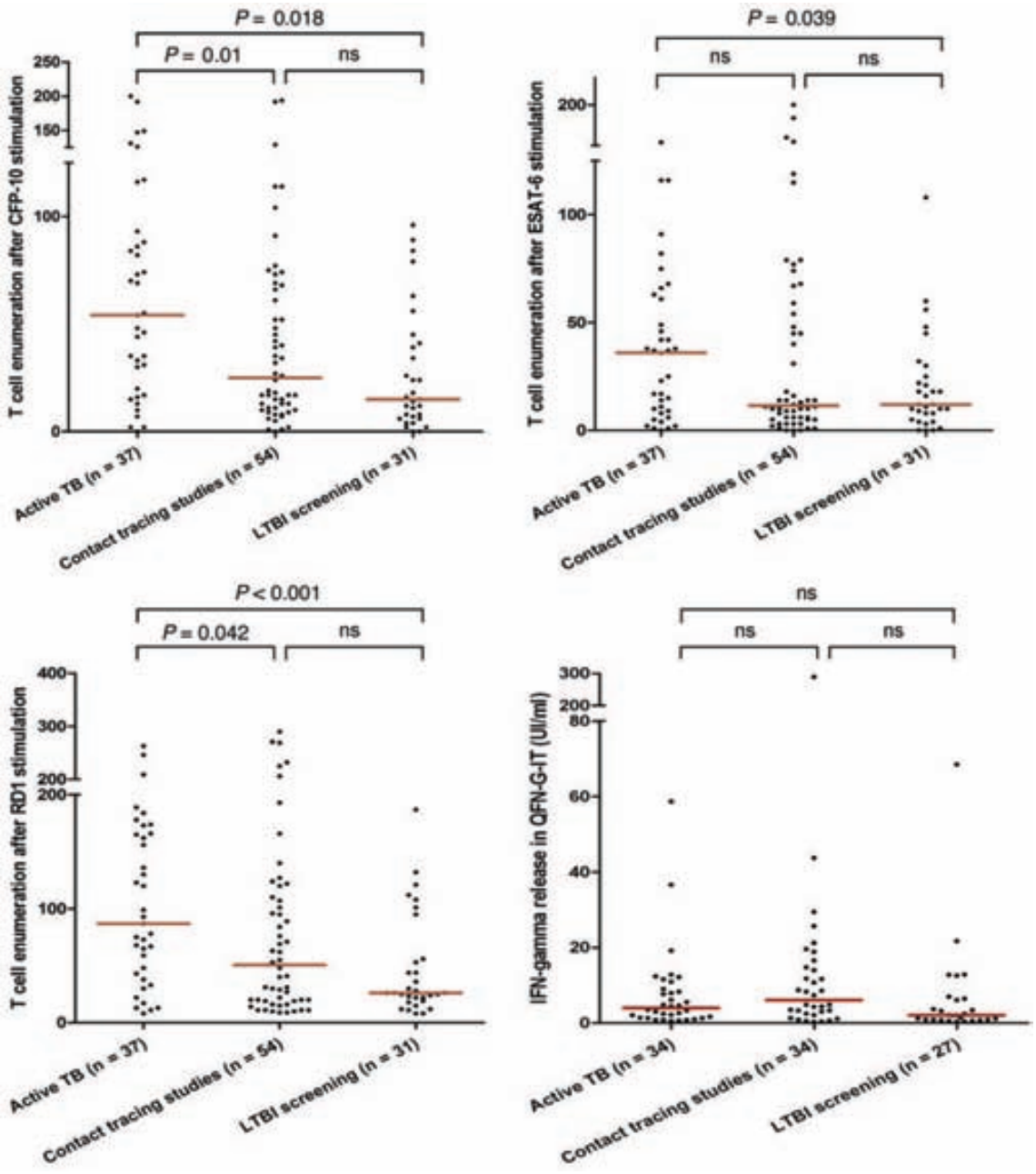

Fig. 1. T-cell enumeration and IFN-γ release after antigen stimulation in the different groups of adults.

The areas under the ROC curve for ESAT-6, CFP-10, RD1, and IFN-γ release were 0.555 (95% CI, 0.355–0.755), 0.534 (95% CI, 0.371–0.697), 0.596 (95% CI, 0.436–0.757), and 0.517 (95% CI, 0.282–0.752), respectively (Fig. 4).

The best threshold point was 65 ESAT-6 T cells, with a sensitivity of 33.3% and a specificity of 80%. However, when we studied RD1 response, the best threshold point was 145 RD1 T cells with a sensitivity of 25% and a specificity of 82%. The cutoff point determined for CFP-10 had a low sensitivity (16.7%) in this population. For QFT-G-IT, we determined 17.10 IU/mL of IFN-γ released with a sensitivity of 33.3% and a specificity of 83.9% (Table 3).

4. Discussion

Although the new generation of IFN-γ immune-based assays have been designed to diagnose LTBI, they have also

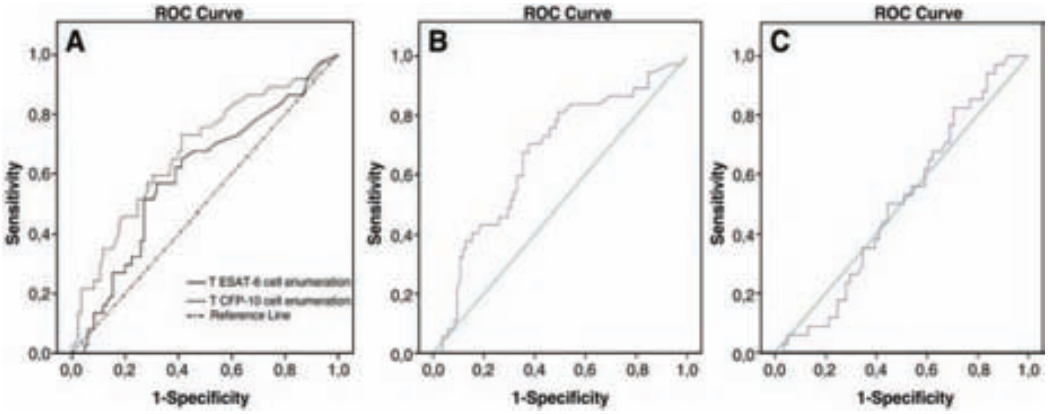

Fig. 2. ROC curve for (A) ESAT-6, CFP-10, (B) RD1 T-cells enumeration, and (C) IFN-γ release between adults with active TB and LTBI.

been explored for use in the diagnosis of active TB, reporting sensitivities between 70% and 90% (Arend et al., 2007; Detjen et al., 2007; Dominguez et al., 2008; Domínguez et al., 2009a; Ferrara et al., 2006; Kang et al., 2007; Lee et al., 2006; Rangaka et al., 2007). Nevertheless, using the cutoff point defined by the manufacturers, IFN-γ tests cannot distinguish between LTBI and active TB when they are performed on blood. We present the results of a study that was designed to evaluate the quantitative specific ESAT-6, CFP-10, and TB7.7 T-cell response in the adult and children populations by means of T-SPOT.TB and QFT-G-IT.

To establish a cutoff point to differentiate between active TB and LTBI, we have assessed the best sensitivity for ROC curve in each case, considering an optimal specificity equal to or higher than 80%. Given that the potential use of these tests would be as a complementary tool for diagnosing active TB patients without any microbiological findings, it is necessary to define a cutoff point that makes the test more specific to avoid false-positive results. However, this consideration could be adjusted according to the prevalence of the *M. tuberculosis* infection in each area. The main problem with using IFN-γ assays to diagnose active TB is their poor specificity for disease. Therefore, when active TB is suspected in adults from countries with a high incidence of TB, as well as high-risk populations in countries with a low incidence of TB (foreign-born or close contacts), the specificity of the tests is very low. Therefore, the positive predictive value will also be low. On the contrary, in patients at very high risk of disease (suspected clinical and radiologic active TB), but with a low probability of latent infection (low-risk populations from low-incidence countries), the positive predictive value is high. Positive IFN-γ assays are very useful in the diagnosis of active TB, especially the positive results above the defined thresholds (Menzies, 2008). In our study, the 14.3%, 61.9%, and 42.9% of adult patients with smear-negative active TB obtained a T-cell count above the ESAT-6, CFP-10, and RD1 described thresholds, respectively.

The overall results show a higher number of positive results for T-SPOT.TB than QFT-G-IT. This is consistent with the results reported previously (Chee et al., 2008b; Domínguez et al., 2009a; Ferrara et al., 2006; Goletti et al., 2006; Kang et al., 2007; Lee et al., 2006). In our experience, in adult patients, the number of responder T cells after RD1 antigens stimulation was significantly higher in active TB than in LTBI patients. However, there is a large amount of overlapping that makes it difficult to distinguish active TB from LTBI.

Fox et al. (2007) studied ESAT-6 and CFP-10 responses for T-SPOT.TB in 183 smear-positive TB cases and 1673 household contacts, finding a higher CFP-10 response in TB cases, indicating that CFP-10 could be an indicator of active TB. They hypothesized that CFP-10 could reflect the number and nature of T-cell epitopes and that CFP-10 responses in

Table 3
Optimal threshold level for ROC curve analysis, sensitivity and specificity in adult and pediatric patients

	Adult population			Pediatric population		
	Threshold[a]	Sensitivity (%)	Specificity (%)	Threshold[a]	Sensitivity (%)	Specificity (%)
T-SPOT.TB						
ESAT-6	61	27	84.7	65	33.3	80
CFP-10	69	45.9	81.2	82.5	16.7	80.3
RD1	116	43.2	81.2	145	25	82
QFT-G-IT						
IFN-γ released	19.04	8.8	86.9	17.10	33.3	83.9

[a] SFU enumeration for T-SPOT.TB and international unit per milliliter of IFN-γ for QFT-G-IT giving an optimal specificity equal to or higher than 80%.

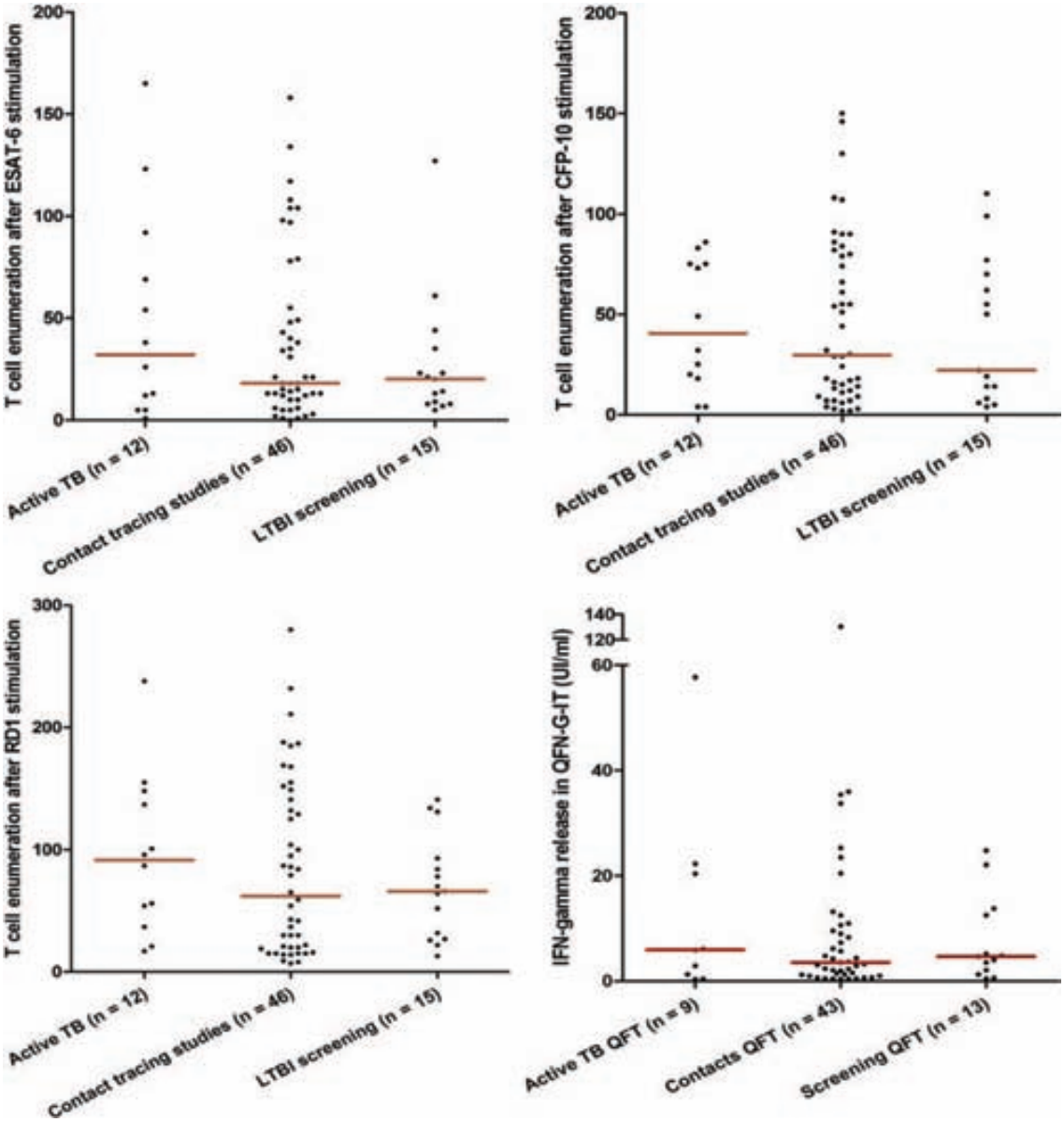

Fig. 3. T-cell enumeration and IFN-γ release after antigen stimulation in the different groups of children.

active TB patients could be more reliable than ESAT-6 responses in CD8–T cells as regards contacts. In our study, the enumeration of responder T cells after stimulation with CFP-10 yielded significant differences between active TB and the other 2 groups of LTBI patients, whereas responder T cells after stimulation with ESAT-6 only yielded differences between active TB and screened LTBI patients. In our experience, a cutoff point of 69 CFP-10 T cells provides a sensitivity of 45.9% with a specificity of 81.2%.

Recently, studying the T-cell response, Kobashi et al. (2008) explained that the quantitative responses to CFP-10 decrease during the treatment of active TB, and that it might be useful as a monitoring marker of clinical efficacy. We have also stated (Domínguez et al., 2009a) that the number of CFP-10 responder T cells decreases during the treatment when compared with the beginning of the therapy, although in our experience, the difference is not significant. However, the response against CFP-10 at the end of the treatment increases, achieving similar values to the one obtained before treatment.

Janssens et al. (2007) evaluated the quantification of RD1 antigen responder T cells for T-SPOT.TB to differentiate active TB from LTBI. They studied contacts with a positive T-SPOT.TB and patients diagnosed with active TB. By ROC

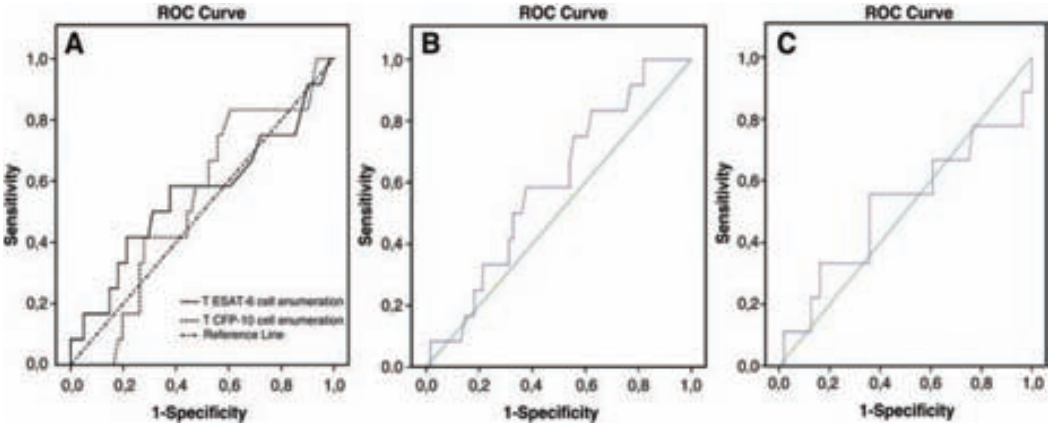

Fig. 4. ROC curve for (A) ESAT-6, CFP-10, (B) RD1 T-cells enumeration, and (C) IFN-γ release between children with active TB and LTBI.

curve analysis, they could establish a threshold value of 49.5 SFUs, with a sensitivity of 83% and a specificity of 74%. Nevertheless, they concluded that the test could not be recommended for diagnosing active TB. The difference in the threshold value between our result and the study by Janssens et al. could be explained by differences in the study population. To calculate the threshold value, Janssens et al. used patients from contact-tracing studies with both positive and negative TST results. As the authors reported, the mean SFU number obtained by negative TST contact patients was lower than that obtained by positive TST contact patients. Therefore, when both groups of patients (negative and positive TST) are analyzed together, the threshold value is lower than if only the positive TST patients are considered. We have proposed as optimum a threshold of 116 RD1 spots with a sensitivity of 43% and a specificity of 81% in adult patients. In our study, using a threshold of 50 SFUs, the sensitivity (70.3%) and the specificity (56.5%) were slightly lower than in the study by Janssens et al.

Recently, Chee et al. (2008a) also compared the quantitative T-cell response measured by the 2 available tests to distinguish active TB from LTBI. They reported that the median number of SFU in response to ESAT-6 and CFP-10 was significantly higher in active TB patients than in LTBI patients; in contrast, the median IFN-γ level of the QFT-G-IT showed no significant differences between the 2 groups. The authors noticed a large degree of overlapping in the range of values obtained by patients diagnosed with active TB or LTBI.

In the adult population, the IFN-γ released by QFT-G-IT did not show significant differences between active TB and LTBI patients. Indeed, the IFN-γ released was higher in contacts than in active TB patients. This result is probably related to the fact that patients with active TB have a low frequency of circulating specific IFN-γ–secreting T cells on peripheral blood (Pathan et al., 2001) because of a sequestration of specific T cells to the site of active TB (Barnes et al., 1993; Hirsch et al., 1999) and because of the fact that IFN-γ released in the QFT-G-IT methodology is diluted in the overall volume of blood. In contrast, T-SPOT.TB is always performed with a standardized number of T cells.

In the pediatric population, both tests have a higher number of responder T cells against the specific antigens and a higher level of IFN-γ released in active TB cases than in LTBI cases, although the differences were not significant. In all cases, the ROC curve analysis offers a very low sensitivity. The absence of significant differences in the response between active TB and LTBI could be explained by the fact that pediatric infection is usually recent. Therefore, the response is still strong, being similar to the one obtained in an active TB. This could explain the slight differences between contacts and screened children. This observation is consistent with the current evidence that T-cell response may indicate mycobacterial burden and disease activity (Chee et al., 2008a). In 1052 healthy household contacts of smear-positive pTB index patients, Hill et al. (2005) reported a high quantitative ELISPOT response in contacts with recent exposure, reflecting *M. tuberculosis* infectious load.

Although the IFN-γ tests were not able to distinguish between active TB and LTBI, given that in childhood, especially in young children, the risk of progression to active TB is high, a rapid and specific diagnosis of LTBI is essential in preventing progression to disease. Moreover, IFN-γ tests may provide useful information in children being evaluated for suspected TB. In a recent study (Connell et al., 2008) on the pediatric population, in the evaluation of 9 children with suspected active TB, QFT-G-IT and T-SPOT.TB were positive in 8 and 9 cases, respectively. In our experience (Dominguez et al., 2008), both tests were also positive in 6 (66.7%) of the 9 children examined with active TB.

Alternative approaches have been developed to use IFN-γ tests as diagnostic tools for active TB. The use of RD1 selected peptides to distinguish patients with active TB from infected individuals has been evaluated. These peptides are a selection of Human Leukocyte Antigen (HLA) class II-

restricted RD1 region from ESAT-6 and CFP-10 MTB proteins. An association between the response to these selected peptides and active TB disease has been described (Goletti et al., 2005, 2006, 2008a; Vincenti et al., 2003). Furthermore, Goletti et al. (2006) evaluated the response to RD1 selected peptides in 23 pTB cases and 32 control patients, obtaining a sensitivity and a specificity of 70% and 91%, respectively. In contrast, the sensitivity and specificity of the 2 commercially available tests were 91% and 59% for T-SPOT.TB and 83% and 59% for QFT-G, respectively.

Recently, Dosanjh et al. (2008) have developed a new IFN-γ test (ELISpot^{PLUS}) that contains ESAT-6 and CFP-10 used in T-SPOT.TB and includes a third new antigen encoded in the genomic segment RD1 called Rv3879c. They studied 389 adults with a moderate to high probability of having active TB. The sensitivity for patients with a culture confirmed result was 85% with T-SPOT.TB and 89% with ELISpot^{PLUS}. This new assay had 4% higher diagnostic sensitivity than the standard T-SPOT.TB and improved the diagnosis of suspected TB to a sensitivity of 99% in combination with the TST. Moreover, the combined use of TST with the ELISpot^{PLUS} assay conferred a rapid exclusion of TB when both tests were negative and the pretest probability was low.

In this regard, in a recent multicenter Tuberculosis Network European Trialsgroup (TBNET) study (Goletti et al., 2008b), 2 commercial IFN-γ tests and the new assay based on RD1 selected peptides were evaluated, obtaining a similar accuracy for diagnosing active TB. They found that the combination of any of the IFN-γ tests evaluated with the TST might allow exclusion of TB.

Another approach is to investigate the use of alternative markers such as CXCL-10, also named IFN-γ inducible protein 10 (IP-10), and interleukin 2 (IL-2) to improve the diagnosis of active TB (Djoba Siawaya et al., 2007; Ruhwald et al., 2007). Interestingly, Ruhwald et al. (2007) studied IP-10, IL-2, and IFN-γ on 12 positive QFT-G-IT patients with confirmed TB and 11 negative QFT-G-IT healthy controls. The results suggested that the expression of IP-10 in active TB patients was 5.6 times higher than in healthy controls. In contrast, in the pediatric population, Whittaker et al. (2008) found that the baseline levels of IP-10 are increased in active TB and in LTBI, but there is no significant difference of stimulated levels of IP-10 between active TB and LTBI. Therefore, they concluded that IP-10 does not distinguish between active TB and LTBI. This observation is consistent with the idea that in the pediatric population, no significant difference in responses between LTBI and active TB children occurs because the infection is always recent, reinforcing the theory that quantitative T-cell assay reflects infectious load. Further studies are needed to establish the use of IP-10 cytokine alone or in combination with IFN-γ for the diagnosis of active TB and LTBI in adult and pediatric patients.

A new open strategy to diagnose the disease is based on detecting the MTB-specific T cell recruited in the site of the infection by means of ELISPOT techniques (Jafari et al., 2008; Losi et al., 2007; Wilkinson et al., 2005). This strategy could be a good alternative for the diagnosis of smear-negative pTB and also some extrapulmonary TB diseases.

In summary, T-cell enumeration after ESAT-6, CFP-10, and RD1 stimulation is high in patients with active TB. Our results demonstrate that the specific antigen CFP-10 response could be a more specific marker of active TB. Moreover, although there is overlapping, in patients with clinically suspected TB, a T-cell count above the described threshold could suggest active TB, especially in patients with a high probability of having active TB and low probability of having LTBI. Further research evaluating the quantitative T-cell response in contact patients and their subsequent development of active TB is required.

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4.4. Artículo 4

IFN-γ response on T-cell based assays in HIV-infected patients for detection of tuberculosis infection.

Latorre I, Martínez-Lacasa X, Font R, Lacoma A, Puig J, Tural C, Lite J, Prat C, Cuchi E, Ausina V, Domínguez J.

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La PT presenta una sensibilidad reducida en pacientes con alteraciones en la inmunidad celular, como por ejemplo, en pacientes infectados por el VIH, ya que puede resultar falsamente negativa debido a la imposibilidad de las células a responder a los antígenos empleados para su estimulación.

En los últimos años, se han publicado resultados prometedores con las técnicas basadas en la detección de IFN-γ en población infectada por el VIH [136-140]. Sin embargo, únicamente se han realizado algunos estudios comparando ambos tests de detección de IFN-γ con la PT en este tipo de pacientes [136, 141-144]. Por lo tanto, para poder entender mejor el papel que juegan estas técnicas *in vitro* en el diagnóstico de la infección tuberculosa en estos pacientes, es necesario disponer de un mayor número de estudios. El objetivo de este apartado de la Tesis fue comparar ambas técnicas de inmunodiagnóstico *in vitro* con la PT para el diagnóstico de la infección tuberculosa en 75 pacientes VIH, y evaluar la influencia del número de células CD4 en la aplicabilidad de estas técnicas.

En nuestro estudio observamos un bajo porcentaje de resultados positivos por las tres técnicas, y al mismo tiempo, una baja concordancia entre ellas. Nuestros resultados evidencian que las técnicas inmunológicas *in vitro* están menos influenciadas que la PT por la vacuna de la BCG, por lo tanto son más específicas. Por lo que respecta al número de positivos en función de las células CD4, ambas técnicas *in vitro* se ven afectadas por el nivel de inmunosupresión. Sin embargo, el T-SPOT.TB parece estar menos afectado por la inmunosupresión severa, ya que observamos por esta técnica un resultado positivo en un paciente con número de CD4 inferior a 100, y ninguno por QFN-G-IT y PT. De este modo, el uso de las técnicas *in vitro* puede ser de utilidad en el diagnóstico de la infección tuberculosa en este tipo de pacientes, además, con estas técnicas se puede detectar ausencia de respuesta inmune ya que presentan controles internos que permiten identificar falta de respuesta en pacientes anérgicos.

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RESEARCH ARTICLE Open Access

IFN-γ response on T-cell based assays in HIV-infected patients for detection of tuberculosis infection

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Abstract

Background: Individuals infected with human immunodeficiency virus (HIV) have an increased risk of progression to active tuberculosis following *Mycobacterium tuberculosis* infection. The objective of the study was to determine IFN-γ responses for the detection of latent tuberculosis infection (LTBI) with QuantiFERON-TB GOLD *In Tube* (QFT-G-IT) and T-SPOT.TB in HIV patients, and evaluate the influence of CD4 cell count on tests performance.

Methods: We studied 75 HIV patients enrolled for ongoing studies of LTBI with T-SPOT.TB, QFN-G-IT and TST. Mean CD4 cell counts ± standard deviation was 461.29 ± 307.49 cells/μl. Eight patients had a BCG scar.

Results: T-SPOT.TB, QFN-G-IT and TST were positive in 7 (9.3%), 5 (6.7%) and 9 (12%) cases, respectively. Global agreement between QFN-G-IT and T-SPOT.TB was 89% (κ = 0.275). The overall agreement of T-SPOT.TB and QFN-G-IT with TST was 80.8% (κ = 0.019) and 89% (κ = 0.373), respectively. We have found negative IFN-γ assays results among 2 BCG-vaccinated HIV-infected individuals with a positive TST. In non BCG-vaccinated patients, QFN-G-IT and TST were positive in 5 cases (7.5%) and T-SPOT.TB in 7 (10.4%). In contrast, in BCG-vaccinated patients, only TST was positive in 4/8 (50%) of the cases. The differences obtained in the number of positive results between TST and both IFN-γ assays in BCG vaccinated patients were significant (95% CI 3-97%, p = 0.046), however, the confidence interval is very wide given the small number of patients. In patients with CD4< 200, we obtained only one (5%) positive result with T-SPOT.TB; however, QFN-G-IT and TST were negative in all cases. On the contrary, percentages of positive results in patients with CD4> 200 were 10.9% (6/55), 9.1% (5/55) and 16.4% (9/55) with T-SPOT.TB, QFN-G-IT and TST, respectively.

Conclusions: IFN-γ tests have the benefit over TST that are less influenced by BCG vaccination, consequently they are more specific than TST. Although our number of patients with advance immunosuppression is limited, our study suggests that IFN-γ assays are influenced with level of immunosuppression. The use of IFN-γ assays could be a helpful method for diagnosing LTBI in HIV population.

Background

Tuberculosis (TB) is still a major cause of morbidity and mortality throughout the world. There is an estimated global incidence of 8.8 million new cases, with a total of 1.6 million deaths [1]. Indeed, individuals infected with human immunodeficiency virus (HIV) have an increased risk of progression to active TB following *Mycobacterium tuberculosis* infection of 5-10% per year [2]. The detection and treatment of active TB is crucial to control the global TB epidemic. Therefore, targeting and treating infected persons with high risk of disease reactivation is a key strategy for an effective control of the spread of TB.

Nevertheless, the diagnosis of latent tuberculosis infection (LTBI) is complicated due to the lack of a gold standard test. Tuberculin skin test (TST) has been used since the last century for diagnosing LTBI. TST measures a cell-mediated immunity as the form of a delayed-type hypersensitivity response to the purified

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protein derivative (PPD) [3]. The main drawback of the TST is its lack of specificity. PPD contains more than 200 antigens that are shared among other mycobacteria such as the Bacille Calmette-Guérin (BCG) vaccine strain and many non-tuberculous mycobacteria (NTM), consequently, false positive TST results can occur due to antigenic cross-reactivity [4]. In addition, the sensitivity of TST is reduced in HIV-positive patients because of false negative results, as a result of cutaneous anergy [5].

New *in vitro* T-cell based assays for the diagnosis of LTBI are now available. These assays measure the IFN-gamma (IFN-γ) released by sensitized T cells after specific *M. tuberculosis* antigen stimulation. These specific antigens are early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), which are encoded in the region of difference 1 (RD1) segment of *M. tuberculosis* genome [6], and TB7.7, encoded in RD11 segment [7].

Nowadays, there are two commercial available IFN-γ T-cell based assays: QuantiFERON-TB GOLD *In Tube* (QFN-G-IT, Cellestis Limited, Carnegie, Australia) and T-SPOT.TB (Oxford Immunotec Limited, Abingdon, UK). Both assays are approved from the U.S. Food and Drug Administration (FDA) as an aid for diagnosing LTBI. QFN-G-IT test stimulates whole-blood with ESAT-6, CFP-10 and TB7.7 in the same tube, and measures the concentration of IFN-γ in supernatants with an enzyme-linked-immunosorbent assay (ELISA). On the other hand, T-SPOT.TB assay stimulates isolated peripheral blood mononuclear cells (PBMCs) with ESAT-6 and CFP-10 separately, and detects number of IFN-γ producing T cells by means of an enzyme-linked immunospot assay (ELISPOT).

Promising results have been published with IFN-γ assays in the diagnosis of LTBI [8-13] and active TB [14-18]. Moreover, in the last years some studies have studied IFN-γ tests in HIV-infected population [19-23]. However, only few studies comparing the performance of both T-SPOT.TB and QFN assays in the same HIV population have been conducted [19,24-27]. However, more studies comparing T-SPOT.TB and QFN-G-IT with TST are required in order to better understand the role of IFN-γ assays in the diagnosis of LTBI in this kind of population, analyzing the impact of the degree of immunosuppression on the antigen-specific T-cell responses.

So, in the present study, we compared the utility of T-SPOT.TB, QFN-G-IT and TST for the diagnosis of LTBI in the same HIV population, and evaluated the influence of CD4 cell count on the different tests performance.

Methods

Study setting and patient recruitment

From January 2006 through November 2009, HIV-positive adults attending to the Hospital Universitari Germans Trias i Pujol and Hospital Universitari Mútua Terrassa were enrolled for ongoing studies of LTBI. The estimated TB community incidence of TB is 23.2/100.000 habitants and the HIV prevalence among those active TB patients is 8.5% [28].

Patients were consecutively recruited, and were enrolled during the course of the routine examinations. Each participant gave written informed consent before blood sampling. Ethics Committees of Hospital Universitari Germans Trias i Pujol and Hospital Universitari Mútua Terrassa approved the study. Information on the following variables was collected completing a detailed questionnaire: age, gender, BCG vaccination, prior TST (date and result), TB contact, history of prior active TB, chest radiography and other medical conditions. In our study, only participants with BCG scars were considered BCG vaccinated. In LTBI patients, active TB was excluded by clinical and radiologic examination. None of the patients included in this study had active TB. Patients were tested during the routine examination with the TST. Blood sampling of IFN-γ assays was performed before TST application. Patients with a previous documented positive TST were excluded.

HIV testing and lymphocyte count

HIV testing was performed in all subjects. Blood samples were taken for HIV serology (ELISA and Western-Blot). CD4 and CD8 cell count were performed on blood samples from all HIV-positive patients.

Tuberculin skin test

Two intradermal tuberculin units of PPD RT23 Tween 80 (Statens Serum Institut, Copenhagen, Denmark) were used to perform the TST, using the Mantoux method. Induration was measured 48-72 h after the application, and the size of the induration was interpreted by trained personnel. According to our national guidelines, an induration equal or higher than 5 mm was considered positive [29].

QuantiFERON-TB GOLD *In Tube*

A total of three tubes of one millilitre each: nil control, positive control (phytohaemagglutinin [PHA]) and TB-specific antigens were drawn by venopuncture from each patient. The tubes were incubated overnight at 37° C, and after incubation plasmas were separated by centrifugation. The production of IFN-γ in whole-blood supernatant was determined by an ELISA.

Raw optical densities were interpreted by using specific software provided by the manufacturer. The result obtained by the nil control was subtracted from the positive control and the antigen-stimulated samples. The cut-off value for a positive test was at least 0.35 IU/mL of IFN-γ in the sample after stimulation with the

specific antigens, regardless of the result of the positive control. The result of the test was considered indeterminate if the antigen-stimulated sample was negative and if the value of the positive control was less than 0.5 IU/mL after subtraction of the value of the nil control; and/or if the negative control was higher than 8.0 IU/mL.

T-SPOT.TB

Eight millilitres of blood were drawn for the isolation of PBMCs in a vaccutainer CPT tube (Beckton Dickinson Diagnostics, Franklin Lakes, NJ). The isolated PBMCs were washed twice by centrifugation with RPMI medium (Invitrogen, Auckland, N.Z.), and later resuspended in AIM-V medium (Invitrogen, Auckland, N.Z.). Finally, viable cells were counted with an inverted microscope using the tripan blue method.

IFN-γ producing T cells were detected by an enzyme-linked immunospot assay (ELISPOT). The test was performed according the manufacturer's instructions. Each subject requires four wells precoated with a monoclonal antibody to IFN-γ. In the first well, cells were incubated with medium alone (control negative), in the second one with PHA (control positive), in the third one with ESAT-6 (Panel A), and in the last one with CFP-10 (Panel B). The assay requires a total of 250,000 cells per well.

On T-SPOT.TB, spots were scored using an automated AID ELISPOT plate reader (Lector AID Elispots, Autoimmun Diagnostiks GMBH, Germany). All readings were also manually verified. Each spot represents the footprint of a cytokine secreting cell and the number of spots obtained provides a measurement of frequency of *M. tuberculosis* sensitized cells. Subjects were considered positive if there was a positive response to one or both of the antigen panels. Test wells were scored as positive if they contained at least six spot-forming cells more than the nil control well and this number was at least twice the number of the nil control well. The result was considered indeterminate if the response to both antigen panels were negative and if the number of spots in the control positive well was less than 20. In addition, the immunoresponse was also considered indeterminate if the number of spots in the negative control was greater than 10.

Statistical methods

Concordance between both tests was assessed using Cohen's Kappa (κ) coefficient. κ values below 0.40 indicate weak correlation, values of 0.41-0.60 indicate good agreement and values above 0.60 indicate strong agreement. Comparison of the number of spots and the IFN-γ released was performed by Mann-Whitney U test analysis. Differences were considered significant when *P* values were less than 0.05. All analyses were made with SPSS statistical software for Windows (SPSS version 15.0; SPSS Inc., Chicago; IL, USA). Graphical representation is based on GraphPad Prism version 4 (GraphPad Software, Inc, Dan Diego, CA).

Results

Patient characteristics

We studied 75 HIV-positive patients who were screened for LTBI. Mean CD4 and CD8 cell counts ± standard deviation were 461.29 ± 307.49 cells/μl and 899.33 ± 649.94 cells/μl respectively. The main demographic characteristics of patients included in the study are summarized in Table 1.

Diagnostic tests performance

The overall number of positive results in HIV-positive individuals screened for LTBI was 7/75 (9.3%), 5/75 (6.7%) and 9/75 (12%) using T-SPOT.TB, QFN-G-IT and TST respectively. There were not significant differences in the percentage of positive results between the three tests. We obtained two indeterminate results, both by T-SPOT.TB and QFN-G-IT, due to an insufficient response to PHA and *M. tuberculosis* specific antigens. In these two cases, TST was negative. Global agreement between T-SPOT.TB and QFN-G-IT was 89% (κ = 0.275; standard error [SE] = 0.184). The overall agreement of T-SPOT.TB and QFN-G-IT with TST was 80.8% (κ = 0.019; SE = 0.123) and 89% (κ = 0.373; SE = 0.173), respectively.

In non BCG-vaccinated patients, QFN-G-IT and TST were positive in 5/67 (7.5%), and T-SPOT.TB in 7/67 (10.4%) of the cases. In BCG-vaccinated patients both IFN-γ assays were negative, but in contrast, we obtained 4/8 (50%) of positive results with TST. The difference between the results obtained by TST in non BCG-vaccinated and BCG-vaccinated was statistically significant (p = 0.006). Furthermore, the differences obtained in the number of positive results between TST and both IFN-γ assays in BCG vaccinated patients were also significant (95% Confidence interval = 3-97%, p = 0.046), however, the confidence interval is very wide given the small number of patients. The number of positive results and the agreement between the assays regarding BCG-vaccination status are shown in Tables 2 and 3.

Influence of CD4 cell count

We analyzed the possible impact of CD4 cell count on T cell responses, stratifying patients into two groups: 20 patients with < 200 CD4 cells/μl and 55 patients with >200 CD4 cells/μl. We found that number of responder T cells to specific *M. tuberculosis* antigens detected by T-SPOT.TB and the IFN-γ released in QFN-G-IT was lower in HIV-positive patients with CD4 cell counts < 200 than >200 cells/μl but not statistically significant,

Table 1 Demographic characteristics of patients included in this study

		All subjects N = 75 (%)	CD4< 200 N = 20 (%)	CD4 >200 N = 55 (%)
Gender				
	Male	53 (70.7)	15 (75)	38 (69.1)
	Female	22 (29.3)	5 (25)	17 (30.9)
Age, mean ± SD		42.41 ± 9.16	42.10 ± 7.96	42.53 ± 9.63
BCG-vaccinated				
	Yes	8 (10.7)	2 (10)	6 (10.9)
	No	67 (89.3)	18 (90)	49 (89.1)
Birth country				
	Immigrants from countries with high prevalence of TB infection	6 (8)	1 (5)	5 (9.1)
	Autochthonous Spanish population	69 (92)	19 (95)	50 (90.9)

as shown in Figure 1. In addition, we studied the PHA T cell responses on QFN-G-IT according CD4 T cells and the differences between < 200 and >200 cell counts were nearly significant (Figure 2). On the other hand, it was impossible to asses the number of responder T cells after PHA stimulation on T-SPOT.TB due to saturation in the control positive well.

The proportion of positive results obtained by T-SPOT.TB and QFN-G-IT were lower in HIV patients with a CD4 cell count < 200 than above >200 cells/μl. In patients with a CD4 cell count below 200, we only obtained an only one (5%) positive result with T-SPOT.TB, that corresponded to one patient with 39 CD4 cells/μl. QFN-G-IT and TST were negative in all cases. In contrast, percentages of positive results in patients with a CD4 cell count above 200 were 10.9% (6/55), 9.1% (5/55) and 16.4% (9/55) with T-SPOT.TB, QFN-G-IT and TST, respectively. Differences in positive results regarding CD4 cell count were not significant for any tests (T-SPOT.TB, QFN-G-IT and TST: p = 0.313, p = 0.123 and p = 0.055, respectively).

The concentration of IFN-γ released in QFN-G-IT and the number of responder ESAT-6 and CFP-10 specific T cells detected by T-SPOT.TB was not correlated with number of circulating CD4 T cells (Spearman's rho [SR] = 0.221, p = 0.056; SR = 0.028, p = 0.813 and SR = 0.013, p = 0.910, respectively), as shown in Figure 3.

Table 2 T-SPOT.TB, QFN-G-IT and TST positive results regarding BCG vaccination status

Diagnostic test	No. (%) of positive results		
	BCG-vaccinated (n = 8)	*Non BCG-vaccinated (n = 67)*	*p**
T-SPOT.TB	0 (0)	7 (10.4)	0.531
QFN-G-IT	0 (0)	5 (7.5)	0.683
TST	4 (50)	5 (7.5)	0.006

*Significance value in percentage of positive results between BCG and non-BCG vaccinated patients.

Discussion

Several authors have studied the responses on T-cell based assays in HIV individuals for the detection of LTBI and active TB. It has been demonstrated in the majority of the studies, that IFN-γ assays have higher number of positive results than TST and a poor agreement with it [19-21,23,30-34]. Nevertheless, only a few studies have performed a direct comparison of T-SPOT.TB, QFN-G-IT and TST to ascertain LTBI in HIV-positive individuals [24-27].

Talati et al [25], compared T-SPOT.TB, QFN-G-IT and TST in 336 HIV-infected persons. They found a low prevalence of LTBI with the three diagnostic tests: 7 (2.1%) had a positive TST, 9 (2.7%) a positive QFN-G-IT and 14 (4.2%) a positive T-SPOT.TB. Overall, agreement between the three diagnostic tests was poor. Furthermore, Richeldi et al [24], performed simultaneously T-SPOT.TB, QFN-G-IT and TST in 116 chronically HIV-infected individuals. They identified a low percentage of individuals as LTBI and also a slight agreement between T-SPOT.TB and TST or QFN-G-IT. Rivas I [27] compared TST and both IFN-γ tests in 139 drug and alcohol abusers, 31% of them being HIV-infected patients. The authors did not found statistically significant associations between HIV serostatus and *in vitro* tests or TST. However, percentages of positive results obtained by T-SPOT.TB and QFN-G-IT in HIV-positive patients were higher for patients with a CD4 count >350 cells/μl than < 350 cells/μl (28.6% and 39.3% *versus* 20% and 10%, respectively).

On the other hand, the evaluation of both T-cell based assays and the TST, in patients with HIV-infection, for the immunodiagnosis of LTBI has been also recently described in a high TB-incidence country. In this sense, Leidl et al [26] enrolled 109 individuals in Uganda with a new diagnosis of HIV-1 infection, and observed that global frequencies of positive results for TST, T-SPOT.TB and QFN-G-IT were 47.2%, 54% and 67.9% respectively. Although there are few differences between

Table 3 Concordance and agreement between TST, T-SPOT.TB and QFN-G-IT results according BCG vaccination status*

	TST vs T-SPOT.TB		TST vs QFN-G-IT		T-SPOT.TB vs QFN-G-IT	
	Concordance (%)	κ (SE**)	Concordance (%)	κ (SE)	Concordance (%)	κ (SE)
HIV-positive						
Overall	59/73 (80.8)	0.019 (0.123)	65/73 (89)	0.373 (0.173)	65/73 (89)	0.275 (0.184)
BCG	4/8 (50)	-***	4/8 (50)	-***	8/8 (100)	-***
Non-BCG	55/65 (84.6)	0.085 (0.154)	61/65 (93.8)	0.567 (0.194)	57/65 (87.7)	0.268 (0.186)

* Indeterminate IFN-γ assays results were not included in the analysis.

** standard deviation

***κ value is not possible to calculate because of T-SPOT.TB and QFN-G-IT results are one constant variable.

numbers of positive results in these previous findings, our results are consistent with those reported in the referred studies, where the commercial IFN-γ tests reached similar number of positive results. Furthermore, according to these studies we observed that concordances between three diagnostic tests were poor.

Intriguingly, we have observed poor agreement between both IFN-γ tests. From the 7 cases with a positive T-SPOT.TB result and the 5 cases with positive QFN-G-IT results, both tests were positive simultaneously in only 2 cases. Diagnostic agreement between tests was moderate (κ = 0.40-0.65). Similar results were described by Richeldi et al [24]. They observed that the agreement decreased in the HIV group when T-SPOT.TB was compared with either TST (κ = 0.16) or QFT-IT (κ = 0.19). In addition they reported highly discordant results (those clearly negative with one IFN-γ assay and clearly positive with another) in all groups of immunosuppressed patients. The analysis of these discordant results needs to be researched further.

It is poorly understood the impact of the HIV-infection in the immune response of LTBI and vice versa. We have observed, as other authors [24], the presence of discordant results between the TST and the IFN-γ tests, and also between both IFN-γ tests (T-SPOT.TB and QFN-G-IT). It is not clear enough the reasons for these kinds of results. In some cases it could be given by BCG vaccination, or by previous NTM infections (discordances between TST and IFN-γ tests). In other cases, it might be due to the different methodologies (TST *vs* QFN-G-IT *vs* T-SPOT.TB). In fact, maybe, the discordant results demonstrate some different immune responses [35], but this hypothesis has not been yet fully explored. In any case, given that a gold standard for LTBI does not exist, it is not possible to know, in case of discordant result, which test gives the true result. So, do we recommend doing TST and IFN-γ tests to all HIV patients? We think that we have to do more diagnostic effort in patients with a high risk of developing active TB if they are infected. Probably we have to use all the tests available in severe immunosuppresed patients, and maybe it is not so necessary in HIV patients with a conserved number of CD4. Therefore, the use of IFN-γ

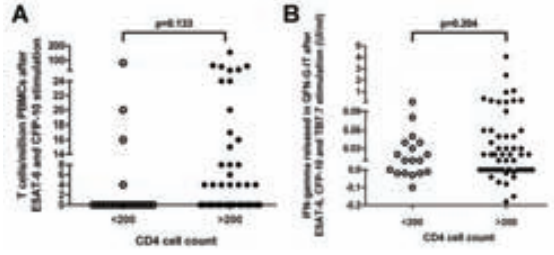

Figure 1 Number of responder T cells to specific antigens in T-SPOT.TB (A) and IFN-γ released after specific antigens (B) stimulation determined by QFN-G-IT in HIV-infected patients stratifying CD4 cell counts into two groups of < 200 and >200 cells/μl.

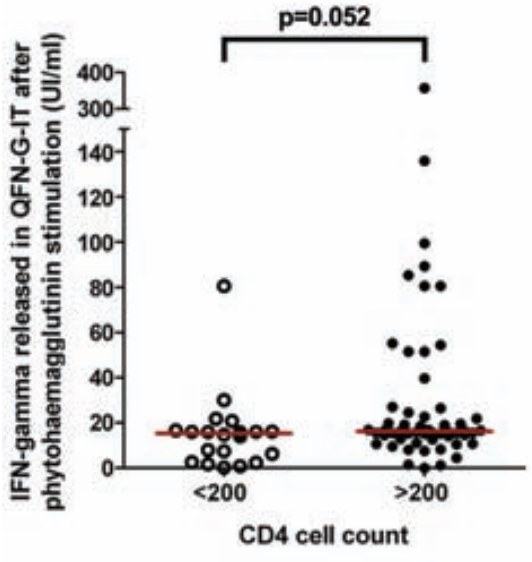

Figure 2 IFN-γ released after PHA stimulation determined by QFN-G-IT in HIV-infected patients stratifying CD4 cell counts into two groups of < 200 and >200 cells/μl.

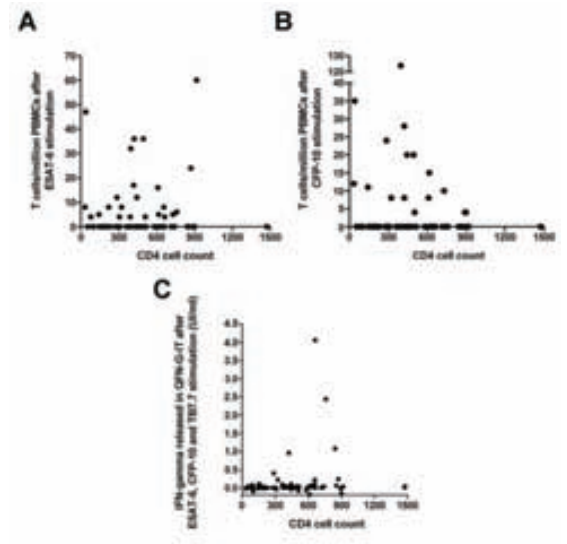

Figure 3 Correlation of numbers of circulating CD4 T cells with responder T cells to ESAT-6 (A) and CFP-10 (B) in T-SPOT.TB, and IFN-γ released in QFN-G-IT (C).

assays in combination with TST could be beneficial for diagnosing LTBI in HIV population severely immunosuppressed. However, probably our results did not provide definite data for supporting these comments.

We only detected two indeterminate results (2.7%) by either IFN-γ assays. This data is consistent with the majority of studies which show low rates of indeterminate results in HIV-infected patients without active TB [22]. One of the indeterminate cases obtained in our study corresponded with a low circulating CD4 cell count patient (103 cells/μl). It has been described that low CD4 cell counts are associated with IFN-γ assays indeterminate results for the diagnosis of LTBI and active TB [23,25,30,31,33,36,37]. However, in our study, 19 of 20 patients with < 200 CD4 cells/μl obtained a valid result. This data differ from other studies where high percentages of commercial IFN-γ tests or in-house ELISPOT assays indeterminate results were found in HIV-infected patients with low CD4 count cells in LTBI screening studies [23,25]. Nevertheless, studies of HIV-infected patients with active TB, generally report higher proportion of indeterminate results [36-38].

Raby et al [37] reported that low CD4 cell counts were associated with both indeterminate and negative QFN-G-IT results. On the contrary, low CD8 cell counts (median 369 cells/μl) were only associated with indeterminate results. They proposed that CD4+ and CD8+ cells could respond to PHA, unfortunately, the MCH class II overlapping peptides used in QFN-G-IT are only restricted to CD4+ cells. Consequently, those patients with high/normal CD8 cell counts (median 999 cells/μl) and low CD4+ cells produced a positive response to PHA but a negative response to *M. tuberculosis* specific antigens. In our study, only one of the two indeterminate results corresponded with a patient with a CD4 cell count < 200 cells/μl and a CD8 cell count < 600 cells/μl (103 and 568 cells/μl, respectively).

Regarding the differences in the IFN-γ assays results in HIV-positive individuals with a CD4 cell count < 200 respect those with a CD4 cell count >200, in our study all tests performed poorly in HIV-infected patients with CD4 cell count < 200. The fact that T-SPOT.TB obtained a positive result in one individual with a CD4 cell count < 200 (39 cells/μl), but none for QFN-G-IT, it is not sufficient to drawn conclusions. Converse et al [39], assessed the effect of HIV immunosuppression on QFN-G, and found that when immunosuppression increased, QFN-G sensitivity decreased, and only 30% (10/23) of HIV-seropositive persons with < 200 CD4 cells were positive.

Some studies have evaluated IFN-γ assays in HIV patients with active TB, and the influence of CD4 cell count. Aabye et al [36], reported that QFN-G-IT sensitivity in HIV-positive patients with active TB increased with high CD4 cell counts. Additionally, there are studies that have determined the diagnostic accuracy of T-cell based assays assessing the ratio of quantitative response of ESAT-6 and CFP-10 to CD4 T cell count, and improving the diagnosis of active TB [40,41]. No HIV-infected patient diagnosed of active TB was included in our study.

Furthermore, we observed that numbers of ESAT-6 and CFP-10 specific T cells in T-SPOT.TB, and concentration of IFN-γ in QFN-G-IT remained constant among patients with different levels of immunosuppression. Our results differ with those obtained in a recent study conducted by Leidl et al [26], where the correlation of the number of CD4 T cells with the IFN-γ released in QFN-G-IT was positive (Spearman's rho = 0.38; p = 0.0001), and constant with the number of ESAT-6 and CFP-10 specific T cells in T-SPOT.TB (Spearman's rho = 0.03; p = 0.77 and Spearman's rho = 0.13; p = 0.21, respectively).

Regarding the BCG-vaccination status, our results evidence that T-cell based assays are less influenced by BCG-vaccination than TST. In addition, we have found negative IFN-γ assays results among 2 non BCG-vaccinated HIV-infected individuals with a positive TST. A possible explanation to these discordant results could be a consequence of a previous NTM sensitization. In fact, in our experience, the utilization of IFN-γ tests could reduce the false diagnosis of LTBI in patients with a NTM sensitization [35,42]. In our study it was impossible to test *in vitro* NTM sensitins given that we didn't have more PBMCs

stored from these patients. However, it is not clear enough the safety of not treating BCG and non BCG-vaccinated patients with a positive TST and negative T-cell based assays in this kind of population, especially in patients with more severe immunosuppression.

The main drawback of our study needs to be reported. Even though we have compared T-SPOT.TB, QFN-G-IT and TST in the same population of HIV-infected individuals, the number of patients, especially those with CD4 cell counts < 200, is limited. Furthermore, we did not detect any significant differences in the overall percentages of positive results between the three tests. However, our results reported in this study are consistent to add valuable data about the utility of the IFN-γ tests in the diagnosis of LTBI in HIV-infected patients, and the influence of the number of CD4 in the results. More studies comparing T-SPOT.TB and QFN-G-IT with TST are required to determine the role of IFN-γ assays for the diagnosis of LTBI in HIV-positive patients.

Conclusions

In conclusion, IFN-γ tests have the benefit over TST that are less influenced by BCG-vaccination, consequently they are more specific than TST. The use of IFN-γ assays in combination with TST could be a helpful method for diagnosing LTBI in HIV population. Our study suggests that IFN-γ assays are influenced with level of immunosuppression. Further studies are required for understanding the meaning of the discrepancies between both IFN-γ tests.

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Authors' contributions

Conceived and designed the experiments: IL, XM-L, VA and JD. *Performed the experiments:* IL, XM-L, RF, AL, JP, CT, JL, CP and EC. *Analyzed the data:* IL, XM-L, RF, AL, JP, CT, JL, CP, EC, VA and JD. *Contributed reagents/materials/analysis tools:* XM-L, CT, JL, CP and JD. *Wrote the paper:* IL, XM-L and JD. All authors read and approved the final manuscript.

Competing interests

None of the investigators have any financial interest in or a financial conflict with the subject matter or materials discussed in this manuscript. None of the Scientific Societies, neither Inverness Medical Ibérica SAU (Barcelona, Spain), Cellestis (Carnegie, Australia) or Oxford Immunotec (Abingdon, UK) had a role in the study design, conduct, collection, management, analysis, or interpretation of the data, or preparation, review, or approval of the manuscript.

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4.5. Artículo 5

Specific *Mycobacterium tuberculosis* T cell responses to RD1-selected peptides for the monitoring of antituberculosis therapy.

Latorre I, De Souza-Galvão, Ruiz-Manzano J, Lacoma A, Prat C, Pérez M, Altet N, Ausina V, Domínguez J.

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La monitorización de la eficacia del tratamiento antituberculoso es complicada, ya que no ha sido descrito hasta el momento ningún marcador específico para poder valorar precozmente si el tratamiento es adecuado y si el paciente progresa correctamente.

Para un mejor control de la expansión de la TB, es necesario identificar nuevos marcadores que nos permitan conocer mejor la respuesta del huésped contra *M. tuberculosis* durante la enfermedad tuberculosa, y también durante el tratamiento. Recientemente, se han descrito unos nuevos péptidos RD1 seleccionados. Estos péptidos son una selección de los antígenos leucocitarios humanos de clase II-RD1 restringidos de las proteínas ESAT-6 y CFP-10 de *M. tuberculosis* y están asociados a la TB activa, y también a la respuesta al tratamiento antituberculoso [145-147].

El objetivo de este apartado de la Tesis, fue determinar la respuesta de IFN-γ de las células T sensibilizadas a los antígenos RD1 seleccionados, mediante una técnica de ELISPOT experimental, en 29 y 24 pacientes con TB activa estudiados en el momento del diagnóstico y durante la terapia tuberculosa, comparando los resultados con el T-SPOT.TB.

En nuestro estudio observamos que el número de células T que responden a los antígenos seleccionados disminuye durante el tratamiento, es más, la conversión a resultados negativos es significativamente mayor para el ELISPOT experimental con los antígenos RD1 seleccionados que para el T-SPOT.TB. Nuestros resultados sugieren que estas técnicas inmunológicas basadas en antígenos seleccionados correlacionan con la carga bacteriana y posiblemente sean buenas herramientas para estudiar la respuesta inmunológica del huésped durante el tratamiento antituberculoso.

Specific *Mycobacterium tuberculosis* T cell responses to RD1-selected peptides for the monitoring of antituberculosis therapy

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Background: Recently, Region of difference (RD) 1 selected peptides have been described. They are a selection of HLA class II-restricted epitopes of ESAT-6 and CFP-10 *Mycobacterium tuberculosis* proteins.

Methods: We evaluated the host IFN-γ T cell response to RD1 selected peptides at the beginning and during anti-tuberculosis therapy. We studied 29 and 24 pulmonary TB patients enrolled at the beginning and during treatment. We performed T-SPOT.TB and ELISPOT with RD1 selected peptides.

Results: The 89.7% and 79.3% of patients included at the beginning of treatment responded producing IFN-γ after antigen stimulation by T-SPOT.TB and RD1 selected ELISPOT, respectively. On the contrary, in patients included during treatment the percentages were 87.5% and 25%, respectively. Differences in sensitivity between patients evaluated at the beginning and during treatment were only significant for RD1 selected ELISPOT ($p<0.0001$).

Conclusions: Host immune response to RD1 selected peptides declined during therapy. Immunological assays based on RD1 selected peptides may be tools for studying immune response during anti-tuberculosis therapy.

The basis for controlling the spread of tuberculosis (TB) consists of the ability to diagnose it in its early stages and the successful treatment of patients with active TB [1]. Notwithstanding, TB diagnosis is difficult.

The diagnosis of active TB is based on clinical and radiographic findings, and is also combined with direct microscopic examination, culture of bacteria and *in vitro* amplification of *Mycobacterium tuberculosis* by PCR-based methods. Nevertheless, the diagnosis of TB often occurs in its advance stages because pulmonary TB could be smear-negative for acid fast bacilli, and culture of the mycobacteria could take several weeks. In addition, the diagnosis of extrapulmonary TB is complicated and often requires invasive diagnostic methods [2, 3].

Until now, the tuberculin skin test (TST) has been used as a complementary tool for the diagnosis of TB. The TST measures a delayed-type hypersensitivity to purified protein derivative (PPD) mediated by the cells. PPD is composed by more of 200 proteins that are shared with other non-tuberculous mycobacteria (NTM) and *Mycobacterium bovis* bacilli Calmette-Guérin (BCG) strain. So, it has a poor specificity that lead to false positive results due to cross-reaction in BCG vaccinated individuals and NTM infected patients.

In the last years, have been developed new assays based on an *in vitro* detection of interferon-gamma (IFN-γ) secreted by sensitized T cells to specific *M. tuberculosis* antigens. Assessment of whether T cells from a given patient have been exposed to *M. tuberculosis* specific antigens provides an alternative approach to TST. These

IFN-γ assays provide a significant improvement for the diagnosis of latent tuberculosis infection (LTBI) in adults and children [4-10]. On the other side, numerous studies have evaluated the utility of these assays in diagnosing active TB [11-14]. These IFN-γ immune-assays use specific *M. tuberculosis* antigens that are encoded in the genomic segment region of difference (RD) 1 (early-secreted antigenic target protein [ESAT-6] and 10-kD culture filtrate protein [CFP-10]) and in RD 11 [TB7.7 (Rv2654)] [15, 16]. These antigens are based on specific *M. tuberculosis* overlapping peptides spanning the whole ESAT-6 and CFP-10 proteins. These overlapping peptides are 15 amino acids long or have a variable length of 16 to 26 amino acids [17] and they are not present in the majority of the NTM and BCG strains. Nowadays, there are two commercial assays that detect IFN-γ secreted by specific T cells: Quantiferon-TB Gold In-Tube (QFN-G-IT, Cellestis Limited, Carnegie, Australia)] and T-SPOT.TB (Oxford Immunotec Limited, Abingdon, UK).

On the other hand, the monitoring of anti-TB treatment is difficult in patients with TB based solely on clinical findings. There is no evidence, besides clinical examination, of a specific marker for evaluating and/or predicting the correct progress of patients during therapy and the assessment of an adequate treatment.

The short period of T cell incubation with specific *M. tuberculosis* antigens in conventional IFN-γ assays reflects the frequencies of effector T cells directly related with the antigen load. In this sense, the number of effector T cells and the quantity of IFN-γ released could be useful for monitoring anti-TB treatment [18]. The impact of anti-TB treatment reducing IFN-γ response has been described elsewhere [11, 19-21]. On the contrary, other studies have shown an increase response during treatment [22-24].

In order to control better the spread of TB, it is necessary to identify new antigenic targets to improve the knowledge of the host response against *M. tuberculosis* during the active disease, and its kinetics during treatment.

Recently, RD1 selected peptides have been described. They are a selection of HLA class II-restricted epitopes of ESAT-6 and CFP-10 *M. tuberculosis* proteins, which were performed by quantitative implemented HLA peptide-binding motif analysis [17, 25] and are associated with the response to active TB [17, 25]. In addition, it has also been described that the IFN-γ response after RD1 selected peptides stimulation fell below the detection level in those patients responding to treatment [26].

Therefore, the aim of the present study was to determine the host IFN-γ T cell responses against these RD1 selected peptides, by means of an experimental ELISPOT, in patients with active TB, at diagnosis and during TB therapy completion, comparing the results with a commercial IFN-γ based assay (T-SPOT.TB).

MATERIAL AND METHODS

Study population

We studied a total of 81 adult patients who attended to *Hospital Universitari Germans Trias i Pujol* and to *Unidad de Prevención y Control de la Tuberculosis* of Barcelona. The main demographic characteristics of patients included in the study are summarized in Table I. We obtained ethics approval for the study from both institution Ethics Committees, and informed consent from all patients. A detailed questionnaire from all patients was collected with the following data: previous TST, BCG vaccination status, history of prior TB, chest radiography and other medical conditions. For those patients with active TB we collected the results and dates of culture, and details of anti-TB treatment. We only considered BCG vaccinated patients with a BCG scar. None of the patients had immunosuppression.

Patients were classified in different groups: **Group 1:** Twenty-nine pulmonary active TB patients at the beginning of the anti-TB treatment. Patients were untreated or received less than 2 weeks of therapy. In all cases *M. tuberculosis* was cultured isolated. **Group 2:** Twenty-four pulmonary active TB patients during the anti-TB treatment. Ten patients received between 2 weeks and 4 months of therapy, and the remaining 14 more than 5 months. In all cases *M. tuberculosis* was cultured isolated. All patients enrolled during treatment had an appropriate clinical response to anti-TB treatment (clinical and radiological improvement; and culture negativization) **Group 3:** Twenty-eight healthy individuals from contact

tracing studies were retrospectively enrolled with negative commercial IFN-γ assays and TST as controls.

Tuberculin skin test

According to our national guidelines [27], TST was performed using two intradermal tuberculin units of PPD RT23 (Statens Serum Institute, Copenhagen, Denmark). The tuberculin was administered following Mantoux method, the size of the indurations was measured after 48-72 hours of the administration by trained workers. TST was performed at the beginning of treatment for pulmonary active TB patients.

T-SPOT.TB

Eight millilitres of blood were drawn in a vaccutainer CPT tube (Beckton Dickinson Diagnostics, Franklin Lakes, NJ) for the isolation of PBMCs. Later, PBMCs were washed twice by centrifugation with RPMI medium (Invitrogen, Auckland, N.Z.), and resuspended in AIM-V medium (Invitrogen, Auckland, N.Z.). Finally, viable cells were counted with an inverted microscope using the tripan blue method.

The IFN-γ produced by PBMCs was detected by an enzyme-linked immunospot assay (ELISPOT) and performed following the manufacturer's recommendations. Cells were incubated with medium alone (control negative), PHA (control positive), ESAT-6 (Panel A) and CFP-10 (Panel B) in different wells precoated with IFN-γ. The assay requires a total of 250,000 cells per well.

Reactive antigen-specific T cells were revealed as a spot on the well, and scored by naked eye with the support of an automated AID ELISPOT plate reader (Lector AID Elispots, Autoimmun Diagnostiks GMBH, Germany). Subjects were considered positive if there was a positive response to one or both of the antigen panels, and negative, when no response was detected for the specific antigens. Test wells were scored as positive if the number of responder cells per million PBMCs minus their number in the control negative was > 24. The result was considered indeterminate if the response to both antigen panels were negative and if the number of cells per million PBMCs in the control positive well was less than 80. The immunoresponse was considered adequate if the number of cells per million PBMCs in the negative control was less than 40.

ELISPOT with RD1 selected peptides

A total of 250,000 isolated PBMCs from each patient were incubated overnight with a pool of selected ESAT-6 and CFP-10 peptides separately and PHA (positive control) in an ELISPOT plate. The RD1 selected antigens were kindly provided by D. Goletti from the National Institute for Infectious Diseases of Rome (Italy). ESAT-6 and CFP-10 selected antigens were incubated with PBMCs at 10 μg/ml and 6 μg/ml, respectively. We also incubated PBMCs with DMSO at 10 μg/ml as negative control because peptides were diluted in this reagent.

The results were interpreted as positive if we obtained more than 34 specific IFN-γ responding T cells per million PBMCs on ESAT-6 and/or CFP-10 selected peptides wells. The result was considered valid, if the specific T cells per million PBMCs were higher than 60 when they were incubated with PHA [17].

Statistical analysis

Comparison of the number of spots and the IFN-γ released, and conversion to negative responses between different groups of patients was performed by Mann-Whitney U test analysis. Differences were considered significant when p values were less than 0.05. We studied the sum of ESAT-6 and CFP-10 T cell enumeration obtained by T-SPOT.TB as an overall RD1 response, as well as for RD1 selected peptides. All analysis were made with SPSS statistical software for Windows (SPSS version 15.0; SPSS Inc., Chicago; IL, USA). Graphical representations were done with GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

RESULTS

Patients at the beginning and during anti-TB treatment. The overall sensitivities using T-SPOT.TB and RD1 selected ELISPOT in patients included at the beginning of treatment were 89.7% and 79.3% (Table II). Differences on sensitivities in those patients enrolled at the beginning of the treatment between RD1 selected ELISPOT and T-SPOT.TB or TST were not significant (p=0.102 and p=129, respectively).

TABLE I. Demographic characteristics of patients included in this study.

VARIABLE		Group of patients		
		Active pulmonary TB		*Healthy controls N=28 (%)*
		Beginning treatment N=29 (%)	*During treatment N=24 (%)*	
Gender				
	Male	18 (62.1)	21 (87.5)	12 (42.9)
	Female	11 (37.9)	3 (12.5)	16 (57.1)
Age mean ± SD		38.86 ± 17.7	39.67 ± 15.08	30.69 ± 8.04
BCG-vaccinated				
	Yes	6 (20.7)	3 (12.5)	4 (14.3)
	No	23 (79.3)	21 (87.5)	24 (85.7)
Birth country				
	Immigrants from countries with high prevalence of TB infection	16 (55.2)	11 (45.8)	4 (14.3)
	Residents in a non-endemic TB country	13 (44.8)	13 (54.2)	24 (85.7)

In contrast, when evaluating patients during treatment, the sensitivities decreased to 87.5% and 25% respectively (Table II). Differences in sensitivity between patients evaluated at the beginning and during the treatment were only significant for RD1 selected ELISPOT ($p<0.0001$). Positive TST results were obtained for 92.3% (24/26 patients) of patients at the beginning of treatment and 100% (7/7 patients) of patients included during treatment.

TABLE II. T-SPOT.TB, RD1 selected peptides and TST results in the different groups of patients.

	Group of patients		
	Active pulmonary TB		*Healthy controls N=28 (%)*
	Beginning treatment N=29 (%)	*During treatment N=24 (%)*	
T-SPOT.TB			
Positive (%)	26 (89.7)	21 (87.5)	-
Negative (%)	1 (3.4)	3 (12.5)	28 (100)
Indeterminate (%)	2 (6.9)	-	-
RD1 selected peptides ELISPOT			
Positive (%)	23 (79.3)	6 (25)	4 (14.3)
Negative (%)	3 (10.3)	18 (75)	24(85.7)
Indeterminate(%)	3 (10.3)	-	-
TST			
Positive (%)	24/26(92.3)	7/7 (100)	-
Negative (%)	2/26 (7.7)	-	28 (100)

The rate of indeterminate results found in active TB patients enrolled at the beginning of therapy was 6.9% (2/29) for T-SPOT.TB and 10.3% (3/29) for RD1 selected peptides ELISPOT. We did not obtain indeterminate results in active TB patients enrolled during treatment

We did not find relation between the IFN-γ response on T-SPOT.TB or RD1 selected ELISPOT, with the length of

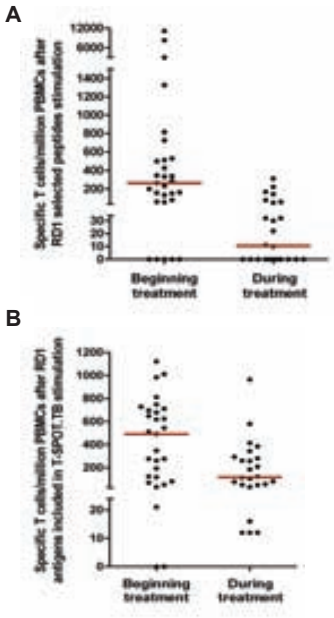

Figure 1. Number of responder T cells by RD1 antigens included in T-SPOT.TB (A) and RD1 selected peptides (B) in active TB patients included at the beginning and during anti-TB treatment.

anti-TB treatment (more than 5 months and less than 4 months of treatment) in those patients that were enrolled during therapy.

Number of responder T cells in TB patients at the beginning of therapy with regard to those studied during treatment was significantly different for both RD1 antigens included in T-SPOT.TB and RD1 selected peptides (Figure 1). However, conversion to negative responses for RD1 selected ELISPOT was significantly higher than for T-SPOT.TB ($p<0.0001$).

Healthy controls. The overall number of negative results by RD1 selected peptides ELISPOT in those individuals with negative commercial IFN-γ assays and TST was 85.7% (Table II). We found four positive results that corresponded to contacts with a smear-positive TB index case. No indeterminate results were observed in this group of individuals.

DISCUSSION

We present the results of an experimental ELISPOT based on ESAT-6 and CFP-10 selected peptides in pulmonary active TB patients enrolled at the time of TB diagnosis and during anti-TB treatment, in order to explore the specific-IFN-γ *M. tuberculosis* T cell responses during the anti-TB therapy.

The utility of HLA class II-restricted RD1 selected peptides has been evaluated in the immunodiagnosis of active TB. Vincenti et al [25], studied the response to ESAT-6 selected peptides, obtaining a sensitivity of 74% and a specificity of 100%. Furthermore, Goletti et al [28], measured the diagnostic accuracy of ESAT-6 and CFP-10 selected peptides for diagnosing TB disease using ELISPOT, and they reported a sensitivity of 70% and a specificity of 91%. The associated RD1 selected response with active TB has been also recently described [29]. In this study, a total of 32 active TB patients and 18 with diseases other than TB were included, and IFN-γ responses were measured with a whole-blood immunologic assay for RD1 selected peptides. They found that the sensitivity of the RD1 selected assay was 84% and the specificity 89%.

Sensitivity of ELISPOT based on selected peptides reported in our study is lower compared with T-SPOT.TB and TST, although the difference was not statistically significant. These differences in sensitivities among the three assays may be related to the amount and the composition of epitopes that are employed in the different tests. Nevertheless, a recent multicenter study of a *Tuberculosis Network European Trials group* (TBNET) [30] found that the combined used of negative results of commercial IFN-γ tests or novel assays based on selected peptides with TST may allow rapid exclusion of active TB.

Carrara et al [26], studied the response to ESAT-6 selected peptides in 18 patients with microbiology confirmed TB when they started treatment and 3 months after therapy. All patients had positive results at the moment of the diagnosis, and only 5 patients presented positive results 3 months later. Additionally, Goletti et al [31], found in 12 patients coinfected with HIV and *M. tuberculosis* a significant decrease of IFN-γ response to selected RD1 peptides after completion of therapy. In our study, we observed that RD1 selected responder T cells diminish during anti-TB therapy; even more, conversion to negative responses for selected peptides ELISPOT was significantly higher than for T-SPOT.TB. These findings and data suggest that the immunological assay based on RD1 selected peptides correlates better with bacterial burden during TB treatment than T-SPOT.TB, and could be also useful for the monitoring of *M. tuberculosis* replication.

In this sense, memory T cells are able to persist in time, even after bacterial clearance. So, they are not able to produce IFN-γ in the short period of time that is incubated with the specific antigens. On the contrary, the frequency of effector T cells that have encountered *in vitro* with the antigens can release IFN-γ in the short period of antigen time exposure; so, they maybe are directly proportional to antigen load and at the same time with the bacterial load. This is probably the reason why IFN-γ responses decrease during an adequate anti-TB treatment [18].

Controversially, it has been recently proposed that the single quantification of IFN-γ responses to conventional antigens is insufficient as a biomarker of micobacterial

load and active TB disease status. In this sense, promising results of IFN-γ measurements with other cytokines like interleukin (IL)-2, IFN-γ inducible protein (IP)-10 or monocytes chemotactic protein (MCP)-2 have been obtained [32-34]. However, it has been recently described that only IFN-γ responses to RD1 selected peptides are associated with active TB, and although the detection of other biomarkers like IL-2, IP-10 and MCP-2 with these selected peptides was also associated with active TB, did not improve the accuracy of the assay [35].

Furthermore, immunogenicity of selected peptides in individuals with LTBI that had a recent exposure to *M. tuberculosis* has been described during the monitoring of isoniazid prophylaxis. In this study, RD1 selected peptides had a higher decrease on IFN-γ response compared to RD1 antigens [36]. Interestingly, we found four positive results with ELISPOT based on selected peptides in healthy controls (4/28 patients) that corresponded to contacts with a smear-positive TB index case. These results may suggest that the immunological assay based on selected peptides could be useful in the LTBI diagnosis of smear positive TB patient contacts, and, therefore, that correlates well with bacterial burden.

The main limitations of our study are the lack of longitudinal follow-up of patients included at the beginning of the treatment, and that TST was only performed on a fraction of the TB patients included (26/29 patients at the beginning and 7/24 patients during treatment). Notwithstanding, from our point of view, results observed with selected RD1 peptides are consistent to strengthen the idea of the utility of these immunological assays for monitoring the effect of anti-TB therapy.

Although the role of immunological IFN-γ assays based on selected peptides for monitoring active TB treatment requires further research, especially in patients that do not respond to therapy, our study has shown that specific *M. tuberculosis* T cell responses to RD1-selected peptides decline during anti-TB treatment. In summary, immunological assays based on selected peptides correlate well with bacterial burden and maybe are a potential tool for studying host immune response during anti-TB therapy.

DECLARATION OF INTEREST

None of the investigators have relevant financial interest in or a financial conflict with the subject matter or materials discussed in this manuscript. None of the Scientific Societies, nor neither Cellestis (Carnegie, Australia) or Oxford Immunotec (Abingdon, UK) had a role in the study design, conduct, collection, management, analysis, or interpretation of the data, or preparation, review, or approval of the manuscript.

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4.6. Artículo 6

A multicentre evaluation of the accuracy and performance of IP-10 for the diagnosis of infection with *M. tuberculosis*.

Ruhwald M, Domínguez J, Latorre I, Losi M, Richeldi L, Pasticci MB, Mazzolla R, Goletti G, Butera O, Bruchfeld J, Gaines H, Gerogianni I, Tuuminen T, Ferrara G, Eugen-Olsen J, Ravn P.

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La búsqueda y el estudio de nuevos biomarcadores, diferentes a la citoquina IFN-γ, podrían permitir aumentar la sensibilidad de las técnicas *in vitro* para el diagnóstico de la infección tuberculosa. Se han estudiado un amplio rango de potenciales biomarcadores candidatos [148-150], de los cuales la IP-10 es el más prometedor.

Los objetivos de este apartado fueron determinar la sensibilidad y especificidad de una técnica de inmunodiagnóstico basada en la detección de IP-10, en 168 pacientes con TB activa confirmada y 101 controles sanos. En todos los pacientes se realizó QFN-G-IT y detección de IP-10 por la técnica de Luminex. Además, en un subgrupo de pacientes también se realizó la técnica de T-SPOT.TB.

En este estudio, hemos observado que la detección de la citoquina IP-10 por la técnica de Luminex es comparable a la del IFN-γ por las técnicas de QFN-G-IT y T-SPOT.TB. Además, se ha demostrado que con el uso combinado de IP-10 y QFN-G-IT se puede conseguir un aumento de la sensibilidad en el diagnóstico de la TB activa, sin que la especificidad se vea alterada.

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DIAGNOSTICS

A multicentre evaluation of the accuracy and performance of IP-10 for the diagnosis of infection with *M. tuberculosis*

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SUMMARY

IP-10 has potential as a diagnostic marker for infection with *Mycobacterium tuberculosis*, with comparable accuracy to QuantiFERON-TB Gold In-Tube test (QFT-IT). The aims were to assess the sensitivity and specificity of IP-10, and to evaluate the impact of co-morbidity on IP-10 and QFT-IT.

168 cases with active TB, 101 healthy controls and 175 non-TB patients were included. IP-10 and IFN-γ were measured in plasma of QFT-IT stimulated whole blood and analyzed using previously determined algorithms. A subgroup of 48 patients and 70 healthy controls was tested in parallel with T-SPOT.TB

IP-10 and QFT-IT had comparable accuracy. Sensitivity was 81% and 84% with a specificity of 97% and 100%, respectively. Combining IP-10 and QFT-IT improved sensitivity to 87% ($p < 0.0005$), with a specificity of 97%. T-SPOT.TB was more sensitive than QFT-IT, but not IP-10. Among non-TB patients IP-10 had a higher rate of positive responders (35% vs 27%, $p < 0.02$) and for both tests a positive response was associated with relevant risk factors. IFN-γ but not IP-10 responses to mitogen stimulation were reduced in patients with TB and non-TB infection.

This study confirms and validates previous findings and adds substance to IP-10 as a novel diagnostic marker for infection with *M. tuberculosis*. IP-10 appeared less influenced by infections other than TB; further studies are needed to test the clinical impact of these findings.

1. Introduction

IFN-γ release assays (IGRAs) are an upgrade to the century old tuberculin skin test (TST). The IGRAs utilize T cell recognition of *Mycobacterium tuberculosis*-specific peptides and are therefore almost exclusively positive in patients infected with the bacteria belonging to the *M. tuberculosis* complex. In contrast to the IGRAs, the TST cross-reacts with the BCG vaccine, therefore the IGRAs are more accurate in vaccinated individuals, and are rapidly becoming the test-of-choice when screening exposed and infected individuals at risk of progression to active TB in high resource settings.[1,2]

The sensitivity of IGRAs is suboptimal. Using confirmed active TB disease as a reference standard for *M. tuberculosis* infection, the

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most recent meta-analysis suggests that the most commonly used IGRA – the QuantiFERON-TB Gold In-Tube test (QFT-IT) – detects approximately 80% (95% CI: 75–84%) of infected individuals[3] and has a specificity of 99% (95% CI: 97.9–99.9%).[4] The IGRAs have a high negative prognostic value for overt TB disease in low endemic regions,[4–6] whereas the positive and negative predictive values for progression to active TB disease in medium and high endemic regions appear less pronounced.[7–9]

In our opinion the shortcomings of the IGRAs could be due to the features of IFN-γ as readout biomarker.[10] IFN-γ is a cytokine expressed at low levels, close to the detection limit of the assays, e.g. 17.5 pg/ml (0.35 IU/ml) for the QFT-IT.[11,12] Alternative biomarkers expressed in higher magnitude may enable a more sensitive test that could detect weak antigen-specific responses that remain undetected with IFN-γ.

We have screened a large panel of potential biomarker candidates[13–15] of which the monocyte derived chemokine IP-10 has shown most promise. In a cohort of 124 controls and 86 patients with active TB, we have established a cut-off for a positive IP-10 test using Luminex,[16] and demonstrated that the IP-10 test performs at least as accurately as the QFT-IT in a clinical study of exposed children[17] and in HIV-infected adults with active TB (Aabye et al. ERJ in press). Other groups have shown similar results in both adults[18] and children,[19,20] and two recent studies in HIV-infected adults have demonstrated that IP-10 detected a greater number of HIV–TB cases than IFN-γ and suggested that IP-10 could be a better alternative marker for diagnosing latent TB infection among immuno-compromised individuals,[21] (Aabye et al. ERJ in press). A limitation of the previous studies is that estimates of sensitivity and specificity were done based on the same cohort used to determine the optimal cut-off.

Although IGRAs are not marketed for the diagnosis of active TB, they are frequently applied as one of several diagnostic tools in suspected patients. Because IGRAs do not discriminate between active and latent TB infection, their usefulness for diagnosing active TB is likely limited in a clinical setting where patients have a high pre-test probability of latent infection.[22]

The present study aimed to investigate two questions. The first was to determine the sensitivity and specificity of the previously developed IP-10 test, to validate its cut-off. For this part of the study we included patients with confirmed TB and healthy presumed unexposed controls. The second question addressed the impact of other diseases on the diagnostic performance. For this part of the study we included a group of sick patients who were suspected of active TB disease, but who received another final diagnosis.

2. Material and methods

2.1. Study population

Patients with active TB (TB), healthy controls (HC) and non-TB patients (non-TB) were included from 9 centres in Europe affiliated to TBNET (Table 1). Except for the Perugia centre that included patients prospectively, TB and non-TB samples were included retrospectively from the participating centres biobanks. Samples are routinely stored in the biobanks to enable confirmation of test results, for quality control issues or for future clinical studies like this. Patient groups were defined based on the following criteria. Active TB: confirmed TB based on positive culture, positive PCR; and/or positive microscopy or histology and a response to treatment. Non-TB patients: sick adults who were initially suspected of active TB, but who ended up not having active TB but other diagnoses (e.g. pneumonia or lung cancer). Non-TB patients were defined by the following criteria: negative microbiological investigations for TB and either a confirmed alternative diagnosis explaining the condition and response to relevant treatment or a confirmed chronic condition such as cancer, or recovery without anti-TB treatment. Demographic and clinical data was collected from patient files. Immuno-suppression was classified according to Lee et al..[23] All patients were IGRA tested as part of the diagnostic procedure in patients with clinical suspicion of TB, among the TB patients blood for IGRA was drawn within the first two weeks of anti-TB treatment. The healthy controls comprised students from a high school in the greater Copenhagen area, Denmark[24] and from the School of Medicine at the University of Modena and Reggio Emilia, all controls had no known exposure to *M. tuberculosis* and no prior TB diagnosis or treatment. We furthermore included samples from non-exposed volunteers among the staff at the Copenhagen and Barcelona centres. Neither IGRA, IP-10 nor TST

Table 1
Overview of centres and participants.

	All (EU)	Modena (I)	Perugia (I)	Rome (I)	Copenhagen (DK)	Stockholm (S)	Barcelona (Sp)	Thessaloniki (Gr)	Terni (I)	Helsinki (Fi)
N	444	149	89	46	41	39	36	27	9	8
Age median (range)	38 (18–92)	28 (18–91)	53 (18–92)	36 (19–81)	18 (18–68)	41 (19-83)	34 (18–85)	56 (23–89)	51 (18–77)	58 (38–64)
Male sex, *n*(%)	263 (59)	97 (66)	52 (58)	29 (63)	16 (39)	21 (54)	23 (64)	16 (59)	4 (44)	5 (63)
Diagnosis										
Tuberculosis										
Pulmonary, *n*(%)	130 (77)	37 (80)	13 (52)	34 (94)	1 (100)	4 (31)	30 (100)	10 (63)	1 (100)	0 (0)
Extrapulmonary, *n*(%)	30 (18)	7 (15)	9 (36)	0 (0)	0 (0)	8 (61)	0 (0)	6 (37)	0 (0)	0 (0)
Pulmonary and extrapulmonary, *n*(%)	8 (5)	2 (4)	3 (12)	2 (6)	0 (0)	1 (8)	0 (0)	0 (0)	0 (0)	0 (0)
Non-TB patients										
Cancer, *n*(%)	13 (7)	0 (0)	5 (8)	0 (0)	1 (9)	1 (4)	0 (0)	3 (27)	1 (13)	2 (25)
Infection, *n*(%)	120 (69)	37 (100)	49 (77)	6 (60)	6 (55)	9 (35)	0 (0)	5 (45)	6 (75)	2 (25)
Autoimmune disease, *n*(%)	5 (3)	0 (0)	3 (5)	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	1 (13)
Other, *n*(%)	17 (10)	0 (0)	7 (11)	2 (20)	1 (9)	3 (12)	0 (0)	0 (0)	1 (13)	3 (38)
Unknown, *n*(%)	20 (11)	0 (0)	0 (0)	2 (20)	3 (27)	12 (46)	0 (0)	3 (27)	0 (0)	0 (0)
Healthy controls	101 (100)	66 (100)	0 (0)	0 (0)	29 (100)	0 (0)	6 (100)	0 (0)	0 (0)	0 (0)

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results were used to define the three groups. The relevant ethical committees at each centre approved the study protocol.

2.2. IP-10 and IFN-γ measurements and test interpretation

The QFT-IT (Cellestis, Carnegie, Australia) and the T-SPOT.TB (Oxford Immunotec, Abingdon, United Kingdom) tests were done in accordance to manufacturer's instructions at the participating centres. After QFT-IT testing, the QFT-IT supernatants were frozen. IP-10 in samples from Barcelona was analyzed on site whereas; samples from the other sites were shipped to Copenhagen and measured there. All samples were run in duplicate by xMAP/Luminex technology as described previously.[16] The same type of hardware, software and batch of IP-10 assay was used at the two sites. The antigen-dependent and mitogen-induced biomarker production were measured by subtracting the concentration measured in the nil. The antigen-dependent and mitogen-induced levels of IP-10 were reduced to positive, negative and indeterminate test outcome using an algorithm previously defined on a cohort of TB patients and healthy controls using ROC curve analysis. The cut-off for positive IP-10 test was 673 pg/ml and for indeterminate IP-10 test was 200 pg/ml.[16] QFT-IT tests were analyzed and interpreted in accordance to manufacturer's instructions. T-SPOT.TB results were analyzed in accordance with the European T-SPOT.TB interpretation algorithm, (≥6 spots in either panel A or B after subtracting the number in the Nil panel was considered positive).

2.3. Data analysis

Data were analyzed using SAS 9.2 (SAS institute, USA). Variables were compared using non-parametric tests where appropriate. Estimates of sensitivity and specificity are presented after excluding indeterminate responders. All tests were two sided and p-values <0.05 were considered significant. For further information on patient inclusion and data analysis refers to Supplementary data file.

3. Results

A total of 168 patients with TB, 101 HC and 175 non-TB patients were included in the study (Table 1). Among the TB patients, 77% (130/168) had pulmonary disease, and 91% (143 of 157 tested) were culture or PCR confirmed (Table 2). Seven percent (11 of 157) TB patients with known HIV status were HIV-infected, and 13% (22 of 166) had another immuno-suppressant condition. The HCs were all from Western Europe, 67% (66/101) were from Italy, 6% (6/101) from Spain and 29% (29/101) were from Denmark. HCs were

Table 2
Baseline table.

		TB patients	Healthy controls	Non-TB patients
N		168	101	175
Age median (range)		37 (18–90)	22 (18–53)	56 (18–92)
Male sex, *n*(%)		96 (57)	60 (59)	107 (61)
Region of birth, *n*(%)	Western Europe	59 (39)	101 (100)	125 (71)
	Eastern Europe	31 (21)	0 (0)	9 (5)
	Africa	27 (18)	0 (0)	27 (15)
	Asia	24 (16)	0 (0)	10 (6)
	South America	9 (6)	0 (0)	4 (2)
Severity, *n*(%)	Outpatient	16 (10)	–	71 (41)
	Required admission	122 (73)	–	102 (58)
	Unknown	30 (18)	–	2 (1)
Immuno-suppression				
HIV status, *n*(%)	Positive	11(7)	1 (1)	26 (15)
	Negative	146 (87)	71 (70)	125 (71)
	Unknown	11 (7)	29 (29)	24 (14)
Other immuno-suppression, *n*(%)	Yes	22 (13)	0 (0)	34 (19)
	No	144 (86)	94 (93)	97 (55)
	Unknown	2 (1)	7 (7)	44 (25)
TB risk factors				
Prior TB, *n*(%)	Prior TB	12 (7)	0 (0)	16 (9)
	No prior TB	114 (68)	101 (100)	139 (79)
	Unknown prior TB	42 (25)	0 (0)	20 (12)
Exposure, *n*(%)	TB exposure	19 (11)	0 (0)	15 (9)
	No exposure	96 (57)	101 (100)	124 (71)
	Unknown exposure	53 (32)	0 (0)	36 (20)
Stay in TB endemic country >2 months, *n*(%)	Yes	69 (41)	0 (0)	44 (25)
	No	47 (28)	97 (96)	111 (63)
	Unknown	52 (31)	4 (4)	20 (12)
TB diagnostic tests				
Culture, *n*(%)	Positive	140 (83)	–	0 (0)
	Negative	13 (8)	–	95 (54)
	Not done	1 (1)	–	20 (11)
	Not available	14 (8)	–	60 (34)
PCR, *n*(%)	Positive	65 (39)	–	0 (0)
	Negative	33 (20)	–	88 (50)
	Not done	13 (8)	–	23 (13)
	Not available	57 (34)	–	64 (37)
Microscopy, *n*(%)	Positive	73 (43)	–	0 (0)
	Negative	81 (48)	–	95 (54)
	Not done	1 (1)	–	21 (12)
	Not available	13 (8)	–	59 (34)

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significantly younger than non-TB and TB patients ($p < 0.0001$). Among the non-TB patients, 69% (120/175) had bacterial (88%) or viral (12%) infection, 10% (17/175) cancer, 3% (5/175) inflammatory diseases, 10% (17/175) had other diseases, and for 11% (20/175) the final diagnosis was unknown, and the patients recovered without anti-TB treatment. Sixty percent (102/173) of the non-TB patients were admitted to hospital; 35% (61/175) had at least one risk factor for TB infection; 17% (26/151) with known HIV status were HIV-infected; and 10% (16 of 155) with available information reported prior TB disease.

3.1. Biomarker levels

Biomarker levels are described in detail in Table 3. The nil IP-10 and IFN-γ levels were significantly lower in HCs compared to TB and non-TB patients, although the differences for IFN-γ were very small. TB patients produced significantly higher absolute levels of antigen-induced IP-10 and IFN-γ compared to the other groups, and non-TB patients produced higher levels than the healthy controls. In paired comparisons, TB patients produced antigen-induced IP-10 in median 29.1 (IQR 10.5–60.0) fold higher magnitude compared with IFN-γ ($p < 0.0001$), and the median signal-to-noise ratio (antigen-induced divided with nil level) for IFN-γ was 25.3 (IQR 5.5–70.0) compared with 19.0 (IQR 4.1–56.7) for IP-10 (0 = 0.07). Mitogen-induced IFN-γ responses were significantly reduced in TB patients compared to HC ($p < 0.05$) and in non-TB patients compared to HC ($p = 0.0002$) whereas the IP-10 responses were not reduced in neither patients with active TB or in non-TB patients. To investigate these differences further we divided the group of non-TB patients into patients with bacterial and viral infection ($n = 120$) and patients with other known diseases (e.g. cancer and autoimmune diseases $n = 35$) and compared responses to healthy controls as reference (Figure 1). The subgroup with bacterial or viral infection had significantly lower levels of mitogen-induced IFN-γ (median 101 pg/ml vs 460 pg/ml, $p < 0.0001$), whereas the mitogen-induced levels were not affected in the group of non-TB patients with other diseases (median 415 pg/ml, $p = 0.823$). In contrast, mitogen-induced IP-10 in infected non-TB patients appeared less and not significantly affected compared to healthy controls (median 1741 pg/ml vs 2216 pg/ml, $p = 0.064$) and the uninfected non-TB patients had a median of 3384 pg/ml, $p = 0.078$. There was no effect on these differences when excluding HIV-infected patients and the impact of infection was not related to viral or bacterial pathogen (data not shown).

3.2. IP-10 and IFN-γ test performance: sensitivity, specificity and indeterminate rate

The TB patients and HC were used to compare the diagnostic accuracy of IP-10 and IFN-γ. IP-10 had a high diagnostic accuracy with an AUC of 0.924 comparable to that of IFN-γ 0.937 ($p = 0.89$, graphs not shown). The specificity of the IP-10 test was 97% at 81% sensitivity; 2% had indeterminate responses (Table 4). In comparison QFT-IT specificity was 100%, at 84% sensitivity; 4% were QFT-IT indeterminate.

The concordance between the IP-10 test and QFT-IT was substantial; both tests were positive in 123/168 (73%), and negative in 18/168 (11%) of the TB patients. Agreement was 97/101 (96%, $k > 0.39$) among healthy controls and 143/168 (85%, $k = 0.57$) among TB patients. When combining the IP-10 test and QFT-IT tests, the sensitivity increased significantly to 145/166 (87%) ($p = 0.005$) without a compromise in specificity (97/100) 97% (Table 4). Twenty-five TB patients had discordant results. Nine were QFT-IT positive and IP-10 test negative, of which three had an immuno-suppressant co-morbid condition. Eight were QFT-IT negative and IP-10 test positive of which one had an immuno-suppressant co-morbid condition. Five patients were IP-10 test negative, QFT-IT indeterminate of which 3 had an immuno-suppressant condition.

3.3. QFT-IT, IP-10 and T-SPOT.TB results

T-SPOT.TB results were available in a subgroup of 48/168 (29%) TB patients and in 70/101 (70%) of the healthy controls (Table 5). IP-10 and T-SPOT.TB detected more patients as positive compared to QFT-IT in this subgroup (41/48 (85%) and 43/48 (90%) vs 37/48 (77%), $p < 0.05$ and $p < 0.04$ respectively), and there were no significant differences between T-SPOT.TB and IP-10 ($p = 0.32$). Of the 7 patients that were T-SPOT.TB positive QFT-IT negative IP-10 detected 4. The 3 T-SPOT.TB positive IP-10 negative TB patients had 36, 17, 9 and 7, 0, 54 spots in A and B panel, respectively and the median number of spots in the T-SPOT.TB and IP-10 concordant positive responders was 42 (IQR 15–90) and 75 (IQR 23–132) for A and B panel, respectively. One patient was QFT-IT positive T-SPOT.TB negative, this patient was also IP-10 positive. There were no significant difference in the rate of negative responders among the controls IP-10:68/70 (97%), T-SPOT.TB: 69/70 (99%) and QFT-IT 70/70 (100%), $p > 0.32$. The control with positive T-SPOT.TB had 17 and 192 spots in the ESAT6 and CFP10 wells, respectively. Combining IP-10 and QFT-IT improved QFT-IT sensitivity significantly ($p < 0.05$), but IP-10 did not have added value when combined with T-SPOT.TB ($p = 0.32$).

3.4. Test performance in non-TB patients

Although IGRAs are not marketed for the diagnosis of active TB, they are frequently applied as one of several diagnostic tools in patients suspected of active TB.[18,25–28] In order to identify potential effects of non-TB diseases on the test performance, we included a heterogeneous group of patients who had been suspected of TB but where TB was excluded and other diagnoses were found. In this group the IP-10 test was positive in 61 (35%) and QFT-IT in 47 (27%), $p < 0.02$. Fourteen percent (24/175) had an indeterminate QFT-IT result and 9% (16/175) an indeterminate IP-10 test ($p = 0.054$), (Table 4). Agreement was 73% (128/175, $k = 0.52$), 46% (81/175) were concordant negative, 22% (39/175) were concordant positive. When combining the IP-10 test and the QFT-IT the number of positive responders increased to 39% (69/175). In order to evaluate

Table 3
Biomarker levels (pg/ml), median (inter quartile range), Kruskal–Wallis test.

		TB patients	Healthy controls	Non-TB patients	HC vs TB	p-values HC vs non-TB	Non-TB vs TB
IFN-γ	Nil	7 (4–16)	5 (3–8)	5 (2–9)	0.0047	0.5179	0.0002
	Antigen-dependent	136 (25–393)	0 (0–1)	0 (0–20)	<0.0001	<0.0001	<0.0001
	Mitogen-induced	391 (75–492)	460 (271–492)	200 (41–492)	0.0487	0.0002	0.0984
IP-10	Nil	207 (120–442)	99 (53–239)	152 (77–329)	<0.0001	0.0337	0.0015
	Antigen-dependent	3414 (873–10,547)	20 (0–79)	182 (13–1496)	<0.0001	<0.0001	<0.0001
	Mitogen-induced	2680 (880–7563)	2216 (1182–5062)	2193 (903–4916)	0.4101	0.5307	0.0771

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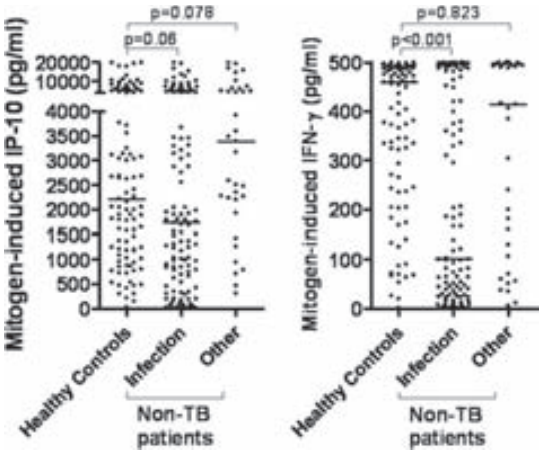

Figure 1. The distribution of mitogen-induced (mitogen subtracted nil) IP-10 and IFN-γ responses in healthy controls and non-TB patients divided into the group with infection ($n = 120$) and a group with known other final diagnosis (e.g. cancer, autoimmune diseases, sarcoidosis ($n = 35$)), Solid lines denote median; significance level was assessed using Kruskal–Wallis test.

whether positive test results among non-TB patients were associated with risk factors for *M. tuberculosis* infection, we calculated the age and sex adjusted Odds Ratio (OR) for positive tests (Table 6). The IP-10 test was significantly increased among patients born in a high endemic region (OR 4.0) and in patients with a prior TB diagnosis (OR 5.9). The QFT-IT was related to the same risk factors; ORs for a positive test were 3.1 and 5.0, respectively. Neither test was associated with a history of prior exposure to a TB patient (OR 2.9), nor prolonged stay in a TB endemic area (OR < 2.1). The OR for the combined IP-10 test and QFT-IT test was higher than IP-10 test alone in patients with prior TB and in patients with history of exposure, but lower among patients born in a TB high endemic country (data not shown).

3.5. Indeterminate responders among the non-TB patients

Fourteen percent had an indeterminate QFT-IT result and 9% had an indeterminate IP-10 test ($p = 0.054$). Among the non-TB patients with a viral or bacterial infection, 13% (16/120) were IP-10 test indeterminate in contrast to none of those with other diseases ($p = 0.01$), for QFT-IT 18% (22/120) were indeterminate in contrast to 6% (2/35) ($p = 0.041$), respectively. In addition 6% (10/175) were treated with corticosteroids of which 20% (2/10) were IP-10 test positive/QFT-IT indeterminate and 10% (1/10) were concordant indeterminate. Among the discordant responders 73% (22/30) were IP-10 test positive QFT-IT negative ($n = 17$) or indeterminate ($n = 5$), and 40% (8/30) were QFT-IT positive IP-10 test negative ($n = 6$) or indeterminate ($n = 2$).

Table 4
Distribution of positive, negative and indeterminate responders with the IP-10 test and QFT-IT and when the tests are combined.

		IP-10 test	QFT-IT	IP-10 test + QFT-IT
TB patients, *n*(%)	+	133 (79)	133 (79)	145 (86)*,†
N = 168	−	32 (19)	26 (15)	21 (13)
	Indet	3 (2)	9 (5)	2 (1)
Healthy controls, *n*(%)	+	3 (3)	0 (0)	3 (3)
N = 101	−	96 (95)	100 (99)	97 (96)
	Indet	2 (2)	1 (1)	1 (1)
Non-TB patients, *n*(%)	+	61 (35)‡	47 (27)	69 (39)*
N = 175	−	98 (56)	104 (59)	97 (55)
	indet.	16 (9)	24 (14)	9 (5)

* $p < 0.0005$ compared to QFT-IT.
† $p < 0.0005$ compared to IP-10 test.
‡ $p < 0.02$ compared to QFT-IT.

Table 5
A three-way comparison of IP-10 QFT-IT and T-SPOT.TB test results in a subgroup of 48 patients and 70 controls.

	N(%)	IP-10	QFT-IT	T-SPOT.TB
Patients	36 (75)	**Positive**	**Positive**	**Positive**
	4 (8)	**Positive**	Negative	**Positive**
	1 (2)	**Positive**	**Positive**	Negative
	1 (2)	Negative	Indeterminate	**Positive**
	1 (2)	Negative	Indeterminate	Indeterminate
	2 (4)	Negative	Negative	**Positive**
	1 (2)	Negative	Negative	Indeterminate
	2 (4)	Negative	Negative	Negative
Controls	67 (96)	**Negative**	**Negative**	**Negative**
	1 (1)	**Negative**	**Negative**	Positive
	2 (3)	Positive	**Negative**	**Negative**

4. Discussion

We report here the results of a comparative evaluation of IP-10 and IFN-γ as biomarkers in diagnostic tests for infection with *M. tuberculosis*. To evaluate sensitivity and specificity we included patients with confirmed TB and healthy presumed unexposed controls, and to evaluate performance in a clinical setting we included a group of patients who were suspected of active TB disease, but where another diagnosis was found. The diagnostic accuracy of IP-10 measured with Luminex was evaluated using a previously determined algorithm and cut-offs.[16]

We obtained two important results. First, we confirmed previous findings that IP-10 is produced in high amounts in stimulated whole blood from TB patients but not from HCs. This gives strength and robustness to IP-10 and to the algorithm we have previously set. Inline with our previous study using the same commercially available Luminex assay,[16] we found that the IP-10 test had comparable performance to QFT-IT and T-SPOT.TB, and that QFT-IT and IP-10 tests could be combined for a significant improvement in sensitivity without a compromise in specificity.

We found a higher proportion of IP-10 positive non-TB patients compared to QFT-IT. Due to the lack of a reference for LTBI we do not know if these patients had LTBI and if IP-10 was more sensitive for LTBI than QFT-IT. The IP-10 positive non-TB patients did however have relevant risk for latent infection suggesting that they were correctly classified with LTBI. We found that the 3 IP-10 positive healthy controls were Italian students from Modena. They did not have any known risk factors for *M. tuberculosis* infection but all produced intermediate to high levels of antigen-dependent IP-10 (1300–2437 pg/ml) which suggests that the responses reflect the presence of a latent infection and not an unspecific signal around the cut-off.[29,30] In contrast the antigen-dependent IFN-γ levels were low in all three students when measured in the QFT-IT. In the subgroup of 70 students with T-SPOT.TB results available one student was T-SPOT.TB positive. Previously the specificity of the IP-10 test has been shown to be almost 100% among healthy subjects in low endemic regions.[16,18,19,31] It can be speculated that IP-10 picks up specific signals from individuals with a well controlled/resolved infection.[32] However, in the absence of a gold standard for TB infection, it remains to be demonstrated whether IP-10 is more sensitive for infection with *M. tuberculosis* or less

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Table 6
The association between risk factors relevant to *M. tuberculosis* infection and test result in non-TB patients. Patients with missing information on risk factors and patients with an indeterminate IP-10 or QFT-IT response were excluded from the respective analysis. Odds ratios were adjusted for sex and age.

	n	IP-10 positive	AOR (95% CI)	*p*-value	*n*	QFT-IT positive	AOR (95% CI)	*p*-value
Born in TB endemic area	159				151			
Yes	49	27 (55)	4.0 (1.7–9.0)	<0.002	45	20 (44)	3.1 (1.3–7.3)	<0.009
No	110	34 (31)	1		106	27 (25)	1	
Exposure	126				119			
Yes	15	9 (60)	2.9 (0.9–9.2)	0.074	14	7 (50)	2.9 (0.9–9.6)	0.087
No	111	41 (37)	1		105	29 (28)	1	
Prior TB	140				132			
Yes	16	12 (75)	5.9 (1.8–20.0)	<0.004	14	9 (64)	5.0 (1.5–16.2)	<0.008
No	124	42 (34)	1		118	31 (26)	1	
Stay in high endemic area >3 months	140				132			
Yes	42	20 (48)	2.1 (0.9–5.0)	0.077	39	14 (36)	1.6 (0.6–3.8)	0.332
No	98	33 (34)	1		93	26 (28)	1	
>1 risk factor	124				116			
Yes	36	21 (58)	4.2 (1.7–10.8)	<0.003	33	15 (46)	3.5 (1.3–9.3)	<0.02
No	88	28 (32)	1		83	20 (24)	1	

specific than QFT-IT in otherwise healthy controls and in non-TB patients, and prospective studies are needed to evaluate how these differences affect the predictive values for progression to active TB in e.g. recently exposed with a positive test.

Our second important finding was the difference between the two biomarkers in the group of non-TB patients, where IP-10 release seemed less affected by bacterial or viral infection. Little is known about the influence of ongoing infectious disease and immune-suppression on IGRA performance in sick patients without active TB.[25,26,33] We and others have recently shown that HIV-infected individuals have a decline in IFN-γ responsiveness to mitogen and in QFT-IT sensitivity in patients with a low CD4 count, although IP-10 was influenced by HIV infection it was in a CD4 independent manner[21] (Aabye et al. ERJ in press). Young children (<5 years) are another important clinical challenge where the IGRAs – especially the QFT-IT – have shown compromised performance[34–36] two studies have indicated that IP-10 performs better in young children with an immature immune system.[17,19] These findings together with results presented here suggest that IP-10 could add diagnostic information in patients with immuno-suppression.

Our findings suggest a different interference of non-TB bacterial or viral infection on the performance of the two tests. Some of the disparities could be explained by the different cellular origin of the biomarkers and their very different role during infection. During severe infection, systemic immune responses coincide with counter-regulatory anti-inflammatory responses and changes in leukocyte number, function and phenotype (reviewed in Ref. [37]). These changes are also reflected in a decreased *ex-vivo* T cell responsiveness of pro-inflammatory cytokines (e.g. IL-2, IFN-γ), inline with our findings on IFN-γ mitogen responsiveness and QFT-IT performance. Monocyte function is also affected, both up- and down regulation of cytokine and chemokine responsiveness may occur.[38–41] The immunological mechanisms underlying these differences in IFN-γ and IP-10 are not fully understood, but they are likely to be attributed to the fact that IFN-γ is a cytokine produced by specific T cells when stimulated by the interaction with an antigen presenting cell (APC); whereas IP-10 is elicited in the APCs by signals from a range of cytokines (IL-1, IL-2, IFN-γ, IFN-α, TNF-α) combined with receptor mediated signals from adjacent T cells. And, as IP-10 is not exclusively dependent on IFN-γ expression, IP-10 can be induced by also by non-IFN-γ producing T cell sub-populations, and potentially lead to a more sensitive measure of T cell recognition.[42–44] Further studies are needed to elucidate the background for these discrepancies and to identify areas of potential synergy.

5. Limitations

The group of non-TB patients was typical cases suspected of active TB seen at both outpatients' clinics and among admitted patients, and is a very heterogeneous group. Apart from the Perugia centre, these patients were not prospectively included which reserved us from drawing conclusions on predictive values. The group of TB suspected non-TB patients however was thoroughly investigated for TB and in 72% of 116 with available information, either PCR or culture for *M. tuberculosis* was done and found negative, therefore we do consider this group to be a relevant control group for evaluating diagnostic tests. The applied IP-10 mitogen cut-off was arbitrarily set and has previously been tested in other studies. Compared to QFT-IT the IP-10 test mitogen cut-off is rather low in respect to the antigen responses, which reflects that the PHA mitogen acts directly on the T cells, whereas IP-10 responses are induced in the monocytes upon stimulation from T cell cytokines in the supernatant. Previously the 200 pg/ml cut-off resulted in a comparable rate of indeterminate responders compared to QFT-IT,[16] but in this study we found significantly less indeterminate IP-10 test results than the QFT-IT. Increasing the mitogen cut-off to e.g. 400 pg/ml resulted in higher rate of indeterminate responders 7/168 (4%), 21/175 (12%) and 4/103(4%) among TB patients, non-TB patients and HCs, respectively. These patients and controls converted from negative to indeterminate and lead to improved concordance with QFT-IT, and higher association between risk factors and positive tests in the non-TB group (data not shown). Further studies are needed to validate the cut-off for indeterminate IP-10 test.

The IP-10 measurements in this study have been acquired using Luminex, a technology that allows quantification of up to 200 different markers in a single 50 μL sample. This versatility comes at a price and new data from our group (unpublished) and by others[18,19,21,31] suggests that IP-10 measured with simpler ELISA technology leads to better reproducibility of the measurements, larger differences between nil and antigen responses and herewith better diagnostic accuracy.

6. Conclusion

In conclusion, this study confirms and validates antigen-specific IP-10 response as a diagnostic marker for infection with *M. tuberculosis* with comparable sensitivity and specificity to the QFT-IT and the T-SPOT.TB. IP-10 detected more non-TB patients as positive,

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although IP-10 responses were associated with relevant risk factors of TB infection it remains unanswered whether this was due to higher sensitivity or lower specificity.

IP-10 seems more robust due to the confirmed magnitude of IP-10 responses and appears less affected by non-TB bacterial or viral infection. The discrepancy between IFN-γ and IP-10 biomarkers needs further detailed characterisation and the potential consequence of these differences in clinical practice prompts further investigation.

Perspectives

The high magnitude of IP-10 suggests that it can be measured with simpler technology e.g. the lateral flow platform known from pregnancy and HIV quick tests. The development of such a device-platform could enable the dissemination of specific tests for TB infection in low resource settings.

Authors' contributions

MR designed the study, included study participants, measured IP-10 and IFN-γ (ELISA), managed the master database, performed the statistics and drafted the manuscript; JD, IL included study participants, compiled clinical information and measured IFN-γ (ELISA and ELISPOT) and IP-10; ML, LR, included study participants, compiled clinical information and measured IFN-γ (ELISA and ELISPOT); MBP, RM, DG, OB, JB, HG, IG, TT and GF included study participants, compiled clinical information and measured IFN-γ (ELISA). JEO supervised the lab work and data interpretation, PR co-designed the study, included study participants, participated in the analysis of the results and helped to draft the manuscript. All authors played a part in drafting the final version of the manuscript and all approved the final version.

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Conflict of interest statement: Morten Ruhwald, Pernille Ravn and Jesper Eugen-Olsen are registered as inventors on patents filed by Hvidovre Hospital disclosing IP-10 as a diagnostic marker for infection with *M. tuberculosis*.

Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.tube.2011.01.001.

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5. DISCUSIÓN

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5.1. Utilidad de las técnicas de detección de IFN-γ en el diagnóstico de la infección tuberculosa en población inmunocompetente adulta y pediátrica

Desde que se desarrollaron las técnicas de inmunodiagnóstico basadas en la detección del IFN-γ tras estimular las células T de un paciente con antígenos específicos de *M. tuberculosis*, se han realizado numerosos estudios con versiones previas del QFN-G-IT y técnicas de ELISPOT *in-house,* evaluando su utilidad en el diagnóstico de la infección tuberculosa en poblaciones de estudio bastante heterogéneas. Sin embargo, es necesario disponer de un mayor número de estudios para poder entender mejor el papel que juegan el T-SPOT.TB y el QFN-G-IT en el diagnóstico de la infección tuberculosa en población inmunocompetente adulta y pediátrica. Hasta el momento, parece que son mucho más específicas que la PT en pacientes vacunados con la BCG y que correlacionan mejor con la exposición a *M. tuberculosis* [123, 135, 151-153]. Asimismo, estudios publicados recientemente demuestran el valor pronóstico de estas técnicas en el desarrollo de TB activa [154-157].

En un estudio llevado a cabo por Ferrara et al. [135], estudiaron simultáneamente 393 individuos con sospecha de TB activa o infección tuberculosa por T-SPOT.TB y una versión previa al QFN-G-IT llamada QFN-G, en la que se estimulaba sangre total en placa con los antígenos ESAT-6 y CFP-10 por separado. Ellos observaron que las técnicas basadas en la detección de IFN-γ estaban menos influenciadas por la vacuna que la PT. Por otro lado, de forma global, obtuvieron más resultados positivos por la técnica de T-SPOT.TB que por la del QFN-G. Del mismo modo, nuestros resultados muestran que la PT está más influenciada por la vacuna de la BCG que las técnicas *in vitro* (**Artículo 1, Tabla 3**). Además, también hemos observado que con la técnica de T-SPOT.TB se obtiene un mayor número de resultados positivos que con el QFN-G-IT en población adulta inmunocompetente (**Artículo 1, Tabla 2**).

En un estudio de Arend et al. [151], se compararon ambas técnicas *in vitro* en 785 adultos no vacunados con la BCG procedentes de un estudio de contactos. La concordancia entre ambas técnicas de IFN-γ fue buena (89.6%, κ=0.59). En consonancia con estos resultados, también hemos observado que la concordancia

entre el T-SPOT.TB y el QFN-G-IT en pacientes no vacunados con la BCG también es alta (**Artículo 1, Tabla 4**).

Las células T detectadas por las técnicas *in vitro* corresponden a células efectoras que han entrado en contacto recientemente con el antígeno y que liberan IFN-γ cuando se reexponen nuevamente al antígeno. En cambio, las células T memoria, que persisten durante mucho tiempo después de la desaparición de *M. tuberculosis*, son relativamente quiescentes y probablemente liberan menor cantidad de IFN-γ durante el corto periodo de incubación de las células T con el antígeno. De esta manera, el estudio mediante las técnicas *in vitro* de las células T efectoras y memoria que liberan IFN-γ puede ser de utilidad para el diagnóstico de infección reciente y remota [158-161]. En un estudio que llevamos a cabo recientemente [161], determinamos la respuesta de las células T por las técnicas basadas en la detección de IFN-γ en personal sanitario, comparando los resultados con la PT; y se analizó la capacidad de estas técnicas *in vitro* para detectar infección reciente y remota estimulando las células memoria (**Figura 19**). En los individuos con una PT positiva previa, el T-SPOT.TB y el QFN-G-IT fueron negativos en el 51.6% y el 62.1%, respectivamente. En cambio, en el personal sanitario al que se le realizó PT y técnicas *in vitro* simultáneamente (por ser la primera vez que se les realizaba la PT o presentar una PT previa negativa) la concordancia del T-SPOT.TB y el QFN-G-IT con la PT fue elevada (κ=0.754 y κ=0.929, respectivamente) (**Anexo VII, Tabla 2**). En el personal sanitario no vacunado con la BCG y con una PT positiva previa, se estimularon las células memoria de 24 individuos con test de IFN-γ negativos. En 2 casos (2/10 resultados válidos) la respuesta fue positiva, sugiriendo que al menos estos sujetos fueron positivos por la PT y negativos por T-SPOT.TB debido a una infección remota (**Anexo VII, Figura 1**).

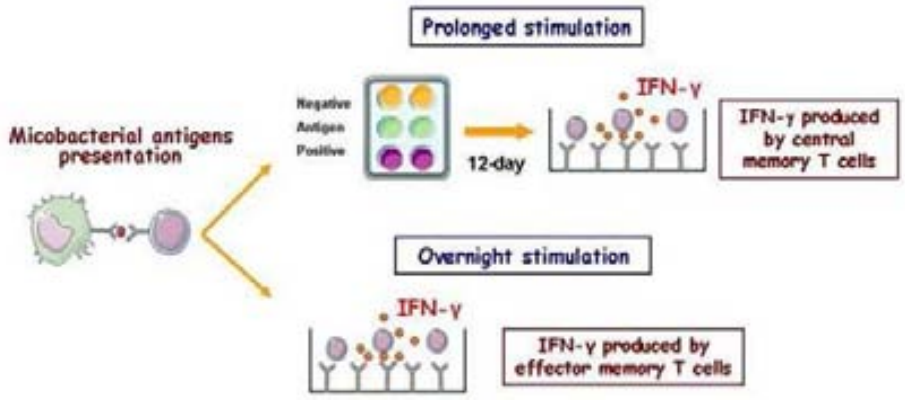

Figura 19. Estimulación de las células memoria y efectoras.

También, se han estudiado estas técnicas *in vitro* en el diagnóstico de la infección tuberculosa en población pediátrica, mostrando su utilidad sobretodo en niños vacunados con la BCG [133, 135, 139, 162, 163].

En nuestro estudio, se han evaluado por T-SPOT.TB y QFN-G-IT 270 individuos procedentes de estudios de contactos, de los cuales 64 eran niños, y hemos observado que ambas técnicas *in vitro* correlacionan mejor que la PT con el riesgo de infección por *M. tuberculosis* (**Artículo 1**). Además, en otro estudio llevado a cabo recientemente por nuestro grupo [164], se han estudiado por ambas técnicas basadas en la detección de IFN-γ 98 niños procedentes de estudios de contactos, observando también una buena correlación con el riesgo de infección y la exposición a *M. tuberculosis* (**Anexo I, Tabla 3**). Estos resultados concuerdan con los resultados de otras publicaciones que evalúan las técnicas de detección de IFN-γ en población pediátrica [133, 162, 163, 165]. Ewer et al. [133], estudiaron la respuesta de las células T por la técnica de ELISPOT tras estimular con antígenos específicos de *M. tuberculosis* en 535 estudiantes, procedentes de un estudio de contactos en un colegio. Ellos observaron que la PT estaba más influenciada por la vacuna de la BCG que el ELISPOT. No obstante, aunque la concordancia del ELISPOT con la PT fue buena (89%, κ=0.72), el ELISPOT correlacionó significativamente mejor que la PT con la exposición a *M. tuberculosis* en función de la proximidad y el tiempo de exposición con el caso índice. Nuestros resultados también muestran que en población pediátrica el T-SPOT.TB y el QFN-G-IT están menos afectados que la PT por la vacuna de la BCG (**Artículo 1, Tabla 3**), y que las concordancias entre la PT y las técnicas de IFN-γ son más elevadas en niños no vacunados que en población vacunada. No obstante, en este tipo de pacientes hemos obtenido una concordancia bastante baja entre la PT y las técnicas *in vitro* (**Artículo 1, Tabla 4)**.

Se ha visto en otros estudios que la concordancia de la PT con el QFN-G o el QFN-G-IT es bastante baja en población pediátrica, además, un elevado porcentaje de niños (vacunados o no vacunados con la BCG) con PT positiva tienen las técnicas basadas en la detección de IFN-γ negativas [134, 165, 166].

Se ha descrito previamente, que existe un elevado porcentaje de PT positivas en población pediátrica debido a infecciones por otras MNT [167]. De hecho, nuestros resultados muestran que un 56.6% de niños no vacunados con la BCG y con una PT positiva, tenían una técnica de T-SPOT.TB negativa (**Artículo 2**). En este sentido,

hemos querido estudiar el papel que juegan las MNT en población pediátrica como factor de discordancia entre la PT y las técnicas *in vitro*. Detectamos la presencia de células sensibilizadas frente a MNT estimulando con sensitinas de *M. avium*, observando que en un 47.6% (10/21) de estos niños se obtuvo una respuesta específica de las células T tras estimular con las sensitinas.

La estimación de infecciones por MNT en nuestra área en niños con una PT positiva entre 5 y 10 mm es del 50% [168]. En nuestro estudio, un 80% (8/10 niños) de los niños en los que se presenciaron células T sensibilizadas frente a sensitinas de *M. avium* tenían una PT entre 5 y 10 mm (**Artículo 2, Figura 1**). Asimismo, en otro trabajo publicado posteriormente por nuestro grupo [164], detectamos células T sensibilizadas tras estimular con sensitinas de *M. avium* en 6 de los 12 niños no vacunados con BCG y con induraciones de la PT entre 5 y 9 mm (**Anexo I**). Sin embargo, otros estudios también han obtenido induraciones superiores a 15 mm [169] y 20 mm [170] en niños infectados por MNT. Esto también concuerda con nuestros resultados, ya que hemos observado en dos de los niños con PT positiva y T-SPOT.TB negativo induraciones superiores a 15 mm.

En nuestro estudio, 8 de los 10 niños del grupo de estudio con resultados positivos para las sensitinas de *M. avium* fueron incluidos durante estudios de cribados escolares de infección tuberculosa, sin evidencia de exposición alguna con un paciente con TB activa (**Artículo 2**). La mayoría de las guías para el diagnóstico y el tratamiento de la TB, recomiendan que en este tipo de población, se considere infección por *M. tuberculosis* cuando una respuesta a la PT sea superior a 10 mm [17]. Por lo tanto, nuestros resultados confirman en parte las recomendaciones de las guías, en el sentido que un resultado de PT inferior a 10 mm en esta población puede considerarse no específico. No obstante, merecen especial consideración los niños que pertenecen a estudios de contactos, ya que tienen más riesgo de desarrollar TB activa, por lo tanto en este grupo de pacientes una PT superior a 5mm indica infección tuberculosa. En este tipo de población, independientemente de que las sensitinas sean positivas y el T-SPOT.TB negativo, el riesgo de progresión a TB activa obliga a considerar al menos una quimioprofilaxis primaria.

Respecto a los 6 resultados discordantes (PT positiva y T-SPOT.TB negativo) en los que se obtuvo una respuesta negativa tras estimular con sensitinas de *M. avium* (**Artículo 2, Tabla 1**), hay al menos tres posibles explicaciones. En primer lugar, podría ser realmente una infección tuberculosa detectada por la PT y no por T-

SPOT.TB. Sin embargo, la sensibilidad de las técnicas basadas en la detección de IFN-γ se considera superior o al menos igual que la de la PT. Segundo, una infección remota detectada por la PT pero no por el T-SPOT.TB, ya que las técnicas *in vitro* detectan células efectoras que han entrado en contacto recientemente con el antígeno, mientras que la PT permanece positiva durante un largo periodo de tiempo. No obstante, en los niños la infección se considera, casi por definición, de adquisición reciente. Finalmente, la tercera explicación sería que la positividad de la PT fuera debido a una infección por otra MNT que no tuviera reacción cruzada con las sensitinas de *M. avium*.

Una limitación importante de este estudio es que no se pudo realizar un *dual test* con sensitinas de *M. avium in vivo* en el momento de la inclusión de los pacientes, y por lo tanto, no se han podido comparar con los resultados *in vitro*.

5.2. Utilidad de las técnicas de detección de IFN-γ en el diagnóstico de infección tuberculosa en población inmunodeprimida

Por otro lado, existe una evidencia cada vez mayor de que las técnicas basadas en la detección de IFN-γ son también robustas cuando se emplean para el diagnóstico de infección tuberculosa en pacientes con déficit en la respuesta inmune celular [139, 171], como podrían ser niños [135, 164, 172], pacientes coinfectados con el VIH [137, 138, 141, 143] y pacientes con enfermedades inflamatorias crónicas, en tratamiento con fármacos inmunosupresores [173-175] (**Anexos VIII y IX**).

Hasta el momento, se ha observado que las técnicas basadas en la detección de IFN-γ presentan en población infectada por el VIH un mayor porcentaje de resultados positivos con una baja concordancia con la PT [136-138, 140, 176-180]. Sin embargo, pocos estudios comparando T-SPOT.TB, QFN-G-IT y PT se han desarrollado en el mismo grupo de pacientes para determinar su utilidad en el diagnóstico de la infección tuberculosa [141-144].

Talati et al. [142], comparó las tres técnicas en 336 pacientes VIH. El porcentaje de positivos (2.1% por la PT, 2.7% por QFN-G-IT y 4.2% por T-SPOT.TB) y las concordancias entre las tres técnicas fueron bajas. Del mismo modo, en un estudio en colaboración con el grupo de Módena (Italia) durante una estancia predoctoral [141], estudiamos las técnicas de T-SPOT.TB, QFN-G-IT y PT en un grupo de pacientes

inmunodeprimidos (candidatos a transplante de hígado, VIH positivos y pacientes con enfermedades hematológicas). Se estudiaron específicamente 116 pacientes VIH positivos, identificándose un bajo porcentaje de pacientes infectados por *M. tuberculosis* y una concordancia moderada entre las tres técnicas (**Anexo II, Tablas 2 y 3**). En nuestra experiencia [144], se comparó la PT con ambos tests basados en la detección de IFN-γ en 139 pacientes con adicciones a alcohol y drogas, de los cuales un 31% estaban infectados por el virus de VIH. Se encontraron por las técnicas *in vitro* porcentajes de positivos más elevados en pacientes con CD4 >350 células/μl que con <350 células/μl (**Anexo III, Tabla 2**). Por otro lado, también han sido evaluadas estas tres técnicas en pacientes VIH positivos residentes en Uganda, un país de alta incidencia de TB [143]. Se estudiaron 109 individuos y se observó que la frecuencia global de resultados positivos fue del 47.2%, 54% y 67.9% por la PT, T-SPOT.TB y QFN-G-IT respectivamente. En nuestro estudio observamos un bajo porcentaje de resultados positivos por las tres técnicas. Además, de los 7 y 5 casos en los que se detectó un T-SPOT.TB y un QFN-G-IT positivo, ambas técnicas fueron simultáneamente positivas en solo 2 casos, encontrando, por lo tanto, una baja concordancia entre ellas (**Artículo 4, Tabla3**).

El análisis de los resultados discordantes necesita ser mejor estudiado. En nuestra experiencia [164], hemos observado que cuando hay discordancias entre ambas técnicas de detección de IFN-γ, existe una correlación negativa entre el número de células T reactivas en el T-SPOT.TB y la cantidad de IFN-γ liberado en el QFN-G-IT, es decir, ambas técnicas son fuertemente discordantes. Estas discordancias podrían sugerir trastornos de la inmunidad celular frente a la infección tuberculosa, relacionándose con un declive de la producción de IFN-γ o bien del número de células T productoras de IFN-γ. Por lo tanto, este grupo de pacientes sería el que más se beneficiaría del tratamiento de la infección tuberculosa, ya que podrían presentar un mayor riesgo de desarrollar TB activa (**Anexo I**).

Por lo que respecta al número de positivos en función de las células CD4, el porcentaje de resultados positivos para el T.SPOT.TB y el QFN-G-IT fue menor en los pacientes con número de CD4 <200 respecto a los >200. No obstante, el T-SPOT.TB parece estar menos afectado por la inmunosupresión severa, ya que se obtuvo al menos un resultado positivo por esta técnica (5%), y ninguno por QFN-G-IT. De todos modos, el número de resultados positivos es limitado para poder establecer conclusiones. Asimismo, Converse et al. [181], evaluaron el efecto de la

inmunosupresión en el QFN-G, y encontraron que a medida que disminuía el número de células CD4, la sensibilidad de QFN-G también diminuía.

Por otro lado, las técnicas basadas en la detección de IFN-γ pueden detectar ausencia de respuesta inmune ya que presentan controles internos que permiten identificar falta de respuesta en pacientes anérgicos. Por lo tanto, un resultado negativo en el mitógeno (control positivo) y negativo frente a los antígenos específicos se ha de considerar un resultado indeterminado. Este resultado implica una falta de respuesta de las células T del paciente. Un paciente con un resultado de la tuberculina negativo y un resultado indeterminado por las técnicas *in vitro* podría significar que nos encontramos frente a un falso negativo de la tuberculina (**Figura 20**). Por lo que respecta a los resultados indeterminados, se ha descrito que están asociados a niños menores de 5 años, inmunosupresión y una PT negativa [135, 182-184].

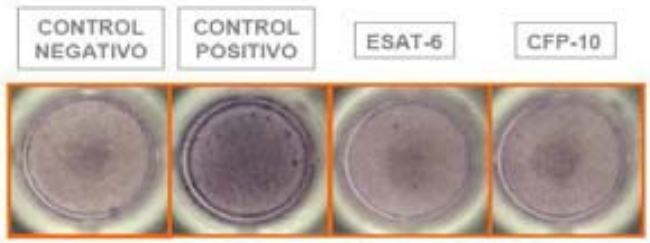

Figura 20. Resultado inderterminado por la técnica de T-SPOT.TB.

En nuestro estudio realizado en pacientes infectados por el VIH, hemos encontrado dos resultados indeterminados (2.4%), ambos por T-SPOT.TB y QFN-G-IT, de los cuales uno pertenecía a un paciente con 103 células CD4. La PT fue negativa en todos los casos. Se ha descrito que los resultados indeterminados en este tipo de población están asociados a un bajo número de células CD4, tanto en el diagnóstico de la infección tuberculosa como en el de la TB activa [140, 142, 176, 177, 179, 185, 186].

No obstante, hemos observado también en población inmunocompetente resultados indeterminados por las técnicas de T-SPOT.TB y QFN-G-IT en un 1.1% y un 0.2% de los casos, respectivamente (**Artículo 1, Tabla 2**).

Por lo que respecta a la vacuna de la BCG, nuestros resultados en población infectada por el VIH evidencian que las técnicas inmunológicas *in vitro* están menos influenciadas que la PT por la vacuna (**Artículo 4, Tabla 2**). Además, hemos

encontrado 2 resultados negativos por las técnicas de IFN-γ en pacientes no vacunados con la BCG y con una PT positiva. Una posible explicación de estos resultados discordantes sería la posible sensibilización por MNT, tal y como se ha comentado anteriormente. En este estudio nos fue imposible estimular *in vitro* con sensitinas de MNT ya que no disponíamos de suficientes PBMCs. No obstante, todavía no está clara la seguridad de no tratar a este tipo de pacientes, con y sin BCG, que tienen una PT positiva y técnicas *in vitro* negativas, especialmente aquellos que tienen inmunosupresión severa. De este modo, el uso combinado de la PT con las técnicas basadas en la detección de IFN-γ puede ser beneficioso para el diagnóstico de la infección tuberculosa en pacientes infectados por el VIH con mayor riesgo de progresión a TB activa.

Por otro lado, también hemos estudiado la utilidad de ambas técnicas *in vitro* en el diagnóstico de la infección tuberculosa en 53 pacientes con enfermedades inflamatorias reumáticas, que van a iniciar tratamiento con anti-TNF-α. En nuestra experiencia, el uso de los tests basados en la detección de IFN-γ es de utilidad en este tipo de pacientes, ya que permiten detectar resultados falsos negativos de la PT, falsos positivos en pacientes vacunados con la BCG y sensibilizados por MNT, y aportan casos adicionales de pacientes infectados por *M. tuberculosis* [187] (*Manuscrito en preparación*).

5.3. Utilidad de las técnicas de detección de IFN-γ en el diagnóstico de la enfermedad tuberculosa

Varios estudios han evaluado la utilidad de las técnicas basadas en la detección de IFN-γ para el diagnóstico de la TB activa, describiendo un amplio rango de sensibilidades [188, 189].

Lee et al. [190] compararon ambos tests basados en la detección de IFN-γ en pacientes con TB activa. Los autores observaron que la sensibilidad del T-SPOT.TB fue mayor que la del QFN-G-IT y la PT. Kang et al. [191] encontraron que la sensibilidad del T-SPOT.TB y el QFN-G-IT en el diagnóstico de la TB activa fue del 92% y el 89%, respectivamente. Asimismo, Chee et al. [192] obtuvieron sensibilidades del 94.1% y 83% para el T-SPOT.TB y QFN-G en pacientes con TB pulmonar. Del mismo modo, los resultados obtenidos por nuestro grupo coinciden con los descritos en estos estudios [132] (**Anexo IV, Tabla 2**), ya que también observamos que la sensibilidad del T-SPOT.TB es mayor que la del QFN-G-IT (**Artículo 1, Tabla 2**).

Existen varios estudios realizados en niños con TB activa, donde se describen rangos alrededor del 60% y el 90% de sensibilidad para las técnicas basadas en la detección de IFN-γ [134, 169, 183, 193, 194]. Nuestros resultados describen que las sensibilidades de las técnicas de T-SPOT.TB y QFN-G-IT en población pediátrica con TB activa son del 66.7% (**Artículo 1, Tabla 2**). Del mismo modo, en el estudio de Altet-Gómez et al. [164] evaluamos 14 niños diagnosticados de TB activa, obteniendo sensibilidades del 78.6% y 64.3% par las técnicas de T-SPOT.TB y QFN-G-IT, respectivamente (**Anexo I**).

Sin embargo, las técnicas basadas en la detección de IFN-γ liberado han sido diseñadas para el diagnóstico de la infección tuberculosa, y no pueden discriminar entre TB activa e infección cuando se realizan con muestras sanguíneas. En este sentido, se han publicado varios estudios en los que se modifican los puntos de corte en el número de *spots* y la concentración de IFN-γ para poder discriminar entre infección tuberculosa y TB activa [195, 196].

En nuestra experiencia, en pacientes adultos, el número de células que responden a los antígenos RD1 es significativamente superior en los pacientes con TB activa respecto a los que estaban infectados (**Artículo 3, Figura 1**). Sin embargo, existe mucho solapamiento en la respuesta tanto de pacientes infectados como enfermos, implicando una baja especificidad.

Janssens et al. [196], evaluó la respuesta a los antígenos RD1 en el T-SPOT.TB con el fin de poder diferenciar entre infección tuberculosa y TB activa. Estudiaron contactos con un T-SPOT.TB positivo y pacientes diagnosticados de TB activa. Pudieron establecer un punto de corte de 49.5 células T reactivas que respondieron a los antígenos RD1, con una sensibilidad del 83% y una especificidad del 74%. El punto de corte propuesto en nuestro estudio fue de 116 células T reactivas en respuesta a los antígenos RD1, con una sensibilidad del 43% y una especificidad del 81% (**Artículo 3, Tabla 3**). Las diferencias entre los puntos de corte de nuestro estudio y el publicado por Janssens et al. podrían ser debidas en parte a que incluyeron pacientes procedentes de estudios de contactos con una PT negativa y positiva, mientras que en nuestro estudio fueron solamente con PT positiva. Los autores encuentran que la media de células T que liberan IFN-γ en los contactos con PT positiva es mayor que la de los contactos con PT negativa. Como consecuencia, cuando este grupo de

contactos (PT negativa y positiva) se analizó para determinar el punto de corte, éste fue más bajo que si se hubieran considerado solamente los contactos con PT positiva.

En un estudio llevado a cabo por Chee et al. [197], observaron que la respuesta de las células T a los antígenos ESAT-6 y CFP-10 fue significativamente superior en pacientes con TB activa respecto a los que estaban infectados, en cambio, no se encontró ninguna diferencia estadísticamente significativa entre los dos grupos por el QFN-G-IT. Finalmente, en un estudio llevado a cabo recientemente [198] también se encontraron respuestas elevadas de IFN-γ asociadas con TB activa.

Fox et al. [199], estudió la respuesta a los antígenos ESAT-6 y CFP-10 en el T-SPOT.TB. Ellos encontraron una mayor respuesta al antígeno CFP-10 en los pacientes con TB activa, indicando que este antígeno podría ser un marcador de TB activa. En nuestro estudio, el número de células T después de estimular con el antígeno CFP-10 fue significativamente mayor en los casos con TB activa respecto los otros dos grupos de pacientes con infección tuberculosa. Sin embargo, el antígeno ESAT-6 únicamente fue significativo cuando se compararon los pacientes con TB respecto los que se estudiaron durante cribados de infección tuberculosa, y no con los de estudios de contactos (**Artículo 3, Figura 1**).

Los resultados de un estudio publicado por Kobashi et al. [200], explican que la respuesta cuantitativa al antígeno CFP-10 disminuye durante el tratamiento antituberculoso, de forma que también podría utilizarse como un marcador de monitorización de la eficacia clínica. En nuestra experiencia [132], la respuesta de las células T al antígeno CFP-10 disminuye durante el tratamiento cuando se compara con el inicio de la terapia antituberculosa, aunque las diferencias no son significativas. Sin embargo, tras la finalización del tratamiento, esta respuesta aumenta alcanzando valores similares a los del inicio del tratamiento (**Anexo IV, Figura 1**).

En nuestros resultados en población adulta no se observan diferencias significativas en el IFN-γ liberado en el QFN-G-IT entre ningún grupo de los pacientes de estudio. Esto puede ser debido a que en los pacientes con TB activa hay una baja frecuencia de células T específicas en sangre [201], debido al secuestro de éstas en el foco de la infección [202, 203]. Otra posible explicación sería metodológica, ya que en la técnica de QFN-G-IT, el IFN-γ liberado queda diluido en todo el volumen de sangre del tubo de extracción. Por lo contrario, el T-SPOT.TB siempre se realiza con un número definido de células T.

Aunque las diferencias en el número de células T reactivas en el T-SPOT.TB y el IFN-γ liberado en el QFN-G-IT no fueron significativas entre los contactos y los pacientes estudiados en cribados de infección tuberculosa, esta respuesta fue mayor en los pacientes procedentes de estudios de contactos, ya que en éstos existe una exposición a *M. tuberculosis* conocida y además, la respuesta de las células T es mayor por proximidad en el tiempo al caso índice (**Artículo 3, Figura 1**).

En población pediátrica, la ausencia de diferencias significativas entre los pacientes con TB activa y aquellos con infección tuberculosa (**Artículo 3, Figura 2**) puede ser atribuida a que las infecciones en los niños son normalmente recientes. Por lo tanto, la respuesta de las células de aquellos niños infectados es casi igual de elevada que en los enfermos, además, el diagnóstico de TB activa siempre se realiza en fases muy incipientes, y por lo tanto la carga bacteriana es muy baja debido a que nunca hay una gran afectación.

Debido a que no existe una técnica *gold standard* para el diagnóstico de la infección tuberculosa, una medida para poder estudiar la sensibilidad de las técnicas basadas en la detección de IFN-γ, es su evaluación en pacientes con TB activa, ya que por definición éstos están infectados. Como consecuencia, las sensibilidades que se obtienen oscilan entre el 60% y el 90%, y no llegan a ser del 100%, ya que un paciente con TB activa es aquel que no ha podido contener inmunológicamente la enfermedad. Además, tal y como ya se ha comentado, en pacientes con TB activa existe un secuestro de las células T sensibilizadas en el foco de la infección, y por lo tanto, disminuye el número de células T sensibilizadas en sangre.

Recientemente, se ha descrito la utilidad para el diagnóstico de la enfermedad tuberculosa detectando las células T sensibilizadas en muestras del foco de infección, como pueden ser muestras obtenidas de lavado broncoalveolar, líquido pleural, líquido cefalorraquideo y líquido articular [204-208]. Esta estrategia podría resultar una buena alternativa en el diagnóstico de la TB pulmonar en pacientes con baciloscopia negativa, y también en pacientes con TB extrapulmonar.

En un estudio piloto de Jafari et al. [204], se estudió la respuesta de las células T específicas en lavado broncoalveolar por la técnica de ELISPOT en 12 pacientes con TB activa y 25 controles con otras enfermedades no tuberculosas. En todos los pacientes con TB activa se obtuvo una respuesta positiva, en cambio, todos los controles fueron negativos. En un estudio multicéntrico realizado en el seno del

TBNET, en el que colaboramos, se ha descrito una sensibilidad del 91% y una especificidad del 80% en el diagnóstico de la TB pulmonar con técnica de ELISPOT en lavado broncoalveolar [207] (**Anexo V**).

5.4. Estudio de nuevos antígenos y citoquinas en el diagnóstico de la TB

Se han desarrollado nuevas propuestas para el diagnóstico de la TB activa utilizando las técnicas basadas en la detección de IFN-γ. Se ha evaluado el uso de péptidos RD1 seleccionados para discriminar entre pacientes con TB activa e infección tuberculosa. Estos péptidos son una selección de los antígenos leucocitarios humanos de clase II-RD1 restringidos de las proteínas ESAT-6 y CFP-10 de *M. tuberculosis* [145, 146, 209, 210].

La utilidad de estos antígenos seleccionados ha sido evaluada previamente en el inmunodiagnóstico de la TB activa. Vicenti et al. [146], estudiaron la respuesta a los péptidos ESAT-6 seleccionados y observaron una sensibilidad del 74% y una especificidad del 100%. Igualmente, en un estudio llevado a cabo por Goletti et al. [209], determinaron la aplicabilidad de los péptidos ESAT-6 y CFP-10 seleccionados con la técnica de ELISPOT para el diagnóstico de la TB activa, indicando una sensibilidad del 70% y una especificidad del 91%.

En nuestra experiencia, la sensibilidad hallada en los pacientes incluidos al inicio del tratamiento en el ELISPOT con RD1 seleccionados es más baja que la del T-SPOT.TB o PT, aunque las diferencias no son significativas (**Artículo 5, Tabla 2**). Estas diferencias en las sensibilidades pueden ser debidas a la cantidad y composición de los epítopos que se utilizan en las diferentes técnicas. En un estudio multicéntrico realizado por miembros del TBNET [211], comparamos las dos técnicas *in vitro* comerciales y la nueva técnica inmunodiagnóstica basada en los péptidos RD1 seleccionados en el diagnóstico de la TB activa. La combinación de cualquiera de los tests basados en la detección de IFN-γ evaluados en el estudio en conjunto con la PT prácticamente excluye la TB activa (**Anexo VI**).

También, se ha estudiado la utilidad de los antígenos seleccionados de las proteínas ESAT-6 y CFP-10 en la monitorización de la eficacia del tratamiento antituberculoso. Los resultados del estudio de Carrara et el. [147] muestran como en 18 pacientes con TB microbiológicamente confirmada y con respuesta positiva al antígeno seleccionado ESAT-6, después de 3 meses de tratamiento antituberculoso, solamente 5 de ellos

seguían siendo positivos. Además, en otro estudio llevado a cabo por el mismo grupo [210], encontraron una disminución de la respuesta de IFN-γ a los péptidos seleccionados en 12 pacientes coinfectados por el VIH y *M. tuberculosis* una vez finalizado el tratamiento antituberculoso. Nuestros resultados refuerzan los estudios previos, ya que observamos que el número de células T que responden a los antígenos RD1 seleccionados disminuye durante el tratamiento (**Artículo 5, Figura 1**), es más, la conversión a resultados negativos es significativamente mayor para el ELISPOT experimental con los antígenos RD1 seleccionados.

La frecuencia de células T efectoras que se encuentran *in vitro* durante el corto periodo de incubación con los antígenos específicos, son capaces de liberar IFN-γ, mientras que las células memoria no. Esta sería una de las posibles teorías que explicarían la reducción de la respuesta de IFN-γ durante el tratamiento, ya que sería directamente proporcional a la carga antigénica y al mismo tiempo a la carga bacteriana [212].

Se ha propuesto que la cuantificación de una respuesta a una sola citoquina de los antígenos específicos de *M. tuberculosis* convencionales, como el IFN-γ, es insuficiente como biomarcador de carga micobacteriana. En este sentido, se han evaluado otras citoquinas como la IL-2 y la IP-10 obteniéndose resultados bastante prometedores [213-215]. No obstante, se ha descrito que únicamente la respuesta de IFN-γ a los antígenos seleccionados se asocia a TB activa [216]. En este estudio, la detección de otros biomarcadores se correlacionaba con la TB activa, aunque no aportaba una mejora en el rendimiento de la técnica.

Por otro lado, la búsqueda y el estudio de nuevos biomarcadores, diferentes a la citoquina IFN-γ, podrían permitir aumentar la sensibilidad de las técnicas *in vitro* para el diagnóstico de la infección tuberculosa y la TB activa.

Se ha estudiado un amplio rango de biomarcadores candidatos [148-150], de los cuales la citoquina IP-10 es la más prometedora. En un estudio llevado a cabo por Ruhwald et al. [214], se evaluó una cohorte de controles y pacientes con TB activa y se encontró un punto de corte para la detección de la IP-10. También, en otro estudio realizado por el mismo grupo, se demostró que un test basado en la detección de IP-10 es como mínimo igual de preciso que el QFN-G-IT [217]. Otros grupos de

investigación han encontrado resultados similares en población adulta [218], pediátrica [219, 220] y pacientes infectados por el VIH [218].

De la misma forma que en el estudio llevado a cabo por Ruhwald et al. [214], hemos observado que la detección de la citoquina IP-10 por la técnica de Luminex es comparable a la del IFN-γ por las técnicas de QFN-G-IT y T-SPOT.TB. Además, se ha observado que con el uso combinado de IP-10 y QFN-G-IT se puede conseguir un aumento de la sensibilidad en el diagnóstico de la TB activa sin que la especificidad se vea alterada (**Artículo 6, Tabla 4**).

En este estudio se ha observado un número de resultados positivos por IP-10 superior a los de QFN-G-IT en pacientes con otras enfermedades no tuberculosas (**Artículo 6, Tabla 4**). Debido a que no existe una técnica *gold standard* para el diagnóstico de la infección tuberculosa, no podemos afirmar con toda seguridad que estos pacientes estén infectados y que, por lo tanto, la detección de IP-10 sea menos específica que la de IFN-γ por QFN-G-IT. No obstante, estos pacientes fueron estudiados, encontrándose un riesgo relevante de infección tuberculosa. Por otro lado, también observamos que 3 controles sanos fueron únicamente positivos para la IP-10 (**Artículo 6, Tabla 4**).

Se conoce muy poco acerca de la influencia que pueden causar otras enfermedades de etiología infecciosa o no infecciosa en la producción de IP-10 e IFN-γ. Se ha demostrado que en pacientes con TB activa infectados por el VIH, la respuesta de IP-10 es mayor que la de IFN-γ. [218]. Además, se ha descrito que la detección de IP-10 podría utilizarse, en combinación con el IFN-γ, para el diagnóstico de la infección tuberculosa y la TB activa en niños con un sistema inmunitario inmaduro (menores de 5 años) [217, 220]. Estos últimos hallazgos, sugieren que la IP-10 es un marcador robusto en pacientes con inmunosupresión. Por otro lado, las discrepancias encontradas en la detección de ambas citoquinas, pueden ser justificadas por el diferente origen celular de ambos biomarcadores, y también por su diferente función durante la infección, aunque todavía los mecanismos inmunológicos de estas discrepancias no son del todo conocidos [221, 222].

6. CONCLUSIONS

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Ability of IFN-γ assays in clinical practice (Article 1)

- IFN-γ tests are more specific than tuberculin skin test because they are less affected by BCG vaccination. So, the utilization of these assays can help to reduce unnecessary latent TB treatment among adult and pediatric populations.

- IFN-γ assays could help in the immunodiagnosis of active TB. Although, T-SPOT-TB gives a higher number of positive results than QFN-G-IT in this population.

- The number of indeterminate results of IFN-γ assays is low in adult and pediatric immunocompetent population.

- Detection of IFN-γ produced by T cells after *M. tuberculosis* specific antigen stimulation is an alternative diagnostic tool for latent TB infection.

Non-tuberculous mycobacteria effect in the TB infection diagnosis (Article 2)

- Previous non-tuberculous mycobacteria sensitization in children induces false-positive results in the tuberculin skin test for diagnosing latent TB infection. Therefore, the use of IFN-γ tests provides a more specific diagnosis of TB infection in childhood.

Quantitatification of T-cell responses in active and latent TB (Article 3)

- In adult patients, the number of responder T cells after RD1 antigens stimulation is significantly higher in active TB than in latent TB infection patients. In contrast, in pediatric population, there is an absence of significant difference in the response between active TB and latent TB infection that could be explained by the fact that pediatric infection is usually recent.

- In patients with clinically suspected TB, a cell count of reactive T cells (after RD1 antigens stimulation) above the described threshold, could suggest active TB, especially in patients with a high suspicion of having active TB in low latent TB infection prevalence countries.

- Nevertheless, there is a large amount of T-cell enumeration overlapping that makes it difficult to distinguish between active TB from latent TB infection.

IFN-γ response in HIV-infected patients for detection of TB infection (Article 4)

- IFN-γ tests have the benefit over tuberculin skin test that they are less influenced by BCG-vaccination in HIV-infected individuals, consequently they are more specific than TST in this kind of population.

- Percentage of positive results of IFN-γ assays seems to be influenced by the level of immunosuppression. Although T-SPOT.TB assay seems to be less affected by the immunosuppression level than QFN-G-IT and tuberculin skin test.

- A low rate of IFN-γ assays indeterminate results has been detected in HIV infected population.

- The agreement between both IFN-γ tests in HIV infected patients is poor.

- The use of IFN-γ assays in combination with TST could be a helpful method for diagnosing latent TB infection in HIV severely immunosuppressed population.

RD1-selected peptides for the monitoring of anti-TB therapy (Article 5)

- Specific *M. tuberculosis* T cell responses to RD1-selected peptides decline during anti-TB treatment.

- Immunological assays based on RD1 selected peptides correlate well with bacterial burden and seem to be promising tools for studying host immune response during anti-TB therapy.

IP-10 for the diagnosis of LTBI and active TB (Article 6)

- Antigen-specific IP-10 response can be useful as a biomarker for latent TB infection and active TB.

- IP-10 and IFN-γ responses could be combined for a significant sensitivity improvement in the diagnosis of active TB, without a compromise in specificity.

In summary, IFN-γ tests are more specific than TST because they are less affected by BCG vaccination and NTM sensitization. So, the utilization of these assays can help to reduce unnecessary LTBI treatment among adult and pediatric populations. Therefore, detection of IFN-γ produced by T cells after *M. tuberculosis* specific antigen stimulation is an alternative diagnostic tool for latent TB infection in immunocompetent and immunosuppressed patients. In addition, IFN-γ assays could help in the immunodiagnosis of active TB. On the other hand, the study of new specific antigens and biomarkers is a potential tool for studying host immune response during anti-TB therapy and finding novel diagnostic markers for active TB and *M. tuberculosis* infection.

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ANEXO I

Diagnosing TB infection in children: analysis of discordances using *in vitro* tests and tuberculin skin test

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Objectives: To study the performance of the IFN-γ tests (QuantiFERON-TB-Gold *In Tube* [QFN-G-IT] and T-SPOT.*TB*) and the tuberculin skin test (TST) in diagnosing tuberculosis infection in children, and to analyse discordant results.

Patients and Methods: A prospective study including 98 children from contact-tracing studies; and 68 children with TST≥5mm recruited during public health screenings.

Results: Positive IFN-γ tests results were associated with risk of exposure (p<0.0001). T-SPOT.*TB* was positive in 11/14 cases with active TB (78.6%) and QFN-G-IT in 9/14 (64.3%). In 6 of 12 children non-BCG-vaccinated, with a TST induration between 5 and 9 mm and both IFN-γ tests negatives, the detection of sensitised T cells against *Mycobacterium avium* was positive. In concordant IFN-γ tests results, a positive correlation was found (p=0.0001) between the number of responding cells and the amount of IFN-γ released; however, in discordant IFN-γ tests results this correlation was negative (p=0.371): an increase in the number of spot forming cells correlated with a decrease in the amount of IFN-γ released.

Conclusions: The use of IFN-γ tests is helpful for the diagnosis of TB infection, avoiding cross-reactions with BCG immunisation and NTM infections. The analysis of highly discordant results requires further investigation to elucidate possible clinical implications.

In 2007 the estimated global incidence of tuberculosis (TB) cases was 9.27 million. Approximately 11% of these cases were children. In the developed world the estimated proportion of children with TB is around 3-6%, but in developing countries this percentage can reach 15-20%, with an approximate mortality of 30% [1]. Latent tuberculosis infection (LTBI) treatment is an essential strategy to eliminate TB [2], though to achieve any epidemiological impact this strategy must target groups with high risk of infection and development of the disease if they get infected. Children merit special consideration since they can develop the disease very quickly after primary infection, with the most severe forms prevailing in younger children [3].

The advantages of the techniques based on the detection of gamma interferon (IFN-γ) secreted by effector T cells stimulated with specific *Mycobacterium tuberculosis* antigens to diagnose LTBI, over the tuberculin skin test (TST) are essentially the lack of cross-reactivity with vaccinal *Mycobacterium bovis* Bacillus Calmette-Guérin strains (BCG), and non tuberculous mycobacteria (NTM), and the absence of booster effect [4, 5]. These antigens are the early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) encoded in the region of difference (RD) 1, and TB7.7 encoded in RD11, absent in all BCG strains and in the majority of NTM. Basically, two commercialized *in vitro* assays based on this technology are currently available: *QuantiFERON-TB GOLD In Tube* (QFN-G-IT) (Cellestis, Carnegie, Australia)

and T-SPOT.*TB* (Oxford Immunotec, Oxford, UK). Studies in adults have shown a high sensitivity and specificity for TB diagnosis [5-8]. In a recent systematic review [5], using active TB as a surrogate for LTBI, sensitivities values were 70% (95% confidence intervals [CI]:63-78) for QFN-G-IT and 90% (95% CI:86-92%) for T-SPOT.*TB*; and specificity: 96% (95%C.I: 94-98%) for QFN-G-IT, and 93% (95% CI:86-100%) for T-SPOT.*TB*.

The objectives of this study were: to compare QFN-G-IT and T-SPOT.*TB* results with those obtained by TST for the diagnosis of TB infection in children in a referral clinical centre for TB control and to analyse their concordant or discordant results.

MATERIALS AND METHODS

Study design. Prospective study in children (≤15 years old) who attended the "Unitat Clínica de Prevenció i Control de la Tuberculosi" in Barcelona (Spain) between September 2005 and September 2007. This study was approved by the Ethics Committees of Fundació Jordi Gol i Gurina and of the Hospital Universitari Germans Trias i Pujol (Spain). Parents were asked to sign an informed consent form.

Children were divided in two groups: a contact group (CG) including children studied due to a close contact with a smear positive active TB patient diagnosed within the last 15 days; and a screening group (SG): healthy children with a positive TST result detected during an epidemiological screening at school or by their paediatrician.

Data were collected by means of a structured interview. A clinical examination, TST result, chest X-rays and both IFN-γ tests were performed. The presence of a BCG scar was recorded. Children were excluded if they had a previous history of any TB treatment.

The risk of infection was classified into three groups: a) High: same household and different household, but with a daily contact of 6 hours or more with the contagious index case; b) Medium: non-daily contact with the contagious index case, minimum once weekly; c) No risk known: children from SG without any TB index case known.

A blood drawn was performed within the 5 days after the TST performance. The study was double-blinded: the clinical diagnosis of TB was made without knowing the IFN-γ test results, and the researchers in the laboratory did not know the clinical data prior to the performance of the tests.

TST. The test was performed with 2 units of purified protein derivative RT23 [1]. TST was considered positive when the induration was ≥5 mm in contacts and in children with abnormal chest radiographs consistent with active TB; and ≥10 mm for children in the SG, irrespective of BCG immunisation.

Active TB diagnosis. We followed national guidelines for the diagnosis of the active TB cases [9, 10] . A TB case was considered as a child with an isolation of *M. tuberculosis* from clinical specimens, or a child with presence of symptoms, signs and/or radiological images compatible with TB (when Chest-X-Ray was doubtful CT thoracic scan was performed); and/or TST positive (as defined previously), and child who clinically responded to anti-tuberculous chemotherapy. The fact of being a close contact of a bacillary TB case was used as a diagnostic support.

T-SPOT.TB. Specific mononuclear cells isolated from peripheral blood (PBMCs) were stimulated with ESAT-6 and CFP-10 separately, according to manufacturer's recommendations. Positive, negative and indeterminate results were strictly interpreted following manufacturer's instructions. Non-stimulated cells were washed with RPMI medium (Invitrogen, Auckland, NZ) and resuspended in freeze medium (80% RPMI and 20% foetal bovine serum [PAA Laboratories GmbH, Pashing, Austria]) adding, drop wise, 10% of DMSO (Merck, Darmstadtd, Germany) and then frozen at -80℃. We considered the sum of spot forming cells (SFCs) obtained after ESAT-6 and CFP-10 stimulation as an overall RD1 response [11].

Ex vivo detection of T-cell sensitised against *M. avium* sensitin. To investigate the influence of NTM infections on non-BCG-vaccinated children, with a TST induration between 5 to 9 mm and both IFN-γ tests with a negative results, we performed an *ex vivo* ELISPOT, stimulating the

cells with *Mycobacterium avium* sensitin. Cells were thawed and re-suspended in RPMI medium. Then, cells were washed, re-suspended and stimulated with medium alone, phytohaemagglutinin and *M. avium* sensitin (10 µg/mL) (Statens Serum Institute, Copenhagen, Denmark) as previously described [12]. Sensitised cells were detected by ELISPOT. The interpretation of the results followed the same criteria as that for detecting ESAT-6 and CFP-10 immunoresponse.

QFN-G-IT. The test detects IFN-γ released from T cells stimulated with the specific antigens in whole blood. QFT-G-IT incorporates specific antigens (ESAT-6, CFP-10, and TB7.7) inside the same blood collection tube. The test was performed and the results were interpreted according to the manufacturer's instructions.

Statistical methods. The qualitative variables description is based on the calculation of the number and its percentage, and for quantitative variables, on calculation of the mean and the standard deviation (SD). The chi-squared test and two-tailed Fisher's exact test were used to compare qualitative variables. The odds ratios (OR) and its 95% confidence intervals (95%CI) were calculated. The associated variables with a value $p<0.05$ were analysed at a multivariate level by means of logistic regression. Non-parametric tests (Mann-Whitney, Kolmogorov-Smirnov, Kruskal-Wallis) were used to compare quantitative variables according to the categories of the group variable. Graphic analysis and Pearson correlation techniques (CC) were used to study the association. Cohen's kappa coefficient (k) was used to analyse the concordance, its p value and standard error (according to Landis and Cock estimation). The area under the receiver operating characteristic (ROC) curve was calculated to compare the diagnostic performance of the TST, T-SPOT.*TB* and QFN-G-IT in the diagnosis of active TB. The data were analysed using SPSS (version 14.0; SPSS Inc., Chicago; IL, USA).

RESULTS

Clinical performance. A total of 166 children were included in the study, 84 (50.6 %) were female. The age (mean±SD) was 9.08±4.85 years. Ninety eight (59%) were contacts and 68 (41%) belonged to the SG. In 149 chldren (89.8%) TST was positive. This high percentage of TST positive results is due to the fact that all children included in the SG group were TST positive. The IFN-γ tests (either one or both) were positive in 72 children (43.4%; 95%CI=35.7-51.3): 54 contacts (55.1%;95%CI=44.7-65.2) and 18 from the SG (26.5%; 95%CI=16.5-38.6). T-SPOT.*TB* was positive in 64 children (38.6%; 95%CI=31.1-46.4) and QFN-G-IT in 61 (36.7%; 95%CI=29.4-44.6) (Table 1). Treatment of LTBI was considered according to the TST result, consequently children who had positive TST and negative IFN-γ tests were treated and conventional follow-up and control was done.

TABLE 1. Variables associated with a positive IFN-γ tests result: bivariate and multivariate analysis in the 166 children included in the study.

Variable	Total	T-SPOT. *TB* (n = 166)					QFN-G-IT[2] (n= 166)				
		Positive (%)	Unadjusted OR [1] (95% CI)	*p*	Adjusted OR (95% CI)	*p*	Positive (%)	Unadjusted OR (95% CI)	*p*	Adjusted OR (95% CI)	*p*
Inclusion group											
Screening	68 (41.0)	16 (23.5)	1		1		14 (20.6)	1		1	
Contacts	98 (59.0)	48 (49.0)	3.3 (1.7– 6.6)	< 0.001	7.2 (3.1-16.5)	<0.001	47 (48.0)	3.6 (1.7– 7.6)	<0.001	6.2 (2.8-13.5)	<0.001
BCG immunization											
Yes	116 (69.9)	39 (33.6)	1		1		36 (31.0)	1		1	
No	50 (30.1)	25 (50.0)	2.0 (1.01 – 3.9)	0.049	1.3 (0.3-5.3)	0.662	25 (50.0)	2.2 (1.1 – 4.4)	0.021	3.03 (1.3-7.03)	0.01
Chest X-ray											
Normal	152 (91.6)	53 (34.9)	1		1		52 (34.2)	1		1	
Compatible with TB	14 (8.4)	11 (78.6)	10.1 (2.15– 47.1)	0.003	12.3 (2.1-70.6)	0.005	9 (64.3)	3.5 (1.1–10.9)	0.033	2.4 (0.6-9.5)	0.217

[1]Unadjusted Odds Ratio (95% confidence interval) for the positivity threshold of TST ≥5 mm

[2]*QuantiFERON-TB- Gold In Tube*

All children considered not TB infected according to the TST result obtained a negative IFN-γ-based tests result. From the 20 non-BCG-vaccinated children from the SG, both IFN-γ tests were negative in 14 children with a TST induration between 5 and 9 mm. There were 48 BCG-vaccinated children in the SG: T-SPOT.*TB* was positive in 11/48 (22.9%) and QFN-G-IT in 9/48 (18.75%). In the 3 of them with TST induration between 5-9 mm both IFN-γ tests were negative. Therefore in the 45 children who had a positive TST (induration ≥10 mm), T-SPOT.*TB* was positive in 11/45 (24.4%), and QFN-G-IT in 9/45 (20%). Distribution of IFN-γ tests and TST results according to BCG and non-BCG-vaccinated status, and contact and screening groups, are shown in figures 1 and 2. No indeterminate results were detected by QFN-G-IT, but by T-SPOT.TB (1.8%) in 3 cases the test failed because the blood volume drawn was insufficient.

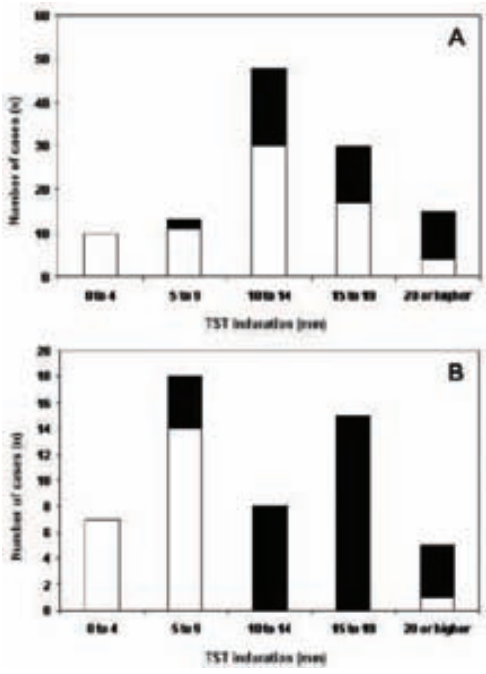

Figure 1: IFN-γ tests results distribution (positive [■] and negative [□]) according to the tuberculin skin test induration in **(A)** BCG and **(B)** non-BCG-vaccinated children from contact and screening group.

IFN-γ tests were in agreement in 146/166 children (Table 2). None of the variables that might have influenced the level of concordance between both tests was significantly associated with the outcome. There were no significant differences in age, gender or study group, between the children with concordant and discordant IFN-γ results (data not shown). IFN-γ tests were discordant in 20 children (12.04%). The 3 failed cases in the T-SPOT.*TB* belonged to the CG (3.06%) and among them there was a 3 year old patient with active TB. The overall agreement was 89.6% (κ=0.778) after excluding the failed cases.

Variables related to the positivity of IFN-γ tests. Variables significantly associated with IFN-γ test positivity are shown in Table 1. In the multivariate analysis a positive T-SPOT.*TB* was associated with the fact of being a contact (p<0.001) and having an abnormal chest X-ray and for the QFN-G-IT was to be a contact (p<0.001) and not to be BCG-vaccinated (p= 0.01) (Table 1).

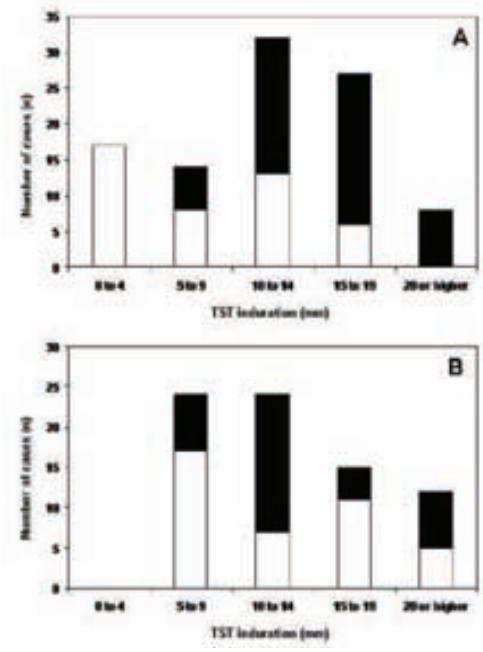

Figure 2: IFN-γ tests results distribution (positive [■] and negative [□]) according to the tuberculin skin test induration in **(A)** contact and **(B)** screening group, including both BCG and non-BCG-vaccinated children.

In Table 3, the positivity of the IFN-γ tests according to the risk of exposure to an infectious source is shown. The probability of a positive IFN-γ test (OR=3.60; 95%CI=1.85-7.04) was significantly associated with an increasing risk of exposure, independent of age and gender in the multivariate analysis (p<0.001). In addition, in the multivariate analysis the main factors associated with a positive T-SPOT.*TB* in the CG were a daily contact over 6 hours (OR=3.5; 95%CI=1.1-12.1; p=0.03) and an exposure time over 30 days (OR=1.9; 95%CI=1.1-6.9; p=0.04). However, no significant associations were found for the QFN-G-IT.

Clinical performance of the IFN-γ tests in active primary TB. Fourteen cases were finally classified as active primary TB. In 4 cases a microbiological confirmation was possible (positive culture for *M. tuberculosis*: 3 in gastric aspirate samples and 1 in sputum sample). In 8 cases the children were from the CG and in 6 from the SG. T-SPOT.*TB* was positive in 11/14 cases (78.6%) and the QFN-G-IT in 9/14 (64.3%). Both IFN-γ tests were positive in 8 children (57.1%) and negative in 2 cases (21.4%). For 1 patient the T-SPOT.*TB* failed and the QFN-G-IT positive; and 3 had a negative QFN-G-IT and a positive T-SPOT.*TB*. On the other hand, the differences in the number of responding T cells after stimulation with the specific antigens in the comparison between children diagnosed with active TB and all children without disease was significant (p=0.01 for ESAT-6 and CFP-10, respectively; and p=0.009 for RD1), but differences in the IFN-γ released did not reach statistical significance (p=0.09).

TABLE 2. Concordance and agreement (Cohen's Kappa coefficient) between the IFN-γ tests results for the different groups of children according to their BCG immunization status.

IFN-γ results/agreements	Initial inclusion group; n(%)				
	Contact group		Screening group		Total (n=166)
	BCG (n=68)	No BCG (n=30)	BCG (n=48)	No BCG (n=20)	
Negative T-SPOT.*TB* and negative QFN-G-IT[1]	36 (52.9)	7 (23.3)	35 (72.9)	15 (75.0)	93 (56.0)
Positive T-SPOT.*TB* and Positive QFN-G-IT	24 (35.3)	17 (56.7)	7 (14.6)	5 (25.0)	53 (31.9)
Negative T-SPOT.*TB* and positive QFN-G-IT	2 (2.9)	2 (6.7)	2 (4.2)	0	6 (3.6)
Positive T-SPOT.*TB* and negative QFN-G-IT	4 (5.9)	3 (10.0)	4 (8.3)	0	11 (6.6)
Failed T-SPOT.*TB* and positive QFN-G-IT	1 (1.5)	1 (3.3)	0	0	2 (1.2)
Failed T-SPOT.*TB* and negative QFN-G-IT	1 (1.5)	0	0	0	1 (0.6)
Overall Concordance[2] (%)	60/68 (88.2)	24/30 (80.0)	42/48 (87.5)	20/20 (100)	146 /166 (88.6)
Cohen's Kappa coefficient	0.765	0.561	0.622	1	0.750
Excluding failed results					
Concordance (%)	60/66 (90.9)	24/29 (82.8)	42/42 (87.5)	20/20 (100)	146/163) (89.6)
Cohen's Kappa coefficient	0.810	0.609	0.622	1	0.778

[1]*QuantiFERON-TB Gold In Tube.* [2] Nº of patients with concordant results/total nº of patients.

TABLE 3. IFN-γ tests results according to the risk of exposure to *M. tuberculosis.*

	Number of children	Positive TST[1] (%)	Positive IFN-γ tests[2] (%)	Unadjusted OR (95% CI)[3]	*p*	Adjusted OR (95% CI)[4]	*p*
No risk known	68	51 (75)	18 (35.3)	1		1	
Medium risk	33	29 (87.9)	18 (62.1)	3.00 (1.06-8.64)	0.037	2.88 (1.22 – 6.80)	0.016
High risk	65	52 (80)	36 (69.2)	4.13 (1.68 –10.27)	0.001	4.29 (2.01 – 9.18)	<0.001

[1]Tuberculin skin testing results (≥ 5 mm in the contact group and TST ≥ 10 mm in the screening group was considered positive); [2]Positive result of one or both IFN-γ tests; [3]Unadjusted odds ratio (95% confidence interval); [4] Adjusted odds ratio (95% confidence interval) adjusted by age and gender.

TABLE 4. Number of spot forming cells after stimulation with ESAT-6, CFP-10 and RD1 antigens and the amount of IFN-γ released measured by T-SPOT.*TB* and *QuantiFERON-Gold In Tube*, respectively, in children diagnosed with active TB and LTBI infected (in both cases, either of the *in vitro* tests or both were positives).

	Active TB		LTBI		
	n	**Median (25- 75 percentiles)**	**n**	**Median (25- 75 percentiles)**	***p***
QFN-G-IT	9	2.09 (0.23-13.23)	52	3.26 (0.65-9.57)	0.52
TS.TB					
ESAT-6	11	26.00 (5.00-69.00)	53	15.00 (6.00-40.00)	0.50
CFP-10	11	32.00 (18.00-75.00)	53	19.00 (7.00-70.00)	0.60
RD1	11	79.00 (37.00-137.00)	53	49.00 (15.00-125.00)	0.31

However, if we exclude from the analysis children who were not diagnosed of LTBI by IFN-γ tests (both T-SPOT.*TB* and QFN-G-IT negatives), then there are not statistical significant differences in the number of responding T cells and the amount of IFN-γ released after antigen stimulation between active and LTBI children (Table 4).

If we consider children diagnosed with active TB as truly infected, and children from CG with a TST <5 mm and children from SG with a TST<10mm as truly not infected, then we could assume that the sensitivity and specificity of the IFN-γ tests is 78.57% (11/14), and 100% (35/35), respectively.

Agreement between IFN-γ tests and TST. The agreement between TST and IFN-γ tests is high in non-BCG-vaccinated children (Table 5). Variables associated with discordant results between TST and IFN-γ tests in multivariate analysis were: belonging to the SG (adjusted OR=6.9; 95%CI=3.4–14.4; p<0.001), being vaccinated with BCG (adjusted OR=10.1; 95%CI=3.3–30.9; p<0.001), and a TST with induration between 5-9 mm (adjusted OR=10.4; 95%CI=3.5–31.1; p<0.001).

Among the 17 autochthonous children from Spain, non-BCG-vaccinated, with a TST result between 5-9 mm of induration and with negative IFN-γ tests, the detection of sensitised T cells against *M. avium* sensitin was performed in 12 cases. In 3 cases the test failed due not having a

sufficient number of cells recovered. It was negative in 3 cases and in the remaining 6 it was positive.

Relationship between number of sensitised T cells and the amount of IFN-γ released. When both IFN-γ tests agreed, high SFCs counts by T-SPOT-*TB* also showed high amounts of released IFN-γ (measured by QFN-G-IT). However, this correlation is negative in those children with a discordant result, where an increase in SFCs correlates with a decrease in IFN-γ released. In this case, the amount of IFN-γ tends to plateau. At this point, few cells produce high quantities of IFN-γ (negative T-SPOT.*TB* and positive QFN-G-IT), whereas the total amount of IFN-γ decreases or remains constant despite an increase in the SFCs (positive T-SPOT.*TB* and negative QFN-G-IT) (Figure 3).

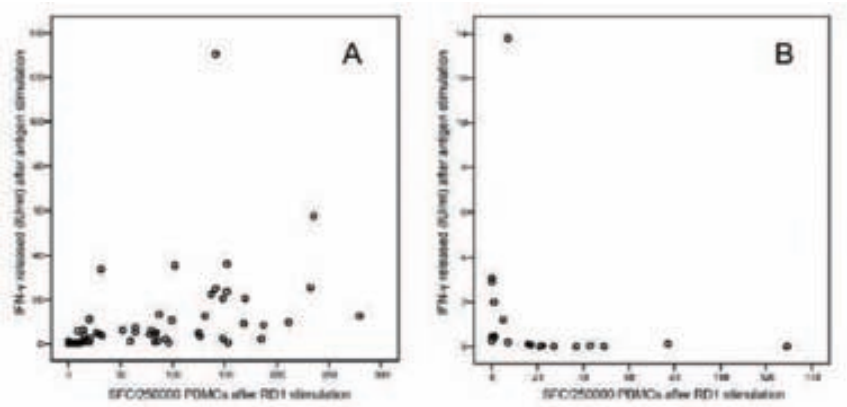

Figure 3: Correlation between the number of spot forming cells (SFCs) after stimulation with specific *M. tuberculosis* antigens and the amount of IFN-γ released in children with concordant **(A)** and discordant **(B)** results between T.SPOT.TB and QFN-G-IT. The RD1 stimulation is the sum of SFCs obtained after ESAT-6 and CFP-10 stimulation. In children with concordant results the Pearson's correlation coefficient was 0.530 (p=0.0001). In children with discordant results the Pearson's correlation coefficient was -0.212 (p=0.371)

TABLE 5. Concordance and agreement (Cohen's Kappa coefficient) between Tuberculin skin test and T-SPOT.*TB* and QuantiFERON-TB Gold *In Tube* results according to the BCG immunization status.

BCG status	Tests compared	Cohen's Kappa coefficient (SD[1])	*p* value
BCG immunized	TST[2] and QFN-G-IT[3]	0.087 (0.155)	0.0048
	TST and T-SPOT.*TB*	0.095 (0.151)	0.0032
Not BCG immunized	TST and QFN-G-IT	0.844 (0.105)	<0.0001
	TST and T-SPOT.*TB*	0.887 (0.062)	<0.0001
Total	TST and QFN-G-IT	0.208 (0.111)	<0.0001
	TST and T-SPOT.*TB*	0.272 (0.092)	<0.0001

On the other hand, as the diameter of the TST induration increases there is an increase in the SFCs (CC=0.09; p<0.0001) and in the total amount of IFN-γ released (CC=0.03; p<0.01); similarly, as the number of SFCs increases there is also an increase in the IFN-γ released (CC=0.27; p<0.0001). However, the correlation between TST induration and the SFCs and the IFN-γ released varies depending on whether the IFN-γ tests agree or not. When

both IFN-γ tests agree, as the diameter of the TST induration increases there is an increase of responding SFCs (CC=0.315; p<0.0001), the regression line slope being 2.986 (p<0.0001); and there is also an increase of the IFN-γ released (CC=0.167; p=0.045), with a regression line slope of 0.343 (p=0.046). When there is no agreement between the IFN-γ tests, there is no correlation between the TST and the SFCs produced (CC=0.065; p=0.786) being the slope of the line almost null 0,189 (p=0.910); neither with the amount of IFN-γ produced (CC=0.362; p=0.117), being the slope of the regression line 0.298 (p=0.069).

DISCUSSION

This study shows the results of IFN-γ tests measurements in children seen in a reference centre for the diagnosis of TB infection, and compares the techniques currently available. Although the specificity for active TB for both tests was 100%, T-SPOT.*TB* obtained more positive results than QFN-G-IT in all groups analysed.

Our results highlight the usefulness of the IFN-γ tests compared to TST in the diagnosis of LTBI in contacts, as an association was found with the increase in the risk of infection and the exposure. These data agree with findings in other studies that have investigated TB outbreaks and study contacts [4, 11, 13-18]. These results also show the usefulness of IFN-γ tests to diagnose LTBI in BCG-vaccinated children when they are screened as part of paediatric or epidemiological control.

We have found that both IFN-γ tests show sensitivity over 75% and specificity of 100% for the diagnosis of active TB. Liebeschuetz et al [19] reported a sensitivity of 83% for T-SPOT.*TB* in African children. Nicol et al [20] described T-SPOT.*TB* positive results in 70% of children with clinical TB. Detjen et al [21] found a sensitivity of 93% for both IFN-γ tests when evaluating children with active TB. In addition, Connell et al [22] also reported positive IFN-γ tests in the 9 children diagnosed with active TB. In contrast with our results, Kampmann et al [23] found better results for QFN-G-IT than for T-SPOT.*TB* in children with culture-confirmed TB. Even if IFN-γ tests have been developed to diagnose LTBI, an alternative approach to the evaluation of the sensitivity of the *in vitro* tests has been to test patients with active TB. Although patients with active TB are infected by definition with *M. tuberculosis*, they do not have a LTBI. In fact, active TB occurs when the host immune responses are unable to contain the latent infection. Therefore, it should be considered that the value of the IFN-γ assays in active TB diagnosis is limited. False negative results of both tests in active TB have been described previously [6, 24, 25]. In addition, it has been reported that young children with severe active TB can have a reduced number of lymphocytes or a reduced lymphocyte function that could affect the sensitivity of the IFN-γ tests. In fact, in our study, in 6 children aged less than 3 years old, the T-SPOT.*TB,* was negative in 3 cases, failed in 2, and was positive in only one case; and the QFN-G-IT was negative in 3, and positive in the remaining 3 cases. However, no very severe TB presentation was diagnosed in children with both IFN-γ tests negative. Other factors also involved could be the release of anti-inflammatory cytokines by PBMC and the temporary depression of T cell responsiveness [26, 27].

On the other hand, we have observed in our study that the IFN-γ assays are not able to distinguish between LTBI and active TB. No significant differences were detected between infected and diseased children in the number of responding T cells and the amount of IFN-γ released after antigen stimulation. The absence of significant differences in the response between active TB and LTBI could be explained by the fact that pediatric infection is usually recent. Therefore, the response is still strong, being similar to the one obtained during active TB [28].

Indeterminate results can be due to different causes, though they are generally due to a failure of the positive control. These results have been associated with immunosuppression, young age (<5 years) and a negative TST [14, 19, 20, 29]. Interestingly new information from different studies suggests that the increased frequency of indeterminate results in young children reflects a performance characteristic of the *in vitro* tests rather than a responding impairment to specific antigens and PHA [17, 29, 30]. An important source of failed results in young children has been related to an inadequate PBMC separation as a consequence of insufficient blood taken (especially in very young children) [13, 17, 23], which is the case in the 3

children who had a failed T-SPOT.*TB* result in our study. From our point of view, this kind of results should be considered since in children (where blood drawn is not always easy) these problems are inherent to the *in vitro* tests. However, given that in the QFN-G-IT assays no T cells count are required, we can not assess the impact of this kind of failure in the performance of the test.

It is difficult to compare the agreement between IFN-γ tests and TST with the results obtained by other authors because in each case positivity cut-off needs to be taken into account. In published studies this threshold can vary greatly, from 5, 10 and up to 15 mm of induration as indicative of TB infection [15, 17, 19, 20, 29] and generally depends on population groups (contacts, level of risk of development of active TB) and specific guidelines of the country.

The variables associated with discordance between the TST and IFN-γ test measurements were BCG immunisation, belonging to SG, and a TST induration of 5 to 9 mm. In the SG an induration ≤ 10 mm is most likely caused by a NTM (non-specific sensitisation). In fact, in our study we detected T cell sensitization against *M. avium* sensitins in 6 out of 9 (66.7%) of these children. The existence of NTM in Spain was shown by Bleiker [31], and recently our group has described its presence in Catalonia [32]. Also, Detjen et al [21] showed the specificity of the IFN-γ tests in infections caused by NTM, and other authors have also described low agreement between IFN-γ tests and positive TST [7, 33]. Our group, in a previous publication, reported that 47.6% (10/21) of children with TST positive and negative T-SPOT.*TB* had sensitised T cells against *M. avium* sensitins [12]. Given that *M. avium* sensitin is not totally specific, we cannot totally exclude the possibility that we are detecting, in some cases, a response of specific T cells against some *M. tuberculosis* antigens different from ESAT6, CFP.10 and TB7.7. In order to reduce as much as possible this possibility we have focused our study on unexposed children with 5 to 9 mm of TST induration. Based in the classical studies performed by Nyboe *et al* [34], the main guidelines in screening children population consider as a cut-off for *M. tuberculosis* infection a TST induration equal to or higher than 10 mm, in order to avoid false positive TST results induced by NTM immunisation [35]. Therefore, our results reinforce, in part, the guidelines [10] in that unnecessary chemoprophylaxis treatment in unexposed population could be avoided, and that IFN-γ based assays could help to confirm a positive TST result. Nevertheless, indurations higher than 15 mm [21] and 20 mm [36] have been reported in children with NTM infections.

The main limitation of our study is that 89.75% of patients included had a positive TST (i.e., all children from the SG). This fact could introduce a bias in the comparison between the TST and IFN-γ tests due to the low number of negative TST results. Nevertheless, despite this limitation, the results obtained are sufficiently consistent to draw some conclusions about their utility in the diagnosis of LTBI in a referral centre.

Both IFN-γ tests have high agreement in our study. Although previous reports have described similar levels of agreement, very few of these studies have been carried out in children. Detjen et al [21] found an agreement of 95.6% (κ=0.91). Ferrara [14] reached a high agreement (κ=0.699), independently of the BCG vaccination status, but T-SPOT.*TB* detected a higher number of positive cases (38%) than QFN-G (26%). Furthermore, Kampmann et al [23] found a poorer agreement of IFN-γ tests (66.7%) in culture-confirmed TB cases, but the agreement was high (92%) in LTBI.

The analysis of discordant results needs to be researched further. This study has shown that when there is disagreement between both IFN-γ tests, a negative correlation exists between the number of SFCs and the amount of IFN-γ produced. In our study, in 11 cases the T-SPOT.*TB* was positive and the QFN-G-IT negative, and in 6 cases the T-SPOT.*TB* was negative but the QFN-G-IT positive. There may have been false positive or false negative IFN-γ tests. But it is also possible that there was an immunological dysfunction in these children. In fact, 3 of the children with a discordant result had discordant IFN-γ tests results again 3 months later. Recently, Richeldi et al [37] performed a comparative study on three different groups of immunocompromised individuals. They described highly discordant results, i.e., those clearly negative with one IFN-γ

test and clearly positive with another, representing 12.1% of the study population. These results suggest an immunological dysfunction related with a decreased production of IFN-γ or a decrease in the number of IFN-γ producing cells. Both situations have been associated with increased risk of developing active TB.

TB infection control in animals and humans is associated with the production of IFN-γ by the Th CD4+ cells [38]. It has been shown, in animal models, that a decreased production of IFN-γ and a decrease in the number of IFN-γ producing cells are predictive of an increased risk of developing TB [39]. In contact patients, it has been observed that the progression to disease was associated with a decrease in IFN-γ and an increase in IL-10 and IL-4 levels [40, 41]. Some data suggest that in individuals with a recent exposure to TB the protective response shifts from Th1 to Th2, even before the clinical symptoms appear [42]. Perhaps children with discordant IFN-γ tests could be a high risk group to developing TB, and therefore this could constitute a group that would benefit most from TB infection treatment.

In conclusion, in the daily practice of a referral centre for TB control, the use of IFN-γ tests is helpful for the diagnosis of TB infection. Its use eliminates the cross-reactions with BCG immunisation and may help to exclude NTM infections. The analysis of highly discordant results requires further investigation to elucidate any possible clinical implications. The use of both techniques simultaneously can contribute to improving the knowledge TB immunity.

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Conflict of Interest. None of the investigators have any financial interest in or a financial conflict with the subject matter or materials discussed in this manuscript. None of the Scientific Societies, nor Inverness Medical Ibérica SAU (Barcelona, Spain), Cellestis (Carnegie, Australia) or Oxford Immunotec (Abingdon, UK) had a role in the study design, implementation, data collection, management, analysis, interpretation of the data, preparation, review, or approval of the manuscript.

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ANEXO II

Performance of Tests for Latent Tuberculosis in Different Groups of Immunocompromised Patients

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Background: **Immunocompromised persons infected with *Mycobacterium tuberculosis* (MTB) have increased risk of tuberculosis (TB) reactivation, but their management is hampered by the occurrence of false-negative results of the tuberculin skin test (TST). The T-cell interferon (IFN)-γ release blood assays T-SPOT.*TB* (TS.TB) [Oxford Immunotec; Abingdon, UK] and QuantiFERON-TB Gold In-Tube (QFT-IT) [Cellestis Ltd; Carnegie, VIC, Australia] might improve diagnostic accuracy for latent TB infection (LTBI) in high-risk persons, although their performance in different groups of immunocompromised patients is largely unknown.**

Methods and results: **Over a 1-year period, we prospectively enrolled patients in three different immunosuppressed groups, as follows: 120 liver transplantation candidates (LTCs); 116 chronically HIV-infected persons; and 95 patients with hematologic malignancies (HMs). TST, TS.TB, and QFT-IT were simultaneously performed, their results were compared, and intertest agreement was evaluated. Overall, TST provided fewer positive results (10.9%) than TS.TB (18.4%; $p < 0.001$) and QFT-IT (15.1%; $p = 0.033$). Significantly fewer HIV-infected individuals had at least one positive test (9.5%) compared with LTCs (35.8%; $p < 0.001$) and patients with HMs (29.5%; $p < 0.001$). Diagnostic agreement between tests was moderate ($\kappa = 0.40$ to 0.65) and decreased in the HIV-infected group when the results of the TS.TB were compared with either TST ($\kappa = 0.16$) or QFT-IT ($\kappa = 0.19$). Indeterminate blood test results due to low positive control values were significantly more frequent with QFT-IT (7.2%) than with TS.TB (0.6%; $p < 0.001$).**

Conclusions: **Blood tests identified significantly more patients as being infected with MTB than TST, although diagnostic agreement varied across groups. Based on these results, we recommend tailoring application of the new blood IFN-γ assays for LTBI in different high-risk groups and advise caution in their current use in immunosuppressed patients.** ***(CHEST 2009; 136:198–204)***

Abbreviations: BCG = bacillus Calmette-Guérin; HM = hematologic malignancy; IFN = interferon; IGRA = interferon-γ release assay; LTBI = latent tuberculosis infection; LTC = liver transplantation candidate; MTB = *Mycobacterium tuberculosis*; QFT-IT = QuantiFERON-TB Gold In-Tube; SFU = spot-forming unit; TB = tuberculosis; TST = tuberculin skin test; TS.TB = T-SPOT.*TB*

Targeting persons at increased risk of disease reactivation once infected with *Mycobacterium tuberculosis* (MTB) is a key factor for an effective tuberculosis (TB) control strategy in low-prevalence countries.[1,2] A weak component of this strategy is the less than ideal performance of the standard diagnostic tool for latent TB infection (LTBI), the century-old tuberculin skin test (TST).[3] False-negative results, mainly due to immunosuppression, preclude the treatment of truly infected persons, and the treatment of individuals with false-positive results, often due to *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccination, reduces the cost-effectiveness of preventive interventions. Because of

their operational advantages and increased specificity, the interferon (IFN)-γ release assays (IGRAs) QuantiFERON-TB Gold In-Tube (QFT-IT) [Cellestis Ltd; Carnegie, VIC, Australia] and T-SPOT.*TB* (TS.TB) [Oxford Immunotec; Abingdon, UK] might improve the efficacy of interventions aimed at TB control.[4] However, the performance of IGRA in various categories of high-risk patients is largely unknown and has been identified as a priority area for research.[5]

We performed a comparative study in three different groups of immunocompromised individuals, in whom the TST is the standard for the diagnosis of LTBI, despite its recognized lack of sensitivity, [3,6] as follows: liver transplantation candidates (LTCs) with end-stage disease[7]; HIV-infected individuals[8]; and patients with hematologic malignancies (HMs).[9]

Materials and Methods

Study participants were evaluated in three different wards of a single referral center (University Hospital of Modena, Italy) during a 12-month period (May 2006 to May 2007). Any consecutive patient with a clinical indication to be tested for LTBI who provided written informed consent was enrolled in the study. Patients with end-stage chronic liver disease in the liver transplantation candidacy period (the LTC group) were enrolled at the solid organ transplant clinic; individuals with chronic HIV infection (the HIV group) were enrolled at the infectious disease clinic; and patients with HMs (the HM group) were enrolled at the hematology clinic. To provide a real-life assessment of patients based on routine clinical practice, no other exclusion criteria were defined, and no patients refused to participate in the study. The research protocol was approved by the local ethics committee.

TST was performed using 5 international units of purified protein derivative (Biocine Test; Chiron; Siena, Italy) immediately after drawing blood samples for IGRA and was read 72 h later. A TST result was considered positive if the skin induration was ≥ 10 mm in patients in the LTC and HM groups, and ≥ 5 mm in the HIV group.[2] Blood samples were processed according to the manufacturer's instructions within 4 h from blood drawing by trained technicians blinded to clinical and demographic identifiers.

Comparisons among tests within the same group were performed using the McNemar test, and comparisons across different groups were performed using the χ^2 test. Bonferroni correction was used for multiple comparisons. Results were considered statistically significant when the p value was < 0.05. The κ statistic measure was used to assess agreement among different tests. This measure provides values from + 1 (perfect agreement) to 0 (no agreement above that expected by chance) to − 1 (complete disagreement). Analyses were performed using a statistical software package (Stata, version 10.0; Stata Corp; College Station, TX).

Results

Three hundred sixty-nine participants were enrolled in the protocol. Fourteen patients (3.8%) were excluded from further analysis because IGRA results (TS.TB, 11 patients; QFT-IT, 3 patients) were deemed invalid due to high negative control values. For 24 patients (6.5%), TST results (LTC group, 6 patients; HM group, 1 patient; HIV group, 17 patients) were not read within the allotted time limit and were thus excluded, leaving 331 subjects for statistical analyses. The mean age was 51.4 years, most patients were white, and a few had been vaccinated with BCG (Table 1). Overall, 24 QFT-IT results (7.2%) and 2 TS.TB results (0.6%; $p < 0.001$) were indeterminate owing to low positive control values; 21 of the indeterminate QFT-IT results (87.5%) were negative using both TST and TS.TB. Compared with TS.TB, there were significantly more indeterminate QFT-IT results in both the LTC and HIV groups ($p = 0.009$ and $p = 0.008$, respectively), but not in the HM group (Table 2). Blood lymphocyte counts on the day of IGRA testing were available for 303 patients. Median lymphocyte counts were significantly lower in patients with indeterminate QFT-IT results (1,200 cells/mL vs 1,820 cells/mL in patients with determinate QFT-IT results; $p = 0.001$). In the LTC group, indeterminate QFT-IT results were significantly associated with a higher median model for end-stage liver disease score (21 vs 13, respectively; $p = 0.002$).

Quantitative responses of IGRA, expressed as international units of IFN-γ or as spot-forming units (SFUs), showed good correlation with the size of the

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Table 1—*Characteristics of the Study Population*

Characteristics	LTC Group (n = 120)	HIV Group (n = 116)	HM Group (n = 95)
Mean age, yr	53.5 (8.7, 26–68)	41.4 (8.5, 20–72)	61.2 (14.4, 26–86)*
Male patients	85 (70.8)	78 (67.2)	52 (54.7)†
Foreign born	6 (5.0)	15 (12.9)‡	2 (2.1)
Recent TB contact	11 (9.2)	11 (9.5)	5 (5.3)
Ethnicity			
White	118 (98.3)	105 (90.5)	95 (100)
Black	1 (0.8)	11 (9.5)‡	0 (0)
Asian	1 (0.8)	0 (0)	0 (0)
BCG vaccinated	4 (3.3)	7 (6.0)	1 (1.1)
Median lymphocytes, cells/mL	1,135§	1,905	2,367

Values are given as the mean (SD, range) or No. (%). Among HIV-infected individuals, 59.5% were receiving highly active antiretroviral treatment and had a mean CD4 count of 461.5/mm^3. In the LTC group, 80% were affected by either alcoholic or hepatitis B or C virus-related cirrhosis, and the median model for end-stage liver disease score was 14. This score is based on objective laboratory variables and predicts the risk of mortality within 3 mo. In the HM group, 82.1% were affected by Hodgkin and non-Hodgkin lymphomas; none was receiving anticancer chemotherapy at the time of testing. Data on peripheral blood lymphocytes were available for 98 LTC, 112 HIV, and 93 HM patients.
*$p < 0.001$ vs LTC and HIV groups.
†$p < 0.05$ vs LTC and HIV groups.
‡$p < 0.05$ vs LTC and HM groups.
§$p < 0.001$ vs HIV and HM groups.

TST skin induration, expressed in millimeters (Fig 1). Overall, however, both TS.TB (18.4%) and QFT-IT (15.1%) identified more positive results than did TST (10.9%; $p < 0.001$ vs TS.TB; $p = 0.033$ vs QFT-IT). Significantly fewer persons were identified as latently infected by any test in the HIV group, compared with the LTC and HM groups (TST, $p = 0.042$; TS.TB, $p < 0.001$; QFT-IT, $p < 0.001$). Considering those patients with at least one positive test result to be infected, LTBI prevalence was significantly lower in the HIV group (9.5%) than in the LTC group (35.8%; $p < 0.001$) and HM group (29.5%; $p < 0.001$). Concordance among tests was substantial, ranging from 80.6% (TST vs TS.TB in LTC) to 95.4% (TST vs QFT-IT in HIV) [Table 3]. However, agreement between tests across different groups was moderate, with the κ statistic ranging from 0.40 to 0.65. In the HIV group, agreement between TS.TB and either TST ($\kappa = 0.16$) or QFT-IT ($\kappa = 0.19$) was slight (Table 3). In the 12 BCG-vaccinated individuals, agreement among all three tests was complete ($\kappa = 1.00$). "Highly discordant" results, that is, those clearly negative with one IGRA and clearly positive with another, were found in all groups and represented 12.1% of the whole population (Table 4). Patients with a positive TST and a negative IGRA (without a history of BCG vaccination) were also represented in all groups (LTC patients: TS.TB, 5 patients; QFT-IT, 4 patients; HIV patients: TS.TB, 5 patients; QFT-IT, 3 patients; HM: TS.TB, 1 patient; QFT-IT, 0 patients). Overall, the fraction of tests with borderline results (*ie*, close to the cutoff for a positive test result) was higher for TS.TB (18 tests with 4 to 5 SFUs) than for QFT-IT (8 tests with 0.20 to 0.34 international units of IFN-γ) [Table 4].

Table 2—*Results of TST, TS.TB, and QFT-IT in the Different Groups of Immunocompromised Patients*

Assay Results	LTC Group (n = 120)	HIV Group (n = 116)	HM Group (n = 95)
TST			
Positive	20 (16.7)*	6 (5.2)	10 (10.5)†
Negative	100 (83.3)	110 (94.8)	85 (89.5)
TS.TB			
Positive	32 (26.7)	4 (3.5)	25 (26.3)‡
Negative	87 (72.5)	112 (96.5)	69 (72.6)
Indeterminate	1 (0.8)	0	1 (1.1)
QFT-IT			
Positive	28 (23.3)	5 (4.3)	17 (17.9)
Negative	80 (66.7)	104 (89.7)	73 (76.8)
Indeterminate	12 (10.0)	7 (6.0)	5 (5.3)

Values are given as No. (%).
*$p = 0.007$ vs TS.TB positive and $p = 0.02$ vs QFT-IT positive in the LTC group.
†$p < 0.001$ vs TS.TB positive and $p = 0.02$ vs QFT-IT positive in the HM group.
‡$p = 0.03$ vs QFT-IT positive in the HM group.

Discussion

We report here the results of a comparative evaluation of all currently available diagnostic tests for LTBI in different categories of immunocompromised patients at increased risk of TB reactivation. We obtained at least three important results. First,

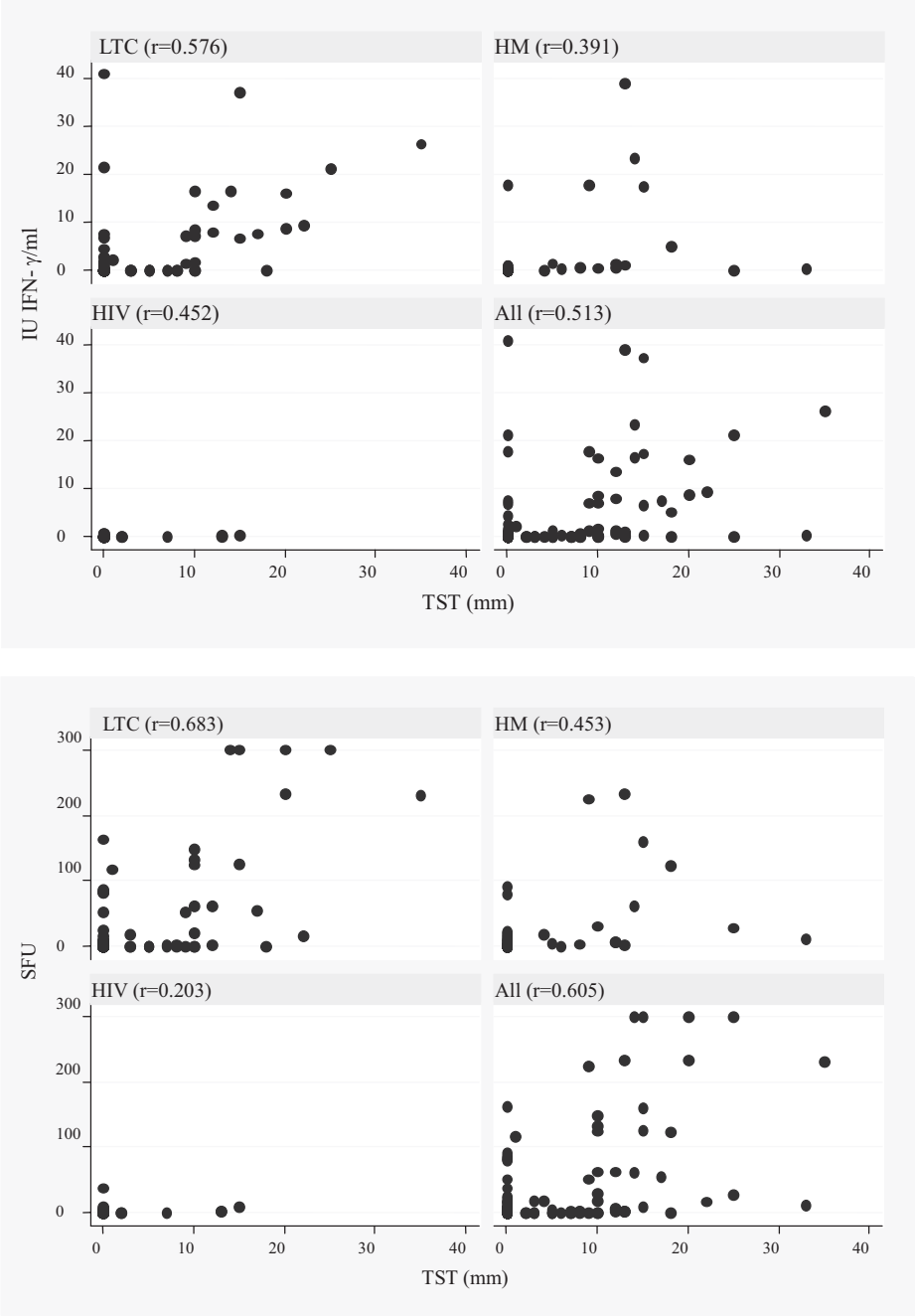

FIGURE 1. Correlation between the IFN-γ response in QFT-IT (*top*) and TS.TB (*bottom*) and the size of the TST induration.

TST detected fewer positive cases in some, but not all, high-risk groups. Therefore, a differential application of TST and IGRA among immunosuppressed patients might be beneficial. For example, in solid organ transplantation candidates, in whom the TST is still recommended as a standard of care,[10] both TS.TB and QFT-IT could replace the TST. However, the lower rate of positive results with all three tests in HIV-infected individuals, which is well explained by the impaired IFN-γ-secreting capacity of MTB-specific CD4 T cells in HIV infection,[11] coupled with the significant discordance among tests (previously reported in various populations of HIV-infected patients in low- and high-incidence settings),[12–14] would support an integrated diagnostic approach based on all three assays. However, in the

Table 3—*Diagnostic Agreement of TST, TS.TB, and QFT-IT in the Different Groups of Immunocompromised Patients*

Assays	Agreement	LTC Group (n = 108)	HIV Group (n = 109)	HM Group (n = 89)
TST vs TS.TB	Concordance, %	80.6	92.7	80.9
	κ value (SE)	0.47 (0.09)	0.16 (0.09)	0.40 (0.09)
TST vs QFT-IT	Concordance, %	85.2	95.4	91.0
	κ value (SE)	0.57 (0.09)	0.52 (0.10)	0.65 (0.10)
TS.TB vs QFT-IT	Concordance, %	84.3	93.6	85.4
	κ value (SE)	0.60 (0.10)	0.19 (0.10)	0.59 (0.10)

Indeterminate IGRA results were not included in the analyses.

particular group of HIV-infected persons included in our study, the lower rates of positive test results may simply reflect a higher degree of immunosuppression and therefore reduced reactivity to the assays.[15]

Our second important result is that the diagnostic agreement among different tests varied across groups of immunocompromised patients. Previous studies[12,16–21] have found that agreement of IGRA with the TST and agreement between QFT and TS.TB may vary from poor to substantial. In the present study, we showed that, among HIV-infected individuals, TS.TB detected a largely different fraction of persons infected by MTB, compared to TST and QFT-IT. Similar results were recently reported[22] in a smaller sample of HIV-infected adults and children in an area in which the disease is endemic. Because identification and treatment of persons coinfected with MTB and HIV are a priority for TB control,[23] these results could be clinically relevant.

To date, few studies are available on the value of IGRA in predicting the reactivation of TB in high-risk groups. Among adult[24] and pediatric[25] contacts of persons with active TB, the IGRA showed a better (or equivalent) positive predictive value than the TST for incident TB cases. However, a recent study from the Republic of The Gambia[26] showed that only the combination of TST and IGRA (with infection defined as at least one positive result among the tests used) improved the detection of subjects at higher risk of progression to active disease. Unfortunately, only a small fraction of the subjects enrolled in the Gambian study were tested for HIV coinfection. Nevertheless, those results lend support to the concept that discordant results in specific risk groups are more likely due to the effects of different impairment of the immune response within the same risk groups on the sensitivity of the assays than to false-positive results due to the technical features of the assays. In addition, we have reported that about a third of discordant results were unlikely to be caused by inaccurate cutoff values, as suggested by others,[21] because they were definitely positive with one assay and clearly negative with the other. These findings support the hypothesis that QFT-IT and TS.TB, based on some methodological differences,[4] might measure slightly different immunologic phenomena. This conclusion is also supported by studies that have reported a different performance (and, most likely, different sensitivities) of the TST and IGRA in patients undergoing screening for LTBI and immunosuppressive treatments for cancer[27] or in patients with autoimmune diseases before anti-tumor necrosis factor-α therapies.[28] All these data suggest again that, in the absence of prospective studies, a combined approach aimed to maximize the efficacy of screening procedures might be preferable in severely immunocompromised patients who have an increased risk of reactivation and development of severe forms of TB.[29]

Finally, as reported for an earlier version of the same assay,[16] indeterminate results of the QFT-IT are more affected by cellular immunosuppression than are those of TS.TB; interestingly, not all indeterminate QFT-IT results were negative with either TS.TB or TST, suggesting that individuals with an indeterminate QFT-IT result might still be infected.

Table 4—*Distribution of TS.TB and QFT-IT Results by Quantitative Responses of the Assays*

TS.TB SFU Value	QFT-IT 0.19 IU	0.20–0.34 IU	0.35–0.40 IU	≥ 0.41 IU	Total
0–3	212	5	0*	12*†	229
4–5	16	0	0*	2*	18
6–7	1*	0*	0	1	2
8–9	7*	0*	0	1	8
≥ 10	12*‡	3*	2	32	49
Total	248	8	2	48	306

For TS.TB, the highest SFU value was considered for any of the two antigens (ESAT-6 and CFP10); for QFT-IT, the IU of IFN-γ in the antigens tube (ESAT-6, CFP10 and TB7.7) was considered. Negative control values were subtracted from antigen response before comparison. Indeterminate results were excluded from the analysis.
*Discordant results between IGRAs.
†Seven LTC patients, one HM patient, and four HIV patients.
‡Five LTC patients, six HM patients, and one HIV patient.

As previously reported for the gold version of the QFT,[30] advanced liver disease is significantly associated also with indeterminate results of the QFT-IT. We have also reported here an association with lower peripheral blood lymphocyte counts. However, technical errors were more frequent with TS.TB than with QFT-IT, reflecting the higher complexity of the enzyme-linked immunospot assay compared with the enzyme-linked immunosorbent assay.

Both the QFT-IT and TS.TB have been recently approved for clinical use by the US Food and Drug Administration, and both tests have been included in guidelines published in several countries, including the United States, Canada, the United Kingdom, France, Italy, Germany, Switzerland, and Australia. These guidelines provide clinicians with various application strategies, although without strong evidence of their efficacy. However, in particular for TB, there is a need for in-field studies of the diagnostic accuracy of newer tools in high-risk populations.[31] Our study shows for the first time that the performance of IGRA, both in terms of rates of positive results and in diagnostic agreement, varies greatly across different categories of patients who are at increased risk of TB reactivation. Because of the importance of targeting such high-risk groups for effective TB control, we advise caution when interpreting the results of IGRA among immunosuppressed patients.

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ANEXO III

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Prospective evaluation of latent tuberculosis with interferon-γ release assays in drug and alcohol abusers

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SUMMARY

In vitro tests have been developed for the diagnosis of tuberculosis (TB) infection. The objective was to analyse latent TB infection in drug and alcohol abusers through two interferon-γ techniques. One hundred and thirty-nine patients were admitted between February 2006 and May 2007. Mean age was 39·8 years [31% HIV positive]. The enzyme immunoassay (EIA) and enzyme-linked immunospot (ELISPOT) interferon-γ assays were positive in 34% of patients with an agreement of 83% ($\kappa = 0{\cdot}63$). Tuberculin skin test (TST) was positive in 29% of patients and the agreement of TST with EIA and ELISPOT interferon-γ assays was 85% ($\kappa = 0{\cdot}62$) and 83% ($\kappa = 0{\cdot}57$), respectively. Almost 50% of patients with history of TB had a positive *in vitro* test. In conclusion, we observed a high prevalence of latent TB and good agreement between the new *in vitro* tests that otherwise may continue to be positive long after developing TB disease.

Key words: Diagnosis of infection, drug abuse, interferon-γ, tuberculosis.

INTRODUCTION

The rate of tuberculosis (TB) in Spain is among the highest of industrialized countries [1–3]. It is well known that drug addicts have a higher risk of TB and that alcoholism and intravenous drug use are among the main factors associated with TB [4–6]. Moreover, intravenous drug use has been the main category of human immunodeficiency virus (HIV) transmission in Spain [7], and the elevated incidence of TB in patients infected with HIV is also known, which increases the risk of developing tuberculosis up to 100-fold [2, 8, 9].

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The diagnosis and treatment of latent infection is very important in the control of TB. For more than 100 years the tuberculin skin test (TST) or Mantoux test has been the only method used in its detection [10]. The TST uses a protein purified derivate (PPD) that is a mixture of antigens, many of which are shared with *Mycobacterium tuberculosis*, *M. bovis*, Bacille Calmette-Guérin (BCG), and other non-tuberculosis mycobacteria; the limitations of TST are well known: low specificity in individuals vaccinated with BCG or in those exposed to other mycobacteria; sensitivity may be lessened in immunocompromised patients. On the other hand a booster effect may be produced by repeated use of TST and errors in the reading and interpretation of the results may occur. A further inconvenience is the need for a second visit for reading the results [11, 12].

Immunodiagnostic methods for detecting TB infection are based on the *in vitro* quantification of the cellular immune response in response to specific *M. tuberculosis* antigens [13, 14].

In the first generation of *in vitro* tests the same antigen was used as in the TST, which caused some limitations [13]. The new versions use antigens more specific for *M. tuberculosis* such as early secretory antigen target-6 (ESAT-6), culture filtrate protein-10 (CFP-10) and TB7.7 antigen; the genes which encode ESAT-6 and CFP-10 are found in a segment named the region of difference 1 (RD1) of the *M. tuberculosis* genome and TB7.7 is encoded in RD11 which are not present in the genome of *M. bovis*, BCG or other non-tuberculosis mycobacteria such as *M. avium* [14]. Other mycobacteria that have been shown to induce T-cell responses to ESAT-6 and CFP-10 are *M. marinum* and *M. kansasii* [15].

There are two tests marketed for the *in vitro* diagnosis of TB based on *M. tuberculosis*-specific antigens: QuantiFERON TB Gold In-Tube (Cellestis Ltd, Australia) which uses enzyme immunoassay (EIA) techniques to measure the production of interferon-γ by the T cells in whole blood in response to ESAT-6, CFP-10 and TB7.7 and the T-SPOT.TB test (Oxford Immunotec Ltd, UK) that uses the enzyme-linked immunospot (ELISPOT) technique to determine the T cells that produce interferon-γ in response to the *M. tuberculosis*-specific antigens ESAT-6 and CFP-10 [12].

The usefulness of the tests in identifying latent infection by *M. tuberculosis* has been established [16, 17]; however, there exists little information in some populations such as immunocompromised patients and drug users.

The objective of this study is to analyse TB infection rates in patients at high risk of infection using two interferon-γ techniques based on TB-specific antigens.

MATERIAL AND METHODS

Study population

Patients admitted to a detoxification unit in a tertiary hospital between February 2006 and May 2007 were included in the study. The patients came from different outpatient centres for the treatment of alcohol and drug abuse in metropolitan Barcelona. All subjects gave their consent for participation in the study.

Methods

Sociodemographic characteristics, as well as information on the history of alcohol and drug abuse were collected from all patients. For medical history patients were asked about previous TB disease or TB infection. Upon admission blood samples were taken for HIV serology (EIA and Western blot), RNA-HIV, CD4 lymphocytes and interferon-γ tests.

TST was carried out by means of intradermal administration of 2 U of PPD-RT23 in the forearm, except where the subject had a history of culture-proven previous TB disease or previous positive TST; the reading of TST was performed 48 h later and was considered positive if the induration was $\geqslant 5$ mm in HIV-positive subjects and $\geqslant 10$ mm in HIV-negative subjects. During admission active TB was ruled out in all cases by means of a chest X-ray and three sputum samples which were analysed using the Ziehl–Neelsen stain and Lowenstein–Jensen culture. If subjects presented with fever and/or constitutional symptoms, extrapulmonary localization of TB was also excluded.

The participants were assessed for TB infection with two interferon-γ techniques based on *M. tuberculosis*-specific antigens (EIA); QuantiFERON-TB Gold In-Tube (Cellestis Ltd) and ELISPOT (T-SPOT.TB) (Oxford Immunotec Ltd).

QuantiFERON-TB Gold In-Tube

Three millilitres of blood were distributed in three test tubes with anticoagulant, one of which contained ESAT-6-, CFP-10- and TB7.7-specific antigens, another with saline solution (negative control) and the third with phytohaemagglutinin (positive control). These were left to incubate at 37 °C overnight. After incubation the plasma was separated by centrifugation and kept frozen (−20 °C) until required.

The production of interferon-γ, expressed in IU/ml, was determined by ELISA (enzyme-linked immunosorbent assay) and analysis software from QuantiFERON was used to obtain the results. The value obtained was deduced in the negative control from the values obtained in the test tubes stimulated with mitogen and with the specific TB antigens. Values $>0{\cdot}35$ IU/ml were considered positive in the sample stimulated with TB antigens. The result of the test was considered indeterminate if the production of interferon-γ after stimulation with phytohaemagglutinin was $<0{\cdot}5$ IU/ml and the sample stimulated with TB antigen was $<0{\cdot}35$ IU/ml.

T-SPOT.TB

The mononuclear cells were separated by density gradient centrifugation from a sample of 8 ml peripheral venous blood, and after cell washing and counting were distributed in wells with ESAT-6- and CFP-10-specific antigens as well as phytohaemagglutinin as positive control and cells only as negative control ($2{\cdot}5 \times 10^5$ cells/well) on a plate covered with anti-interferon-γ antibodies which were left to incubate overnight. After washing the plate a conjugate was added against the antibodies used, an enzyme substrate was also added.

The number of spots was determined using an automatic reader (AID ELISPOT, AIDSystem, Germany) as well as with visual assistance. The result was considered positive if the number of spots in any well with antigen was $\geqslant 6$ after subtracting the number of spots from the negative control. Negative results with ESAT-6 and CFP-10 antigens and phytohaemagglutinin were considered indeterminate.

Statistical analysis

The descriptive statistics were expressed as mean $\pm$ S.D. for the quantitative variables and absolute frequencies and percentages for the qualitative variables. The comparisons were made by means of χ^2 test and Student's t test. Kappa (κ) statistics were used to evaluate the agreement between the diagnostic tests. Values from $P < 0{\cdot}05$ were considered statistically significant. The data analyses were performed with SPSS software, version 11.5 (SPSS Inc., USA).

RESULTS

In total, 139 patients were admitted to detoxification between February 2006 and May 2007; the results of the interferon-γ release assays (IGRAs) were not valid in four cases. The mean ($\pm$ S.D.) age of the 135 patients analysed was $39{\cdot}8 \pm 8{\cdot}0$ years (83·7% males). The main drug abused was alcohol for 59 patients (45%), cocaine for 46 (35·1%) and opiates for 26 (19·9%). Sixty-one patients (45·2%) were current injecting drug users (IDUs); 42 patients (31·1%) tested positive for HIV infection upon admission; the characteristics of the patients with and without HIV infection are shown in Table 1. In HIV-positive patients the RNA-HIV was $\geqslant 400$ cp/ml in 38·1% of cases; 18 patients (42·9%) were undergoing antiretroviral treatment.

In terms of history of TB, 13 patients (9·6%) had TB disease before admission and 20 (14·8%) had history of positive TST before admission. TST was performed on 100 patients and was positive in 29 patients globally (29%). Chest X-rays were performed for 113 patients and were normal in 96 (85%); eight patients (7·1%) presented with residual lesions and nine (7·9%) with findings attributable to chronic obstructive pulmonary disease.

Table 1. *Characteristics of the study population according to HIV serostatus*

	HIV(–) n/N (%)	HIV(+) n/N (%)
Male	80/93 (86·0)	33/42 (78·6)
Age (mean $\pm$ S.D.)	$40{\cdot}3 \pm 8{\cdot}9$	$38{\cdot}9 \pm 5{\cdot}6$
Current or past IDU	23/93 (24·7)	38/42 (90·5)
Duration of drug use (months) (mean $\pm$ S.D.)	$157{\cdot}5 \pm 111{\cdot}2$	$161{\cdot}4 \pm 107{\cdot}3$
Previous imprisonment	14/73 (19·2)	16/25 (64·0)
Hepatitis C virus infection	28/89 (31·5)	39/40 (97·5)
CD4 cell count (mean $\pm$ S.D.) ($n = 125$)	$1144 \pm 465{\cdot}8$	$536{\cdot}5 \pm 347{\cdot}9$
Haemoglobin (g/dl) (mean $\pm$ S.D.) ($n = 111$)	$14{\cdot}7 \pm 1{\cdot}6$	$13{\cdot}9 \pm 1{\cdot}5$
Body mass index (mean $\pm$ S.D.) ($n = 101$)	$25 \pm 4{\cdot}7$	$22{\cdot}5 \pm 3{\cdot}1$
History of TST(+)	10/93 (10·8)	10/42 (23·8)
History of TB	6/93 (6·5)	7/42 (16·7)

IDU; Injecting drug user; TST, tuberculin skin test.

Fifty-seven (42·2%) patients tested positive in at least one of the interferon-γ tests. With EIA, 46 patients (34·1%) tested positive, two were indeterminate (1·5%), and one was positive with ELISPOT and the other negative. ELISPOT was positive in 46 patients (34·1%) and indeterminate in one case, which was positive with EIA. The agreement between EIA and ELISPOT was 83% [$\kappa = 0{\cdot}63$, 95% confidence interval (CI) 0·50–0·76].

Prevalences of interferon-γ tests and TST in HIV-negative patients and HIV-positive patients with CD4 $\geqslant 350$ cells and < 350 cells are shown in Table 2; there were no statistically significant associations between HIV serostatus and *in vitro* or *in vivo* tests.

Of the 100 patients given the TST test upon admission, the agreement between TST and EIA was 85% ($\kappa = 0{\cdot}62$, 95% CI 0·45–0·80) and was 83% between TST and ELISPOT ($\kappa = 0{\cdot}57$, 95% CI 0·39–0·75). Figure 1 shows the frequency distribution of the results from the two *in vitro* tests according to TST. Of the 29 patients who tested positive with TST, 20·7% (six cases) had negative results with IGRA. Of the 71 patients who tested negative with TST, 15·6%

Table 2. *Prevalence of* in vitro *and* in vivo *tests for the diagnosis of latent tuberculosis according to HIV infection and CD4 cell count*

	HIV(–) *n*/*N* (%)	HIV(+) CD4 ⩾ 350 *n*/*N* (%)	HIV(+) CD4 < 350 *n*/*N* (%)
EIA(+)	33/93 (35·5)	11/28 (39·3)	1/10 (10·0)
ELISPOT(+)	35/93 (37·6)	8/28 (28·6)	2/10 (20·0)
EIA(+) or ELISPOT(+)	41/93 (44·1)	13/28 (46·4)	2/10 (20·0)
TST(+)*	27/77 (35·1)	2/15 (13·3)	0/5 (0·0)
EIA(+) or TST(+)	31/77 (40·3)	4/15 (26·7)	0/5 (0·0)
ELISPOT(+) or TST(+)	32/77 (41·6)	5/15 (33·3)	0/5 (0·0)

EIA, Enzyme immunoassay; ELISPOT, enzyme-linked immunospot technique; TST, tuberculin skin test.
* ⩾5 mm in HIV(+) patients; ⩾10 mm in HIV(–) patients.

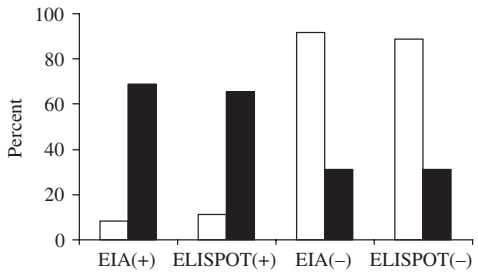

Fig. 1. Agreement of two interferon-γ release assays with tuberculin skin test (TST) at admission. □, TST(–); ■, TST(+). EIA, Enzyme immunoassay; ELISPOT, enzyme-linked immunospot technique.

Table 3. *Characteristics of patients with previous tuberculosis disease and results of IGRA tests*

Sex	Age (yr)	HIV	CD4 cells	Years between TB disease and an IGRA test	EIA	ELISPOT
F	43	–	796	8	–	–
F	55	–	716	30	–	–
M	41	–	668	9	+	–
M	43	–	1061	13	+	+
M	41	–	974	8	+	+
M	45	–	1129	9	+	+
F	38	+	185	13	–	–
M	43	+	667	15	–	–
M	43	+	602	11	–	–
M	43	+	694	11	–	–
F	43	+	217	15	–	+
M	43	+	1014	10	+	+
M	42	+	150	4	+	+

IGRA, Interferon-γ release assay; EIA, enzyme immunoassay; ELISPOT, enzyme-linked immunospot technique.

(11) tested positive with IGRA, four of which were HIV positive.

Table 3 shows the results of the interferon-γ tests in those patients with a history of TB disease. With EIA, positive results were obtained in 6/13 patients (46·1%) and six cases (46·1%) were also positive with ELISPOT with an agreement of 85%. Of those whose IGRA tests were positive, the time between TB disease and the interferon-γ test was 8·8 years for EIA and 9·8 years for ELISPOT compared to 14·7 years and 13·8 years for those whose EIA and ELISPOT interferon-γ results were negative.

In the univariate analysis, age, sex, HIV infection, hepatitis C virus infection, previous imprisonment, being an IDU and the main type of drug were not associated with positive results with IGRA testing.

DISCUSSION

The results from this cross-sectional study show an elevated prevalence of latent TB in current alcohol and drug abusers independently of the *in vitro* method used. This fact is unsurprising since the level of TB rates in Spain have been high until recent years. In drug users, studies developed with the first generation of interferon-γ techniques that used stimulation with PPD found more than double the positive results in the *in vitro* test than in the skin test [13, 18]. Another study in patients receiving methadone showed a 17% prevalence of first-generation interferon-γ test [19]. Only one study in drug addicts has used new interferon-γ assays with antigens specific for TB [20]. To our knowledge this is the first study using IGRA testing in alcohol and drug abusers from Spain. The results described in a setting characterized by high

prevalence of drug abuse and TB infection will allow better estimation of the burden of latent infection when using the new *in vitro* techniques. The observed agreement between the two *in vitro* techniques is good ($\kappa = 0{\cdot}63$) and coincides with that which has been observed in other populations [21].

The interferon-γ techniques that used TB-specific antigens have shown an elevated specificity (98% with EIA and 92% with ELISPOT) [14, 22] in low-risk populations; in terms of sensitivity, the results are controversial: while some studies find better sensitivity than in patients with TB disease [21–23], in others, the sensitivity of EIA is less than that of TST for the diagnosis of TB [24]. In immunocompromised subjects, ELISPOT has been associated with higher sensitivity than TST [14, 16, 22, 25].

In the present study the agreement between TST and EIA and the agreement between TST and ELISPOT was 85% ($\kappa = 0{\cdot}62$) and 83% ($\kappa = 0{\cdot}57$), respectively. The discordance between IGRA and TST in the cases where TST was positive and IGRA negative could be ascribed to previous BCG vaccination, environmental mycobacteria, or an increased sensitivity of TST with respect to IGRAs. The cases where TST was negative and IGRA positive suggest higher sensitivity with new *in vitro* tests. However, the absence of a reference test for latent TB makes the evaluation of the sensitivity and specificity difficult to determine with IGRA.

The frequency of an IGRA test with indeterminate results is lower than observed in previous studies [21, 26]. It is well known that indeterminate results are associated with immunodepression [16, 27] and the association between the number of CD4 cells and indeterminate results [26]. The mean of CD4 cells in HIV-positive patients in this study was >500 cells/μl and only two of the 42 HIV-positive patients presented with CD4 counts <100 cells/μl, which would explain the low (with EIA) or null (with ELISPOT) number of indeterminate results. CD4 cell counts >500 cells/μl in HIV-positive patients might also explain the fact that no significant differences were observed for EIA and ELISPOT according to HIV serostatus at admission.

The results of the interferon-γ tests were positive in $>50\%$ of patients previously diagnosed and treated for TB disease. The same number of positive IGRA results (6/13) was obtained with the two tests in the subgroup that had TB disease before admission and the agreement between them was good. The duration of a positive IGRA test after TB (an average of 9 years in EIA-positive cases and 10 years in ELISPOT-positive cases), is even longer than that described in other studies [26]. In this sense, it is not well defined when interferon-γ tests become negative after treatment for TB; our results indicate that the IGRA test can remain positive for years after the illness which could limit its usefulness in differentiating current or past infection and should be an indication to perform additional tests for active TB.

We found no associations between a positive IGRA test and HIV infection, age, sex, substance of abuse, intravenous drug use or antecedent of imprisonment. Other studies in wider populations are necessary to assess the risk factors for TB infection when using new *in vitro* tests.

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DECLARATION OF INTEREST

None.

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ANEXO IV

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Mycobacteriology

T-cell responses to the *Mycobacterium tuberculosis*-specific antigens in active tuberculosis patients at the beginning, during, and after antituberculosis treatment

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Abstract

The objectives of the study were to assess the performance of the QuantiFERON-TB Gold In-Tube (QFN-G-IT) and the T-SPOT.TB tests in the immunodiagnosis of active tuberculosis (TB) in adult patients, and to study the T-cell interferon γ (IFN-γ) responses during treatment and in patients who have recovered after curative treatment and self-healed TB patients. When only analyzing patients included at the beginning of treatment, the sensitivity was 83.3% for T-SPOT.TB and 69.4% for QFN-G-IT. In contrast, when evaluating patients during treatment, the sensitivity of the T-SPOT.TB and QFN-G-IT decreased to 69.8% and 48.8%, respectively. The response to the specific antigens increased after finishing the treatment compared with the values during the treatment. The T-SPOT.TB was more sensitive in diagnosing active TB than the QFN-G-IT. The IFN-γ tests could be used as a complementary method in the diagnosis of active TB.

Keywords: Active tuberculosis; *Mycobacterium tuberculosis*

1. Introduction

The basis of tuberculosis (TB) control programs consists of the diagnosis and correct treatment of patients with active TB. One essential factor for controlling the spread of this disease is the ability to diagnose it in its early stages. Patients with pulmonary TB could be smear-negative for acid-fast bacilli, and mycobacterial culture can take several weeks. In addition, the diagnosis of extrapulmonary TB is complicated and often requires invasive diagnostic methods (Migliori et al., 2006; Valdes et al., 1998).

In an effort to develop more accurate tests for the immunodiagnosis of *Mycobacterium tuberculosis* infection and also for vaccine design strategies, 2 specific region of difference (RD) 1 antigens have been described. These antigens are the early secreted antigenic protein 6 kDa (ESAT-6) and the culture filtrate protein 10 kDa (CFP-10) that are present in *M. tuberculosis* but absent from all *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) vaccine strains and from most environmental mycobacteria (Andersen et al., 2000).

In vitro assays to measure T-cell–mediated immune responses to these antigens have been evaluated. Infected individuals are identified by the interferon γ (IFN-γ) released by their T cells after being stimulated by *M. tuberculosis*-

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specific antigens. Several studies have published promising results for the utility of T-cell assays in the diagnosis of latent TB infection (Dominguez et al., 2008; Eum et al., 2008; Ewer et al., 2003; Lalvani et al., 2001a; Piana et al., 2006; Porsa et al., 2007).

Basically, 3 in vitro IFN-γ assays that incorporate RD1-specific antigens are available: QuantiFERON-TB Gold assay (QFN-Gold) and QuantiFERON-TB Gold In-Tube (QFN-G-IT) (Cellestis Limited, Carnegie, Australia), and the T-SPOT.TB (Oxford Immunotec Limited, Abingdon, UK). QuantiFERON-TB Gold assay and QFN-G-IT detect IFN-γ production after stimulation of whole blood samples with the specific antigens; in contrast, T-SPOT.TB detects the number of IFN-γ–producing T cells after stimulation of isolated peripheral blood mononuclear cells. The main difference between QFN-Gold and QFN-G-IT is that in the latter, the specific *M. tuberculosis* antigens are included in the same blood sample collection tube with the addition of a 3rd specific antigen, TB7.7 (Rv2654). This new antigen is encoded in RD11 and is missing from the BCG strains as well as most common nontuberculous mycobacteria (Brock et al., 2004).

Numerous studies have evaluated the utility of these tests in diagnosing active TB (Pai and Menzies, 2007) and have described a wide range of sensitivities (Pai et al., 2008). In addition, they have shown that the IFN-γ response decreased with treatment (Carrara et al., 2004; Goletti et al., 2006a; Lalvani et al., 2001a; Pathan et al., 2001), suggesting that this response could be used to monitor the efficacy of therapy. On the other hand, there are only few studies using tests based on specific antigens in patients who have recovered from active TB.

The objectives of the present study were to assess the accuracy and reliability of QFN-G-IT and the T-SPOT.TB tests in the immunodiagnosis of active TB in adult patients at the moment of diagnosis, and to study the T-cell IFN-γ responses to the specific antigens during the treatment and in patients who have recovered after a curative treatment.

2. Materials and methods

2.1. Study population

We enrolled adult patients between September 2004 and June 2007 who went to Hospital Universitari Germans Trias i Pujol, the TB Control and Prevention Unit of Barcelona (CAP Drassanes), and the Serveis Clinics (Barcelona). Ethics approval for this study was provided by the corresponding ethics committees.

We obtained informed consent from all patients before blood sampling. A detailed questionnaire about clinical information was completed. Patients were asked to indicate the results of any previous tuberculin skin test (TST), whether they had received BCG vaccination, details of previous diagnosis of active TB, and whether they had any other medical conditions. All subjects were tested for human immunodeficiency virus (HIV) infection. Data were also collected from medical records of chest radiography. The results and dates of culture and details of treatment of TB were also collected.

We included different patients during various stages of the anti-TB treatment (at the beginning, and during and after treatment) to evaluate the effect of the antibiotic therapy on the immune response and on the IFN-γ test results. Patients at the beginning of treatment had received less than 2 weeks of therapy or were untreated at the time of giving blood for both assays. Patients studied during treatment were at later stages of their treatment course. The TST was performed at the beginning of the treatment on all patients diagnosed with pulmonary TB. The patients were classified into 5 groups.

2.1.1. Group 1

Group 1 is composed of 79 patients diagnosed with pulmonary TB. Thirty-six cases were at the beginning of the treatment and 43 during the treatment. In all cases, *M. tuberculosis* was isolated by culture. Ten patients were followed longitudinally, all of whom were HIV negative and received curative treatment. Treatment was successful in these 10 participants as evidenced by no clinical or radiologic evidence of current disease, the completion of antituberculous chemotherapy, and sterile mycobacterial cultures.

2.1.2. Group 2

Group 2 is composed of 14 patients with extrapulmonary TB (lymphadenitis [4], pleural [3], peritoneal [1], disseminated [6]). Two cases were recruited at the beginning of the treatment and 12 cases during the treatment. In all cases, *M. tuberculosis* was isolated by culture.

2.1.3. Group 3

Group 3 is composed of 10 patients diagnosed with pulmonary TB (5 cases at the beginning of the treatment and the remaining 5 cases during the treatment) and 6 patients diagnosed with nonrespiratory TB (lymphadenitis [3], pleural [2], and cutaneous [1]) recruited during the treatment. Although the disease was not microbiologically confirmed, the physician decided that the patients suffered from TB based on clinical, pathologic, and radiologic findings, and the patients clinically and radiologically responded to a full course of anti-TB treatment.

2.1.4. Group 4

Group 4 is composed of 6 healed pulmonary TB patients after a curative treatment. In all cases, the disease was microbiologically diagnosed and successfully treated.

The main demographic characteristics of the TB patients included in the study are shown in Table 1. The technicians performing the TST and the IFN-γ tests were not made aware of the patients' clinical status and times of sample collection.

2.2. Tuberculin skin test

Tuberculin skin test was performed by the Mantoux method using 2 intradermal tuberculin units of PPD RT23 (Statens Serum Institut, Copenhagen, Denmark). Size of

Table 1
Demographic characteristics of TB patients studied

Variable	Group 1	Group 2	Group 3		Group 4
	Pulmonary TB confirmed microbiologically, *n* = 79 (%)	Extrapulmonary TB confirmed microbiologically, *n* = 14 (%)	Pulmonary TB not confirmed microbiologically, *n* = 10 (%)	Extrapulmonary TB not confirmed microbiologically, *n* = 6 (%)	Healed pulmonary TB after curative treatment, *n* = 6 (%)
Gender					
Female	25 (31.6)	5 (35.7)	1 (10)	2 (33.3)	2 (33.3)
Male	54 (68.4)	9 (64.3)	9 (90)	4 (66.7)	4 (66.7)
Age, mean ± SE	38.87 ± 18.5	40.24 ± 19.60	39.50 ± 12.7	28.67 ± 6.5	48.8 ± 11.98
BCG vaccinated					
Yes	16 (20.3)	1 (7.1)	6 (60)	2 (33.3)	2 (33.3)
No	63 (79.7)	13 (92.9)	4 (40)	4 (66.7)	4 (66.7)
Immunosuppression					
AIDS patients	2 (2.5)	6 (42.9)	1 (10)	0 (0)	0 (0)
Treatment with systemic steroids	1 (1.3)	0 (0)	0 (0)	0 (0)	0 (0)
No	76 (96.2)	8 (57.1)	9 (90)	6 (100)	6 (100)
Country of birth					
Immigrants from countries with high prevalence of TB infection	44 (55.7)	5 (35.7)	6 (60)	5 (83.3)	2 (33.3)
Autochthonous Spanish population	35 (44.3)	9 (64.3)	4 (40)	1 (16.7)	4 (66.7)
Groups of patients					
At the beginning of treatment	36 (45.5)	2 (14.3)	5 (50)	0 (0)	–
During the treatment	43 (54.5)	12 (85.7)	5 (50)	6 (100)	–

the induration was measured 48 to 72 h later. The TST results were noted and read according to the Spanish Society of Pneumology guidelines (Blanquer et al., 2008) by experienced staff who perform these duties regularly. In our study, TST indurations ≥5 mm were classified as positive results.

2.3. T-SPOT.TB

The test was performed following the manufacturer's recommendations. Cells were stimulated in each well by medium alone (as nil control), phytohemagglutinin (as positive control), and 2 peptide panels containing the antigens ESAT-6 and CFP-10. The presence of reactive antigen-specific T cells was revealed as a spot on the well. Spots were scored by the naked eye and, in doubtful cases, with the support of an automated AID ELISPOT plate reader (Lector AID Elispots; Autoimmun Diagnostiks, Germany). Test wells were scored as positive if they contained at least 6 spot-forming cells more than the nil control well, and this number was at least twice the number of the nil control well on one or both of the antigen panels. A result was considered indeterminate if the antigen-stimulated sample was negative and if the value of the positive control was less than 20 spots.

2.4. QuantiFERON-TB Gold In-Tube

This test was also performed according to the manufacturer's instructions. Briefly, a total of 3 mL of blood was taken from each patient and collected in 3 tubes of 1 mL each (nil control, positive control, and TB-specific antigens). The amount of IFN-γ released was measured by ELISA. Raw optical densities were interpreted using the specific software provided by the manufacturer. The result obtained by the nil control was subtracted from the mitogen control and the antigen-stimulated samples. Regardless of the result of the mitogen control, the cutoff value for a positive test was at least 0.35 IU/mL of IFN-γ in the sample after simultaneous stimulation with the specific antigens. The result of the test was considered indeterminate if an antigen-stimulated sample was negative and if the value of the positive control was less than 0.5 IU/mL.

2.5. Statistical analysis

Concordance between the tests was assessed using Cohen's κ coefficient. We used the McNemar test to compare the proportion of negative and positive results among the QFN-G-IT, T-SPOT.TB, and TST. The differences in functions of the time of collecting the sample and of smear results were calculated using the nonparametric Mann–Whitney *U* test. Comparison of the number of spots and the IFN-γ released was performed by Kruskal–Wallis 1-way analysis. Differences were considered significant when the *P* value was less than 0.05. All analyses were made with SPSS statistical software for Windows (SPSS version 14.0; SPSS, Chicago, IL).

3. Results

The number of positive results in each group of patients obtained by both IFN-γ assays and the TST are shown in Table 2.

3.1. Results in pulmonary TB patients

The overall sensitivity in microbiologically confirmed patients, including both those at the beginning and during the treatment, was 75.9% using T-SPOT.TB and 58.2% using QFN-G-IT. T-SPOT.TB produced significantly more positive results than QFN-G-IT (P = 0.003), with poor agreement (κ = 0.44; standard error [SE] = 0.096). However, when only analyzing patients included at the beginning of the treatment, the sensitivity increased to 83.3% for T-SPOT.TB and 69.4% for QFN-G-IT. Differences between both tests were not significant (P = 0.125, κ = 0.43, SE = 0.153). In these patients, the sensitivity of the TST is 94.4%, with insignificant differences with T-SPOT.TB (P = 0.125, κ = 0.46, SE = 0.216) but significant with QFN-G-IT (P = 0.004, κ = 0.24, SE = 0.142). In contrast, while evaluating patients during treatment, the sensitivity of the T-SPOT.TB and QFN-G-IT decreased to 69.8% and 48.8%, respectively. In this case, the difference in the number of positive results between the 2 tests was also significant (P = 0.022, κ = 0.42, SE = 0.127).

Although the differences in sensitivity between patients evaluated at the beginning and during the treatment were not significant for T-SPOT.TB (P = 0.209) or for QFN-G-IT (P = 0.078), the number of responding T cells after stimulation with ESAT-6 (Fig. 1) and the IFN-γ released after specific antigen stimulation was significant (P = 0.004 and P = 0.030, respectively) (Fig. 1). The number of responding T cells after stimulation with CFP-10 did not obtain significance (Fig. 1); however, we observed differences when comparing patients at the beginning with patients recruited after more than 5 months of treatment. We did not observe a relationship between negative results for IFN-γ tests and the length of treatment time (data not shown).

However, the results obtained in the 10 patients monitored from the beginning and during the treatment showed that there is considerable interindividual variation in terms of the rate of decline of the response (Fig. 2 A-C).

In addition, the difference in the percentage of positive results for IFN-γ tests was not significant for smear-positive or negative (P = 0.063) (Table 2). However, the agreement between the IFN-γ tests in smear-positive patients was better than in smear-negative (κ = 0.62, SE = 0.182, and κ = 0.21, SE = 0.181, respectively), with

Table 2
QuantiFERON-TB Gold In-tube, T-SPOT.TB, and TST results in the different groups of TB patients

Groups of patients	Diagnostic tests							
	T-SPOT.TB			QFN-G-IT			TST	
	Positive (%)	Negative (%)	Indet[a] (%)	Positive (%)	Negative (%)	Indet (%)	Positive (%)	Negative (%)
Group 1								
Pulmonary TB confirmed microbiologically (n = 79)	60 (75.9)	16 (20.3)	3 (3.8)	46 (58.2)	31 (39.3)	2 (2.5)	55/58 (94.8)	3/58 (5.2)
At the beginning of treatment (n = 36)	30 (83.3)	4 (11.1)	2 (5.6)	25 (69.4)	10 (27.8)	1 (2.8)	34 (94.4)	2 (5.6)
Smear-positive (n = 19)	14 (73.7)	3 (15.8)	2 (10.5)	14 (73.7)	4 (21.1)	1 (5.2)	17 (89.5)	2 (10.5)
Smear-negative (n = 17)	16 (94.1)	1 (5.9)	0 (0)	11 (64.7)	6 (35.3)	0 (0)	17 (100)	0 (0)
During the treatment (n = 43)	30 (69.8)	12 (27.9)	1 (2.3)	21 (48.8)	21 (48.8)	1 (2.4)	21/22 (95.5)	1/22 (4.5)
Group 2								
Extrapulmonary TB confirmed microbiologically (n = 14)	7 (50)	7 (50)	0 (0)	7 (50)	7 (50)	0 (0)	2/2 (100)	0 (0)
At the beginning of treatment (n = 2)	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)
During the treatment (n = 12)	5 (41.7)	7 (58.3)	0 (0)	5 (41.7)	7 (58.3)	0 (0)	–	–
Group 3								
Pulmonary TB without microbiological confirmation (n = 10)	8 (80)	2 (20)	0 (0)	7 (70)	3 (30)	0 (0)	10 (100)	0 (0)
At the beginning of treatment (n = 5)	4 (80)	1 (20)	0 (0)	5 (100)	0 (0)	0 (0)	5 (100)	0 (0)
During the treatment (n = 5)	4 (80)	1 (20)	0 (0)	2 (40)	3 (60)	0 (0)	–	–
Extrapulmonary TB not confirmed microbiologically	6 (100)	0 (0)	0 (0)	4 (66.7)	2 (33.3)	0 (0)	4/4 (100)	0 (0)
During treatment (n = 6)								
Group 4								
Healed pulmonary TB after curative treatment (n = 5)	5 (83.3)	1 (16.7)	0 (0)	5 (83.3)	1 (16.7)	0 (0)	5 (83.3)	1 (16.7)

[a] Indeterminate result.

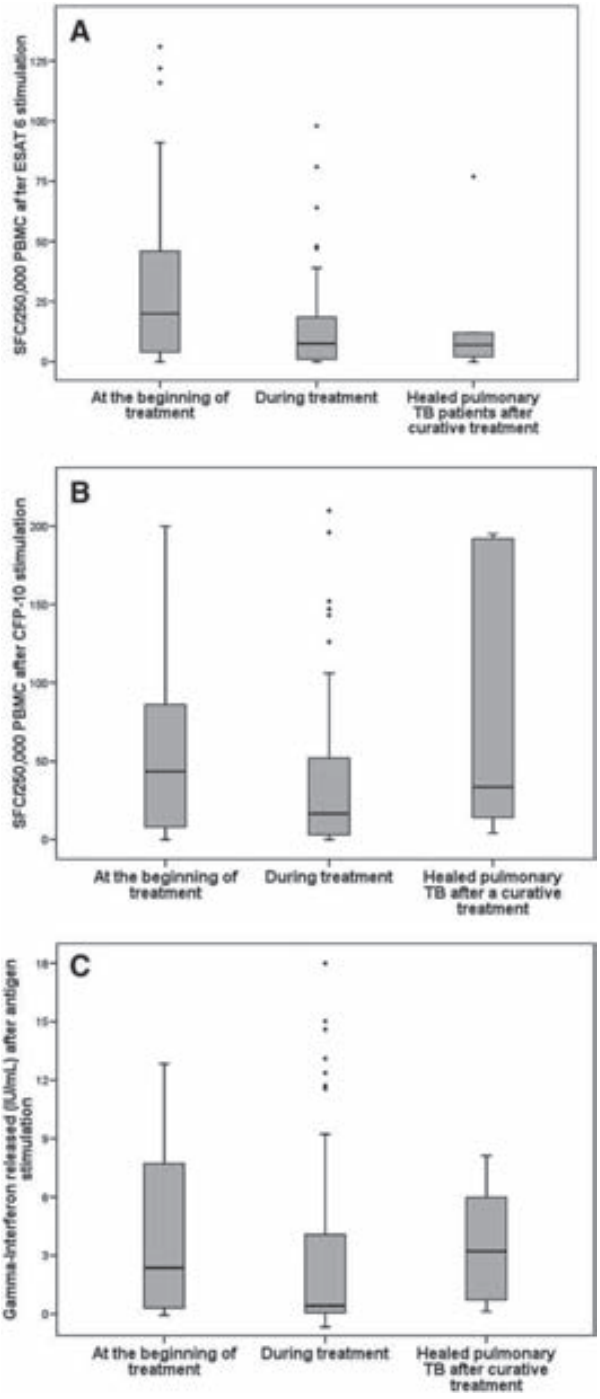

Fig. 1. Number of spot T cells formed and IFN-γ released after specific antigen stimulation for patients at the beginning, during, and after curative treatment (A, after ESAT-6 stimulation; B, after CFP-10 stimulation; C, IFN-γ released after specific antigen stimulation).

T-SPOT.TB being more sensitive than the QFN-G-IT in smear-negative patients. On the other hand, the differences in responding T cells after stimulation with ESAT-6 and CFP-10, and the IFN-γ released were not significant (P = 0.621, 0.640, and 0.459, respectively) between those patients with confirmed pulmonary TB and those that were not microbiologically confirmed.

Comparing the overall results obtained for the group of patients treated successfully and the results of patients at the beginning and during the treatment, the differences were not significant (P = 0.945 and P = 0.719 for T-SPOT.TB, and P = 0.713 and P = 0.260 for QFN-G-IT, respectively). However, although the differences were not significant, the

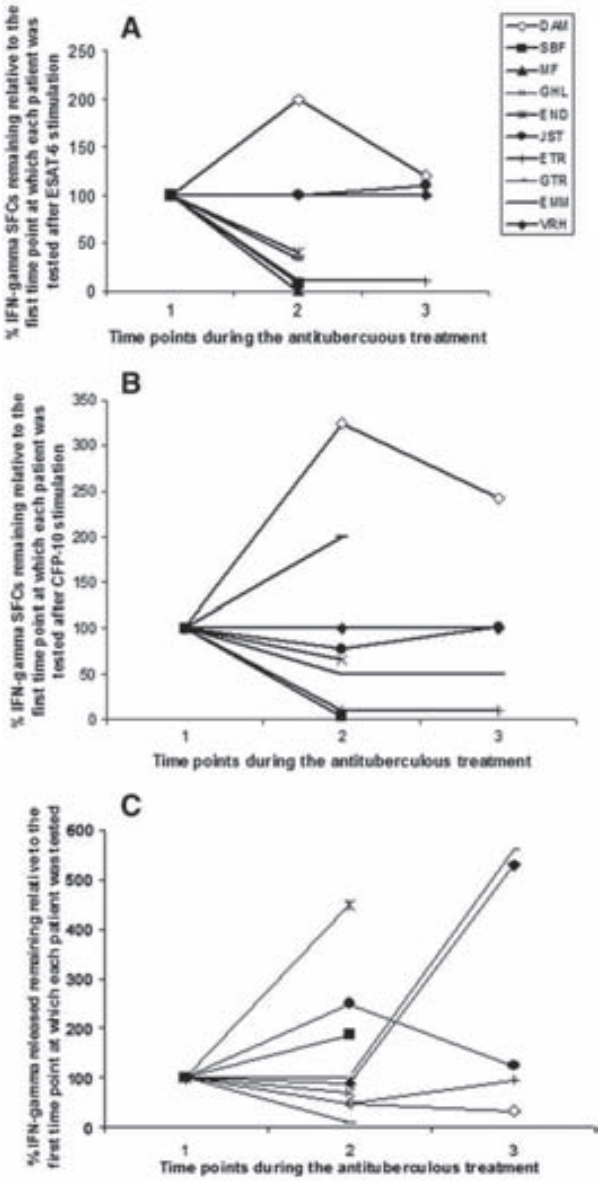

Fig. 2. Percentage of spot T cells and IFN-γ released remaining at various time points relative to the original at the time at which each patient was 1st tested (1, at the time of TB diagnosis; 2, between 1 to 3 months of antituberculous therapy; and 3, more than 3 months of antituberculous treatment) (A, after ESAT-6 stimulation; B, after CFP-10 stimulation; C, IFN-γ released after specific antigen stimulation).

Table 3
Median and 5 to 95 percentiles of T-cell IFN-γ responders and IFN-γ released against specific *M. tuberculosis* antigens in TB patients at the beginning, during, and after finishing the antituberculous treatment, and in self-healed patients

	No. of T cells IFN-γ responders against ESAT-6 antigens			No. of T cells IFN-γ responders against CFP-10 antigens			Amount of IFN-γ released after stimulation with specific antigens		
	Median (5–95 percentiles)	P^a	P^b	Median (5–95 percentiles)	P^a	P^b	Median (5–95 percentiles)	P^a	P^b
At the beginning of the treatment	20.00 (0.35–119.90)	–	0.004	43.50 (0.70–176.95)	–	0.145	2.36 (0.01–30.13)	–	0.030
During the treatment	7.50 (1–66.35)	0.004	–	16.50 (3–147.5)	0.145	–	0.42 (0.05–15.46)	0.030	–
After curative treatment	7.00 (1.50–28.25)	0.069	0.623	33.50 (4–192.75)	0.954	0.373	3.12 (0.12–6.51)	0.772	0.275

[a] Significance value between the responses in patients included at the beginning of the treatment and each group of patients.
[b] Significance value between the responses in patients included during the treatment and each group of patients.

T-cell count after CFP-10 stimulation and the IFN-γ released increase after finishing the treatment compared with the values during the treatment (Table 3).

3.2. Results in extrapulmonary TB patients

When analyzing all extrapulmonary TB patients, both IFN-γ tests were positive for the 2 patients included at the beginning of the treatment (100%), decreasing to 11 (61.11%) and 9 (50%) positive results for the 18 patients included during the treatment by T-SPOT.TB and QFN-G-IT, respectively (Table 2). The concordance of both IFN-γ tests in these patients was 80% (κ = 0.588, SE = 0.180). No relation between negative results and the amount of time under treatment was observed. Distribution of IFN-γ test results considering the type of extrapulmonary TB cases is shown in Table 4.

4. Discussion

Several authors have studied IFN-γ tests in diagnosing active TB, showing a wide range of sensitivities (Goletti et al., 2007; Pai and Menzies, 2007; Ravn et al., 2005; Richeldi, 2006). However, only 5 studies have made a direct comparison between the T-SPOT.TB and the QFN-Gold in the same study population (Chee et al., 2008; Ferrara et al., 2006; Goletti et al., 2006b; Kang et al., 2007; Lee et al., 2006).

Lee et al. (2006) compared T-SPOT.TB and QFN-Gold tests in 87 active TB patients. They found that T-SPOT.TB was the more sensitive test (96.6%), whereas the sensitivities of QFN-Gold and TST were 70.1% and 66.7%, respectively.

Table 4
QuantiFERON-TB Gold In-Tube and T-SPOT.TB results in patients diagnosed with extrapulmonary TB

Extrapulmonary TB	Positive results (%)		Concordance (%)
	T-SPOT.TB	QFN-G-IT	
Lymphadenitis (n = 7)	4 (57.1)	4 (57.1)	5/7 (71.4)
Pleural (n = 5)	5 (100)	5 (100)	5/5 (100)
Peritoneal (n = 1)	1 (100)	1 (100)	1/1 (100)
Disseminated (n = 6)	2 (33.3)	1 (16.7)	5/6 (83.3)
Cutaneous (n = 1)	1 (100)	0 (0)	0/0 (0)
Total (n = 20)	13 (65)	11 (55)	16/20 (80)

Ferrara et al. (2006) studied 24 active TB patients (13 pulmonary and 11 extrapulmonary TB). Of the 11 patients with culture-proven pulmonary TB, the T-SPOT.TB was positive in 81.8% cases, whereas the QFN-Gold was positive in 72.7% of cases. Regarding the extrapulmonary TB cases, the T-SPOT TB identified all patients (100%), whereas QFN-Gold and TST identified 73%. Goletti et al. (2006b) studied the 2 commercially available tests in 23 pulmonary TB cases obtaining a sensitivity for T-SPOT.TB of 91% and for QFN-Gold of 83%. Kang et al. (2007) conducted a prospective study enrolling 144 participants with suspected pulmonary TB and compared the results of the IFN-γ with the final confirmed diagnoses. They found that the sensitivities for T-SPOT.TB and QFN-Gold were 92% and 89%, respectively. Finally, Chee et al. (2008) reported a sensitivity for the T-SPOT.TB of 94.1% and for QFN-G-IT of 83.0% (P = 0.001), evaluating 286 patients diagnosed of pulmonary TB.

Although there are few differences between the sensitivities of the tests in the 5 studies, in all studies, the sensitivities are in the same range, with the T-SPOT.TB being more sensitive than QFN-Gold. In general, our results are consistent with the results reported by the 5 referenced studies. In our study, the sensitivity in patients with pulmonary TB at the beginning of the treatment was higher for T-SPOT.TB (83.3%) than for QFN-G-IT (69.4%). More recently, Dosanjh et al. (2008) developed an ELISPOT [ELISPOT-(PLUS)] incorporating a novel RD1 antigen, Rv3879c, for diagnosing active TB. They found that the ELISPOT(PLUS) assay is more sensitive than the standard ELISPOT, obtaining, when combined with TST, high sensitivity for rapid exclusion of active TB.

An alternative approach to the evaluation of the sensitivity of the in vitro tests has been to test patients with active TB. The false-negative result of both tests in active TB has been described previously (Dewan et al., 2007; Richeldi, 2006). Some hypotheses have been proposed to explain the negative results of the IFN-γ assays in immunocompetent patients with active TB at the beginning of the treatment. First of all, IFN-γ assays have been developed to diagnose latent TB infection (Lalvani et al., 2001b; Mazurek et al., 2001). Although patients with active TB are infected by definition with *M. tuberculosis*, they do not have a latent infection. Nevertheless, active TB occurs when the host

immune responses are unable to contain the latent infection (Pai and Menzies, 2007; Richeldi, 2006). Other factors involved could be the release of anti-inflammatory cytokines by peripheral blood mononuclear cells and the temporary depression of T-cell responsiveness (Chen et al., 2007; Wilkinson et al., 1998).

On the other hand, these results could be also explained by the recruitment of specific T-cells during active TB. Antigen-specific cells clonally expand and migrate to the site of infection (Barnes et al., 1993; Kosters et al., 2008; Wilkinson et al., 2005). In fact, the detection of specific T cells in samples collected from the site of infection has been explored. Jafari et al. (2006) found specific T cells in the bronchoalveolar lavage of all patients with pulmonary TB by T-SPOT.TB. In other studies, the use of an ex vivo characterization of ESAT-6–specific T cells at sites of active disease in pleural (Losi et al., 2007; Wilkinson et al., 2005) or cerebrospinal fluids (Kosters et al., 2008) suggests a good alternative for diagnosing pleural TB and the meningitis, respectively.

The impact of the antituberculous treatment on the IFN-γ tests in reducing the response to the specific antigens has been described elsewhere (Carrara et al., 2004; Dheda et al., 2007; Goletti et al., 2006a; Hirsch et al., 1999; Lalvani et al., 2001a; Pathan et al., 2001). T cells enumerated by both assays are effector cells that have recently been in contact with the antigen in vivo and can release IFN-γ when they are reexposed to the antigen in vitro. It is thought that the frequencies of effector T cells are directly related to the antigen load. Consequently, the count of these effector T cells and the quantity of IFN-γ released reflects a dynamic process of the antigen load. During TB treatment, the number of specific T cells decreases progressively, suggesting that the frequency of ESAT-6–specific T cells is related to antigen load (Ferrand et al., 2005; Pathan et al., 2001). It has been proposed that the IFN-γ could be used to monitor the patients' response to the treatment (Lalvani, 2004).

In our study, there was an important decrease in the proportion of positive responders to both IFN-γ tests during the treatment compared with that at the beginning of the therapy. The decrease in IFN-γ released and in the count of specific responder T-cells was significant. The effect of the antituberculous treatment was observed in both pulmonary and extrapulmonary TB cases. These results strengthen the idea of the utility of these tests for monitoring the response to the treatment. However, it has recently been demonstrated that the sole utilization of IFN-γ secretion by T cells is an insufficient biomarker of antigen load and clinical disease status (Millington et al., 2007). In our experience, the results obtained individually for the patients monitored at the beginning of and during the treatment confirmed the variation in responses between individuals (Fig. 2). Furthermore, we have also observed that the response becomes higher after finishing treatment than during treatment. The increased IFN-γ production after treatment with these assays likely reflects the fact that antigen-specific lymphoproliferation is inhibited by the nonspecific immunosuppression associated with active untreated TB. Therefore, other measures of T-cell function will be required in addition to the IFN-γ. In this sense, promising results studying the dynamic relationship between IFN-γ and interleukin 2 profiles have been obtained (Millington et al., 2007).

In our experience, the CFP-10 responder T cells during the treatment decrease in comparison with that at the beginning of the treatment, but the difference is not significant. After finishing the treatment, the achieved values were similar to those obtained at the beginning of the treatment (Table 3). The persistence of high levels of CFP-10–specific T cells in patients after a curative treatment has been recently described by Fox et al. (2007). In fact, they observed that CFP-10 responder T cells were higher in active TB cases than in cases of latent TB infection. In addition, 6 months after completing antituberculous treatment, the rates of these T cells were similar to those in the healthy contacts. They hypothesized that this differing immunogenicity of CFP-10 could reflect the nature of T-cell epitopes, and that CFP-10 responses could be more reliant on CD8-T cells in active TB than in contacts.

In our study, the high sensitivity obtained by the TST in patients diagnosed with pulmonary active TB could be surprising. The low sensitivity of the TST is directly related with immunosuppressive treatments, with old patients, and with extrapulmonary TB (Holden et al., 1971; Pina et al., 2002). Differences in the sensitivity found by our study and others could be explained by differences in the characteristics of the patients.

The gold standard of a positive culture is sometimes not obtained or at least not early enough. It has been described that about one-third of those treated for pulmonary TB do not have a positive culture for *M. tuberculosis*. In our experience, we did not observe differences between patients with or without microbiologic diagnosis in either of the assays studied. Interferon γ tests can also be especially useful in diagnosing extrapulmonary TB, where about half of the cases have not been microbiologically diagnosed, and which often required complex and invasive diagnostic tools. In addition, it could be useful in pulmonary smear-negative patients. We found that in patients who were sputum smear-negative, the T-SPOT.TB and the QFN-G-IT were positive in the 94.1% and 64.7% of cases, respectively. Ravn et al. (2005) obtained a sensitivity of 87% using QFN-Gold in patients who were negative by culture or microscopy. The contribution of the IFN-γ tests in the clinical work is improved by a higher specificity than TST in detecting real *M. tuberculosis* infection, avoiding cross-reactions with atypical mycobacteria or with the BCG strains.

The main drawback of our study is the lack of longitudinal follow-up in all the patients included at the beginning of the treatment. This was not possible for logistic reasons. However, in our point of view, the differences

obtained between patients at the beginning of the treatment and patients during treatment are sufficiently consistent to draw conclusions.

In summary, the T-SPOT.TB has been more sensitive in diagnosing active TB than the QFN-G-IT. The response to the specific antigens decreases during the treatment, increasing again at the end of the curative therapy to similar levels to those shown at the beginning of the treatment. The role of the CFP-10–specific T cells in patients after the antituberculous treatment and their utilization in the diagnosis of active TB requires further research. The IFN-γ tests could be used as a complementary method in the diagnosis of active TB. It is especially useful in low endemic areas where the ratio of latent TB infection is very low in special populations where culture-proven cases are difficult and also in extrapulmonary TB.

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ANEXO V

Bronchoalveolar Lavage Enzyme-linked Immunospot for a Rapid Diagnosis of Tuberculosis

A Tuberculosis Network European Trialsgroup Study

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***Rationale*: The rapid diagnosis of pulmonary tuberculosis (TB) is difficult when acid fast bacilli (AFB) cannot be detected in sputum smears.**

***Objectives*: Following a proof of principle study, we examined in routine clinical practice whether individuals with sputum AFB smear-negative TB can be discriminated from those with latent TB infection by local immunodiagnosis with a *Mycobacterium tuberculosis*–specific enzyme-linked immunospot (ELISpot) assay.**

***Methods*: Subjects suspected of having active TB who were unable to produce sputum or with AFB-negative sputum smears were prospectively enrolled at Tuberculosis Network European Trialsgroup centers in Europe. ELISpot with early-secretory-antigenic-target–6 and culture-filtrate-protein–10 peptides was performed on peripheral blood mononuclear cells (PBMCs) and bronchoalveolar lavage mononuclear cells (BALMCs). *M. tuberculosis*–specific nucleic acid amplification (NAAT) was performed on bronchoalveolar lavage fluid.**

***Measurements and Main Results*: Seventy-one of 347 (20.4%) patients had active TB. Out of 276 patients who had an alternative diagnosis, 127 (46.0%) were considered to be latently infected with *M. tuberculosis* by a positive PBMC ELISpot result. The sensitivity and specificity of BALMC ELISpot for the diagnosis of active pulmonary TB were 91 and 80%, respectively. The BALMC ELISpot (diagnostic odds ratio [OR], 40.4) was superior to PBMC ELISpot (OR, 10.0), tuberculin skin test (OR, 7.8), and *M. tuberculosis* specific NAAT (OR, 12.4) to diagnose sputum AFB smear-negative TB. In contrast to PBMC ELISpot and tuberculin skin test, the BALMC ELISpot was not influenced by previous history of TB.**

***Conclusions*: Bronchoalveolar lavage ELISpot is an important advancement to rapidly distinguish sputum AFB smear-negative TB from latent TB infection in routine clinical practice.**

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The rapid diagnosis of active pulmonary tuberculosis is difficult when acid-fast bacilli (AFB) cannot be detected in sputum smears. Local immunodiagnosis by *Mycobacterium tuberculosis*–specific enzyme-linked immunospot assay (ELISpot) is a promising method for the rapid identification of patients with sputum AFB smear-negative tuberculosis.

What This Study Adds to the Field

In a prospective multicenter TBNET-study, patients with sputum AFB smear-negative pulmonary tuberculosis could rapidly be distinguished from patients with latent tuberculosis infection by the *M. tuberculosis*–specific ELISpot on cells from the BAL fluid with a high diagnostic sensitivity and specificity. These findings may have significant implications for the rapid decision to initiate antituberculosis treatment where bronchoscopy is routinely performed for individuals suspected to be affected by sputum AFB smear-negative tuberculosis.

Tuberculosis (TB) is among the leading causes of morbidity and mortality worldwide (1). Pulmonary TB is the major manifestation of the disease (2). Despite constant diagnostic improvements, the rapid diagnosis of pulmonary TB is still difficult in a substantial proportion of cases (3). Identification of *Mycobacterium tuberculosis* by culture is the diagnostic gold standard for active TB, but culture growth of *M. tuberculosis* may take 2 or more weeks on average (4), and its sensitivity is only approximately 80% (5).

Microscopy for the identification of acid-fast bacilli (AFB) is rapid and inexpensive (6), but AFB are undetectable from the sputum smear in 85 to 90% of children (7) and in approximately 50% of adults (8) with active pulmonary TB. In these cases, the decision to initiate anti-TB treatment can be difficult, especially because sensitivity estimates for the nucleic acid amplification technique (NAAT) to detect nucleic acids of *M. tuberculosis* from respiratory specimen are too variable and too low to be used to exclude the diagnosis of TB (9).

If combined test results are negative, immunodiagnosis by peripheral blood IFN-γ release assays (IGRAs) and tuberculin skin testing (TST) may be used as rule out tests for active TB in patients with a negative sputum smear result (10, 11). However, positive IGRA results (when performed on peripheral blood) and/or a positive TST result are of limited value because immunodiagnostic tests cannot distinguish individuals with active TB from those with latent TB infection (LTBI) (10, 12).

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In active TB, *M. tuberculosis*–specific lymphocytes are concentrated at the site of the infection (13, 14). Comparison of systemic and local immune responses against antigens of *M. tuberculosis* may be useful to rapidly distinguish sputum AFB smear-negative cases of active TB from individuals with LTBI. In a pilot study, *M. tuberculosis* region of difference-1 early secretory antigenic target (ESAT)-6 and culture filtrate protein (CFP)-10 peptide–specific mononuclear cell responses were detectable by enzyme-linked immunospot (ELISpot) assay in bronchoalveolar lavage (BAL) mononuclear cells (BALMCs) only from patients with sputum AFB smear-negative pulmonary TB, whereas these responses were absent in patients with LTBI, in patients with a history of TB without current reactivation, or in patients with pulmonary infiltrates of other origin (15). By enumerating ESAT-6– and CFP-10–specific BALMCs, patients with active TB could be fully distinguished from patients with pulmonary infiltrates of other origin. However, the numbers of enrolled individuals were low in this study, and the setting was monocentric.

To better evaluate the role of BAL ELISpot for the rapid diagnosis of sputum AFB smear-negative TB in countries of low TB incidence, where bronchoscopy and IGRA techniques are available, we performed a large prospective multicenter clinical study within several European centers participating in the TBNET.

METHODS

Patients

After we obtained written informed consent and local ethical committee approval, HIV-seronegative individuals having negative sputum AFB smear results on three consecutive examinations or being unable to produce sputum with pulmonary infiltrates on chest radiography, a medical history, clinical signs, or symptoms compatible with TB were prospectively enrolled between September 2006 and September 2008 at the Medical Clinic of the Research Center Borstel (Germany), the Hospital Großhansdorf (Germany), the Thorax Clinic Heidelberg (Germany), the Diakonessenhuis Utrecht (The Netherlands), the University Hospital of Modena (Italy), the National Institute for Infectious Diseases Rome (Italy), and the Hospital Universitari Germans Trias i Pujol Badalona (Spain).

Standard diagnostic procedures were performed, including PBMC ELISpot, TST (following the national guidelines (16), TST was not performed at the center in The Netherlands; patients with former TB did not receive a TST), bronchoscopy with BAL for microscopy and *M. tuberculosis* culture, BAL ELISpot, and NAAT (if requested by the treating physician). Transbronchial biopsies were taken for further examinations by the decision of the operating physician.

ELISpot Assays

Venous blood was drawn in preheparinized tubes, and peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll Hypaque density gradient centrifugation.

M. tuberculosis–specific ELISpot (T-SPOT.*TB* test) was performed based on the recommendations for blood according to the manufacturer's guidelines (OxfordImmunotec Ltd., Abingdon, UK) with 250,000 mononuclear cells per well. BAL was performed with 200 to 300 ml of normal saline from an affected lung segment for mycobacteriological culture, *M. tuberculosis*–specific ELISpot (T-SPOT.*TB* test), and *M. tuberculosis*–specific NAAT. The BAL ELISpot was performed as previously described (14, 15). Mononuclear cells were obtained by passing the BAL fluid through a stainless steel sieve with a mesh aperture of 0.5 mm (Teesieb-Profi-Plus; WMF, Geislingen, Germany). ELISpot assay results were considered positive if more than five spot-forming cells (SFCs) were counted in the ESAT-6 or the CFP-10 well after subtraction of the number of SFCs in the negative control well and if the total number of SFCs in the ESAT-6 or CFP-10 well was at least twice the number of SFCs in the negative control well. ELISpot assay results were considered negative if they did not meet the definition for a positive result and if the number of SFCs in the positive control well was more than 20 SFCs after subtraction of the number of spots in the negative control well and had at least twice the number of spots of the negative control well. Results that did not meet the criteria of positive or negative were considered to be indeterminate. Treating physicians were blinded to the results of the ELISpot assays until the decision for or against anti-TB treatment was made.

Tuberculin Skin Test

Bioequivalent tuberculin skin testing was performed in Germany and Spain with 0.1 ml (2 TU) of tuberculin RT23 (Statens-Serum-Institut, Copenhagen, Denmark) and in Italy with 0.1 ml (5 TU) of tuberculin Biocine (Chiron, Siena, Italy) according to nationally licensed products.

M. tuberculosis–specific Nucleic Acid Amplification Technique

Three different specific NAAT systems were used in the seven centers: (*1*) a BD Probe Tec ET system (BD Diagnostic Systems, Sparks, MD), (*2*) an Amplified-MTD (GenProbe, San Diego, CA), and (*3*) an in-house PCR assay targeting the IS6110 gene (17). All laboratories at the centers participate in regular external quality control surveys.

Statistical Analysis

Data were analyzed using Stata 9.0 (StataCorp, College Station, TX). Comparisons between proportions were performed using χ^2 test; the Student's t test was used for continuous variables, and its nonparametric version (Wilcoxon-Mann-Whitney test) was used when appropriate. Differences were considered to be significant when $P < 0.05$. To avoid a mathematical error, we assigned values of "0" a value of "0.1" when calculating ratios of ESAT-6– and CFP-10–specific cells among lymphocytes from the blood and the BAL.

Reporting of the research findings followed the STARD (standards for the reporting of diagnostic accuracy) guidelines (18).

RESULTS

Eleven patients with HIV infection considered for enrolment were excluded. Overall, 347 suspects of active TB unable to produce sputum or with three consecutive negative AFB sputum smears were enrolled in this study. Seventy-one of the subjects with suspected TB were finally diagnosed with active pulmonary TB by one of two case definitions: In 40 (56.3%) subjects, *M. tuberculosis* was recovered by culture; in 31 (43.7%) patients, TB was diagnosed clinically after alternative diseases were ruled out and patients received anti-TB therapy, which had been prescribed by the treating physician on clinical grounds after the subjects showed no clinical response to antibiotic therapy, in accordance with WHO definitions (19).

Of the 276 patients in the non-TB group, 250 patients had a definitive alternative diagnosis other than active TB (50 individuals had bacterial pneumonia or lung abscess, 13 had nontuberculous mycobacteria infections, 48 had sarcoidosis, 32 had pulmonary malignancies, 25 had a former history of TB who were re-evaluated for possible reactivation, 15 had cryptogenic organizing pneumonia, 15 had idiopathic pulmonary fibrosis, 15 had collagen vascular diseases, 10 had bronchiectasis, and 27 had miscellaneous identified pulmonary diseases). In 26 individuals, the final alternative diagnosis could not be established. All of these patients did not receive anti-TB therapy and did not develop TB within a 6-month follow–up period. None of the patients enrolled in this study was HIV seropositive. Demographic and microbiological characteristics are shown in Table 1. In 123 patients (41 with TB and 82 from the non-TB group), transbronchial biopsies were taken during bronchoscopy. If available, biopsies were used to make a diagnosis in patients with and without TB.

ELISpot Results

In patients with active TB, ELISpot results on PBMCs were positive in 65 out of 71 (91.5%) and negative in 6 out of 71

TABLE 1. DEMOGRAPHIC, MICROBIOLOGICAL, AND NUCLEIC ACID AMPLIFICATION TECHNIQUE CHARACTERISTICS OF 347 SUBJECTS SUSPECTED TO BE AFFECTED BY ACTIVE TUBERCULOSIS WITH NEGATIVE ACID-FAST BACILLI SMEARS

Variables	Tuberculosis (*n* = 71)	Nontuberculosis (*n* = 276)	*P* Value
Males, %	45 (63.4)	179 (64.9)	0.87
Age, years (mean ± SD)	42.4 ± 2	56.6 ± 0.95	<0.001
Positive BAL microscopy result for AFB, n (%)	3/64 (4.7)	0/240 (0)	—
Positive BAL NAAT result for *M. tuberculosis*, n (%)	16/56 (28.6)	6/192 (3.1)	<0.001
Tuberculin skin testing, mm (mean ± SD)	16.3 ± 9.8 (n = 43)	5.5 ± 8.4 (n = 115)	<0.001
Culture confirmation from sputum, BAL or biopsy, n (%)	40/71 (56.3)	0/276 (0)	—

Definition of abbreviations: AFB = acid-fast bacilli; BAL = bronchoalveolar lavage; NAAT = nucleic acid amplification technique.

(8.5%), respectively. In patients in the non-TB group, ELISpot results on PBMCs were positive in 127 out of 245 (51.8%) and negative in 118 out of 245 (48.2%), respectively. Indeterminate results in the PBMCs ELISpot were observed in 31 out of 347 (8.9%) patients, all belonging to the non-TB group.

ELISpot results on BALMCs were positive in 60 out of 66 (90.9%) and negative in 6 out of 66 (9.1%) of patients with active TB, respectively. In patients in the nonTB group, ELISpot results on BALMCs were positive in 50 out of 249 (20.1%) and negative in 199 out of 249 (79.9%), respectively (Figures 1 and 2; Table 2). In 5 out of 71 (7.0%) of individuals with TB and in 27 out of 276 (9.8%) of individuals without TB, the BALMCs ELISpot results were indeterminate.

When patients with culture-confirmed sputum AFB smear-negative TB were analyzed separately, sensitivity and specificity of the ELISpot on BALMCs were 87.2 and 79.9%, respectively (area under ROC curve, 0.83; 95% CI, 0.78–0.89).

The diagnostic odds ratio (OR) for the ELISpot on BALMCs was 27.1 (95% CI, 10.1–72.7; $P < 0.001$). ELISpot results from blood and BAL were not significantly different between culture-positive and culture-negative patients with active TB.

Differentiation of Active TB versus Latent TB Infection

In patients with a positive blood ELISpot result, BAL ELISpot was positive in 55 out of 60 (92%) patients with active TB and negative in 82 out of 116 (71%; $P = 0.0012$) patients without active TB. A positive result in the BAL ELISpot was highly discriminative to differentiate patients with active TB from individuals with LTBI (OR, 26.5; 95% CI, 9.8–72; $P < 0.001$).

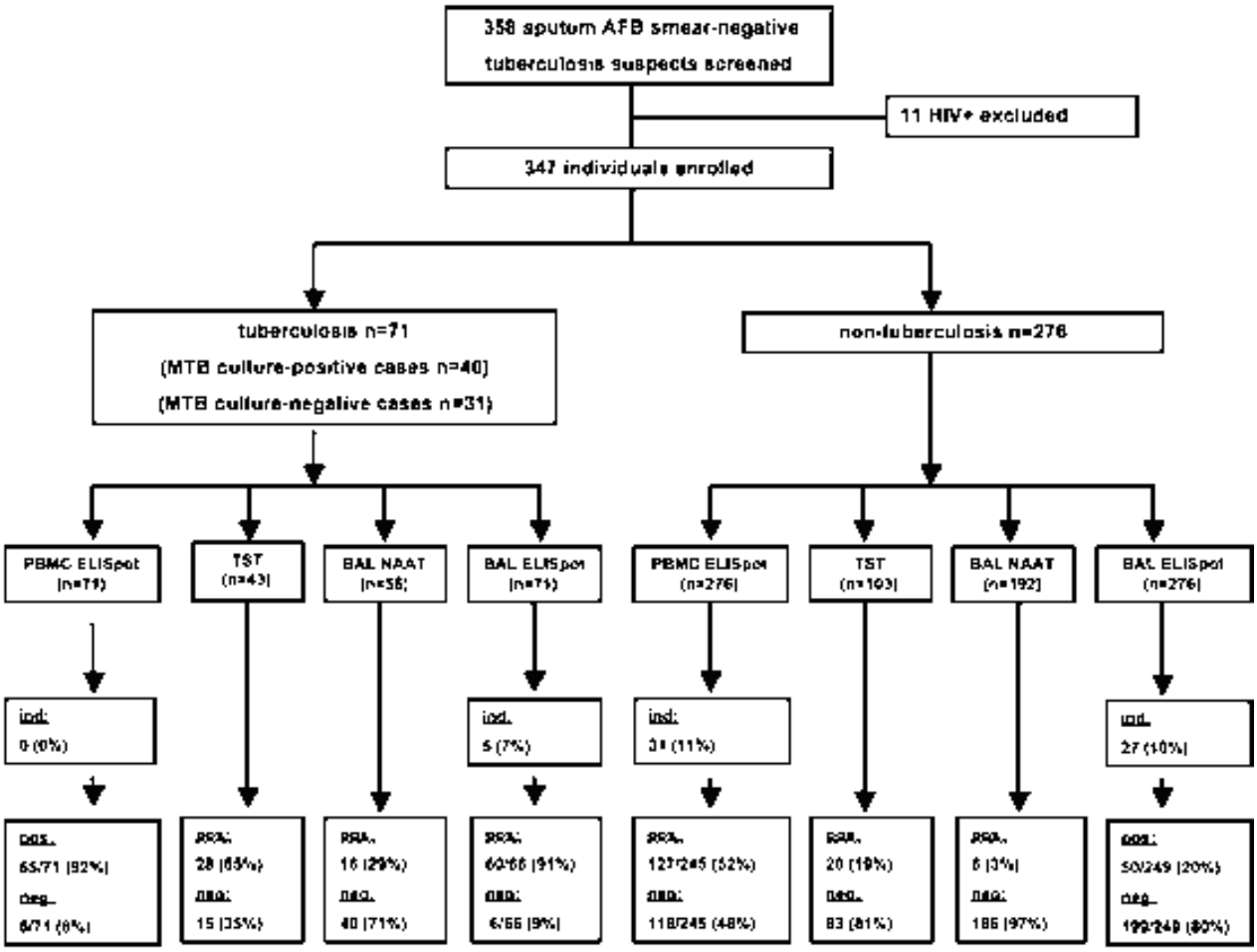

Figure 1. Study design and main outcome.

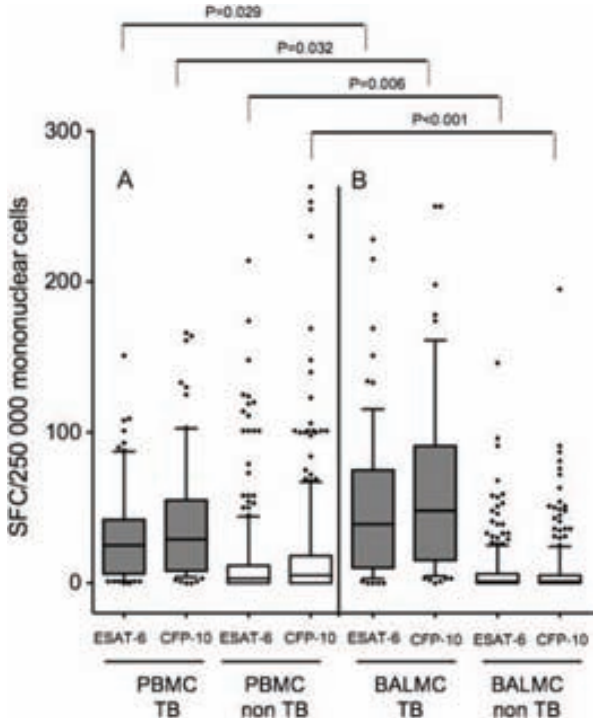

Figure 2. Early secretory antigenic target (ESAT)-6– and culture filtrate protein (CFP)-10–specific enzyme-linked immunospot with (*A*) peripheral blood mononuclear cells (PMBCs) and (*B*) bronchoalveolar lavage mononuclear cells (BALMCs; B) in suspects with sputum acid-fast bacilli (AFB) smear-negative pulmonary tuberculosis (TB). *Gray bars* and *white bars* represent numbers of spot forming cells (SFC) per 250,000 PBMCs and BALMCs in patients with TB and in patients with alternative pulmonary diseases, respectively. *Horizontal lines* represent median values; *whiskers* represent 10th to 90th percentiles.

Concentration of Antigen-specific Lymphocytes at the Site of Infection

Individuals with positive *M. tuberculosis*–specific immune responses in PBMCs in the non-TB group were considered to be latently infected with *M. tuberculosis*. In these individuals, frequencies of ESAT-6– and CFP-10–specific lymphocytes were slightly increased among BAL lymphocytes compared with blood lymphocytes (Figure 3). The mean ratios of ESAT-6– and CFP-10–specific lymphocytes in BAL/blood of patients in the non-TB group were 3.3 and 2.6, respectively ($P < 0.0001$ for both antigens).

In patients with sputum AFB smear-negative TB, numbers of ESAT-6– and CFP-10–specific lymphocytes were clearly elevated among BAL-lymphocytes (Figure 3). The mean ratios of ESAT-6– and CFP-10–specific lymphocytes in BAL/blood were 16.3 and 16.0, respectively, demonstrating a concentration of antigen-specific T cells at the site of the infection in active TB (Figure 4).

Tuberculin Skin Test

TST was performed in 146 out of 347 (42.1%) patients, 43 with active TB and 103 with alternative diseases. In 28 out of 43 (65.1%) patients with active TB and 20 out of 103 (19.4%; $P < 0.0001$) with alternative diseases, the TST was positive, corresponding to a sensitivity and specificity of 65.1 and 80.6%, respectively (area under ROC curve, 0.73; 95% CI, 0.65–0.81) (Table 2). The diagnostic OR for TST was 7.8 (95% CI, 3.5–17.2; $P < 0.001$).

Nucleic Acid Amplification Technique

Results for the NAAT were available for 248 patients (56 with active TB and 192 with other diseases). In 16 out of 56 (28.6%) of patients with active TB and in 6 out of 192 (3.1%; $P < 0.001$) of patients with alternative diseases, the results of the *M. tuberculosis*–specific NAAT were positive, corresponding to a sensitivity and specificity of NAAT for the diagnosis of paucibacillary active TB of 29 and 97%, respectively (OR, 12.4; 95% CI, 4.6–33.6; $P < 0.001$; area under ROC curve, 0.62; 95% CI, 0.56–0.68) (Table 2).

Direct Comparison of *M. tuberculosis*–specific BAL and NAAT for the Diagnosis of Active TB

In 228 out of 347 (65.7%) patients (50 with active TB and 178 with alternative diseases), *M. tuberculosis*–specific ELISpot and NAAT were performed in parallel on BAL.

In 16 out of 50 (32%) of patients with active TB, NAAT on BAL was positive, and in 6 out of 178 (3.4%) of patients with alternative diseases, NAAT on BAL was positive, corresponding to a sensitivity and specificity of *M. tuberculosis*–specific NAAT on BAL of 32 and 97%. In 45 out of 50 (90%) of patients with active TB, ELISpot on BAL was positive and in 30 out of 178 (16.9%; $P < 0.001$) of patients with alternative diseases, ELISpot on BAL was positive, corresponding to a sensitivity and specificity of *M. tuberculosis*–specific ELISpot on BAL of 90 and 83% (OR, 44.4; 95% CI, 16.3–121.2; $P < 0.001$; area under ROC curve, 0.87) (Table 3).

When comparing the assay agreement between BAL ELISpot and NAAT, Cohen's kappa index was 0.012, corresponding to slight agreement according to the interpretation of Landis and Koch. The percentage of patients with TB not diagnosed by NAAT was 64.8, whereas 14.1% patients with TB were not diagnosed by BAL ELISpot. The percentage reduction is 50.7% (95% CI, 36.9–64.5). The inverse of the percentage reduction, comparable to the number needed to treat, is 2. This means that about one in every two patients with AFB smear-negative pulmonary TB will "benefit" from BAL ELISpot (95% CI, 1.6–2.7).

TABLE 2. COMPARISON OF METHODS FOR THE DETECTION OF ACTIVE TUBERCULOSIS IN SPUTUM ACID-FAST BACILLI SMEAR-NEGATIVE CASES

Parameter	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Positive Likelihood Ratio	Negative Likelihood Ratio	Area under ROC Curve
Blood ELISpot (n = 316)	0.92	0.48	0.34	0.95	1.77	0.18	0.69
BAL ELISpot (n = 316)	0.91	0.79	0.55	0.97	4.53	0.11	0.85
TST (n = 146)	0.65	0.81	0.58	0.85	3.35	0.43	0.76
NAAT (n = 248)	0.29	0.97	0.73	0.82	9.14	0.74	0.62

Definition of abbreviations: BAL = bronchoalveolar lavage; ELISpot = enzyme-linked immunospot; NAAT = nucleic acid amplification technique; TST = tuberculin skin test.

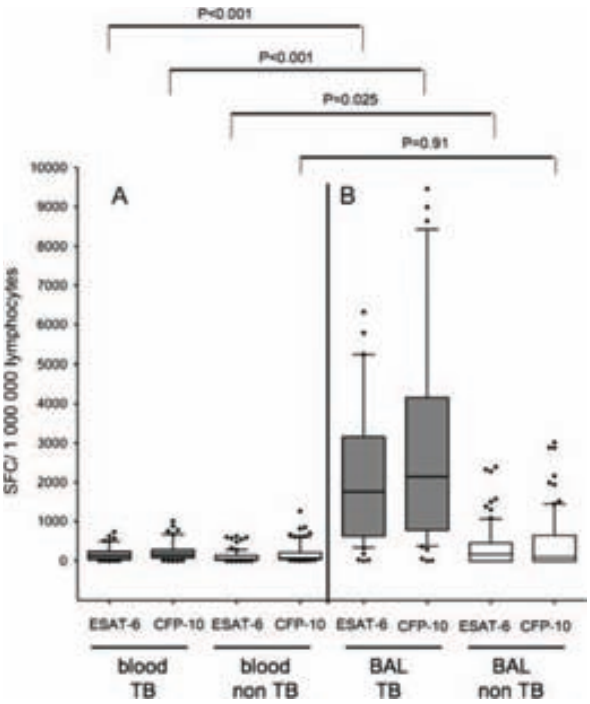

Figure 3. Numbers of early secretory antigenic target (ESAT)-6- and culture filtrate protein (CFP)-10–specific (*A*) peripheral blood lymphocytes and (*B*) bronchoalveolar lavage lymphocytes per 1,000,000 lymphocytes in individuals suspected to be affected by sputum acid-fast bacilli (AFB) smear-negative pulmonary TB. All individuals shown had positive *M. tuberculosis* specific enzyme-linked immunospot assay results in the blood, compatible with either latent TB infection or active tuberculosis. *Gray bars* represent patients with sputum AFB smear-negative pulmonary TB; *white bars* represent patients with alternative pulmonary diseases. *Horizontal lines* represent median values; *whiskers* represent 10th to 90th percentiles.

Comparison of Methods for the Rapid Detection of Sputum AFB Smear-negative Active TB

In logistic regression, the OR of a positive BAL ELISpot result to be associated with active TB was 40.4 (95% CI, 16.5–98.9; $P < 0.001$), compared with 12.4 for the BAL-NAAT (95% CI, 4.6–33.7; $P < 0.001$), 7.8 for the TST (95% CI, 3.5–17.2; $P < 0.001$), and 10.1 for the blood ELISpot (95% CI, 4.2–24.1; $P < 0.001$) (Table 4).

The correlation between TST results and BAL ELISpot and blood ELISpot results was only moderate (Spearman's rho, 0.32 and 0.3, respectively). A poor relationship was found when BAL NAAT results were correlated to the BAL ELISpot (qualitative variable; Spearman's rho, 0.08).

Influence of Previous TB on Blood and BAL ELISpot Results

In patients with a previous medical history of active TB, *M. tuberculosis*–specific PBMC and BALMC ELISpot results were positive in 29 out of 39 (74.4%) and 8 out of 39 (20.5%), respectively. In patients without a previous medical history of active TB, *M. tuberculosis*–specific PBMC ELISpot and BALMC ELISpot results were positive in 39 out of 101 (38.6%; $P = 0.0001$) and in 18 out of 115 (15.7%; $P = 0.49$), respectively. Previous active TB was significantly related to a positive *M. tuberculosis*–specific ELISpot result in the blood but not in the BAL fluid (Table 5).

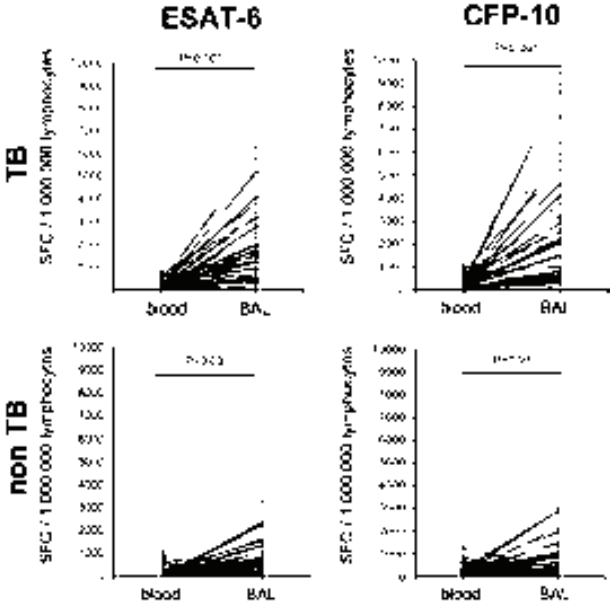

Figure 4. Comparison of numbers of early secretory antigenic target (ESAT)-6- and culture filtrate protein (CFP)-10–specific peripheral blood lymphocytes and bronchoalveolar lavage lymphocytes in patients with sputum acid-fast bacilli (AFB) smear-negative tuberculosis (TB) (*upper row*) and alternative pulmonary diseases (*bottom row*) by enzyme-linked immunospot.

DISCUSSION

The results from this study suggest that local immunodiagnosis by *M. tuberculosis*–specific ELISpot is an important advancement to rapidly distinguish sputum AFB smear-negative active TB from LTBI in routine clinical practice in countries with low TB incidence.

IGRAs, as ELISA (QuantiFERON-TB-Gold In Tube; QFT-GIT test; Cellestis, Carnegie, Australia) and as ELISpot (T-SPOT.*TB* test; Oxford Immunotec, Abingdon, UK), have been approved in many countries as advanced tools for the immunodiagnosis of LTBI. However, for the diagnosis of active TB, IGRAs are of little clinical value because immunodiagnostic tests are not likely to distinguish active TB from LTBI when performed on cells from the peripheral blood alone (10, 20). A plausible explanation for the lack of discrimination of active TB from LTBI by IGRAs is the limitation relating to the use of peripheral blood for the assays. Immune responses assayed on blood mononuclear cells may only provide background information about effector memory T-cell activity in active TB (21). In contrast, *M. tuberculosis*–specific T cells are recruited to and expanded among lymphocytes from pleural effusion (13, 22–24), ascites (13), pericardial effusion (25), and cerebrospinal fluid (26) in patients with AFB smear-negative pleural, peritoneal, pericardial, and meningeal TB. Because IFN-γ–secreting T lymphocytes are also expanded in human lungs in active pulmonary TB (27, 28), IGRA responses assayed in mononuclear cells from BAL should provide better discrimination of active TB from LTBI than responses with PBMC alone.

In a pilot study (15), it was reported that sputum AFB smear-negative active TB was highly likely when *M. tuberculosis*–specific lymphocytes were detectable by ELISpot among cells from the BAL fluid. However, the study was too small to draw definitive clinical conclusions (29). Results from this much

TABLE 3. COMPARISON OF *MYCOBACTERIUM TUBERCULOSIS*–SPECIFIC NUCLEIC ACID AMPLIFICATION TECHNIQUE AND ENZYME-LINKED IMMUNOSPOT FROM BRONCHOALVEOLAR LAVAGE FOR THE DIAGNOSIS OF SPUTUM ACID-FAST BACILLI SMEAR-NEGATIVE TUBERCULOSIS*

Parameter	BAL ELISpot	BAL NAAT
Sensitivity	0.9	0.32
Specificity	0.83	0.97
Positive predictive value	0.6	0.73
Negative predictive value	0.97	0.84
Positive likelihood ratio	5.34	9.49
Negative likelihood ratio	0.12	0.7
Area under ROC curve	0.87	0.64
Odds ratio	44.4	13.49

Definition of abbreviations: BAL = bronchoalveolar lavage; ELISpot = enzyme-linked immunospot; NAAT = nucleic acid amplification technique.

* Smear-negative tuberculosis, n = 228; tuberculosis, n = 50; alternative diseases, n = 178.

TABLE 4. LOGISTIC REGRESSION ANALYSIS FOR THE DIAGNOSIS OF SPUTUM ACID-FAST BACILLI SMEAR-NEGATIVE TUBERCULOSIS

Method	Diagnostic Odds Ratio (95% confidence interval)	*P* Value
Blood ELISpot	10.1 (4.2–24.09)	<0.001
BAL ELISpot	40.4 (16.54–98.93)	<0.001
TST	7.8 (3.5–17.15)	<0.001
NAAT	12.4 (4.56–33.65)	<0.001

Definition of abbreviations: BAL = bronchoalveolar lavage; ELISpot = enzyme-linked immunospot; NAAT = nucleic acid amplification technique; TST = tuberculin skin test.

larger prospective multicenter study by the TBNET confirm that in the majority of patients suspected to be affected by active TB without detectable AFB in sputum smears, *M. tuberculosis*–specific ELISpot with peptides of ESAT-6, and CFP-10 performed on mononuclear cells from the BAL fluid can rapidly distinguish patients with active TB from those with LTBI. Although the results of this study are not as clear cut as in the pilot trial, the sensitivity and specificity of the BAL ELISpot for the detection of sputum AFB smear-negative pulmonary TB were 91 and 80%, respectively. The diagnostic accuracy of this BAL ELISpot assay for the diagnosis of sputum AFB smear-negative pulmonary TB is comparable with results from a recent trial using a technically more demanding flow cytometry assay (30).

In patients with a positive blood ELISpot result, the diagnostic OR for active TB versus LTBI was 26.5 (95% CI, 9.8–72) if the BAL ELISpot assay result was also positive. Although a previous history of active TB was a confounder for a positive blood ELISpot assay result, BAL ELISpot results were independent of previous TB. When compared with the blood ELISpot, the TST and the *M. tuberculosis*–specific NAAT on BAL, BAL ELISpot was superior for rapidly identifying patients with sputum AFB smear-negative TB. When compared with *M. tuberculosis*–specific NAAT, local immunodiagnosis for mycobacteria-specific T cells was markedly more sensitive for the rapid diagnosis of sputum AFB smear-negative TB, confirming previous findings (15, 30). However, because of the very high specificity of NAAT of 97%, the positive likelihood ratio of NAAT was superior to the BAL ELISpot for the diagnosis of sputum AFB smear-negative TB.

Monocytes and dendritic cells are included together with lymphocytes in the ELISpot assays as antigen presenting cells, but their contribution to IFN-γ production is probably negligible. By adjusting the numbers of spot-forming cells to the numbers of lymphocytes in the blood and BAL ELISpot assays using differential blood and BAL cell counts, we observed a concentration of ESAT-6– and CFP-10–specific lymphocytes in the lungs versus the blood by a factor of 16 in patients with active TB who did have a positive blood ELISpot result. These findings are consistent with previous observations (13, 14). *M. tuberculosis* antigen-specific lymphocytes were also slightly expanded among BAL lymphocytes versus blood lymphocytes in individuals with LTBI who did not have active TB, presumably because of antigen stimulation at the site of *M. tuberculosis* persistence in LTBI. It is interesting to speculate whether individuals with positive blood ELISpot responses and absent BAL ELISpot responses may be more likely to have cleared latent *M. tuberculosis* infection in the lungs completely.

Bronchoscopy is indicated in all individuals suspected to be affected by active TB with negative AFB smears in countries with low incidence of TB (31) because alternative diagnoses, including sarcoidosis, bronchoalveolar carcinoma, and cryptogenic organizing pneumonia, must be considered (32). Only one out of five individuals suspected to be affected by sputum AFB smear-negative TB in this multicenter study was eventually diagnosed with active TB. Simple immunodiagnostic assays that enable to establish a rapid diagnosis of active TB in sputum AFB smear-negative cases would be welcome, but the frequencies of ESAT-6– or CFP-10–specific T cells in induced sputum are too low to be reliably detected by currently available techniques (33).

The limitations of our study need to be addressed. Active TB was only proven in 56.3% of cases that fulfilled the case definition for active sputum AFB smear-negative TB. Although the results of the ELISpot and NAAT investigations were comparable in the culture-positive and culture-negative cases of sputum AFB smear-negative TB, some patients with negative *M. tuberculosis* cultures may have been misclassified. In 9.2% of BAL-ELISpot assays, indeterminate results were found. Unstimulated IFN-γ production is frequently observed in BALMCs. To avoid an impairment of the test, we expanded the manufacturer's definition for test results in PBMCs to the BALMCs.

Following national guidelines, TST was not performed at the center in the Netherlands. The tuberculin licensed for the TST in Italy differs from the tuberculin used in Germany and Spain, although the products are thought to be bioequivalent.

The sensitivity of *M. tuberculosis*–specific NAAT for the detection of sputum AFB smear-negative TB was low in this study. Although the ELISpot method was standardized at all centers participating, different NAAT systems were used according to local practice, causing a limitation in comparability. A

TABLE 5. INFLUENCE OF PREVIOUS ACTIVE TUBERCULOSIS ON TEST RESULTS OF *MYCOBACTERIUM TUBERCULOSIS*–SPECIFIC ELISPOT IN BLOOD AND BRONCHOALVEOLAR LAVAGE

	Positive Blood ELISpot		Positive BAL ELISpot	
Patients	n (%)	*P* Value	n (%)	*P* Value
With a previous diagnosis of active tuberculosis	29/39 (74.4)	<0.001	8/39 (20.5)	0.49
Without a previous diagnosis of active tuberculosis	39/101 (38.6)		18/115 (15.7)0.49	

Definition of abbreviations: BAL = bronchoalveolar lavage; ELISpot = enzyme-linked immunospot.

recent metaanalysis reported a high variability of NAAT sensitivity among different clinical trials in individuals with sputum AFB smear-negative TB (9).

None of the individuals enrolled in this study had a positive HIV serostatus. Although sputum AFB smear-negative pulmonary TB can be identified by BAL ELISpot in persons with immunosuppression (34), the results from this study cannot be extended to persons with HIV infection without further investigations.

Finally, in individuals living in areas of high incidence of TB, where frequent exposure to *M. tuberculosis* is likely, pulmonary immune responses to antigens of *M. tuberculosis* could be different from those observed in individuals from areas of low incidence of TB who were enrolled in this study.

In conclusion, *M. tuberculosis*–specific ELISpot on cells from the BAL fluid is an important advancement to distinguish sputum AFB smear-negative pulmonary TB from LTBI in routine clinical practice, although the specificity of the test is suboptimal and inferior to NAAT. One in every two patients evaluated in this study would have benefited from BAL ELISpot for the rapid diagnosis of AFB smear-negative pulmonary TB. This approach may be most applicable for a rapid decision to initiate anti-TB treatment where bronchoscopy is routinely performed for individuals suspected to be affected by sputum AFB smear-negative TB and where the technology for ELISpot can be established. However, the gold standard for the diagnosis of pulmonary TB continues to be the direct detection and isolation of *M. tuberculosis* from respiratory tract specimen because species identification and drug susceptibility testing is currently not possible by immune-based tests.

Conflict of Interest Statement: C.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.T. has a patent for the Cross-spot technique for recognizing antigens using immune-effector cells. G.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.G. has patent that is submitted on the use of RD1 selected peptides for the discrimination of active TB from LTBI. J.A.D.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.B. received up to $1,000 from bioMerieux in lecture fees and has a patent for the Cross-spot technique for recognizing antigens using immune-effector cells. I.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.B.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. U.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.R. received $1,001 to $5,000 from Cellestis Ltd and $1,001 to $5,000 from Oxford Immunotec in consultancy fees. M.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.L. received $1,001 to $5,000 from AstraZeneca, $1,001 to$5,000 from GlaxoSmithKline, $1,001 to $5,000 from Pfizer, $1,001 to $5,000 from Oxford Immnotec, and $1,001 to $5,000 from Chiesi in lecture fees.

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ANEXO VI

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PLoS one

Accuracy of Immunodiagnostic Tests for Active Tuberculosis Using Single and Combined Results: A Multicenter TBNET-Study

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Abstract

Background: The clinical application of IFN-γ release assays (IGRAs) has recently improved the diagnosis of latent tuberculosis infection. In a multicenter study of the Tuberculosis Network European Trialsgroup (TBNET) we aimed to ascertain in routine clinical practice the accuracy of a novel assay using selected peptides encoded in the mycobacterial genomic region of difference (RD) 1 for the diagnosis of active tuberculosis in comparison with tuberculin skin test (TST), QuantiFERON-TB GOLD In-Tube (Cellestis Ltd., Carnegie, Australia) and T-SPOT.*TB* (Oxfordimmunotec, Abingdon, UK).

Principal Findings: 425 individuals from 6 different European centres were prospectively enrolled. We found that sensitivity of the novel test, TST, QuantiFERON-TB GOLD In-Tube and T-SPOT.*TB* was respectively 73.1%, 85.3%, 78.1%, and 85.2%; specificity was respectively 70.6%, 48.0%, 61.9% and 44.3%; positive likelihood ratios were respectively 2.48, 1.64, 2.05, and 1.53; negative likelihood ratios were respectively 0.38, 0.31, 0.35, 0.33. Sensitivity of TST combined with the novel test, QuantiFERON-TB GOLD In-Tube and T-SPOT.*TB* increased up to 92.4%, 97.7% and 97.1%, respectively. The likelihood ratios of combined negative results of TST with, respectively, the novel test, QuantiFERON-TB GOLD In-Tube and T-SPOT.*TB* were 0.19, 0.07 and 0.10.

Conclusions: The assay based on RD1 selected peptides has similar accuracy for active tuberculosis compared with TST and commercial IGRAs. Then, independently of the spectrum of antigens used in the assays to elicit mycobacterial specific immune responses, the novel test, IGRAs, and the TST do not allow an accurate identification of active tuberculosis in clinical practice. However, the combined use of the novel assay or commercial IGRAs with TST may allow exclusion of tuberculosis.

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Competing Interests: DG, SC, MA and EG have a patent pending on T-cell assay based on RD1 selected peptides.

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Introduction

Tuberculosis control is based on the consequent use of preventive chemotherapy in individuals with latent tuberculosis infection (LTBI) who are at risk of developing active disease and on the rapid diagnosis and effective treatment of infectious cases [1–3]. While the identification of patients with active tuberculosis can rapidly be established by the detection of alcohol acid fast bacilli (AFB) on sputum smears, early diagnosis of infectious cases by sputum microscopy is only possible in approximately 50% of cases [4]. The sub-optimal performances of existing diagnostic tools [4], in terms of both speed and sensitivity, delayed diagnosis and, consequently, treatment of active tuberculosis.

The recent introduction of T-cell-based interferon (IFN)-γ release assays (IGRAs), using antigens belonging to *M. tuberculosis* region of difference (RD) 1 (including early secreted antigenic target [ESAT]-6 and culture filtrate protein 10 [CFP]-10) represents a significant step towards improved LTBI diagnosis [5–9]. There is growing evidence that in low incidence settings both the commercial IGRAs currently available, the Quantiferon-GOLD In-tube assay (Cellestis Ltd., Carnegie, Australia) and the T-SPOT.*TB* assay (Oxfordimmunotec, Abingdon, UK) are less affected by bacillus Calmette-Guerin (BCG) vaccination than the tuberculin skin test (TST) and that they are more specific and correlate better with exposure to an infected index case [10–13]. Although these commercial assays provide an accurate diagnosis of

M. tuberculosis infection and detect active tuberculosis disease, they cannot discriminate between active tuberculosis and LTBI. Thus, further clinical workup is required to rule out active tuberculosis after a positive response to these tests.

Recently the design of a novel *in vitro* immune diagnostic enzyme-linked immunospot (ELISPOT) and whole blood ELISA (WBE) for IFN-γ using multiepitopic peptides that are selected by computational analysis from CFP-10 and ESAT-6 as stimulating antigens has been reported [14]. It has been shown that the response to RD1 selected peptides can be detected in subjects with ongoing *M. tuberculosis* replication, such as during active tuberculosis and/or recent infection [15–17]. This response is mediated by $CD4^+$ T effector cells, shown to undergo clonal expansion during *M. tuberculosis* replication, followed by a contraction phase after efficacious therapy culminating in the generation of $CD4^+$ memory T-cells [18,19]. These studies were conducted at one center in Italy, a country with a low tuberculosis incidence of <10/100.000 [20]. The aims of this multicenter study were: i) to evaluate whether this assay based on RD1 selected peptides may help in providing evidence of active tuberculosis; ii) to compare the response to this novel assay with TST and the commercially available RD1 tests, individually and in combination for the diagnostic work-up of active tuberculosis [21].

Materials and Methods

Study design

Following obtaining of informed consent, patients with a clinical suspicion of tuberculosis (abnormal chest radiograph suggestive of tuberculosis and/or other signs and symptoms such as persistent cough, haemoptysis, weight loss, fever) were prospectively recruited at participating centers of the Tuberculosis Network European Trialsgroup (TBNET): Bulgaria (Department of Immunology and Allergology, National Center for Infectious and Parassitic Diseases, Sofia), Germany (Medical Clinic, Research Centre Borstel, Borstel), Italy (INMI and University La Sapienza, Rome; Scientific Institute San Raffaele, Milan) and Spain (Hospital Universitari Germans Trias i Pujol; Barcelona) between November 2005 and March 2008.

Patients underwent clinical and microbiological examinations including chest radiographs to confirm or exclude the diagnosis of tuberculosis. Briefly, 3 sequential respiratory expectorated or 2 induced sputum smears over the first 7 days following clinical evaluation were collected. AFB smear and culture (on both, Lowenstein-Jensen and Bactec MGIT, BD Biosciences Division, Sparks, Maryland, USA) were performed on each specimen. Additionally *M. tuberculosis*-specific RNA amplification was performed on specimens from patients with a high likelihood for tuberculosis in which examinations for AFB were negative [(Gen-Probe® Amplified™ Mycobacterium Tuberculosis Direct (MTD) Test, San Diego, CA, USA)]. TSTs were administered by the Mantoux method with bioequivalent 5 Tuberculin Units (Biocine, Chiron, Siena, Italy) or 2 Tuberculin Units (RT23, Statens Serum Institute, Copenhagen, Denmark) [22,23] or 5 Units of PPD Tuberculin Mammalian (BulBio-NCIPD, Sofia, Bulgaria). Indurations were measured 48–72 hours following tuberculin administration by the ballpoint technique. Individuals with an induration ≥10 mm [24] or in Bulgaria ≥15 mm [25–26] for those with past BCG vaccination were classified as TST-positive [22].

For extra-pulmonary tuberculosis, *M. tuberculosis*-specific RNA amplification (MTD Test) and/or nucleic acid amplification test (NAT) for *M. tuberculosis*-specific DNA based on a commercial test (BD ProbeTec ET system; BD Diagnostic Systems, Sparks, MD) or based on a homemade version developed from the literature [27] was performed on biopsy specimens and/or biological fluids; moreover histology and AFB staining were performed on biopsies.

Enrolled patients were classified as "confirmed tuberculosis" if the diagnosis was based: i) in those with pulmonary tuberculosis by a positive culture for *M. tuberculosis*; ii) in those with extra-pulmonary tuberculosis by a) positive *M. tuberculosis*-specific RNA amplification and/or *M. tuberculosis*-specific NAT from biological specimens *or* b) by histological pathological finding consistent with tuberculosis and presence of AFB in a tissue sample *or* c) by positive culture for *M. tuberculosis* in clinical samples (pleural fluid and abscesses). Conversely, patients were classified as "clinical tuberculosis" if the diagnosis was based on clinical and radiologic criteria (having excluded other disease) including appropriate response to anti-tuberculosis therapy.

We defined patients without tuberculosis as those admitted with a suspicion of active tuberculosis, who subsequently showed negative sputum for AFB smear and culture for *M. tuberculosis* with either a resolution of clinical symptoms and radiographic abnormalities after an antibiotic therapy not involving *M. tuberculosis* active drugs, or presenting a confirmed alternative diagnosis (e.g.: lung cancer).

Following admission, a 10–20 ml (depending on the center) heparin venous blood sample was drawn from all enrolled individuals. ELISPOT or WBE based on RD1 selected peptides was performed. In a subgroup of patients the test was done in parallel with the commercially available immune assays for tuberculosis. Clinicians were blinded to the results of *in vitro* assays and laboratory personnel were blinded to the status of the patient. The study was approved by the ethics committee at all the institutions in which the study was performed.

RD1 selected peptides and stimuli used for cell cultures

The selection of Human Leukocytes Antigens (HLA)-class II restricted epitopes of ESAT-6 and CFP-10 *M. tuberculosis* proteins was performed by quantitative implemented HLA peptide-binding motifs analysis as previously described for ESAT-6 [14,15]. Peptides were synthesized as free amino acid termini using Fmoc chemistry (ABI, Bergamo, Italy). Lyophilized peptides were diluted in DMSO at stock concentrations of 10 mg/mL for each peptide and stored at −80°C. RD1 selected peptides were used as follows: a pool of the two ESAT-6 peptides (at 10 μg/mL each), a pool of the three CFP-10 peptides (at 2 μg/mL each). DMSO was used as negative control at 10 μg/mL. As positive control we used Phytohemagglutinin (PHA) (Sigma, St Louis, MO, USA) at 5 μg/mL. RD1 selected peptides from the same batch were provided to the all centers with a detailed protocol. Four out of the 5 external centers received personal training from INMIs' laboratory personnel for at least 2-days. Inter-site communication was present all over the performance of the study to solve any potential problem.

ELISPOT. 2.5×10^5 peripheral blood mononuclear cells (PBMC) were separated, washed twice and plated in the T-SPOT.*TB* plates stimulated with or without RD1 selected peptides and PHA, as described above and previously [14,15]. Cell cultures were incubated overnight at 37°C, with 5% CO2. On the next morning, the cells were washed off, and the ELISPOT was developed following the manufacturer's instructions (Oxford Immunotec, UK). Spots were then counted by an automated ELISA-Spot assay video analysis system (AELVIS, Hannover, Germany). Evaluated spots had a size >15 U (1 U = 50 μm2). Indeterminate results were defined by values in the PHA-stimulated samples below 34 spot-forming cells per million PBMC. The RD1 selected peptide responses were scored as positive if above 34 spot-forming cells/million PBMC. This cutoff value was determined by constructing a receiver operator

characteristic (ROC) curve by means of LABROC-1 software. To obtain the absolute value, the number of spot-forming cells in the negative controls was subtracted from the number of spot-forming cells in the stimulated cultures. Clinicians were blinded to the laboratory test results and laboratory personnel were blinded to the status of the patients.

WBE. Briefly, aliquots of 0.5 ml per well of heparinized blood were seeded in a 48-well plate and stimulated with or without RD1 selected peptides and PHA, as described above. Samples were then incubated for 24 hours at 37°C in presence of 5% CO_2 when an amount of 100 µl of plasma was harvested. IFN-γ levels in culture supernatants were assessed by a commercially available kit (QuantiFERON-CMI kit, Cellestis). For the results scoring, a cut-off value of 0.7 IU/mL was chosen for all stimuli by constructing a ROC curve. Indeterminate results were defined by values in the PHA-stimulated samples below 0.7 IU/mL.

Commercially available assays

T-SPOT.*TB* and QuantiFERON-TB GOLD In-Tube assays were performed and their results were scored as indicated by the manufacturers.

Statistical Analysis. The tests performance was evaluated by using categories of confirmed tuberculosis, clinical tuberculosis, and no active tuberculosis. Cases with indeterminate responses to *in vitro* assays were not included in the analysis. Sensitivity, specificity and likelihood ratios with their 95% Confidence Interval (CI), were computed for each test overall and according to the diagnostic categories and tuberculosis localization. Proportions were compared by using Fisher exact test and, for paired data, McNemar chi-square test. Sensitivities of 2 tests used in combination were obtained assuming that a positive result is given by a positive response to at least one assay.

Furthermore the accuracy of two tests used in combination was analyzed by computing the likelihood ratios, together with the distribution of subjects with and without active tuberculosis, according to the responses to the tests. Sensitivities and specificities of diagnostic tests were compared by using a logistic regression model with robust standard errors to account for the correlation between observations. Two-tailed *P* values are reported.

Results

We consecutively enrolled 425 consenting adult patients from 6 different centres in Europe. Complete data were unavailable from 1 patient. Results from 11 (2.5%) subjects were found to be indeterminate by *in vitro* assays based on RD1 selected peptides and/or QuantiFERON-TB GOLD In-Tube (Figure 1). Among them, 4 had active tuberculosis and 7 were without active tuberculosis. These patients were similar to those without indeterminate results in terms of age, sex, ethnicity, immune suppressive therapy intake and presence of comorbidity conditions (data not shown).

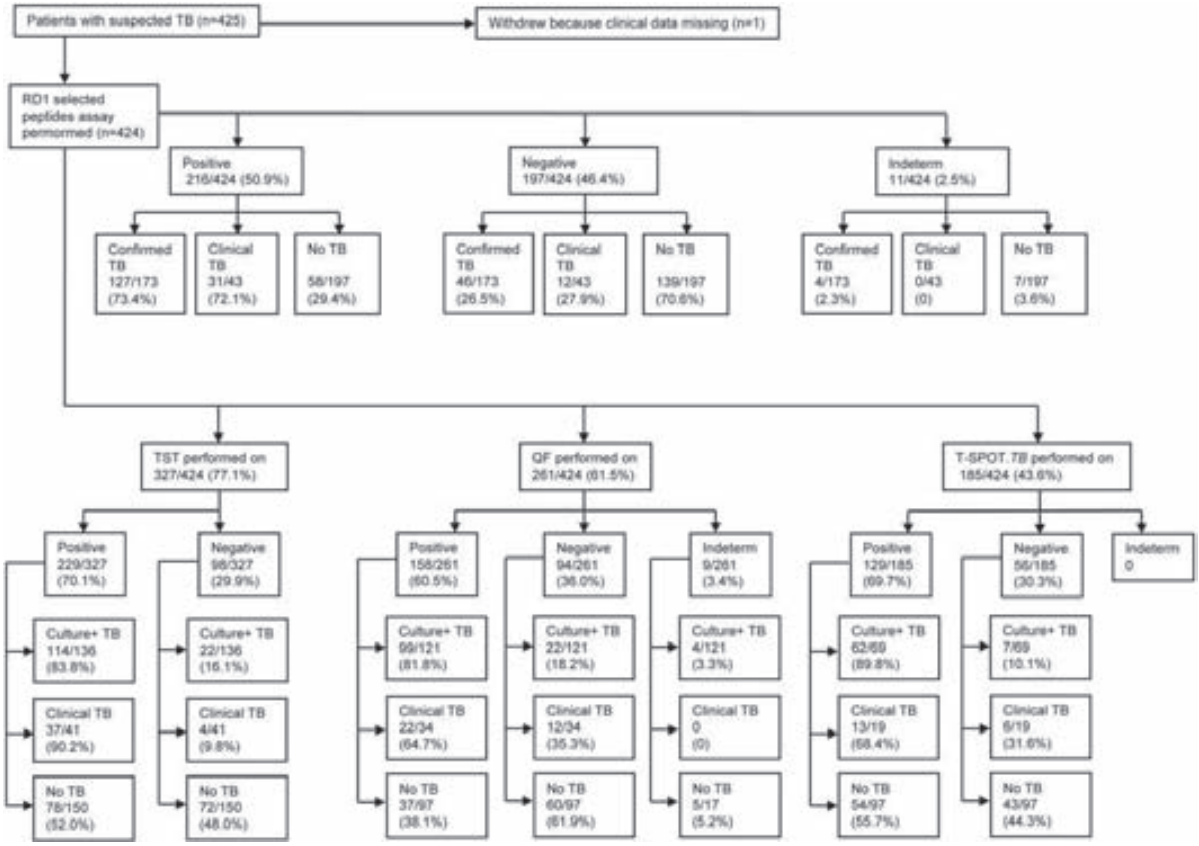

Figure 1. Study flow diagram. Abbreviations: TB: tuberculosis; RD: Region of Difference; Indeterm: indeterminate; TST: tuberculin skin test; QF: QuantiFERON-TB GOLD In-Tube.
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Hereafter, the data analysis was performed only in the 413 samples with valid *in vitro* test results. Demographic characteristics of these subjects are shown in Table 1. Among them we classified 173 patients (41.9%) as having confirmed tuberculosis and 43 (10.4%) as having clinical tuberculosis. We excluded active tuberculosis in 197 patients (47.7%). Based on localization site, 146 (67.6%) were classified as having pulmonary tuberculosis, 56 (25.9%) extrapulmonary tuberculosis and 14 (6.5%) had both pulmonary and extrapulmonary localization (Table 2).

Response to RD1 selected peptides assay and comparison with the other tests

WBE and ELISPOT readouts significantly correlate for the detection of the responses to RD1 selected peptides. Evaluation of the response to RD1 selected peptides was performed by 2 different readouts, the ELISPOT and the WBE that we previously demonstrated to significantly correlate with each other [15]. Also in this study, 138 samples were run in parallel with a significant correlation (percentage of agreement: 80.4%; $p = 0.0001$). Moreover no differences were found in terms of detection of positive results in those with active tuberculosis among the patients from the different centers ($p>0.5$). Given the concordance of the results, the data were pooled together and analyzed as a whole.

Response to immunological tests for tuberculosis: assay based on RD1 selected peptides, TST, commercial IGRAs

For confirmed and clinical tuberculosis cases, diagnostic test sensitivities were 73.1% (95% CI, 66.7–78.9%) with RD1 selected peptides test, 85.3% (95%CI, 79.2–90.2%) with TST, 78.1% (95% CI, 70.7–84.3%) with QuantiFERON-TB GOLD In-Tube, and 85.2% (CI, 76.1–91.9%) with T-SPOT.*TB* (Table 2).

To investigate whether inclusion of patients with clinical tuberculosis in the analysis affected performance estimates, we re-estimated sensitivity by using only confirmed cases. Sensitivity remained stable and was 73.4% (CI, 66.2–79.8) with RD1 selected peptides test, 83.8% (CI, 76.5–89.6) with TST, 81.8% (CI, 73.8–88.2%) with QuantiFERON-TB GOLD In-Tube, and 89.9% (CI,80.2–95.8%) with T-SPOT.*TB* (Table 2). No differences were found between the results obtained considering confirmed tuberculosis vs. clinical tuberculosis cases with the exception of T-SPOT.*TB* for which a higher proportion of positive results was observed for confirmed tuberculosis (62/69) vs. clinical tuberculosis (13/19, $p = 0.03$). Results of immune responses were therefore evaluated for patients with confirmed and clinical tuberculosis pooled together, unless differently specified, and were defined as patients with active tuberculosis.

Among patients with active tuberculosis, the RD1 selected peptides assay was less sensitive than TST (in the 170 patients with

Table 1. Demographic and clinical characteristics of the subjects enrolled in the study.

	Confirmed TB	Clinical TB	No Active TB	Total
	N. 173 (%)	N. 43 (%)	N. 197 (%)	N. 413 (%)
Age years (median)	34	36	48	40
Gender				
Female	69 (39.9)	15 (34.9)	67 (34.0)	151 (36.6)
Male	104 (60.1)	28 (65.1)	130 (66.0)	262 (63.4)
BCG				
Yes	105 (60.6)	7 (16.2)	54 (27.4)	166 (46.0)
No	54 (31.2)	34 (79.0)	107 (54.3)	195 (54.0)
Unknown	14 (8.0)	2 (0.04)	36 (18.2)	52 (12.5)
Origin				
Africa	29 (16.8)	6 (14.0)	11 (5.6)	46 (11.1)
Asia	17 (9.8)	10 (23.3)	9 (4.6)	36 8.7)
Eastern Europe	66 (38.2	5 (11.6)	31 (15.7)	102 (24.7)
South America	17 (9.8)	0	10 (5.1)	27 (6.5)
Western Europe	44 (25.4)	22 (51.2)	136 (69.0)	202 (48.9)
Past TB				
Yes	0	0	27 (13.7)	27 (6.5)
No	173 (100)	43 (100)	170 (86.3)	386 (93.5)
Immune suppressive therapy				
Yes	4 (2.3)	2 (4.7)	4 (2.0)	10 (2.4)
No	169 (97.7)	41 (95.3)	193 (98.0)	403 (97.6)
HIV status				
Yes	3 (1.7)	0	4 (2.0)	7 (1.7)
No	155 (89.5)	43 (100)	167 (84.7)	365 (88.3)
Unknown	15 (8.6)	0	26 (13.2)	41 (9.9)

Abbreviations:
TB: tuberculosis; BCG: Bacillus Calmette and Guerin; HIV: Human Immunodeficiency Virus.
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Table 2. Accuracy for the diagnosis of active tuberculosis.

Sensitivity*		RD1 selected peptides test	TST	QuantiFERON-TB GOLD In-Tube	T-SPOT.*TB*
		Positive over total (%) [CI]			
According to diagnostic criteria	**Confirmed TB**	127/173 (73.4)	114/136 (83.8)	99/121 (81.8)	62/69 (89.9)
		[66.2–79.8]	[76.5–89.6]	[73.8–88.2]	[80.2–95.8]
	Clinical TB	31/43 (72.1)	37/41 (90.2)	22/34 (64.7)	13/19 (68.4)
		[56.3–84.7]	[76.9–97.3]	[46.5–80.3]	[43.4–87.4]
According to TB localization	**Pulmonary**	109/146 (74.7)	100/115 (87.0)	88/107 (82.2)	49/56 (87.5)
		[66.8–81.5]	[79.4–92.5]	[73.7–89.0]	[75.9–94.8]
	Extra-pulmonary	40/56 (71.4)	41/47 (89.4)	26/39 (66.7)	17/23 (73.9)
		[57.8–82.7]	[76.9–96.5]	[49.8–80.9]	[51.6–89.8]
	Pulmonary and extra-pulmonary	9/14 (64.3)	9/15 (60.0)	7/9 (77.8)	9/9 (100)
		[35.1–87.2]	[32.3–83.7]	[40.0–97.2]	[71.7–100.0]
Total		158/216 (73.1)	151/177 (85.3)	121/155 (78.1)	75/88 (85.2)
		[66.7–78.9]	[79.2–90.2]	[70.7–84.3]	[76.1–91.9]
Specificity**					
		139/197 (70.6)	72/150 (48.0)	60/97 (61.9)	43/97 (44.3)
		[63.7–76.8]	[39.8–56.3]	[51.4–71.5]	[34.2–54.8]
Positive likelihood ratio***					
		2.48	1.64	2.05	1.53
		[1.97–3.1]	[1.39–1.94]	[1.57–2.67]	[1.26–1.87]
Negative likelihood ratio***					
		0.38	0.31	0.35	0.33
		[0.30–0.48]	[0.21–0.45]	[0.25–0.50]	[0.19–0.58]

*evaluated on the total number of positive results over the total number of patients with active tuberculosis disease.
**evaluated on the total number of negative results over the total number of patients without active tuberculosis disease.
***evaluated on the total number of tuberculosis cases (confirmed and clinical tuberculosis).

Abbreviations:
TB: tuberculosis; RD: region of difference; TST: tuberculin skin test; CI: confidence interval.

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results from both tests) (p = 0.004) and T-SPOT.*TB* (in the 88 patients with results from both tests) (p = 0.008), but not the QuantiFERON-TB GOLD In-Tube (in the 154 patients with results from both tests) (p = 0.16).

We also evaluated the sensitivities of the different tests based on tuberculosis localization. In Table 2, sensitivities for pulmonary, extra-pulmonary and disseminated tuberculosis (pulmonary and extra-pulmonary tuberculosis) are shown. Considering each test per se, no significant difference in proportion of positive results was observed in patients with active tuberculosis according to tuberculosis localization [with the exception of TST for which the highest proportion of positive results was recorded among patients with pulmonary tuberculosis (p = 0.027)].

Of the 132 patients with culture confirmed pulmonary tuberculosis, 28 had a negative sputum smear. Among these patients, sensitivity results were 64.3% (18/28; CI, 44.1–81.4) for RD1 selected peptides test, 84.6% (22/26; CI, 65.1–95.6) for TST, 88.0% (22/25; CI, 68.8–97.5) for QuantiFERON-TB GOLD In-Tube, and 83.3% (5/6; CI, 35.9–99.6) for T-SPOT.*TB*. Compared to RD1 selected peptides test, the sensitivity for active tuberculosis was significantly higher only for QuantiFERON-TB GOLD In-Tube (p = 0.037). *M. tuberculosis*-specific RNA amplification was performed in 22 of these 27 subjects (8 sputa and 16 broncholavage) and resulted positive in 75% of sputa (6/8; CI, 34.9–96.8) and in 93.8% of broncholavages (15/16; CI, 69.8–99.8) with an overall sensitivity of 86.4% (19/22; CI, 65.1–97.1). Among the 118 patients with smear positive culture confirmed pulmonary tuberculosis the sensitivity results were 77.1% (91/118; CI, 68.5–84.3) by RD1 selected peptides test, 87.6% (78/89; CI, 79.0–93.7) by TST, 80.5% (66/82; CI, 70.3–88.4) by QuantiFERON-TB GOLD In-Tube, and 88.0% (44/50; CI, 75.7–95.5) by T-SPOT.*TB*. No statistical difference was found between the single tests' results obtained in those smear positive vs those smear negative.

Specificity for active tuberculosis was 70.6% (CI, 63.7–76.8%) with RD1 selected peptides test, 48.0% (CI, 39.8–56.3%) with TST, 61.9% (CI, 51.4–71.5%) with QuantiFERON-TB GOLD In-Tube, and 44.3% (CI, 34.2–54.8%) with T-SPOT.*TB* (Table 2). The specificity for active tuberculosis was significantly higher for the assay based on RD1 selected peptides compared with TST (p<0.001) and T-SPOT.*TB* (p<0.001).

Then we assessed whether the pair-wise combination of the tests could lead to a better evaluation of active tuberculosis diagnosis and we calculated the probabilities for the potential outcomes (double positive, double negative and discordant results) given the disease status.

The probability of a positive result of at least one of the two tests considered was higher than sensitivities of each test (Table 3). In particular the assay based on RD1 selected peptides combined with TST led to a sensitivity of 92.4% (CI, 87.3–96.0%), with QuantiFERON-TB GOLD In-Tube of 85.7% (CI, 79.2–90.8%), and with T-SPOT.*TB* of 88.6% (CI, 80.1–94.4%) (Table 3). To note that the highest sensitivities were obtained by using the combination of TST with either QuantiFERON-TB GOLD In-Tube [sensitivity of 97.7% (CI, 93.0–99.5%)], or T-SPOT.*TB* [sensitivity *of* 97.1% (CI, 89.9–94.4%) (Table 3)]. Then we estimated the likelihood ratios for the combination of the tests. Positive results from both tests provided likelihood ratios of 2.92 for the RD1 selected peptides test combined with TST [CI, 2.15–3.98], 3.21 with QuantiFERON-TB GOLD In-tube, [CI, 2.11–4.89], and 2.20 with T-SPOT.*TB* [CI, 1.59–3.07], (Table 4).

Negative results on combined tests were associated with lower negative likelihood ratios compared to that obtained by single assay especially when a blood test was associated with TST. In particular the negative likelihood ratio of the combination of RD1 selected peptides test with TST was 0.19 (CI, 0.11–0.33), with QuantiFERON-TB GOLD In-Tube was 0.25 (CI, 0.16–0.38) and with T-SPOT.*TB* was 0.27 (CI, 0.14–0.50) (Table 4). To note that better negative likelihood ratios were obtained by the combination of commercial tests with TST (Table 4).

Discussion

We present the results of a prospective multicenter trial of the TBNET that was designed to investigate the performance of a novel blood test based on RD1 selected peptides for the immunodiagnosis of active tuberculosis.

The novel assay had a higher specificity for active tuberculosis than the TST and commercial IGRAs, but it had a lower sensitivity. Although the novel assay had a higher likelihood ratio, none of the tests evaluated was accurate enough to discriminate patients with active tuberculosis from those without, probably because of the high levels of LTBI in the population studied. Combined use of TST with either the RD1 selected peptides test or with the other commercial IGRAs improved the diagnostic accuracy for active disease, especially when considering the combination of negative results, contributing to rapid exclusion of tuberculosis. However, *M. tuberculosis* culture remains the diagnostic gold standard for active tuberculosis and is required for identifying drug resistance. Consequently, active tuberculosis should not be ruled out in a high-risk individual without a thorough microbiological work-up for tuberculosis disease.

The specificity of the assay based on RD1 selected peptides was lower in this multicenter trial compared with earlier, smaller studies of more limited patient groups [15,16]. Nevertheless, also in the present study the test based on RD1 selected peptides maintains the higher specificity compared to commercial IGRAs and TST which is not unexpected. In fact, the commercial IGRAs and TST use a greater variety of epitopes to elicit *M. tuberculosis* immune responses by effector memory T-cells [28–29] being TST a crude preparation of several mycobacterial antigens, and commercially IGRAs based on pools of overlapping peptides spanning the whole length of CFP-10 and ESAT-6 proteins [5]. Conversely the selective approach of the design of the test based on RD1 selected peptides reduces false positive test results at the cost of a loss of diagnostic sensitivity [14–16]. Which would be more acceptable between false positive test results that may lead to overtreatment or false negative test results that potentially lead to missing of cases with active tuberculosis to be treated, is a matter of debate and is largely dependent upon the prevalence of *M. tuberculosis* infection and the pre-test probability of tuberculosis in a community.

Specificity of commercial IGRAs is considerably lower in the present study compared to what was reported in a recent updated meta-analysis [30]. This may be due to the fact that this report involve the enrollment of patients with a suspicion of active tuberculosis that could by affected also by LTBI, while the literature reported in the meta-analyses [30] enclosed low-risk subjects with no known tuberculosis exposure in low incidence settings. Conversely sensitivity results were similar to those recently reported in the literature because based on patients with active disease [5,16,30].

In terms of parameters used to evaluate the accuracy of diagnostic tests it is important to consider that while sensitivity and specificity are easy and straightforward measures, they are limited and must be considered as surrogates for patient-important outcomes. There is still lack of adequate data on important outcomes such as accuracy of diagnostic algorithms (rather than single tests), incremental or added value of IGRAs, impact of IGRAs on clinical decision-making and therapeutic choices, and the prognostic ability of IGRAs to accurately identify individuals with LTBI who are at the highest risk for progressing to active tuberculosis and therefore are most likely to benefit from preventive therapy. These issues need to be evaluated for further studies.

New tools for a rapid diagnosis of active tuberculosis are needed especially for the smear negative and extra-pulmonary cases. In the smear negative tuberculosis group, the overall response to RD1 selected peptides was 64.3% and, among the tests used for comparison, a significantly higher sensitivity was found only for the QuantiFERON-TB GOLD In-Tube. Conversely the sensitivity for extra-pulmonary tuberculosis was 71.4% for the RD1 selected peptides and this value did not significantly differ from the results obtained by the other tests, with the exception of TST. However, in general, the size of these sub-groups of patients was small and no definitive conclusion can be drawn.

The rate of indeterminate results in our study was similar to the 3% to 4% rates observed in other studies [31–33]. Moreover, the

Table 3. Estimates of sensitivities for the combination of the diagnostic tests studied.

	Combined tests % [CI]		
	TST	**QuantiFERON-TB GOLD In-Tube**	**T-SPOT.*TB***
RD1 selected peptides test	92.4 [87.3–96.0]	85.7 [79.2–90.8]	88.6 [80.1–94.4]
TST	-	97.7 [93.0–99.5]	97.1 [89.9–99.6]
QuantiFERON-TB GOLD In-Tube	-	-	87.0 [76.7–93.9]

Abbreviations:
RD: region of difference; TST: tuberculin skin test; CI: confidence interval.
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Table 4. Estimates of likelihood ratios for the combination of the diagnostic tests studied according to the disease status.

Combined tests	Subjects with Active TB n (%)	Subjects without Active TB n (%)	Combined likelihood ratio [CI]
RD1 test pos/TST pos	116 (68.1)	35 (23.3)	2.92 [2.15–3.98]
RD1 test neg/TST neg	13 (7.7)	60 (40.0)	0.19 [0.11–0.33]
RD1 test pos/TST neg	11 (6.5)	12 (8.00)	0.81 [0.37–1.78]
RD1 test neg/TST pos	30 (17.7)	43 (28.7)	0.62 [0.41–0.93]
Total	170 (100.0)	150 (100.0)	
RD1 test pos/QuantiFERON pos	99 (64.3)	19 (20.0)	3.21 [2.11–4.89]
RD1 test neg/ QuantiFERON neg	22 (14.3)	55 (57.8)	0.25 [0.16–0.38]
RD1 test pos/ QuantiFERON neg	12 (7.8)	3 (3.2)	2.47 [0.71–8.52]
RD1 test neg/ QuantiFERON pos	21 (13.6)	18 (19.0)	0.72 [0.40–1.28]
Total	154 (100.0)	95 (100.0)	
RD1 test pos/T-SPOT.*TB* pos	60 (68.1)	30 (30.9)	2.20 [1.59–3.07]
RD1 test neg/T-SPOT.*TB* neg	10 (11.4)	41 (42.3)	0.27 [0.14–0.50]
RD1 test pos/T-SPOT.*TB* neg	3 (3.4)	2 (2.1)	1.65 [0.28–9.67]
RD1 test neg/T-SPOT.*TB* pos	15 (17.1)	24 (24.7)	0.69 [0.39–1.23]
Total	88 (100.0)	97 (100.0)	
QuantiFERON pos/TST pos	81 (65.8)	23 (30.7)	2.15 [1.49–3.09]
QuantiFERON neg/TST neg	3 (2.4)	27 (36.0)	0.07 [0.02–0.22]
QuantiFERON pos/TST neg	19 (15.5)	9 (12.0)	1.29 [0.61–2.70]
QuantiFERON neg/TST pos	20 (16.3)	16 (21.3)	0.76 [0.42–1.38]
Total	123 (100.0)	75 (100.0)	
QuantiFERON pos/T-SPOT.*TB* pos	50 (72.5)	20 (41.7)	1.74 [1.21–2.51]
QuantiFERON neg/T-SPOT.*TB* neg	9 (13.0)	24 (50.0)	0.26 [0.13–0.51]
QuantiFERON pos/T-SPOT.*TB* neg	2 (2.9)	0 (0.0)	NA
QuantiFERON neg/T-SPOT.*TB* pos	8 (11.6)	4 (8.3)	1.39 [0.44–4.36]
Total	69 (100.0)	48 (100.0)	
TST pos/T-SPOT.*TB* pos	50 (72.4)	29 (36.7)	1.97 [1.43–2.73]
TST neg/T-SPOT.*TB* neg	2 (2.9)	23 (29.1)	0.10 [0.02–0.41]
TST pos/T-SPOT.*TB* neg	5 (7.3)	10 (12.7)	0.57 [0.21–1.59]
TST neg/T-SPOT.*TB* pos	12 (17.4)	17 (21.5)	0.81 [0.42–1.57]
Total	69 (100.0)	79 (100.0)	

Abbreviations:
TB: tuberculosis; RD: region of difference; RD1 test: test based on the RD1 selected peptides; TST: tuberculin skin test; CI: confidence interval; pos: positive; neg: negative; QuantiFERON: QuantiFERON-TB GOLD In-Tube.
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proportion of patients with tuberculosis was no higher among those with indeterminate results. As expected, false-negative results to any of the immune assays considered were associated with factors known to cause anergy such as disseminated disease.

Taken together, our results suggest that none of the tests considered is accurate enough to be used in clinical practice to diagnose active tuberculosis, and new approaches should be considered. Recently, it has been shown that the discrimination of active tuberculosis from LTBI may be ameliorated by documenting recruitment of *M. tuberculosis*-specific lymphocytes to the site of the infection by RD1-specific ELISPOT assays [34–36] which may open a new strategy for the distinction of the two different status of tuberculosis.

Another recent study suggests that the combination of different immunodiagnostic tests may improve their diagnostic accuracy [21]. In fact it has been shown that, T-SPOT.*TB* or a new ELISPOT assay incorporating the Rv3879c to RD1 antigens, when used in combination with TST have an increased positive and negative likelihood ratio compared with single tests suggesting that this approach can be used to exclude active tuberculosis in patients with moderate to high pre-test probability of disease [21]. The results of the present study confirm and extend these findings. In fact, we confirmed the diagnostic performance of the commercial version of T-SPOT.*TB* when combined to TST, and additionally, we substantiated the data analyzing the results obtained by the assay based on RD1 selected peptides and the QuantiFERON-TB GOLD In-Tube. The likelihood ratio of a negative test result that was 0.38 with the assay based on RD1 selected peptides, became 0.19 when combined with a negative TST. Similarly, the combination of a negative result of the QuantiFERON-TB GOLD In-Tube assay with a negative TST scoring reached the lowest negative likelihood ratio of 0.07. This means that while a negative result to RD1 selected peptides test reduces 2.6-fold the odds of tuberculosis, a negative result to both tests, RD1 selected peptides and TST, would reduce the odds of tuberculosis 5.3-fold and using the combination of TST and

QuantiFERON-TB GOLD In-Tube by 14.2-fold which may currently be the best available option to rapidly exclude tuberculosis by immunodiagnostic tests. Conversely, a positive result of the assay based on RD1 selected peptides with a corresponding likelihood ratio of 2.48 is of limited value and it is not significantly modified in those with positive results from either the assays based on RD1 selected peptides or the TST or QuantiFERON-TB GOLD In-Tube (likelihood ratio goes up to 2.92 and 3.21 respectively). Similarly, results on other tests combinations improve the positive likelihood ratio of the single test per se, but do not increase significantly that obtained by the combination of RD1 selected peptides with TST or QuantiFERON-TB GOLD In-Tube.

The higher sensitivity of combined use of the novel assay or commercial IGRAs with TST reflects the fact that patients who had a false-negative result with one test were distinct from those who had a false-negative result with the other. This implies that distinct immunologic processes underlie failure of these different, yet complementary, immune-based tests.

The study has some limitations. Not all individuals were tested by all the assays in parallel, as not all techniques for the different tests were established in the participating centers. In addition, the restricted number of immunocompromised patients does not allow a generalization of the results to this patients group. However, the prospective and multicenter design of the study, the high consistency of data across the different centers and the large number of patients enrolled to evaluate the diagnostic accuracy of different immune based tests in clinical practice render the results robust.

In conclusion, current approaches to elicit *M. tuberculosis*-specific immune responses in PBMC or in the skin by using either a broad or narrow spectrum of epitopes of RD1 mycobacterial antigens have a limited value for the diagnosis of active tuberculosis, as these tests do not reliably distinguish patients with active tuberculosis from those without. This is important to be considered in populations with a high pre-test probability of *M. tuberculosis* infection. However, the combined use of negative test results obtained by IGRAs or the test based on RD1 selected peptides with TST may enable rapid exclusion of tuberculosis.

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Author Contributions

Conceived and designed the experiments: DG GBM CL EG. Performed the experiments: OB ME IS EB RHD JD IL. Analyzed the data: DG SC VV DMC RKM CA AN EG. Contributed reagents/materials/analysis tools: DG MA VV DMC RKM NP FNL GI. Wrote the paper: DG SC CA GBM CL EG.

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ANEXO VII

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PLoS one

Evaluation of Interferon-Gamma Release Assays in the Diagnosis of Recent Tuberculosis Infection in Health Care Workers

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Abstract

Background: Health care workers (HCWs) are a group at risk of latent tuberculosis infection (LTBI). The aims of this study were to determine IFN-γ response by Quanti*FERON*-TB GOLD *In Tube* (QFN-G-IT) and T-SPOT.TB in HCWs, comparing the results with tuberculin skin test (TST); and to analyze the capacity of IFN-γ tests to detect recent *versus* remote LTBI with a prolonged stimulation test (PST).

Methodology/Principal Findings: A total of 147 HCWs were enrolled; 23 of whom were BCG vaccinated. 95 HCWs (64.6%) had a previous positive TST and were not retested; and 52 HCWs had a previous negative TST or were tested for the first time. When we analysed individuals without previous positive TST, the number of positive results for T-SPOT.TB was 12/52 (23.1%); and for QFN-G-IT, 9/52 (17.3%). The global concordance (κ) between T-SPOT.TB and QFN-G-IT with TST was 0.754 and 0.929 respectively. Of individuals with previous positive TST, T-SPOT.TB and QFN-G-IT were negative in 51.6% (49/95) and 62.1% (59/95) respectively, decreasing the concordance to 0.321 and 0.288, respectively. In non-BCG vaccinated HCWs with previous positive TST a positive IFN-γ test was associated with degree of exposure and diameter of TST. PST was performed in 24 HCW with previous positive TST and negative IFN-γ tests. PST was developed in 3 cell cultures stimulated with medium alone, ESAT-6 and CFP-10, respectively. In the third and sixth day of incubation period, part of the supernatants were replaced with complete medium supplemented with (rIL)-2. On day 9, ELISPOT assay was performed. In 14 samples PST was not valid due to not having enough cells. In 8 cases, the response was negative, and in 2 cases positive, suggesting that these patients were infected with *Mycobacterium tuberculosis* in some point in the past.

Conclusions: Both IFN-γ tests showed a similar number of positive results, and concordance between the tests was excellent. None of the tests was affected by prior BCG vaccination. IFN-γ tests are a useful tool for detecting recent infection in HCW population.

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Introduction

Health care workers (HCWs) are one of the groups at risk of *Mycobacterium tuberculosis* infection through occupational exposure [1]. However, the risk varies widely among the various occupational groups and according to their exposure to active tuberculosis (TB) patients. Therefore, the screening of HCWs for latent tuberculosis infection (LTBI) is crucial in an infection control program [2]. Periodical tuberculin skin testing (TST) has been recommended as part of surveillance [3].

However, TST has some known limitations. TST measures cell-mediated immunity in the form of a delayed-type hypersensitivity response to the purified protein derivative (PPD) [4]. However, its specificity is limited due to PPD cross reactivity with the vaccination strain of *Mycobacterium bovis bacilli Calmette-Guérin* (BCG), and several non-tuberculous mycobacteria (NTM) [5]. Therefore, individuals sensitized by previous exposures to NTM or vaccinated with BCG may respond immunologically to PPD.

Tests for *in vitro* diagnosis of LTBI based on the measurement of interferon-gamma (IFN-γ) production from peripheral blood mononuclear cells (PBMCs) and whole blood in response to specific *M. tuberculosis* secreted antigens have been developed. The recent use of the 6-kD *M. tuberculosis* early-secreted antigenic target protein (ESAT-6) and the 10-kD culture filtrate protein (CFP-10)

[6] encoded in RD1 (Region of Difference) and TB7.7 [7] encoded in RD11, absent in the BCG strain and in the majority of NTM as stimulating antigens has improved the specificity of the tests.

On the basis of this technology, two commercial IFN-γ tests are essentially available: Quanti*FERON*-TB Gold *In Tube* assay (QFN-G-IT) (Cellestis Limited, Carnegie, Victoria, Australia) and T-SPOT.TB assay (Oxford Immunotec, Abingdon, UK). Both tests have received the final approval from the US Food and Drug Administration (FDA) as a tool for diagnosing *M. tuberculosis* infection. There are some differences between the two tests. QFN-G-IT test is whole blood assay that detects IFN-γ produced by T cells in response to ESAT-6, CFP-10 and TB7.7 using an enzyme-linked immunosorbent assay (ELISA) to measure IFN-γ concentrations in supernatants. In contrast, T-SPOT.TB detects the number of IFN-γ producing T cells after stimulating a definite number of isolated peripheral blood mononuclear cells with ESAT-6 and CFP-10 by means of an enzyme-linked immunospot assay (ELISPOT). In commercially available tests, the whole blood and the isolated T cells are short-stimulated with the specific antigens (16–24 h).

Since the development of the IFN-γ assays, promising results in diagnosing LTBI [8–15] and active TB [12,16,17] have been published. IFN-γ test results are not affected by BCG vaccination or infection by the most common NTM, and its results have shown to be closer than TST in relation to the degree of exposure to *M. tuberculosis* [14,18,19]. In addition, in a large-scale cohort studies recently published [20–22], positive IFN-γ assays predicted development of active TB in individuals with recent TB contact. However, limited information is available on the performance of the IFN-γ tests, specially T-SPOT.TB in occupational medicine, when used for screening of HCWs [23–28]. No head-to-head comparisons between the two available IFN-γ tests have been performed in HCWs.

On the other hand, the IFN-γ assays are thought to reflect more recent, rather than remote TB infections. This is because activated lymphocytes and effector T cells that produce IFN-γ persist for a limited time in the circulation once the antigen is cleared [29]. It is thought that central memory T cells, but not effector ones, may take several days (rather than hours) to produce effector cytokines [30,31]. Therefore, contrary to the findings of the TST, in cases of remote infection, the IFN-γ level did not increase during the short period of exposure to the antigen in the ex vivo IFN-γ assay at baseline.

In the present study we investigated the performance of both commercially available IFN-γ tests (QFN-G-IT and T-SPOT.TB) and TST for detecting LTBI in HCWs. Concordance between both test results and association with known risk factors for LTBI were studied. We have also analyzed the capacity of the IFN-γ tests to detect recent *versus* remote TB infection, assessing the effector and memory T cell profiles by means of a prolonged stimulation test.

Materials and Methods

Study design and setting

We conducted a cross-sectional study from November 2004 to July 2005 at the Hospital Universitari Germans Trias i Pujol in Badalona, Barcelona, Spain. This is a general hospital with more than 600 beds. Approximately fifty TB patients are treated each year at the hospital and the estimated community incidence of TB is 18.6/100.000 habitants [32].

Study Population

HCWs were recruited in the course of the routine examinations at the time of the study. HCWs were not enrolled consecutively. Each participant gave written informed consent. The study was approved by the Ethics Committee of the Hospital Universitari Germans Trias i Pujol. Information on the following variables was gathered using a standardised questionnaire: age, gender, reason for testing, degree of occupational exposure to TB (with High being defined as HCWs from wards with≥5 contagious patients per year, HCWs from microbiology laboratory and autopsy wards, and from emergency departments; Medium as HCWs from wards with 2 to 4 contagious patients per year; and Low as HCWs from wards with a maximum of 1 contagious patient per year [33]), BCG vaccination and BCG scar, prior TST (date and result), job category, service and years in the health profession.

Tuberculin skin test

All new HCWs, who did not have a documented TST result, are tested with the TST during the routine examination at the time of employment in the Preventive Medicine Department [34]. If HCWs had a previous positive TST, we took note of the place and the year, but they were no retested. The Spanish national guideline only recommend repeating TST in HCWs with a previous negative TST [34]. TST was performed by the Mantoux method using 2-TU of PPD RT23 (Statens Serum Institut, Copenhagen, Denmark). Induration was measured 48–72 h after the application. Following Spanish national guideline a diameter equal or greater than 5 mm was considered positive (in BCG vaccinated equal or more than 15 mm) [34]. To reduce the risk of confusion between a booster effect and tuberculin conversion on subsequent testing, individuals with negative initial test results were re-tested within 7 to 10 days and the results of this second test were recorded as the definitive result (two-step tuberculin testing procedure) [35]. TST was administered and read by experienced HCWs. Blood for IFN-γ tests was collected before TST application.

T-SPOT.TB

Briefly, eight millilitres of blood was drawn from each subject by venopuncture in a vacutainer CPT tube (Beckton Dickinson Diagnostics, Franklin Lakes, NJ). PBMCs were isolated by centrifugation. After centrifugation, PBMCs were washed with GIBCO RPMI 1640 medium (Invitrogen, Auckland, N.Z.) and finally, were re-suspended in GIBCO AIM-V (Invitrogen, Auckland, N.Z.). The test was performed following manufacturer's recommendations. Four wells with a membrane pre-coated with monoclonal antibody to IFN-γ were used for each subject. The assay requires a total of 250,000 viable cells per well. Cells were stimulated in each well with medium alone (as nil control), phytohaemagglutinin (as positive control) and different peptide panels from the specific MTB antigens ESAT-6 (panel A) and CFP-10 (panel B). Plates were incubated for 16–20 hours at 37°C with 5%CO_2. After the incubation, wells were washed four times with PBS and incubated for 1 hour at 2–8°C with a monoclonal antibody to IFN-γ conjugated with alkaline-phosphatase. After another four washing steps and adding a chromogenic substrate, the presence of reactive antigen specific T cells was revealed as a spot on the well.

Spots were scored by an automated AID ELISPOT plate reader (Lector AID Elispots, Autoimmun Diagnostiks GMBH, Germany). All readings were also manually verified. Subjects were considered positive if there was a positive response to one or both antigen panels. Test wells were scored as positive if they contained at least six spot forming cells (SFC) more than in the nil control well and if this number was at least twice the number of the nil control well. The immunoresponse of each individual was considered adequate if the number of spots in the positive control was 20 or more. The

result was interpreted as indeterminate if the number of spots in the positive control well was less than 20 and if the antigen specific wells were negative.

QuantiFERON-TB-GOLD *In Tube*

Briefly, a total of 3 ml of blood was drawn for each patient in three tubes of 1 ml each (nil control, positive control and *M. tuberculosis* specific antigens [ESAT-6, CFP-10 and TB7.7]). Samples were incubated with the stimulating antigens during 16–24 h at 37°C. Afterwards, plasma samples were harvested and the amount of IFN-γ released was measured by an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. Raw optical densities were interpreted by using specific software provided by the manufacturer. The result obtained in the nil control was subtracted from the mitogen control and the antigen stimulated samples. The *cut off* value for a positive test was 0.35 IU/mL of IFN-γ in the sample after stimulation with the specific antigens, regardless of the result of the mitogen control. The result of the test was considered indeterminate if an antigen-stimulated sample was negative and if the value of the positive control was less than 0.5 IU/ml after subtraction of the value of the nil control.

Prolonged T cell stimulation test

The prolonged T cell stimulation test was performed using a protocol previously described and validated to our requirements [36]. The assay was performed with frozen PBMCs, that were thawed and resuspended in 10 ml of RPMI medium (Invitrogen, Auckland, N.Z.). Later, cells were washed and cultured in complete medium (90% RPMI and 10% FBS [PAA Laboratories GmbH, Pasching, Austria] supplemented with penicillin and streptomycin) in wells of 96-well round-bottomed microtiter plates at 37°C with 5% CO_2. For each patient we prepared three different cell cultures. In the first culture, cells were incubated in the absence of antigen (medium alone); in the second one stimulated with ESAT-6 (Panel A); and in the last one with CFP-10 (Panel B). The final volume of each culture was 0.5 mL. After 3 and 6 day incubation period, 0.25 mL of each culture supernatant was removed and replaced with fresh complete medium supplemented with recombinant human interleukin (rIL)-2 (Roche Diagnostics GmbH, Mannheim, Germany) at a final concentration of 5 U/mL. On day 8, cells were washed one time and cultured again in complete medium without rIL-2. On day 9, 250,000 cells were transferred by well to an ELISPOT plate and stimulated with medium alone (negative control), phytohaemagglutinin (positive control), ESAT-6 (Panel A) and CFP-10 (Panel B) during 16–20 hours at 37°C with 5%CO_2. On day 10, ELISPOT assay was developed according to manufacturer's instructions.

Statistical analysis

Concordance between both tests was assessed using the Kappa coefficient. Kappa (κ) values below 0.40 indicate weak correlation, values of 0.41–0.60 indicate good agreement and values above 0.60 indicate strong agreement. The difference in means was detected using Students' t-tests. The difference between number of positives (percentage) among different groups was assessed using Pearson's Chi-square test. Risk factors for a positive test result were defined using an odds ratio (OR). To adjust for multiple variables we used a logistic regression model with IFN-γ tests and TST results as the outcomes. All variables included in the multivariate analysis were determined *a priori* based on an estimation of their significance during the unvariate analysis and biological plausibility. Differences were considered significant when the p value was less than 0.05. All analyses were performed using the SPSS statistical software for windows (SPSS version 15.0; SPSS Inc, Chicago, IL, USA).

Results

A total of 147 HCWs agreed to take part in the study, and 129 of them (87.1%) were screened with TST at least once in the past. As shown in Table 1, the majority of the participants were women (76.9%) and the median age was 43.3 (range: 22–63 years). Only 23 of the HCWs (15.6%) had received BCG vaccination. The mean duration of years spent in the health care profession was 18.4 (range: 1–43 years), and the exposure was particularly high in 16 cases (10.9%).

95 HCWs (64.6%) had a previous positive TST and were not re-tested. The positive TST result was obtained in 9 cases (9.5%) in the last 5 years, in 10 cases (10.5%) in the last 5 to 9 years, in 14 cases (14.7%) in the last 10 to 14 years, in 59 cases (62.1%) more than 14 years ago, and in 3 cases (3.2) the date was not reported. Of the 52 HCWs with a previous negative TST or who were tested for the first time, 8 (15.4%) resulted TST positive, and 44 (84.6%) were TST negative.

When we excluded the individuals with previous positive TST, the number of positive results for T-SPOT.TB was 12/52 (23.1%); and for QFN-G-IT, 9/52 (17.3%) (Figure 1). The overall agreement between T-SPOT.TB and TST excluding the patient with indeterminate IFN-γ result was 92.1% (47/51) (κ:0.754; se:0.11) and between QFN-G-IT and TST it was 98% (50/51) (κ:0.929; se:0.07). The agreement between the T.SPOT.TB and the QFN-G-IT was 90.2% (46/51) (κ:0.702; se:0.12). Only 4 HCW were BCG-vaccinated in this subgroup (Table 2).

Among individuals with a previous positive TST, the number of positive results for T-SPOT.TB and QFN-G-IT was 45/95 (47.4%) and 34/95 (35.8%), respectively (Figure 1). Regarding BCG status the number of positive results for T-SPOT.TB in BCG-vaccinated individuals was 7/19 (36.8%) and in non BCG-vaccinated was 33/70 (47.1%). For QFN-G-IT, the number of positive results in BCG-vaccinated was 4/19 (21.1%) and in non BCG-vaccinated population it was 28/70 (40.01%) (Table 2).

In non BCG vaccinated HCWs with a previous positive TST, we evaluated, by both univariate and multivariate analysis (Table 3), the relationship between a positive IFN-γ tests and the risk factors for LTBI. In univariate analysis (P = 0.03; OR: 3.0; 95% Confidence interval [CI]: 1.13–8.15), as well as, in multivariate analysis (P = 0.03; OR: 3.67; 95%CI: 1.07–12.59), only the occupational degree exposure was significant when the outcome was a positive T-SPOT.TB result. For QFN-G-IT, the occupational degree exposure was important but not statistically significant (OR: 2.62; 95%CI: 0.81–8.42).

The diameter of TST induration was important in the univariate analysis in both IFN-γ tests, but not significant. The highest diameter of indurations had the greatest percentage of positive IFN-γ tests; however, only 29 of the 95 HCW with previous positive TST had the diameter of induration registered. On the other hand, the results showed no significant association between positive IFN-γ tests and years since the previous positive TST. Nevertheless, the number of responder T cells and the amount of IFN-γ released was higher in the HCWs with previous positive TST results performed in the last 5 years (data not shown).

Of the non BCG-vaccinated HCWs with a previous positive TST and a negative IFN-γ test, a prolonged T cell stimulation test was performed in 24 of them to detect remote infection. In 14 cases the test was not valid because there were not enough cells recovered after the thawing process. For the remaining 10 samples, in 8 cases, although the controls ran well, no response

Table 1. Participants characteristics (n = 147).

	Individuals without previous positive TST[1] (n = 52)		Individuals with previous positive TST (n = 95)		Total (n = 147)	
	n	%	n	%	n	%
Gender						
Women	40	76.9	73	76.8	113	76.9
Men	12	23.1	22	23.2	34	23.1
Age						
18–29	7	13.5	1	1.1	8	5.5
30–39	29	55.7	14	14.8	43	29.3
40–49	13	25.0	38	40.0	51	34.6
>50	3	5.8	42	44.1	45	30.6
Years in the health care profession						
1–4	7	13.5	1	1.1	8	5.5
5–9	8	15.4	6	6.3	14	9.5
10–14	17	32.7	7	7.4	24	16.3
15–24	16	30.7	44	46.3	60	40.8
>24	4	7.7	37	38.9	41	27.9
BCG vaccination						
No	48	92.3	73	76.8	121	82.3
Yes	4	7.7	19	20.0	23	15.6
Unknown	0	0	3	3.2	3	2.0
Occupational TB[2] degree exposure						
Low	20	38.5	48	50.5	68	46.3
Medium	24	46.1	39	41.1	63	42.8
High	8	15.4	8	8.4	16	10.9
Job category						
HCW[3]	51	98.1	83	87.4	134	91.2
No HCW	1	1.9	12	12.6	13	8.8
TST results						
Negative	44	84.6	0	0	44	29.9
Positive	8	15.4	95	100.0	103	71.1
Diameter induration TST						
<5 mm	44	84.6	0	0	44	29.9
5–9 mm	5	9.7	16	16.8	21	14.3
10–14 mm	1	1.9	10	10.5	11	7.5
>14 mm	2	3.8	4	4.2	6	4.1
Unknown	0	0	65	68.5	65	44.2
T-SPOT.TB						
Negative	39	75.0	49	51.6	88	59.9
Positive	12	23.1	45	47.3	57	38.8
Indeterminate	1	1.9	1	1.1	2	1.3
QFN-Gold-IT[4]						
Negative	43	82.7	59	62.1	102	69.4
Positive	9	17.3	34	35.8	43	29.3
Indeterminate	0	0	2	2.1	2	1.3

[1]Tuberculin skin test;
[2]Tuberculosis;
[3]Health care Worker;
[4]Quanti*FERON*-TB Gold *In Tube*.
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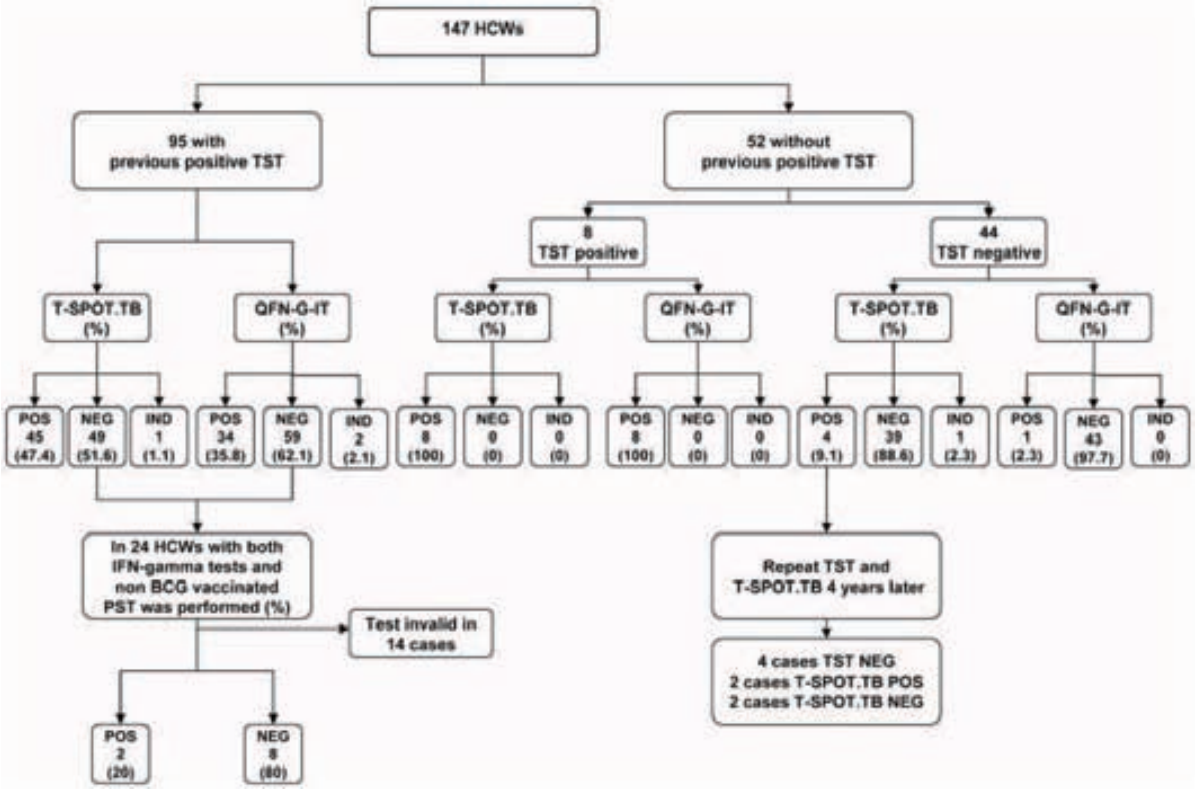

Figure 1. Study flow diagram summarizing study sequence and results. HCW: Health care worker; TST: Tuberculin skin test; QFN-G-IT: Quantiferon-TB Gold *In Tube*; PST: Prolonged stimulation test; POS: Positive; NEG: Negative; IND: Indeterminate.
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Table 2. Agreement between the tuberculin skin test, T-SPOT.TB and *QuantiFERON*-TB Gold *In Tube* in the different group of patients (excluding indeterminate results).

	TST[1] vs T-SPOT.TB			TST vs QFN-G-IT[2]			T-SPOT.TB vs QFN-G-IT		
	Agreement (%)	**Kappa**	**SE**	**Agreement (%)**	**Kappa**	**SE**	**Agreement(%)**	**Kappa**	**SE**
All subjects (n = 143)	90/143 (62.9)	0.321	0.061	84/143 (58.7)	0.288	0.052	123/143 (86.0)	0.692	0.063
BCG vaccination (3 excluded)									
Non BCG vaccinated	76/117 (64.9)	0.349	0.07	74/117 (63.2)	0.345	0.06	101/117 (86.3)	0.701	0.068
BCG vaccinated	11/23 (47.8)	0.169	0.09	8/23 (34.7)	0.085	0.05	20/23 (86.9)	0.650	0.177
Subjects without previous									
positive TST result (n = 51)	47/51 (92.1)	0.754	0.11	50/51 (98.0)	0.929	0.07	46/51 (90.2)	0.702	0.123
BCG vaccination									
Non BCG vaccinated	43/47 (91.5)	0.749	0.116	46/47 (97.8)	0.928	0.07	41/47 (87.2)	0.695	0.125
BCG vaccinated	4/4 (100)	-	-	4/4 (100)	-	-	4/4 (100)	-	-
Subjects with previous									
positive TST result (n = 92)	43/92 (46.7)	-	-	34/92 (36.9)	-	-	77/92 (83.7)	0.668	0.077
BCG vaccination (3 excluded)									
Non BCG vaccinated	33/70 (47.1)	-	-	28/70 (40.0)	-	-	59/70 (84.2)	0.682	0.087
BCG vaccinated	7/19 (36.8)	-	-	4/19 (21.0)	-	-	16/19 (84.2)	0.627	0.183

SE: standard error.
[1]Tuberculin skin test;
[2]Quanti*FERON*-TB Gold *In Tube*.
doi:10.1371/journal.pone.0006686.t002

Table 3. Association between tuberculosis risk factors and positive T-SPOT.TB and *QuantiFERON*-TB Gold *In Tube* results in non BCG vaccinated subjects with previous positive tuberculin skin test (n = 70) by means of univariate and multivariate analysis.

Risk factors	T-SPOT.TB		QFN-G-IT[4]		T-SPOT.TB	QFN-G-IT
	Positive n (%)	OR (CI 95%) unadjusted	Positive n (%)	OR (CI 95%) unadjusted	OR (CI 95%) adjusted	OR (CI 95%) adjusted
Gender						
Women	24 (42.1)	1	21 (36.8)	1	1	1
Men	9 (69.2)	3.09 (0.85–11.27)	7 (53.8)	2.0 (0.59–6.75)	0.27 (0.06–1.18)	0.60 (0.16–2.25)
Age years	-	1.01(0.95–1.07)	-	1.03 (0.96–1.09)	1.08 (0.97–1.22)	1.09 (0.98–1.22)
Occupational TB[1] degree exposure						
Low	13 (34.2)	1	12 (31.6)	1	1	1
High	19 (61.3)	3.0 (1.13–8.15)	15 (48.4)	2.03 (0.76–5.42)	3.67 (1.07–12.59)	2.62 (0.81–8.42)
Years since the previous positive TST[2]	-	1.02 (0.97–1.07)	-	0.99 (0.94–1.05)	1.03 (0.97–1.10)	0.98 (0.92–1.04)
Years in health care profession	-	0.97 (0.92–1.04)	-	1.0 (0.95–1.07)	0.92 (0.82–1.02)	0.96 (0.86–1.06)
Diameter of TST induration						
5–10 mm	4 (33.3)	1	4 (33.3)	1	-	-
>10 mm	5 (50.0)	2.0 (0.35–11.23)	5 (50.0)	2.0 (0.35–11.2)	-	-
Job category						
Non HCW[3]	5 (55.6)	1	3 (33.3)	1	1	1
HCW	28 (45.9)	0.67 (0.16–2.77)	25 (41.0)	1.38 (0.31–6.08)	0.67 (0.10–4.15)	0.51 (0.07–3.56)

CI: Confidence interval; OR: Odds ratio.
[1]Tuberculosis;
[2]Tuberculin skin test;
[3]Health care worker;
[4]Quanti*FERON*-TB Gold *In Tube*.
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against specific antigen stimulation was obtained after prolonged T cell assay, rendering a negative result. In 2 cases, response against both ESAT-6 and CFP-10 was detected in one case, and only against CFP-10 in the other case. Therefore, the results suggested that in the 20% of cases these individuals were infected in the past.

Concordance between both test results and association with known risk factors for LTBI were also analysed in all the population included in the study. TST was positive in 71.1% (103/147) (Figure 1). Table 4 shows the risk factors associated with a positive TST result. Univariate analysis showed a statistically significant association between positive TST, age and number of years in health care profession (p = 0.001). Interestingly, the non-HCWs showed a higher rate of TST and IFN-γ tests positivities than that shown by HCW. The most likely explanation is that non-HCWs were significantly older than HCWs (P = 0.013), with the mean and SD being 50.4 years (9.5) and 42.6 years (8.7), respectively. There was also association with previous BCG vaccination (OR: 2.37; 95%CI: 0.75–7.46) and gender (OR: 1.80; 95% CI: 0.72–4.55), but the associations were not statistically significant.

Positive T-SPOT.TB results were obtained in 38.8% of all studied HCW (57/147) in comparison with 29.3% for QFN-G-IT (43/147). T.SPOT.TB was indeterminate in 2 cases and QFN-G-IT was indeterminate in other 2 cases (Figure 1). Table 4 shows the risk factors associated with a positive IFN-γ tests result. On univariate analysis, age, gender and degree of occupational exposure were statistically significant for positive T-SPOT.TB results; in contrast, for QFN-G-IT, only the age was statistically significant. Gender and degree of occupational exposure were important factors but were not statistically significant (OR: 2.0; 95%CI: 0.91–4.63; and OR: 1.82; 95%CI: 0.88–3.80, respectively).

In the multivariate analysis, age showed significant association for positive TST (OR: 1.26; 95%CI: 1.05–12.20) and QFN-G-IT (OR: 1.15; 95%CI: 1.05–1.25) and T-SPOT.TB (OR: 1.14; 95%CI: 1.05–1.24), but occupational TB degree showed significant association only for positive IFN-γ tests (OR: 4.59; 95%CI:1.68–12.51; and OR: 2.72; 95%CI: 1.04–7.13, respectively).

Data on agreement between the TST and IFN-γ test results were available for 143 participants (not including the 4 indeterminate results) (Table 2). The highest number of positive IFN-γ tests was obtained in patients with positive TST over 10 mm, although the differences in the number of positive IFN-γ tests in patients with a TST between 5–9 and over 10 mm were not significant. Regarding the BCG-vaccination status, the overall agreement in the non-vaccinated population was higher than in the BCG-vaccinated population, but the κ values indicate weak agreement in both cases (Table 2). The difference in concordance between TST and QFN-G-IT in non BCG and BCG vaccinated HCWs was significant (P = 0.021); in contrast, between TST and T-SPOT.TB; and between both IFN-γ tests it was not significant (P = 0.189 and P = 0.801, respectively).

Discussion

Serial TST testing as part of a surveillance of HCWs may induce a boosting phenomenon complicating the TST interpretation. A booster reaction may occur as a result of remote *M. tuberculosis* infection, infection with NTM or prior BCG vaccination [37,38]. In individuals who undergo serial TSTs, it is possible that, after a negative result in the initial TST, a positive result in the second year of testing may have been the result of a boosted reaction. Although the booster phenomenon is less frequent if the second test is administered more than 2 months after the first TST, it has been described after intervals of 1 year [35,39] and possible longer [37].

Table 4. Association between tuberculosis risk factors and positive tuberculin skin test, T-SPOT.TB and *QuantiFERON*-TB Gold *In Tube* results by means of univariate analysis.

Risk factors	TST[1]			T-SPOT.TB			QFN-G-IT[2]		
	Positive n(%)	OR (CI 95%)	P	Positive n (%)	OR (CI 95%)	P	Positive n (%)	OR (CI 95%)	P
Gender									
Women	74 (67.3)	1	NS	35 (31.8)	1	0,004	29 (26.4)	1	NS
Men	26 (78.8)	1.80 (0.72–4.55)		20 (60.6)	3.2 (1.47–7.37)		14 (42.4)	2.0 (0.91–4.63)	
Age years	-	1.18(1.11–1.25)	0.0001	-	1.04(1.0–1.08)	0.03	-	1.04(0.99–1.08)	0.05
Occupational tuberculosis degree exposure									
Low	48 (70.6)	1	NS	20 (29.4)	1	0,04	16 (23.5)	1	NS
High	52 (69.3)	0.94 (0.46–1.93)		35 (46.7)	2.1 (1.05–4.19)		27 (36.0)	1.82 (0.88–3.80)	
Years in health care profession	-	1.12(1.06–1.18)	0.0001	-	1.0(0.97–1.04)	NS	-	1.0(0.96–1.05)	NS
BCG vaccination									
No	78 (66.7)	1	NS	45 (38.5)	1	NS	37 (31.6)	1	NS
Yes	19 (82.6)	2.37 (0.75–7.46)		7 (30.4)	0.7 (0.26–1.83)		4 (17.4)	0.45 (0.14–1.43)	
Job category									
Non HCW[2]	12 (92.3)	1	NS	7 (53.8)	1	NS	4 (30.8)	1	NS
HCW	88 (67.7)	0.17.(0.02–1.38)		48 (36.9)	0.5 (0.16–1.58)		39 (30.0)	0.97 (0.28–3.32)	

NS: Non significant differences; OR: Odds ratio; CI: Confidence interval.
[1]Tuberculin skin test;
[2]Quanti*FERON*-TB Gold *In Tube*;
[3]Health care worker.
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In this respect, IFN-γ tests seem to be a promising alternative to the TST for the diagnosis of LTBI in HCWs. The IFN-γ tests have potential advantages, beyond greater specificity. These include logistical convenience (only require one visit to the healthcare facility, the test result can be available within one day), more objective interpretation of the results, and the ability to perform serial testing without inducing the boosting phenomenon.

When considering HCWs without previous positive TST only, the prevalence of LTBI in this study by IFN-γ tests was higher (T-SPOT.TB: 23.1%, and QFN-G-IT: 17.3%) than by TST (15.4%) with an excellent level of agreement. In univariate analysis, age and number of years in heath care profession were associated with an increased risk for a positive TST. In contrast, for positive IFN-γ tests associations were found with age and the degree of occupational exposure and were not related to previous BCG vaccination. These findings are consistent with previous studies showing that an increased degree of exposure [23,40], and age [27,41] were significant risk factors for positive QFN-G tests. Contrary to our results, several studies obtained more positive results by TST than by QFN-G tests, although in the majority of cases the results could be explained by the effect of prior BCG vaccination [23,24,26,27,41]. Barsegian et al [42] obtained a 1% of positive results by T-SPOT.TB in German radiologist HCWs, and a 34% and 27% of positive TST using>5 mm or>10 mm as a *cut off*, respectively. Authors found that the induration of the TST was significantly higher by foreign births (P<0.001) (all HCWs studied came from areas with a high TB incidence) and previous TST (P = 0.001). Although BCG vaccination did not reach significance, the induration size in vaccinated HCWs increased threefold. In contact studies involving HCWs, TST also obtained a higher number of positive results than QFN-G, however this is attributed to the impact of the BCG vaccination [43,44]. It has been described that a positive QFN-G result was associated with higher exposure groups [45].

Interestingly, the excellent concordance between T-SPOT.TB and QFN-G-IT with TST (92.1% and 98%, respectively) in patients without previous positive TST, falls dramatically to 46.7% and 36.9%, respectively when compared to those HCWs with previous positive TST. A proportion of negative IFN-γ test cases could be explained by the lack of specificity of TST, with some of the previous positive TST being false-positive results as a consequence of prior BCG vaccination or NTM infection [5]. Indeed, the agreement between TST and IFN-γ test results in non BCG vaccinated HCWs was higher than in BCG vaccinated individuals. Furthermore, we have to consider as potential explanation the fact that we cannot confirm the accuracy of the previous positive TST results performed in other institutions, because the tests were not done under study conditions, and maybe some of them were erroneously considered as positive (the milimetres of induration of the previous positive TST were not recorded). Another limitation is that we have not repeated the TST in the HCWs with a previous positive TST. Therefore, in these individuals we have not compared the results of the IFN-γ with the current TST result. In this respect we asked some HCWs about the possibility of repeating the TST and they all refuse. However, although the capacity to respond positively to tuberculin does no remain constant over the course of an individual's lifetime, and that it can weaken over time; it can never fully disappear. These specific facts could introduce a bias to the study adding false-positive TST results, and thereby increasing discordance.

However, another interesting hypothesis resides in the fact that TST is able to detect both remote and recent TB infection while IFN-γ tests only detect recent infection [28,46,47]. This is because after an overnight incubation only activated effector memory T cells that are present in the circulation while the antigen is not cleared have enough time to produce IFN-γ. In contrast, the longer intradermal stimulation of PPD might evoke central memory T cells. Consequently, the IFN-γ released by central memory T cells could be produced and detected by *in vitro* methods after a prolonged stimulation. Therefore, HCWs infected in the past would not respond to *M. tuberculosis* antigen

stimulation in a short-incubation period, but might be reactive after a prolonged incubation period [28,30,36,46,48]. Leyten et al [46] described that among TST positive patients with a history of exposure to *M. tuberculosis*, a 6-day lymphocyte stimulation test was more frequently positive (92% of cases) than the T-SPOT.TB (46%) and QFN-G-IT (33%) with the usual overnight stimulation. In our experience, the 10-day stimulation assay in non-BCG vaccinated HCWs with a previous positive TST and negative IFN-γ tests was positive in the 20% (2/10) of cases, suggesting that these patients were infected with *M. tuberculosis* in some point in the past. Pollock et al [28] reported 19% (7/36) of positive results after an extended stimulation assay in TST-positive HCWs with a negative QFN-G and T-SPOT.TB result. Ferrand RA [48] reported that the 6-day T cell responses to ESAT 6 were greater than responses obtained by ex vivo short-stimulation ELISpot. Recently, Schuck et al [49] exploring new antigens to be candidate biomarkers of LTBI, detected that, in contrast to the short-term single stimulation assay, latency-associated antigens induced IFN-γ expression in memory T cells from the majority of LTBI in the long-term re-stimulation assay.

Although, the hypothesis that short-incubation mainly detects recent or ongoing infection by *M. tuberculosis*, while prolonged-incubation tests seem to be more sensitive for the diagnosis of LTBI has not been totally demonstrated, the published results and our own results make the theory plausible. Indeed, there are findings in accordance with this line of thought from a study of hepatitis C virus showing that short-term ELISPOT responses were not influenced by depletion of memory cells, while the depletion of these memory cells did decrease the antigen-specific responses after prolonged culture [50].

Given that the risk of developing active TB is higher in the first 2 years after infection, the detection of recent TB infection by means of IFN-γ tests in HCWs (mainly immunocompetent) seems to be very useful for targeting the high risk population that really need LTBI.

In our experience, we have detected 5 HCWs with a positive result by IFN-γ test (4 by T-SPOT.TB, and 1 by QFN-G-IT) and a negative TST. These results could be considered as a false-positive IFN-γ result, or as a true LTBI not detected by TST. Indeed, this kind of discrepancy has been previously obtained by other authors. Nienhaus et al [27] in a study that comprised 261 HCWs with exposure to *M. tuberculosis*, 40% of positive QFN-G-IT had negative TST; and Herrmann et al [43], in a contact study involving HCWs, described that, in 2 of 19 cases QFN-G-IT was positive and TST negative. In our study, the patients with negative TSTs and positive IFN-γ tests showed no sign of active TB and were allocated to a 6-month clinical follow-up, without medical therapy. We have continued monitoring the 4 cases with negative TST and positive T-SPOT-TB (the HCW with a negative TST and positive QFN-G-IT stopped working at our institution), and today, 4 years later, all remain healthy, their TST continue to be negative, the T-SPOT-TB is positive in 2 cases, but revert to negative in the other 2 cases. Reversion of QFN-G-IT results [25] has been previously described in a follow-up cohort study of Indian HCWs, although the authors explained that these reversions were related to borderline positive results of QFN-G-IT at the baseline determination. In our study, the two reversions are not associated with a previous borderline T-SPOT-TB result. In one case, the initial response to ESAT-6 and CFP-10 antigens was 4 and 14 SFCs/250,000 cells, respectively, and in the second determination no response was detected. In the other case, the initial response to ESAT 6 antigen was 38 SFCs/250,000 cells, and against the CFP10 antigen was 35. In the second determination after 4 years the no response against the ESAT 6 antigen stimulation was produced, and against CFP-10 antigen only 4 SFCs/250,000 was detected. In addition, spontaneous clearance of TB infection cannot be rejected [51].

The use of IFN-γ tests for serial follow-up of HCWs in order to detect recent infection and avoid the booster effect seems to be an alternative to TST. However, some factors should be taken into consideration: Firstly, it has also been reported that levels of IFN-γ measured by QFN-G-IT remain persistently elevated after treatment for LTBI among HCWs in India [52]. Secondly, Choi et al [53] have described in HCWs QFN-G-IT conversion 2–4 weeks after performing a TST test in positive TST population, but not in the negative ones. Recently, van Zyl-Smit et al [54] have also reported some IFN-γ tests result conversions on day 7 after TST administration. However, they stated that when using a two-step screening strategy it appears safe to develop IFN-γ tests within 3 days of performing the TST. Richeldi et al [55] did not obtain conversion after performing serial TST in negative TST individuals either. Nevertheless, the HCWs who need serial testing will be those with previous negative TST. For serial testing of negative TST HCWs the IFN-γ will be appropiate [25,53], although further investigation is required.

The main limitation of our study is that we have included a small number of HCWs that allowed the detection of a limited number of significant associations between some risk factors and IFN-γ positivity, especially in patients with a previous positive TST. Nevertheless, despite this limitation, the results obtained are sufficiently consistent to draw conclusions. Our paper not only corroborates the previously published data confirming the use of QFN-G-IT as an *in vitro* test for detecting LTBI in HCWs, but, additionally, we provide new information regarding the use of the T-SPOT.TB and its agreement with the QFN-G-IT and TST highlighting its capacity to detect remote *versus* recent infection.

In summary, both IFN-γ tests showed a similar number of positive results, the concordance between the tests was excellent. In addition, none of the tests were affected by prior BCG vaccination. The decision to select T-SPOT.TB or QFN-G-IT in HCW population will depend essencially on the resources available. The *in vitro* tests required an expert laboratory with trained personnel. The results indicate that the IFN-γ tests are a useful tool for detecting recent infection in HCWs population. The use of IFN-γ tests in the follow-up of negative TST HCWs requires further studies which analyze the meaning of the conversions and reversions results.

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Author Contributions

Conceived and designed the experiments: IC IL ME JRM VA JD. Performed the experiments: IC IL ME JRM DR CP IGO AL. Analyzed the data: IC IL ME JRM DR CP IGO AL VA JD. Contributed reagents/materials/analysis tools: IC IL ME JRM CP IGO AL VA JD. Wrote the paper: IC IL JD.

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ANEXO VIII

Review

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EXPERT REVIEWS

IFN-γ-release assays to diagnose TB infection in the immunocompromised individual

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The tuberculin skin test (TST) is used for diagnosing latent TB infection (LTBI). The main limitation of TST is its low sensitivity in populations with the highest risk of progression to active TB: immunosuppressed patients and young children. New IFN-γ-based tests appear as an alternative to the TST. IFN-γ-based tests seem more specific than the TST, being closely associated with LTBI factors, and not being affected by bacillus Calmette–Guérin vaccination. Indeterminate results are mainly related to immunosuppression. Looking at the available data, it seems prudent to recommend the utilization of IFN-γ-based tests after a negative TST result, in order to increase the sensitivity of detecting LTBI cases in severely immunosuppressed patients. In summary, IFN-γ-based tests appear to be a valuable tool, in combination with the TST, for diagnosing TB infection in immunosuppressed patients.

KEYWORDS: active TB • diagnosis • IFN-γ-release assays • immunocompromised patient • latent TB infection • *Mycobacterium tuberculosis* • TST • tuberculin skin test

TB is still a major cause of morbidity and mortality throughout the world. Indeed, there is an estimated global incidence of 8.8 million new cases, with a total of 1.6 million deaths annually [1]. The detection and treatment of active TB is crucial to control the global TB epidemic. The diagnosis of active TB is based on the study of compatible clinical and radiographic signs, combined with direct microscopic examination, culture of *Mycobacterium tuberculosis* and the *in vitro* amplification of mycobacterial target DNA by PCR-based methods. However, in order to better control the spread of TB, it is also necessary to identify and treat infected individuals before they become infectious to others through the progression to active TB.

Since the end of the 19th Century, the tuberculin skin test (TST) has been used for diagnosing latent TB infection (LTBI) and for assisting in the diagnosis of active TB. The TST attempts to measure cell-mediated immunity in the form of a delayed-type hypersensitivity response to the purified protein derivative (PPD) [2]. The PPD contains more than 200 antigens that are widely shared among mycobacteria other than *M. tuberculosis*, including the vaccinal strain of *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) and many nontuberculous mycobacteria (NTM) [3]. As a result, individuals sensitized by previous exposure to NTM or vaccinated with BCG respond immunologically to PPD. Consequently, unnecessary LTBI treatments are prescribed. In addition, errors in the administration of tuberculin and subjective reading of the results also confound accurate interpretation. Nevertheless, the main limitation of the TST is its low sensitivity in detecting LTBI in the group of individuals with a high risk of progression to active TB: immunosuppressed patients (especially with deficient cellular immunity) and young children [4].

Immunodiagnostic methods based on the *in vitro* quantification of the cellular immune response for diagnosing LTBI have been developed. The detection of IFN-γ released by sensitized T cells stimulated with specific *M. tuberculosis* antigens enables the identification of infected individuals. The main antigens used are the 6-kD *M. tuberculosis* early-secreted antigenic target (ESAT)-6 protein and the 10-kD culture filtrate protein (CFP-10), coded in the region of difference (RD) 1, which is present in *M. tuberculosis* but not in any BCG strain nor in the majority of NTM [5].

New *in vitro* diagnostic technology has been rapidly adapted from initial basic in-house methods to three commercially available techniques: QuantiFERON®-TB Gold (QFN-G) assay, QuantiFERON-TB Gold In Tube (QFN-G-IT)

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assay (Cellestis Ltd, Carnegie, Victoria, Australia) and T-SPOT.TB assay (Oxford Immunotec, Oxford, UK). The three tests have received final approval from the US FDA for use as an aid in diagnosing *M. tuberculosis* infection. There are some differences between the three tests. QFN tests are whole-blood assays that detect IFN-γ produced by T cells in response to ESAT-6 and CFP-10 using ELISA to measure IFN-γ concentrations in supernatants. The main differences between the QFN-G and QFN-G-IT assays are that, in the QFN-G assay the blood is stimulated in separate wells with ESAT-6 and CFP-10, respectively, and in the QFN-G-IT assay, both specific *M. tuberculosis* antigens are already included inside the same tube. Furthermore, in the QFN-G-IT assay, a third stimulating antigen has been included: TB7.7. This new antigen is encoded in RD11 and is not present in BCG strains and common NTM [6]. By contrast, the T-SPOT.TB assay detects the number of IFN-γ-producing T cells after stimulating a definite number of isolated peripheral blood mononuclear cells with ESAT-6 and CFP-10 by means of an enzyme-linked immunospot assay (ELISPOT). In the T-SPOT.TB assay, cells are also stimulated in separate wells. In all commercially available tests, the whole blood and isolated T cells were stimulated with the specific antigens over 16–24 h (overnight incubation) (Figure 1).

The three tests include a positive control (cells stimulated using phytohemagglutinin as mitogen) that detects the capacity of T cells to produce IFN-γ after stimulation. If no response against the mitogen is obtained, no negative result after specific

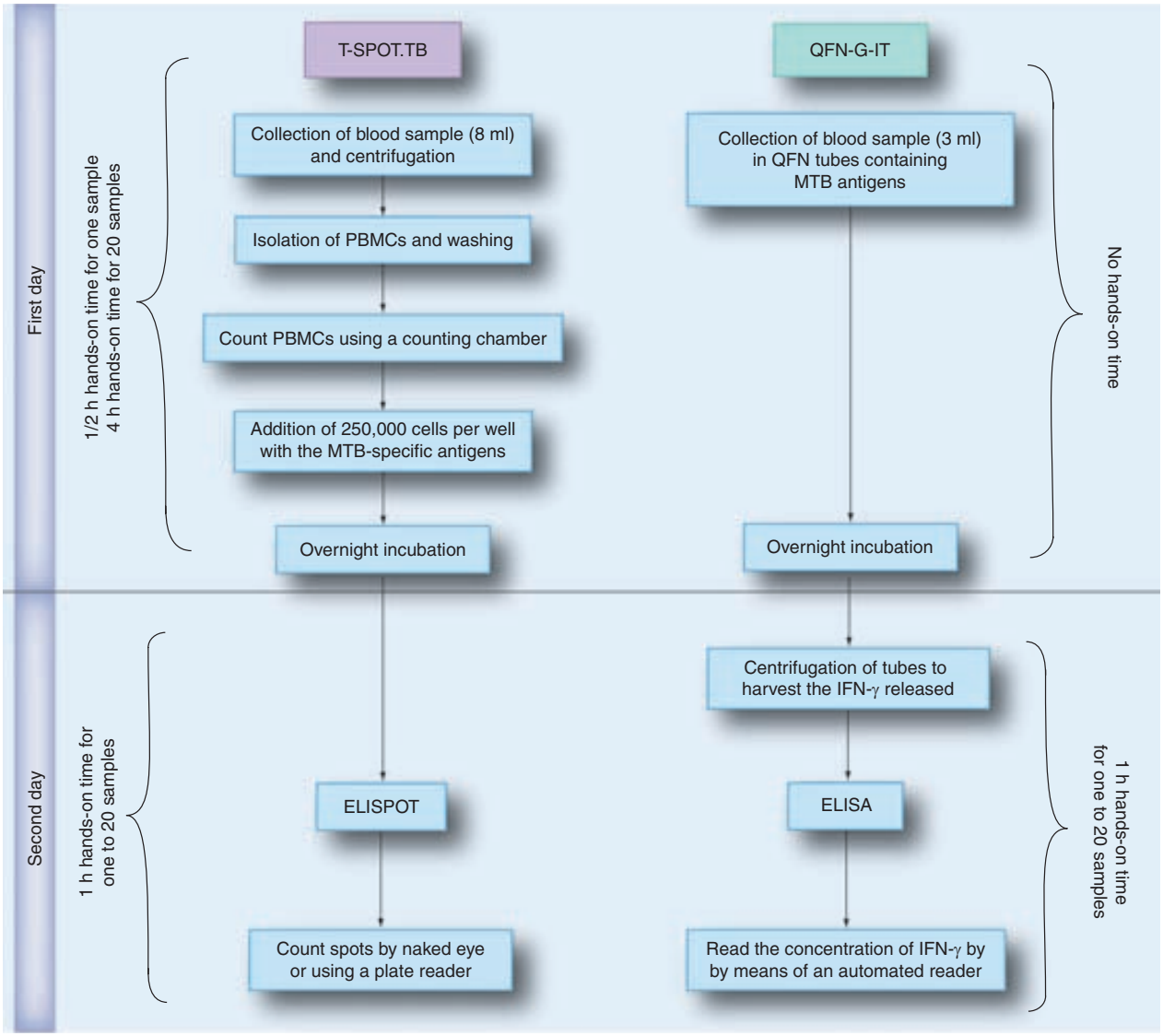

Figure 1. Comparison of T-SPOT.TB and QuantiFERON-TB Gold In Tube methodology.
ELISPOT: Enzyme-linked immunospot assay; MTB: *Mycobacterium tuberculosis*; PBMC: Peripheral blood mononuclear cell; QFN-G-IT: QuantiFERON-TB Gold In tube.

M. tuberculosis antigen stimulation could be considered. The test result is considered indeterminate if an antigen-stimulated sample is negative and if the value of the positive control is also negative after the subtraction of the value of the nil control. This positive control is especially useful for immunosuppressed patients, whose immunological response could be diminished, accounting for the detection of a lack of response after stimulation. However, if a specific lack of response occurs, response to TB antigens may not necessarily correlate with response the to the mitogen.

Given that there is an increased risk for progression from LTBI to active TB in immunosuppressed patients, it is crucial to identify and treat these infected patients. The risk of progression to active TB is higher in children, especially in very young children (<2 years of age) [7], suggesting T-cell response immaturity to contain TB infection. The sensitivity of TST in young children is unknown, but the existence of immature conditions indubitably induces a lower cutaneous response. To date, current guidelines recommend the use of IFN-γ-based tests for the diagnosis of LTBI in individuals with a high possibility of having a false-negative TST result, such as patients with suppressed cellular immunity, and also children [8–10]. This article discusses the potential utility of IFN-γ-based tests in the diagnosis of TB infection in immunocompromised patients: HIV-infected patients, patients receiving immunosuppressive therapies, patients with other immunocompromising conditions and children (not exclusively <2 years of age).

General studies including the immunosuppressed population

Although the sensitivity of IFN-γ-based tests in immunocompromised patients and the effect of immunosuppression in the management of the tests were identified as priority areas for research [11], it remains far from being clearly defined. Three studies have been carried out, including different groups of immunocompromised patients. In a prospective study conducted by the Modena group (Italy) [12], T-SPOT.TB and QFN-G tests were compared. A total of 393 patients were studied for suspected latent or active TB. A varied group of immunosuppressed patients made up 38% of the study population: patients with any form of cancer (independently if they were under chemotherapy), HIV infection, chronic renal failure, patients receiving immunosuppressant therapies (systemic steroids) or biological treatments, children under 5 years of age, elderly patients, and patients awaiting solid transplantation. Analyzing the overall results, the authors reported that IFN-γ-based tests were affected by factors potentially associated with reduced functioning of the cellular immune system, such as age or immunosuppressive treatments. Indeed, they noticed that indeterminate results for both IFN-γ-based tests were significantly more frequent in patients undergoing cancer chemotherapy than in participants not treated with chemotherapy. In general, indeterminate results were more frequent in QFN-G (11%) than in T-SPOT.TB (3%). In addition, QFN-G had a higher number of indeterminate results than T-SPOT.TB in all subgroups of immunosuppressed patients.

Similarly, a study by Kobashi *et al.* focused on patients with immunocompromising conditions (malignant diseases, immunosuppressive treatments, diabetes mellitus, chronic renal failure and HIV infection) described that QFN-G reached a rate of positive results for TB infection higher than the TST (78.1 versus 50%) [13]. They noted that indeterminate results (13%) were more frequent in patients receiving immunosuppressive therapy (particularly with lymphocytopenia in the peripheral blood) than in those who had other underlying diseases.

Recently, Richeldi *et al.* conducted a prospective study, which in a 1-year period enrolled 369 immunosuppressed patients (patients with end-stage chronic liver disease in the liver transplant candidacy period, individuals with chronic HIV infection and patients with hematologic malignancies) [14]. They observed that IFN-γ-based tests detected significantly more patients as being infected by *M. tuberculosis* than did the TST, although the results varied across the groups. In patients waiting for liver transplantation, the IFN-γ-based tests could replace the TST, but in HIV patients the low rate of positive results obtained by the TST and IFN-γ-based tests support an integrated diagnostic based on *in vivo* and *in vitro* assays. The authors concluded that, in accordance with the data, a combined approach to maximize the efficacy of LTBI infection should be recommended in severely immunocompromised patients.

HIV-infected patients

Patients co-infected with HIV and *M. tuberculosis* are more prone to a reactivation of LTBI and to the development of disseminated disease than immunocompetent individuals. TST sensitivity is low in HIV-infected patients. In one study developed in Zambia [15], only 30% of HIV-positive patients had a positive TST result compared with 62% of HIV-negative individuals. The presence of skin anergy means that false-negative TSTs have been reported in 26–41% of HIV-infected patients who are screened for LTBI [16]. Therefore, there is a need for an accurate test for LTBI detection that remains effective in HIV-co-infected individuals.

In the majority of studies, IFN-γ-based tests show a poor agreement with TST results [17–21]. The studies evaluating T-SPOT.TB and its precommercial version [18,19,22,23], and the QFN tests [23–26], seem to demonstrate that *in vitro* tests show a higher number of positive results than the TST in diagnosing LTBI [27].

In the last few years, some studies have compared IFN-γ-based tests in an HIV-infected population (Table 1) [17,18,21,23,27–29]. Rangaka *et al.* did not obtain significant differences in the proportion of positive T-SPOT.TB or QFN-G tests in HIV-infected persons [17], and Vincenti *et al.* found that the commercial tests reached a similar sensitivity [23]. By contrast, Mandalakas *et al.* conducted a cross-sectional study where they found that T-SPOT.TB reached a higher number of positive results for LTBI detection compared with the QFN-G test and TST [18], and in a prospective study Stephan *et al.* reported that the T-SPOT.TB (25.2%) and QFN-G (20.0%) assays showed more positive test results than the TST (12.8%) [27].

Some studies have found a correlation between QFN test results and risk factors for LTBI. Brock *et al.* found that 78% of HIV-infected patients with a positive QFN-G-IT (27/590) had risk factors such as long-term residence in a high TB-endemic area (odds ratio [OR]: 5.7; 95% CI: 2.6–12.5; $p < 0.0001$), known

Table 1. Recent studies analyzing the results of IFN-γ-based tests by comparison with the tuberculin skin test in HIV infection.

Study	Location	Population	Patients (n)	BCG (%)	Median CD4/mm^3	Test	Number of positives (%)	% IFN-γ-based test versus TST concordance (κ)	% IFN-γ-based test concordance (κ)	Indeterminate results (%)	Ref.
Dheda *et al.* (2005)	UK	LTBI screening	29	NR	361	TST T-SPOT.TB	NR 6.9	- NR	- -	- 3.39	[29]
Brock *et al.* (2006)	Denmark	LTBI screening	590	NR	523	TST QFN-G-IT	ND 4.6	- -	- -	- 3.4	[24]
Rangaka *et al.* (2007)	South Africa	LTBI screening	74	51	392	TST (5 mm) T-SPOT.TB QFN-G	52 52 43	- 80 (0.60) 79 (0.58)	- 67 (0.34)	- 1 7	[17]
Jones *et al.* (2007)	NY, USA	LTBI screening	203	2	452.7	TST (5 mm) QFN-G	6.4 5.4	- 88 (0.38)	- -	- 4.9	[26]
Luetkemeyer *et al.* (2007)	CA, USA	LTBI screening	294	6	363	TST (5 mm) QFN-G-IT	9.3 8.5	- 89.3 (0.37)	- -	- 5.1	[20]
Clark *et al.* (2007)	UK	Active TB and LTBI screening	201	49	213	TST (5 mm) T-SPOT.TB	NR 24.9	- 89.06 (0.74)	- -	- 2.5	[30]
Mandalakas *et al.* (2008)	OH, USA	LTBI screening	20	70	787.7	TST T-SPOT.TB QFN-G	62.5 72.2 35.3	- 0.43 0.46	- 64.3 (0.36)	- 10 15	[18]
Stephan *et al.* (2008)	Germany	LTBI screening	286	6.64	408	TST (5 mm) T-SPOT.TB QFN-G	12.8 25.2 20	- NR (0.201) 0.335	- 67.9 (0.15)	- 3.1 0.3	[27]
Karam *et al.* (2008)	Senegal	LTBI screening	285	72.6	179.5	TST (5 mm) In-house ELISPOT	21.5 50.6	- 61.1 (0.23)	- -	- 13.3	[19]
Balcells *et al.* (2008)	Chile	LTBI screening	116	88.1	393	TST (5 mm) QFN-G-IT	10.9 14.8	- 90.8 (0.59)	- -	- 0	[25]
Raby *et al.* (2008)	Zambia	Active TB	59	NR	212	TST (5 mm) QFN-G-IT	55 63	- NR	- -	- 17	[34]
Aichelburg *et al.* (2009)	Austria	LTBI screening	830	NR	194	TST (5 mm) QFN-G-IT	ND 5.3	- NR	- -	- 5.7	[37]
Talati *et al.* (2009)	GA, USA	LTBI screening	336	7.4	334	TST (5 mm) T-SPOT.TB QFN-G-IT	2.1 4.2 2.7	- 0.16 0.23	- 0.23	- 14 1.8	[21]
Aabye *et al.* (2009)	Tanzania	Active TB	93	NR	519	TST QFN-G-IT	ND 65	- -	- -	- 22	[35]

BCG: Bacillus Calmette–Guérin vaccination; ELISPOT: Enzyme-linked immunosorbent spot; LTBI: Latent TB infection; ND: Not done; NR: Not reported; QFN-G: QuantiFERON®-TB Gold; QFN-G-IT: QuantiFERON-TB Gold In tube; TST: Tuberculin skin test.

TB exposure (OR: 4.9; 95% CI: 2.0–11.8; p = 0.001) or previous TB disease (OR: 4.9; 95% CI: 1.7–14.1; p = 0.007) [24]. Jones *et al.* evaluated the QFN-G assay and TST in 207 HIV-infected patients, obtaining a poor concordance between the TST and QFN-G assay, and the QFN-G assay results showed a statistically significant association between the number of risk factors for LTBI (TB exposure, homelessness, drug use, prison, healthcare worker, long-term care facility and foreign-born) and a positive test result, but not the TST (OR: 1.6; p = 0.039) [26].

Regarding the indeterminate results, in most studies the T-SPOT.TB and in-house ELISPOT assays appear relatively unimpaired by low CD4 cell counts [27,29–33]. On the contrary, when using the QFN-G-IT assay, a strong correlation between low CD4 T-cell count and a low mitogen response was detected [24].

It has been described that patients with a low CD4 cell count had more indeterminate QFN-G assay results. Luetkemeyer *et al.* found that patients with a CD4 count less than 100 cells/mm^3 had a relative risk ratio of indeterminate results of 4.24 compared with those with a CD4 count of 100 or more [20]. Jones *et al.* noticed that all indeterminate results occurred in patients with CD4 counts of less than 200 cells/mm^3 [26]. Furthermore, Raby *et al.* observed that with falling CD4 count there was a decrease in positive QFN-G-IT results, with a relative increase of negative and indeterminate results [34]. This was particularly marked at counts less than 100 cells/mm^3; in the study by Brock *et al.*, 24% (4/17) of patients with CD4 cell counts of less than 100 cells/mm^3 had indeterminate result compared with only 2.8% (16/573) of patients with CD4 cell counts of over 100 cells/mm^3 [24], and Aabye *et al.* reported that the number of indeterminate results using QFN-G-IT significantly increased with the decrease in the CD4 cell count [35]. Nevertheless, Balcells *et al.* described that, at least among subjects with a negative TST and a low CD4 cell count, the QFN-G test was positive in 8.2% of cases [25].

However, although indeterminate results have been more frequently described for QFN tests than for the T-SPOT.TB assay, Stephan *et al.* reported the opposite: that T-SPOT.TB provided significantly more indeterminate results than the QFN-G assay (eight vs one in 256 patients) [27]; Karam *et al.* found that the proportion of patients with a positive result for an in-house ELISPOT test decreased significantly with declining CD4 counts [19] and Talati *et al.* described a higher number of indeterminate results by the T-SPOT.TB than by the QFN-G-IT assay, being a CD4 count less than 200/µl associated with indeterminate results of the T-SPOT.TB assay, but not with the QFN-G-IT assay [21].

Interestingly, Raby *et al.* assessed QFN-G-IT utility in patients with active TB [34]. In the study, T-lymphocyte counts were estimated (CD3, CD4 and CD8). A total of 17% of the indeterminate results were obtained by the QFN-G-IT assay. Although a low CD4 count was associated with both negative and indeterminate results, CD8 count was high or normal in those with negative results but low in those with indeterminate results. Given that the overlapping peptides used as antigens in the QFN-G-IT assay, owing to their length, are essentially MHC class II-restricted, only CD4 cells will respond. Nevertheless, subjects with low CD4 counts in conjunction with high/normal CD8 counts react to phytoheamagglutinin but not to the specific antigens, generating negative results and consequently suppression of both cell lines, leading to indeterminate results. This observation adds further useful clinical information to an indeterminate result.

Regarding the possibility of using IFN-γ-based tests for diagnosing active TB, Clark *et al.* proposed that a combination of TB antigen-specific IFN-γ responses and CD4 T-cell counts could differentiate active TB from latent TB in HIV-infected patients [30]. The authors obtained a positive predictive value of 86 and 79% for diagnosing active TB when the ratio of the combined number of ESAT-6 and CFP-10 IFN-γ spot-forming cells per million of peripheral blood mononuclear cells for CD4 and CD8 T-cell count, respectively, was higher than the value of 1.5. Indeed, Rangaka *et al.* previously noticed that with in-house ELISPOT, the response to ESAT-6 and CFP-10 was higher in the group of HIV-infected subjects with TB, although this group had lower CD4 cell counts [36]. They concluded that a ratio of the ELISPOT response divided by the CD4 cell count higher than 1.0 had 88% sensitivity and 80% specificity for active pulmonary TB in HIV-infected individuals.

Aichelburg *et al.* conducted a prospective and longitudinal study involving 830 HIV-infected patients [37]. They screened LTBI by means of the QFN-G-IT assay at baseline and then followed patients up for a mean time of 19 months. They detected patients that developed active TB only among the patients with a positive QFN-G-IT in the baseline (3 out of 44). Any of the 44 patients accepted prophylaxis at the baseline. The authors concluded that the QFN-G-IT assay is a sensitive tool for the detection and prediction of active TB in HIV-infected individuals.

Until now, an association between positive results by IFN-γ-based tests and the presence of risk factors to LTBI has been described. Furthermore, in HIV-infected patients that have a high incidence of NTM infections, IFN-γ-based tests increase the specificity in diagnosing LTBI. Therefore, the accumulated evidence discussed here presents IFN-γ-based tests as useful tools for diagnosing LTBI in HIV-positive individuals. In highly immunocompromised patients, the QFN-G test seems impaired by low CD4 T-cell counts.

Chronic immune-mediated inflammatory disease

Biological agents, especially anti-TNF-α agents, have emerged as an effective treatment in patients with chronic inflammatory diseases such as Crohn's disease, ulcerative colitis, rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis [38,39]. TNF-α is one of the key molecules involved in granuloma formation and the maintenance of TB infection. Consequently, patients undergoing TNF-α inhibition are at an increased risk (28–54 per 100,000 population) of developing active TB [40]. A strong association between anti-TNF-α antibody treatment (infliximab) and reactivation of LTBI has been described [39,41]. Before starting a treatment based on TNF-α inhibition, the appropriate screening for LTBI and exclusion of active TB has become mandatory [42]. However, most patients with chronic inflammatory diseases are already under corticosteroid and/or immunosuppressive drugs prior to anti-TNF-α therapy. It is well known that this group of patients may not be able to produce

an adequate delayed-type hypersensitivity reaction to the TST owing to their deficient cell-mediated immunity [38,42]. Therefore, in this population the utility of the IFN-γ-based tests, although promising, is still limited at present [43,44].

In this type of population, poor agreement between the TST and IFN-γ-based tests [45–50] has been found. The discordant positive TST and negative IFN-γ-based test results in BCG-vaccinated populations have been attributed to the BCG vaccination effecting the TST result [46,48,51–54]. However, the discordant negative TST and positive IFN-γ-based test results have been related to the immunosuppressive therapy that patients are receiving [47–49,51]. In general (Table 2), the available data suggest that the IFN-γ-based tests show a higher number of positive results than the TST in this population [47,52,55]. It has been described that IFN-γ-based assays, but not the TST, are closely associated with the presence of LTBI risk factors [45,49]. In the study by Matulis *et al.*, the following factors were closely associated with QFN-G-IT: being born or a resident in a high-prevalence country (OR: 11.7; 95% CI: 2.11–65.0; $p < 0.001$), history of household contact (OR: 17.8; 95% CI: 2.06–154; $p < 0.001$), chest x-ray suggestive of previous active TB (OR: 66.8; 95% CI: 10.1–441; $p < 0.001$) and a history of active TB (OR: 179; 95% CI: 6.69–4787; $p < 0.001$) [49]. Bocchino *et al.*, in a group of 15 patients with risk factors for LTBI, found that the TST and IFN-γ-based tests were positive in eight cases, and in the remaining seven the TST was negative, but at least one of the IFN-γ-based tests (QFN-G-IT and T-SPOT.TB assays) were positive [45]. The rate of indeterminate results ranged from 1.9 to 11.5%, being higher than those observed in healthy controls [45,47,56]. Indeterminate results were obtained in all IFN-γ-based tests, being impossible to conclude, at the moment, if any of them are more or less affected by this specific category of immunosuppression. However, Murakami *et al.* found that the TST was more strongly attenuated than an in-house ELISPOT assay by corticosteroid therapy in patients with rheumatoid arthritis [53]. In a study involving 398 consecutive subjects with immunomediated inflammatory diseases, Bartalesi *et al.* described that, by multivariate analysis, the use of conventional disease-modifying antirheumatic drugs was not associated with the results of the TST or QFN-G-IT tests, while the use of steroids was associated with a lower probability of a positive result [57].

There is scarce information regarding the utility of IFN-γ-based tests for monitoring TB infection during anti-TNF-α therapy. Matulis *et al.* found that the OR for a positive IFN-γ test (QFN-G-IT) were lower in patients treated with TNF-α inhibitors [49]. Similarly, Hamdi *et al.* described that treatments with TNF-α inhibitors (infliximab and etanercept) decreased IFN-γ release [58]. Moreover, in the Bartalesi *et al.* study, treatment including TNF-α inhibitors significantly decreased the positive outcome of the TST (OR = 0.3; 95% CI: 0.1–0.6; $p = 0.004$) without affecting QFN-G-IT results (OR: 0.9; 95% CI: 0.4–2; $p = 0.8$) [57]. However, more data are required to define their role in this setting.

Another interesting issue would be to establish the prognostic value of a positive IFN-γ-based test result for the subsequent development of active TB for patients undergoing treatment with TNF-α inhibitors. In this sense, Chen *et al.* prospectively followed up 43 rheumatoid arthritis patients who received adalimumab therapy and underwent serial TST and QFT-G assays [59]. Among the 35 patients with negative TST results, two patients developed active TB after 12 months of treatment and both patients had initially had QFN-G test results. Pratt *et al.* screened 101 patients with rheumatoid arthritis and seven cases had a positive QFN-G-IT test result [60]. Four of them subsequently started anti-TNF-α treatment. Of the patients that were followed-up, none developed active TB within 6–30 months.

According to the available data, IFN-γ-based assays seem to be useful for LTBI screening in patients with chronic inflammatory disease before starting treatments. Studies have demonstrated a higher number of positive results for the IFN-γ-based tests than the TST. However, in these patients with a significant risk of progression to active TB, the combined utilization of TST and IFN-γ-based tests, for confirming a negative TST by an IFN-γ-based test, should be recommended to reduce the possibility of failure in the LTBI diagnosis. At the moment, there are not enough studies available for stating its utility in monitoring during anti-TNF-α therapy. In this sense, further studies are required to establish the exact role of specific corticosteroids and/or immunosuppressive drugs in the IFN-γ-based test results.

Other immunocompromised situations

Recipients of solid organ transplants

For transplant recipients, the incidence of active TB is 20–74-times higher when comparing with the general population. In this immunosuppressed population, owing to anergy, the TST is frequently negative [61]. Studies assessing the utility of the IFN-γ-based tests have only been performed in patients awaiting liver transplantation. In these patients, accurate diagnosis of TB infection is very important as active TB can cause severe complications, and because active and latent TB infection treatments are very hepatotoxic. Manuel *et al.* found a good sensitivity for QFN-G in detecting LTBI [62]. They studied 153 patients prior to liver transplantation, obtaining a similar number of positive results: 37 for the TST and 34 for the QFN-G assay. However, they described discordant results (12 TST-positive/QFN-G-negative; and nine TST-negative/QFN-G-positive) that were not associated with prior BCG vaccination. By contrast, indeterminate results for QFN-G test were obtained in 7.8% of patients, all of them with a negative TST. Codeluppi *et al.* reported a case of pulmonary TB 55 days after the liver transplant [63]. In this case, the TST was negative prior to the transplantation, and the QFN-G test was also negative before and after transplantation.

Recently, Lindemann *et al.* studied the performance of the T-SPOT.TB and TST tests in 48 patients awaiting liver transplantat [64]. In their experience, four patients had a positive T-SPOT.TB result. Given that all patients were TST-positive, the T-SPOT.TB was repeatedly positive and they reported TB exposures, all of which were considered TB infected. In one patient, shortly after the transplantation, the reactivity against the TB-specific antigens was lost. This scenario should be considered when regarding the possibility of using IFN-γ-based tests for monitoring patients after transplantation.

Table 2. Summary of studies analyzing the values of IFN-γ-based tests by comparison with the tuberculin skin test in patients with chronic immune-mediated inflammatory disease prior to anti-TNF-α therapy.

Study	Location	Population	Patients (n)	BCG (%)	Test	Number of positives (%)	% IFN-γ-based test versus TST concordance (κ)	% IFN-γ-based test concordance (κ)	Indeterminate results (%)	Ref.
Cobanoglu *et al.* (2007)	Turkey	LTBI screening	68	100	TST (10 mm) QFN-G-IT	60.6 14.7	- 50.8	- -	- 10.3	[46]
Sellam *et al.* (2007)	France	LTBI screening	13	100	TST (10 mm) In-house ELISPOT	61.5 84.6	- 46.1	- -	- 0	[52]
Takahashi *et al.* (2007)	Japan	LTBI screening	14	NR	TST (20 mm) QFN-G	28.6 28.6	- 64.3	- -	- 5	[55]
Pratt *et al.* (2007)	UK	LTBI screening	101	78.5	TST QFN-G-IT	ND 7	- -	- -	- 9.9	[60]
Bocchino *et al.* (2008)	Italy	LTBI screening	69	3	TST (5 mm) T-SPOT.TB QFN-G-IT	26 30.4 31.8	- 78.4 (0.21) 80.5 (0.26)	- NR	- 5.8 2.8	[45]
Ponce de Leon *et al.* (2008)	Peru	LTBI screening	101	80	TST (5 mm) QFN-G-IT	26.7 44.6	- 70 (0.37)	- -	- 1.9	[47]
Vassilopoulos *et al.* (2008)	Greece	LTBI screening	70	40	TST (5 mm) T-SPOT.TB	38.6 22.8	- 72.8 (0.38)	- -	- 0	[48]
Matulis *et al.* (2008)	Switzerland	LTBI screening	142	83	TST (5 mm) QFN-G-IT	32.4 12	- 64 (0.17)	- -	- 6	[49]
Schoepfer *et al.* (2008)	Switzerland	LTBI screening	168	70.2	TST (5 mm) QFN-G-IT	18 8.3	- NR (-0.03)	- -	- 3	[51]
Greenberg *et al.* (2008)	NY, USA	LTBI screening	61	27.8	TST (5 mm) QFN-G	21.3 18	- NR	- -	- 11.5	[56]
Chen *et al.* (2008)	Taiwan	LTBI screening	43	100	TST (5 mm) QFN-G	18.6 13.9	- 60 (0.21)	- -	- 4.6	[59]
Murakami *et al.* (2009)	Japan	LTBI screening	71	100	TST (5 mm) In-house ELISPOT	21.4 14.1	- NR	- -	- NR	[53]
Bartalesi *et al.* (2009)	Italy	LTBI screening	398	4	TST (NR) QFN-G-IT	19 13	- 87.7 (0.55)	- -	- 1.2	[57]
Martin *et al.* (2009)	Ireland	LTBI screening	150	82	TST (5 mm) T-SPOT.TB QFN-G	18 9.8 7.1	- NR NR	- 98.2 (NR)	- 4.7 2.8	[54]
Behar *et al.* (2009)	MA, USA	LTBI screening	179	4.7	TST (5 mm) T-SPOT.TB	1.1 5.6	- 93.3 (-0.019)	- -	- 0	[50]

BCG: Bacillus Calmette–Guérin vaccination; ELISPOT: Enzyme-linked immunosorbent spot; LTBI: Latent TB infection; ND: Not done; NR: Not reported; QFN-G: QuantiFERON®-TB Gold; QFN-G-IT: QuantiFERON-TB Gold In tube; TST: Tuberculin skin test.

The IFN-γ-based tests appear reliable for diagnosing LTBI in patients awaiting liver transplants, being beneficial in detecting infected patients and for avoiding unnecessary anti-TB treatments. However, the presence of discordant results, failure in detecting LTBI in one patient that progressed to active TB and reversion after transplantation makes it necessary to interpret results with caution. Further studies are compulsory to establish the utility of the IFN-γ-based tests in other solid organ transplants. The specific immunosuppressant drugs used in each setting for avoiding organ rejection are likely to have a different effect on the performance of the IFN-γ-based tests.

End-stage renal disease

Regarding chronic renal conditions, current guidelines recommend LTBI screening for hemodialysis patients. In this sense, in contrast to a positive TST, positive IFN-γ-based tests have been associated with established TB risk factors. Passalent *et al.* compared the T-SPOT.TB assay with the TST in 203 patients with end-stage renal disease (ESRD) [65]. T-SPOT.TB was positive in 78.6% of patients with a history of active TB (OR: 7.24; 95% CI: 1.70–30.8; p = 0.007) and in 72.7% of patients with radiographic markers of previous infection (OR: 5.48; 95% CI: 1.20–25.1; p = 0.03); by contrast, the TST was only positive in 21.4% (OR: 2.73; 95% CI: 0.65–11.5; p = 0.17) and 18.2% (OR: 1.21; 95% CI: 0.24–6.21; p = 0.82), respectively. In a recently published study [66], enroling a total of 100 ESRD patients, the number of positive results for the TST, an in-house ELISPOT and QFN-G were similar (26, 27 and 21 positive results, respectively). Nevertheless, patients with contact to a TB case were more likely to have a positive ELISPOT (OR: 2.7; 95% CI: 1.0–7.2; p = 0.04) and QFN-G (OR: 2.8; 95% CI: 0.9–8.4; p = 0.02), whereas no association was found for a positive TST. By contrast, Triverio *et al.* found that, after adjusting for age and BCG administration, the OR of having a positive QFN-G-IT was 4.6-fold (p = 0.029) higher in patients with LTBI risk factors (chest x-ray suggestive of prior TB infection and/or contact with a patient with contagious active TB) than those without LTBI, but no association was found between LTBI risk factors and a positive TST or a positive T-SPOT.TB assay [67]. Similarly, Lee *et al.* described that the possibility of positive QFN-G increases in those patients with past TB disease and/or evidence of past TB disease on the chest x-ray [68]. None of the factors were associated with a positive TST or a positive ELISPOT result.

It has been reported that hemodialysis reduces the IFN-γ production level. Hursitoglu *et al.* described seven predialysis-positive and two indeterminate QFN-G-IT results in patients that became negative after hemodialysis [69]. This fact should be considered in order to schedule the IFN-γ-based test determination before and not after the hemodialysis process.

Malignant hematological disease

The T-SPOT.TB test has demonstrated its utility in diagnosing LTBI in a large contact study with immunosuppressed hematological patients [70]. T-SPOT.TB obtained a higher number of positive results (44.2%) than the TST (17.4%), and reached an overall rate of 4.3% of indeterminate results. Considering white blood cell counts, they did not detect differences in the number of indeterminate results by T-SPOT.TB, neither the number of positive results between patients with pathological white blood cell counts nor patients with normal counts. By contrast, for TST the level of positive results fell from 25.9 to 14.5%, although the difference was not significant. The study results suggest that the T-SPOT.TB assay was not affected by the immunosuppressive status.

A summary of the reported studies analyzing the value of IFN-γ-based tests by comparison with the TST in these immunocompromised conditions is shown in Table 3.

Pediatric population

Children represent 11–15% of the global TB burden [1]. In the pediatric population, TB infection is usually recent. In addition, BCG vaccination, especially in TB-endemic areas, affects the specificity of the TST. In this sense, a higher specificity has been reported for IFN-γ-based tests by comparison with the TST in children. However, the sensitivity of the IFN-γ-based tests diagnosing active TB in children has shown contradictory results among the different studies (Table 4) [22,71–77]. Nicol *et al.* reported, using in-house ELISPOT, that positive results obtained at diagnosis were higher in patients with a definite TB case (83.3%), than in patients with probable (72.3%) or possible TB cases (45.5%) [71]. Recently, Davies *et al.* also reported that a significantly higher proportion of HIV-infected children with definite or probable TB have a positive ELISPOT compared with a positive TST (p = 0.005) [78]. The authors noticed that, in contrast with TST, results from ELISPOT were not affected by young age or severe immunosuppression. In some cases it was impossible to obtain a microbiological diagnosis of active TB, often in developed countries, where the primary lesions are closed, small, with a low number of bacilli, being very difficult to get positive sputum, or gastric aspirate smears and cultures. This made it difficult to establish definite conclusions about the real IFN-γ-based test sensitivity and specificity.

Similarly, IFN-γ-based tests have also obtained discordant results in studies for diagnosing LTBI (Table 4). For interpretation of the results, especially the discordant ones, it is important to take into account the millimetres of induration considered positive in the TST, but also factors that could increase the false-positive immune response of the PPD, such as the number of BCG-vaccinations or the number of previous TSTs performed. ELISPOT has correlated more closely with measures of exposure to *M. tuberculosis*, such as duration and proximity to an active TB index case, than the TST [79–85]. However, in some studies, positive IFN-γ-based test and TST results were both equally frequent when exposure to the index case increased [86], or when the index case was a smear-positive patient [87]. In general, results seem to indicate that the ELISPOT test reaches a higher number of positive results than the TST or the QFN assay when detecting LTBI. Nevertheless, both IFN-γ-based test results have demonstrated independence regardless of the BCG-vaccination status [76,79,82,88]. Surprisingly, some studies have detected no influential impact of BCG in TST results [75,86]. However, these results have been obtained in countries with a high TB prevalence and

Table 3. Studies on number of positives, concordance with tuberculin skin test and number of indeterminate results of IFN-γ-based tests in different immunocompromised situations.

Study	Location	Population	Patients (n)	BCG (%)	Test	Number of positives (%)	% IFN-γ-based test versus TST concordance (κ)	% IFN-γ-based test concordance (κ)	Indeterminate results (%)	Ref.
Liver transplantation										
Manuel *et al.* (2007)	Canada	LTBI screening	153	82	TST 5 mm TST 10 mm QFN-G	24.2 17.6 22.2	TST 5 mm: 85.1 (0.60) TST 10 mm: 82.2 (0.48)	- - -	- - 7.8	[62]
Lindemann *et al.* (2008)	Germany	LTBI screening	48	29.2	TST (5 mm) T-SPOT.TB	12.8 8.3	- NR	- -	- NR	[64]
End-stage renal disease										
Passalent *et al.* (2007)	Canada	LTBI screening	203	NR	TST (10 mm) T-SPOT.TB	12.8 35.5	- 70.4 (0.25)	- -	- 5.4	[65]
Winthrop *et al.* (2008)	GA, USA	Contact study	100	NR	TST (5 mm) In-house ELISPOT QFN-G	26 27 21	- 71 (NR) 79 (NR)	- 87 (NR)	- NR NR	[66]
Hursitoglu *et al.* (2008)	Turkey	LTBI screening	56	NR	TST QFN-G-IT	NR 58.9	- NR	- -	- 13.6	[69]
Triverio *et al.* (2009)	Switzerland	LTBI screening	62	22.6	TST (5 mm) T-SPOT.TB QFN-G-IT	19 29 21	- NR (0.32) NR (0.16)	- NR (0.60)	- 11 8	[67]
Lee *et al.* (2009)	Taiwan	LTBI screening	32	71.9	TST (10 mm) T-SPOT.TB QFN-G	62.5 46.9 40	- 65.5 (0.32) 60 (0.25)	- 76.7 (0.53)	- 0 6.3	[68]
Hematological patients										
Piana *et al.* (2006)	Italy	Contact study	138	1.5	TST (5 mm) T-SPOT.TB	17.4 44.2	- 67.8	- -	- 4.3	[70]

BCG: Bacillus Calmette–Guérin vaccination; ELISPOT: Enzyme-linked immunosorbent spot; LTBI: Latent TB infection; NR: Not reported; QFN-G: QuantiFERON®-TB Gold; QFN-G-IT: QuantiFERON-TB Gold In tube; TST: Tuberculin skin test.

Table 4. Published studies comparing the number of positive results, the concordance and the number of indeterminate results between tuberculin skin test and IFN-γ-based tests in children with latent TB infection and active TB.

Study	Location	Population and mean or median years of age (range)	Patients (n)	BCG (%)	Test	Number of positives (%)	% of IFN-γ-based test versus TST concordance (κ)	% of IFN-γ-based test concordance (κ)	Indeterminate results (%)	Ref.
Ewer *et al.* (2003)	UK	Contact study Mean age 13.1 (11–15)	535	87.3	Heaf Test In-house ELISPOT	38.7 27.5	- 89 (0.72)	- -	- NR	[79]
Richeldi *et al.* (2004)	Italy	Contact study Neonates	41	0	TST (5 mm) In-house ELISPOT	0 4.9	- 0	- -	- NR	[81]
Liebeschuetz *et al.* (2004)	South Africa	Suspect TB Median 3 (3–6)	262	NR	TST (5 mm) In-house ELISPOT	46.8 68.6	- 64.2 (NR)	- -	- NR	[22]
Nicol *et al.* (2005)	South Africa	Active TB Median 2.6 (0.25–13)	70	NR	TST (NR) In-house ELISPOT	NR 70	- NR	- -	- NR	[71]
Nakaoka *et al.* (2006)	Nigeria	Contact study Mean 7.4 (1–14)	161	36	TST (5 mm) QFN-G-IT	31.6 39.8	- NR	- -	- 0	[87]
Hill *et al.* (2006)	The Gambia	Contact study Median 7.0 (0.5–14)	718	46	TST (10 mm) In-house ELISPOT	32.5 32.3	- 83 (0.62)	- -	- NR	[86]
Connell *et al.* (2006)	Australia	Suspected LTBI or active TB Median 9.2 (0.6–18)	101	49	TST (5 mm) QFN-G	50.5 19.8	- 57.4 (0.3)	- - -	- 17	[72]
Dogra *et al.* (2007)	India	Suspected TB or LTBI Median 6 (1–12)	105	82	TST (10 mm) QFN-G-IT	9.5 10.5	- 95.2 (0.73)	- -	- 0	[75]
Detjen *et al.* (2007)	Germany	Active TB Median 2.3 (0.3–7)	28	14.2	TST (5 mm) T-SPOT.TB QFN-G-IT	100 93 93	- 92.9 (0.00) 92.9 (0.00)	- 95.6 (0.91)	- 0 0	[90]
Lewinsohn *et al.* (2008)	Uganda	Contact study <5 years of age	296	79.4	TST (5 mm) ELISA	65.2 61.5	- 72 (0.39)	- -	- NR	[85]
Bakir *et al.* (2008)	Turkey	Contact study Mean 7.5 (0.08–16)	908	80	TST (5 mm) In-house ELISPOT	60.6 42	- 71.7 (NR)	- -	- 0	[98]

*Results in casual contacts, control subjects (not history of exposure) and children with suspected TB are also reported in the study.
‡5 mm in children with suspected active TB or recent TB contact, and 10 mm in children who were younger than 4 years of age or were tested because of recent immigration from an area of high TB prevalence, regardless of BCG status.
§In-paired tests with QFN-G-IT (1.5%) and QFN-G (2.3).
¶In-paired tests with T-SPOT.TB.
BCG: Bacillus Calmette–Guérin vaccination; ELISPOT: Enzyme-linked immunosorbent spot; LTBI: Latent TB infection; NR: Not reported; QFN-G: QuantiFERON®-TB Gold; QFN-G-IT: QuantiFERON-TB Gold In tube; TST: Tuberculin skin test.

Table 4. Published studies comparing the number of positive results, the concordance and the number of indeterminate results between tuberculin skin test and IFN-γ-based tests in children with latent TB infection and active TB.

Study	Location	Population and mean or median years of age (range)	Patients (n)	BCG (%)	Test	Number of positives (%)	% of IFN-γ-based test versus TST concordance (κ)	% of IFN-γ-based test concordance (κ)	Indeterminate results (%)	Ref.
Connell *et al.* (2008)	Australia	Suspected latent TB Mean 10.2 (0.7–18.8) Active TB Mean 8.2 (1.8–13.6)	100	47	TST (5 mm) T-SPOT.TB QFN-G-IT	60 25 29	- 75 (0.51) 75 (0.50)	- 93 (0.83)	- 14 3	[73]
Hesseling *et al.* (2008)	South Africa	Contact study Mean 2.9 (NR)	29	100	TST (10 mm) T-SPOT.TB QFN-G	54 89 39.6	- 46.1 (0.15) 88.9 (0.78)	- 56.2 (0.03) -	- 3.6 14.3	[83]
Winje *et al.* (2008)	Norway	LTBI screening (positive TST) Born in 1991 (14–15)	511	46.2	TST (5 mm) QFN-G-IT	- 9	- -	- -	- 0	[84]
Domínguez *et al.* (2008)	Spain	LTBI screening; contact study Median 9 (1–17)	125	68	TST (5 mm) T-SPOT.TB QFN-G-IT	85.8 38.1 37.3	- 51.2 (0.18) 47.2 (0.12)	- 87.1 (0.71)	- 2.2 0	[76]
Mandalakas *et al.* (2008)	OH, USA	HIV infected. LTBI screening Mean 4.4 (NR)	23	91.3	TST (5 mm) T-SPOT.TB QFN-G-IT	26.1 52.2 16.7	- NR (-0.02) NR (0.44)	- 66.4 (0.33)	- 0 0	[18]
Chun *et al.* (2008)*	Korea	Close contacts Median 1.7 (0–12.8)	42	100	TST (5 mm) QFN-G-IT	62 19	- 57.1 (0.19)	- -	- 0	[88]
Lighter *et al.* (2009)	Finland	LTBI screening and active TB Mean 9 (1–5)	207	36	TST (10 mm) QFN-G-IT	56 15.2	- 55 (0.17)	- -	- 1.4	[92]
Nicol *et al.* (2009)	South Africa	Suspected latent or active TB Median 1.5 (1–2)	243	100	TST (10 mm) T-SPOT.TB	38.8 25.2	- 79.9 (0.55)	- -	- -	[93]
Bergamini *et al.* (2009)	Italy	Suspected latent or active TB Mean 11.1 (0–19)	496	38.9	TST (5/10 mm)‡ T-SPOT.TB QFN-G QFN-G-IT	NR 14.3 (22/154) 18.8 (34/181) 17.1 (54/315)	- NR (0.52) NR (0.43) NR (0.35)	- - NR -	- 1.5 and 2.3§ 12.6¶ 16.4¶	[94]
Kampmann *et al.* (2009)	UK	LTBI screening Mean 6.9 (0.25–16) Active TB Mean 9.2 (0.5–15)	118 91	74 60.4	TST (5 mm) T-SPOT.TB QFN-G-IT TST (5 mm) T-SPOT.TB QFN-G-IT	68.4 31.03 32.2 37.4 37.8 46.2	- 75 (0.49) 77 (0.53 - 71.6 (0.42) 80.5 (0.61)	- 92 (0.82) - 82.7 (0.66)	- 8.6 5.1 - 8.9 8.8	[77]

*Results in casual contacts, control subjects (not history of exposure) and children with suspected TB are also reported in the study.
‡5 mm in children with suspected active TB or recent TB contact, and 10 mm in children who were younger than 4 years of age or were tested because of recent immigration from an area of high TB prevalence, regardless of BCG status.
§In-paired tests with QFN-G-IT (1.5%) and QFN-G (2.3).
¶In-paired tests with T-SPOT.TB.
BCG: Bacillus Calmette–Guérin vaccination; ELISPOT: Enzyme-linked immunosorbent spot; LTBI: Latent TB infection; NR: Not reported; QFN-G: QuantiFERON®-TB Gold; QFN-G-IT: QuantiFERON-TB Gold In tube; TST: Tuberculin skin test.

cannot be extrapolated to countries with moderate or low TB prevalence. Indeed, a decreased T-cell response against ESAT-6 stimulation was reported in household contact children exposed to *Mycobacterium africanum* (responsible for up to half of TB cases in western Africa), rather than that observed in children exposed to *M. tuberculosis* [89].

Regarding the specificity of tests in NTM infection, Detjen *et al.* analyzed 23 children with bacteriological NTM lymphadenitis and 22 with other nonmycobacterial respiratory tract infections, and reported a specificity for the T-SPOT.TB and QFN-G-IT tests of 100% [90]. Although the specificity of IFN-γ-based tests is excellent, frequent discordant results with the TST have been described [72,73,76,77,83,84,91–93]. Among 511 TST-positive children screened for LTBI at school (14–15 years old) in Norway [84], only 44 (9%) had a confirmed positive QFN-G result. In another study conducted in Barcelona (Spain) among children who were not BCG-vaccinated, QFN-G-IT was negative in 60.4% of children with a positive TST, and T-SPOT.TB assay in 56.6% of cases [76]. In fact, a priority research area is to understand discordant TST and IFN-γ-based test results, including the role of NTMs [11].

With regard to indeterminate results, Ferrara *et al.* noted that QFN-G obtained in children under 5 years gave a higher proportion of indeterminate results than the T-SPOT.TB (32 vs 0%) [12]. A higher ratio of QFN-G-indeterminate results (17%) was also obtained by Connell *et al.* [72]. However, in a later study, Connell *et al.* reported a higher proportion of indeterminate results by the T-SPOT.TB than the QFN-G-IT assay [73]. The available results seem to indicate that indeterminate results using QFN-G are age-dependent, being that IFN-γ is released in significantly lower quantities in response to phytohemaglutinin in young children [72,92]. By contrast, T-SPOT.TB, except in the first weeks of life [81], seems not to be age-dependent [12]. Nevertheless, Nicol *et al.* [93] reported a decrease in the number of children below the age of 1 year who scored positive T-SPOT.TB, whereas the TST results were unaffected by age. On the other hand, the QFN-G-IT test seems to offer a lower number of indeterminate results than the QFN-G test [75,76]. Indeed, recently, Bergamini *et al.*, in a retrospective study involving 496 children, reported that indeterminate results were associated with younger age (<4 years of age) for both QFN-G and QFN-G-IT, but not for T-SPOT.TB [94]. Lewinsohn *et al.*, using an in-house IFN-γ-based test based on ELISA detection, found that young household contact children (<2 years old) produced IFN-γ responses comparable to adults [85]. Liebeschuetz *et al.* reported that ELISPOT was less affected by HIV co-infection, malnutrition or age under 3 years old, than the TST [22]. Probably one of the main reasons that can explain the indeterminate results is the impossibility of isolating a high number of T cells in children if less of 4 ml of blood is collected.

One unresolved issue with IFN-γ-based tests is reversions and conversions observed in contact patients [95,96]. Hill *et al.* conducted a longitudinal study in the Gambia comparing ELISPOT results with those of the TST [96]. They included 740 contacts of 177 TB patients, all contacts being at least 15 years old. 3 and 18 months after the moment of recruitment, ELISPOT was repeated in contact patients. 3 months after recruitment ELISPOT reversion was detected in 40% (54 out of 134) of contacts with a previous positive ELISPOT, and 18 months after recruitment reversion occurred in 36% (28 out of 78). On the contrary, the ELISPOT conversion rate was 27% (20 out of 75) at 18 months. Intriguingly, the rate of TST reversion was lower than in the ELISPOT, and the rate of TST conversion at 18 months was higher (50%). One plausible explanation is that reversion might reflect the clearance of bacilli from the organism [95], or might reflect the transition into latency [96]. In addition, the different rate of conversion between the ELISPOT and TST may also be due to a combination of factors: increased TST sensitivity, a different interval between initial exposure and test conversion, boosting the effect of TST results, and early ELISPOT reversion [96]. Therefore, more studies are needed to determine the biological variability of IFN-γ responses over time in the absence of TB exposure, and also to define the exact criteria for ELISPOT reversion and conversion. Further research is required for better understanding the meaning of these reversions and conversions [97]. All these facts should be considered when preparing IFN-γ-based test guidelines.

Bakir *et al.*, in a study conducted in Turkey involving more than 900 children and adolescents with recent household TB exposure, found that among the 381 contacts with positive ELISPOT, 11 developed active TB, and among the 550 with positive TST, 12 developed active TB [98]. They concluded that a positive ELISPOT result predicted the development of active-TB as well as the TST.

In children, IFN-γ-based tests have demonstrated to be useful in diagnosing LTBI, showing association between positive ELISPOT results with exposure to *M. tuberculosis* and not being affected by BCG vaccination. They could also be a useful tool for helping in the diagnosis of active TB. It is necessary to cautiously interpret a positive TST in screening studies, as the high ratio of discordant results with IFN-γ-based tests is probably due to NTM infection.

Diagnosing active TB

Some studies [12,13,23,30,34,99] and case reports [63,74,100–102] referring to the utility of IFN-γ-based tests in diagnosing active TB in immunocompromised patients have been reported. Nowadays, IFN-γ-based tests can be used as a complementary tool in the diagnosis of active TB, being helpful in areas where the prevalence of LTBI infection is very low. In addition, these tests can also be of use in diagnosing extrapulmonary TB, where approximately half of the cases have not been microbiologically diagnosed, and which often require complex and invasive diagnostic tools. Finally, tests could also be useful for pulmonary smear-negative patients. However, IFN-γ-based assays, as they are currently designed, cannot distinguish between active and latent TB infection.

Nevertheless, a novel approach has been proposed for diagnosing active TB. It is based on applying ELISPOT in samples collected directly from the site of infection. The recruitment of specific T cells during active TB and how antigen-specific cells clonally expand and migrate to the site of infection have been described [103].

Indeed, the utility of the ELISPOT in diagnosing active TB in immunocompromised patients using samples different from blood has already been reported. Richeldi *et al.* described how

an ELISPOT based on RD1 antigens on a pleural effusion sample accelerated the diagnosis of disseminated TB in a leukemic patient with persistent fever and a negative TST [104]. In this case, the ELISPOT on blood was positive 5 weeks before the TST became positive; and the ELISPOT on the pleural fluid sample was also positive 1.5 weeks before the mycobacteria isolated on the peripheral blood was identified as *M. tuberculosis*. Lange *et al.* also described the diagnosis of active TB in a patient receiving anti-TNF-α therapy by means of T-SPOT.TB on a pleural effusion sample [105]. The TST was negative (0 mm), the smear of sputum and bronchoalveolar lavage (BAL) fluid was also negative, and only the sputum culture and the pleural biopsy study (histopathology and nucleic acid-amplification technique) confirmed active TB. However, the sputum culture result was delayed between 7 and 35 days, and the nucleic acid-amplification technique on pleural biopsy was performed retrospectively.

By contrast, Baba *et al.* designed a study for validating the QFN-G assay on pleural fluid samples, in a group of HIV-infected patients with confirmed TB, probable TB and non-TB pleuritis [106]. The authors found that the QFN-G assay obtained 52% of indeterminate results, mainly caused by the high background observed in the negative control of both TB groups.

The detection of reactive T cells against the *M. tuberculosis*-specific antigens in BAL fluid by ELISPOT has been also described as compatible with the diagnosis of pulmonary active TB [107]. Regarding the experience in immunocompromised patients, its utility has been described in a patient receiving immunosuppressive therapies for a mixed connective tissue disease [108]. The patient had a negative TST, and smear and nucleic acid-amplification techniques were also negative in both the sputum and BAL fluid. The treatment was initiated, based on the ELISPOT result obtained from BAL fluid, 18 days before *M. tuberculosis* was isolated in the sputum culture.

Expert commentary

Since the development of IFN-γ-based assays as an *in vitro* alternative method to the TST for the immunodiagnosis of LTBI, promising results in adults and also in children have been published [12,70,72,76,79,80,109–111]. Although IFN-γ-based tests are designed for diagnosing LTBI, they have also been evaluated as an aid in the diagnosis of active TB [109,112–114]. A high specificity of the IFN-γ-based tests has been demonstrated, being that the IFN-γ-based tests were not affected by BCG vaccination or infection by the most common NTM. In addition, in the absence of a gold standard test to determine LTBI, IFN-γ test results have demonstrated to be closer than the TST to the degree of exposure to *M. tuberculosis* [80,100,115]. One of the key questions for its utilization is establishing the capacity of these tests in predicting the development of disease. In studies recently published [98,116,117], positive IFN-γ-based assays predicted development of active TB in adult and children with recent TB contact.

IFN-γ-based tests offer general advantages over the TST [118]: avoidance of cross-reaction with BCG-vaccinated individuals and with NTM infection, logistical convenience (result available in 24 h), avoidance of poorly reproducible measurements, no follow-up visit required and the result remains confidential. Furthermore, an injection of PPD for the TST can boost subsequent TST responses, primarily in NTM-infected or BCG-vaccinated individuals. On the contrary, IFN-γ-based tests could be performed serially without inducing the boosting phenomenon.

According to cumulative evidence, the T-SPOT.TB assay showed a higher number of positive results, and a lower number of indeterminate results than the QFN tests. Given that the number of cells stimulated in the T-SPOT.TB instead of QFN test is standardized to 250,000 per culture, negative and indeterminate results associated with low lymphocyte counts could be reduced.

Nevertheless, although the T-SPOT.TB test seems to be more sensitive than the QFN-G-IT test, some operational aspects have to be noted for the performance of both IFN-γ-based tests (Figure 1). The T-SPOT.TB assay requires same-day processing of specimens, given the early and often inconvenient time limits for sample collection. This is especially important if a courier service is involved. Recently, the T-SPOT.TB manufacturer introduced a complementary reagent to allow the isolation of lymphocytes from whole blood up to 32 h following venopuncture. However, although the manufacturer claims that there is not a significant decrease in peripheral blood mononuclear cell yields or T-cell populations when comparing with whole-blood samples stored for less than 8 h, the procedure has not been extensively assessed and further experience is required.

Furthermore, T-SPOT.TB technical performance makes it difficult to run a large number of samples in a single day. In this sense, the QFN-G-IT test allows more flexible timing for specimen collection and transport owing to the fact that the antigens are already incorporated in the sample collection tube. The QFN-G-IT test also makes it possible to store the samples after stimulation with the antigens and to run batches. However, there are few studies evaluating whether stimulation of T cells with antigens together instead of separately could affect the sensitivity or specificity of the test. In a previous study evaluating LTBI in a rural area of South Africa, the QFN-G-IT test (201/358; 56%) obtained more positive results than the conventional QFN-G test (137/358; 38%) [119]. Furthermore, T-SPOT.TB requires more expertise in the readout of the results than QFN-G-IT, especially when the result is close to the cut-off value.

For immunosuppressed patients, additional advantages are required, such as a low ratio of indeterminate results, a high predictive value and a higher sensitivity than TST for avoiding false-negative results due to anergy.

Indeterminate results were mainly associated with immunosuppression, especially related to immunosuppressant therapy, and in patients with suppressed cellular immunity. Nevertheless, it is important to remark that in immunosuppressed patients with a negative TST result, an indeterminate IFN-γ test result should be considered as very useful clinical information.

The fact that IFN-γ-based tests measure effector T-cell responses rather than memory T cells seems to indicate that IFN-γ-based tests are a good indicator of recent LTBI, allowing the identification of patients with remote LTBI. Although this fact could be useful for selecting candidates to receive prophylaxis in an immunocompetent population, at the moment it is recommended to treat all immunocompromised patients with LTBI (both recent and remote infections).

Therefore, although among immunosuppressed patients the percentage of negative TST results is higher, and IFN-γ-based tests obtain more positive results with a higher specificity, the presence of discordant results between TST and IFN-γ-based tests, and the high risk of progression to active TB in this population, it seems prudent to recommend the utilization of IFN-γ-based tests after a negative TST result in order to increase the sensitivity of detecting LTBI cases in severely immunosuppressed patients. According to the data available, there is no risk of TST boosting in the IFN-γ-based test response if these tests are performed after a negative TST [120–124]. The possibility of using IFN-γ-based tests alone to avoid a large number of false-positive TST results should be considered for BCG-vaccinated nonsevere immunocompromised patients.

Although more studies comparing IFN-γ-based tests with the TST are needed to sufficiently expand knowledge, IFN-γ-based tests seem to be, in combination with the TST, a useful method for diagnosing TB infection in immunocompromised patients and children, according to their exact role in the different immunocompromising situations, the degrees of immunosuppression and the specific risks of LTBI for each patient.

Five-year view

In the next 5 years, the evolution of the IFN-γ-based tests in diagnosing TB infection will require advancements in two directions. First, it will be necessary to perform studies with a large number of patients to establish the prognostic value of positive test results in the different populations of immunosuppressed patients, especially in those that undergo an anti-TNF-α therapy.

Guidelines for IFN-γ-based test utilization in each group of immunocompromised patients and in each individual situation will be established. Given that *in vitro* assays depend on the secretion of IFN-γ, which is largely produced by CD4 T cells, more studies will be developed in order to define the CD4 threshold at which the performance of these assays declines, in particular when CD4 T-cell counts are under 50 cells/μl. These studies should explore the necessity and accuracy of new cut offs for diagnosing LTBI in immunosuppressed patients, and for distinguishing between active and latent TB infection.

Furthermore, specific studies will be performed in order to understand the discordant results between IFN-γ-based tests and the TST, especially in the pediatric population where the effect of NTM infection may play a very important role. The knowledge of the meaning of conversion and reversion results will also increase, and the clinical explanation of discordant and indeterminate results between IFN-γ-based tests will be expanded.

Second, technical modifications will be performed on IFN-γ-based tests [125]. The refinement of IFN-γ-based tests will include the exploration of alternative readouts to measure IFN-γ release [125], the utilization of new *M. tuberculosis*-specific antigens [6,126], and the simultaneous measurement of chemokines [127] and interleukins [128]. The next generation of IFN-γ-based tests will significantly enhance diagnostic sensitivity without diminishing specificity, and reduce the number of indeterminate results. In addition, the commercial IFN-γ-based test manufacturers should increase efforts to simplify technology and enhance its applicability in resource-limited settings.

Similarly, the detection of *M. tuberculosis*-specific T cells in nonblood samples using ELISPOT is a promising tool for the diagnosis of active TB in immunocompromised patients with negative smears. The definition of methodological procedures and the establishment of an accurate cut off will also be performed.

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Key issues

- IFN-γ-based tests produce more positive results than the tuberculin skin test (TST) for diagnosing latent TB infection (LTBI) in immunosuppressed patients, but less than in immunocompetent patients.
- Concordance between the TST and IFN-γ-based tests is poor, but IFN-γ-based test results are more likely associated with risk factors for LTBI than the TST.
- T-SPOT.TB shows a higher number of positive results than the QuantiFERON®-TB (QFN) test.
- Available data suggest that the T-SPOT.TB assay is less influenced by immunosuppression status than the QFN test.
- The T-SPOT.TB assay has a lower number of indeterminate results than the QFN test.
- Differences in the performance of both commercial tests should be considered when selecting the adequate IFN-γ-based test for each population and setting.
- In children, IFN-γ-based tests show a higher number of positive results than the TST, and have an especially higher specificity than the TST.
- The role of nontuberculous mycobacteria infection in children requires further studies for determining their effect in discordant results between the TST and IFN-γ-based tests.
- IFN-γ-based tests seem to be, in combination with the TST, a useful method for diagnosing TB infection in severely immunosuppressed patients.

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ANEXO IX

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VIEWPOINT

Role of the T-cell interferon-gamma release assays in preventing reactivation of latent tuberculosis infection in immunosuppressed patients in treatment with anti-TNF agents

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Chronic inflammatory diseases such as Crohn's disease or ulcerative colitis, are major autoimmune disorders with increasing incidence.[1] Although conventional therapies based on corticosteroids and immunosuppressants play a major role in the management of these diseases, the corticoisteroid-associated adverse events and the existence of immunosuppressant-refractory patients have prompted the development of new disease-modifying therapies. Biological agents, especially the anti-Tumor Necrosis Factor (TNF)-α agents, have emerged as an effective treatment for these diseases.[1] One of the primary concerns with anti-TNF-α agents is the possibility of collateral effects on host defence mechanisms. Patients undergoing TNF-α inhibition are at increased risk of developing severe infections. A temporal association between anti-TNF-α antibodies and reactivation of latent tuberculosis (TB) infection has been established.[2,3] In fact, TNF-α is one of the key molecules involved in granuloma formation and maintenance. Therefore, previous to start TNF-α inhibition, appropriate screening of latent TB infection and early active TB diagnosis has become mandatory.[4]

The current guidelines for screening latent TB are based on the tuberculin skin test (TST) that has been used since the late 1800s for diagnosing latent TB infection. TST attempts to measure cell-mediated immunity as assessed by a delayed-type hypersensitivity response to its components.[5,6] TST is produced by steaming cultures of *Mycobacterium tuberculosis* in a sterilizer and purifying the proteins (purified protein derivative [PPD]) by repeated precipitation with neutral ammonium sulphate.[7] In the TST, 0.1 ml of PPD solution are injected intradermically in the forearm of the patient. After 48 h, the diameter of induration is measured.

A TST induration >5mm is considered positive, independently of the *Mycobacterium bovis* bacilli Calmette-Guérin (BCG) vaccination status. In case of induration <5mm, it is necessary to repeat the TST between 1–2 weeks later (booster effect), also considering as positive an induration >5mm[8]. The booster effect is based on the phenomenon of increased TST reactions after retesting as a result of a recall of waned cell-mediated immunity.[9]

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The biggest drawback of TST is the fact that PPD contains more than 200 antigens that are widely shared among mycobacteria other than *M. tuberculosis*, including BCG bacilli and many environmental mycobacteria. As a result, individuals sensitised by previous exposure to non-tuberculous mycobacteria (NTM) or vaccinated with BCG respond immunologically to PPD.[6,10] In addition, in BCG vaccinated individuals or those infected by NTM but not truly infected by *M. tuberculosis*, the repeated TST (booster effect) could promote a false positive induration.[9]

On the other hand, although the intrinsic sensitivity of TST for detecting latent infection is not known, because there is not a definitive gold standard for comparison, there is evidence of a low sensitivity in presence of immunosuppressive therapy and in young children. Therefore, the population at high risk of progressing to active TB might remain undiagnosed.[11,12] It is well known that patients with chronic inflammatory diseases may not be able to produce an adequate delayed type hypersensitivity reaction to TST because of their deficient cell mediated immunity.[1,4]

Recently, in an effort to develop more sensitive and specific tools for the immunological diagnosis of latent *M. tuberculosis* infection, a 6-kD *M. tuberculosis* early-secreted antigenic target protein (ESAT-6) and the 10-kD culture filtrate protein (CFP-10), coded in the region of difference 1 (RD1), have been described as being present in *M. tuberculosis* but not in any BCG strain or in the majority of environmental mycobacteria.[13]

In vitro assays for measuring T cell mediated immune responses after RD1 antigen stimulation have been developed.[14,15] In these assays, infected individuals are identified by the detection of interferon-γ (IFN-γ) released by the T cells that are sensitized. On the basis of this technology, two commercial IFN-γ tests are available: Quantiferon-TB Gold *In tube* assay (QFN-G-IT) (Cellestis Limited, Carnegie, Victoria, Australia) and the T-SPOT.TB assay (Oxford Immunotec, Oxford, UK). QFN-G-IT has received final approval from the U.S. Food and Drug Administration (FDA) as an aid for diagnosing *M. tuberculosis* infection. T-SPOT.TB has been approved for sale in Europe.

QFN-G-IT detects IFN-γ production by enzyme-linked immunosorbent assay (ELISA) after stimulation of whole blood samples with the specific antigens; in contrast, T-SPOT.TB detects the number of IFN-γ producing T cells by enzyme-linked immunospot assay (ELISPOT) after stimulation of isolated peripheral blood mononuclear cells. One of the main differences between T-SPOT.TB and QFN-G-IT is that in the latter, specific *M. tuberculosis* antigens are included together in the stimulation of the blood. In addition, in the QFN-G-IT, a third stimulating antigen has been included: TB7.7 (Rv2654). This new antigen is encoded in RD11 and is lacking from the BCG strains as well as most common environmental mycobacteria.[16] Interestingly, both tests include a positive control (stimulation of the cells using phytohaemagglutinn as mitogen). A summary with the technical and performance characteristics of each IFN-γ test are shown in Table 1.

The result of the tests is considered indeterminate if an antigen-stimulated sample is negative and if the value of the positive control is also negative after subtraction of the value of the nil control. Therefore, the inclusion of positive control allows these tests the detection of anergy. This control is especially useful in immunossuppressed patients where the response could be diminished by therapies and maybe even

Table 1 Comparison of technical and performance characteristics of the IFN-γ tests

Variable	T-SPOT.TB	QFN-G-IT
Technical characteristics		
Setting of test	*In vitro*	*In vitro*
Antigens	ESAT-6 and CFP-10, used separately	ESAT-6, CFP-10 and TB7.7, used together
Sample stimulated	Peripheral blood mononuclear cells	Whole blood
Readout Units	IFN-γ spot forming cells	International units of IFN-γ released
Reading system	ELISPOT	ELISA
Positive internal control	Yes	Yes
Time required for results	18–24 h	18–24 h
Methodology and reagents standardized	Yes	Yes
Laboratory infrastructure required	Yes (moderate to high)	Yes (low to moderate)
Trained personnel required	Yes (moderate to high)	Yes (low to moderate)
Possibility to run batches	No	Yes
Performance characteristics		
Need of second visit	No	No
Cross-reactivity with BCG vaccination	No	No
Cross-reactivity with non-tuberculous mycobacteria	No[a]	No[a]
Boosting phenomenon in repeated tests	No	No
Correlation with exposure intensity	Yes	Yes
Positive predictive value for active TB development during follow-up	Limited evidence but seems high	Limited evidence but seems high

[a]ESAT-6 and CFP-10 antigens encoded in the RD1 of the *M. tuberculosis* are also present in *Mycobacterium kansassi*, *Mycobacterium szulgai* and *Mycobacterium marinum*. The influence of these species in the IFN-γ tests has inadequate evidence.

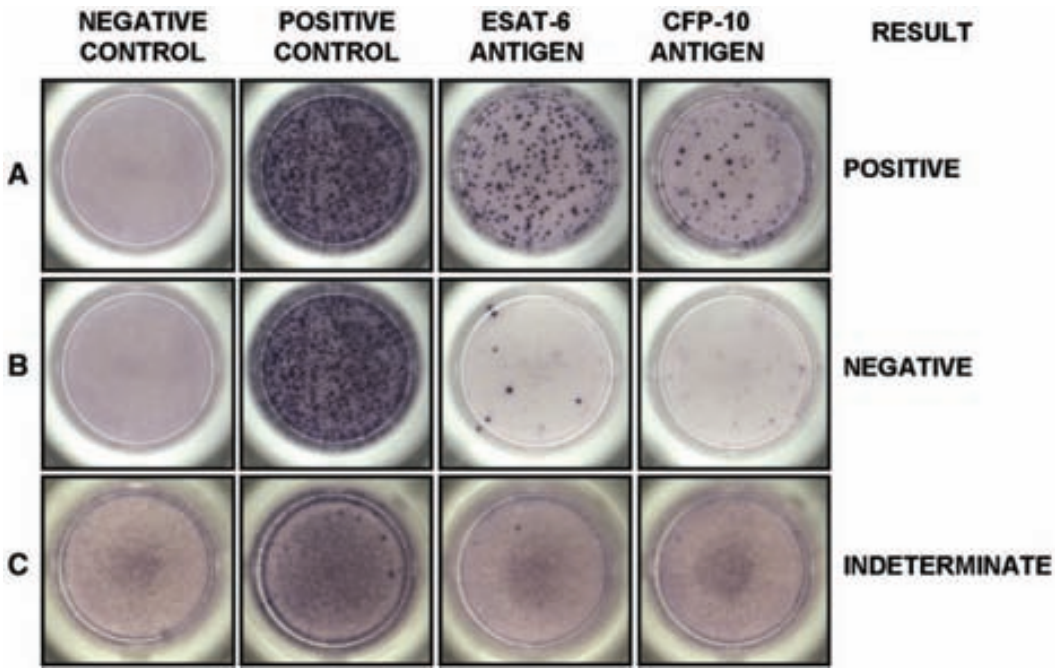

Figure 1 T-SPOT.TB detects the IFN-γ produced by peripheral blood isolated mononuclear cells by means of ELISPOT method, after stimulation with ESAT-6 and CFP-10. Subjects are considered positive if there is response to one or both of the specific antigens (A). The result is considered negative when there is not response for any of the specific antigens (B). The presence of reactive antigen-specific T cells is revealed as a spot on the well. Spots can be scored manually or also with the aid of an automated ELISPOT plate reader. Test wells are scored as positive if they contain at least six spot-forming cells more than the negative control well and this number is at least twice the number of the negative control well. The result of the assay is considered indeterminate if the number of spots in the positive control is less than 20, and the response to both of the specific antigens is negative (C).

as part of the disease itself.[11,17] Examples of T-SPOT-TB results are shown in Fig. 1, including an indeterminate result.

Several studies have been performed using IFN-γ assays based in stimulating specific antigens and, although the results vary widely, they have demonstrated the utility in the diagnosis of latent TB infection and active TB in immunocompetent patients, and their high specificity in BCG vaccinated patients.[18–23] There is also emerging evidence that IFN-γ tests are robust in people with immature cellular immune systems (young children) and HIV-TB co-infected patients.[24–26] However, the experience in patients with chronic inflammatory conditions (receiving immunosuppressive drugs) although promising, is still limited at present.[27–32]

Few case reports have been published describing the higher sensitivity of the IFN-γ tests as compared to the TST in diagnosing latent TB infection[28,29] and active TB[27] in patients with rheumatoid arthritis, and diagnosing latent TB infection in patients with Crohn's disease[30,32].

Richeldi et al[32] reported how a positive T-SPOT.TB test helped in diagnosing active TB in an asymptomatic, immunosuppressed adult with a negative result on a TST. The patient was receiving azathioprine therapy for Crohn's disease. High-resolution computed tomography of the chest showed suggesting images, and bronchoalveolar lavage culture confirmed microbiologically the active TB.

Cobanoglu et al[30] studied 38 healthy individuals and 68 patients with chronic inflammatory diseases for latent TB infection before the use of TNF-α blockers. All subjects included in the study were BCG vaccinated. The authors report poor agreement between the TST and the QFN-G-IT. According to the results, they conclude that only 8 of the 49 subjects who received prophylaxis against latent TB infection actually needed it. In addition, they obtained seven indeterminate results by QFN-G-IT in patients with chronic inflammatory diseases (being Crohn's disease the diagnosis in two cases); in six of them, the TST was negative.

In a study conducted in Japan, Takahashi et al[31] reported their experience comparing QFN-G-IT and conventional methods (TST, imaging and medical history) in 14 rheumatoid arthritis patients treated with anti-TNF-α therapy. In seven cases the latent TB infection was confirmed by at least one method. QFN-G-IT was the only positive method in two cases. They concluded that the QFN-G-IT should be employed in parallel with conventional procedures.

Although in immunosuppressed patients T-SPOT.TB seems more sensitive than QFN-G-IT[21], some operational challenges have to be noted for the performance of both IFN-γ tests. T-SPOT.TB requires same-day processing for specimens, given the early and often inconvenient cut-off times for sample collection. Furthermore, its technical performance makes it difficult to run a large number of samples. Given that in the QFN-G-IT, the antigens are incorporated in the sample collection tube, the QFN-G-IT test allows more flexible timing of specimen collection and transport. QFN-G-IT also makes it possible to store samples and to run batches. T-SPOT.TB performance requires a better-equipped laboratory than QFN-G-IT.

One of the main drawbacks could be the cost of the assays, which are more expensive than TST. However, preliminary studies[33] have shown that, in terms of overall cost-effectiveness, there is a benefit to use the new techniques instead of TST due to their saving the cost of unnecessary chemoprophylaxis (isoniazide; and analytical and radiographic controls) to false positive TST subjects; and also the cost to diagnose and treat active TB in patients previously infected and not detected by the TST.

There are some unanswered questions regarding IFN-γ tests.[34,35] One of the unsolved questions is how to explain the discrepancies between negative TST and positive IFN-γ test results that become negative a few months later without any tuberculous treatment. Ewer et al[36] suggested that this reflects an acute resolving infection. However, Pai et al[19] observed that reversions were more frequent in those with baseline results close to the diagnostic cut-off.

Probably, the key issue regarding the utility of the IFN-γ is if they are better than the TST in predicting the progression to active TB. Answering this question would require long-term longitudinal cohort studies that follow clinical outcomes of tested individuals. Recently, Diel R et al[37] have conducted an study comparing the QFN-G-IT with the TST in exposed close contacts of active TB with respect to their development of TB disease within 2 years. Their results suggest that QFN-G-IT determines more accurately than TST the presence of latent tuberculosis infection, with a high ratio of progression to active tuberculosis of those QFN-G-IT positive (14.6%), far greater than the 2.3% found for those TST positive.

IFN-γ tests have several advantages over the TST: 1) in BCG vaccinated individuals, the IFN-γ tests are more specific than the TST in diagnosing latent TB infection. It is important to note that a false-positive result by TST can lead to inappropriate initiation of chemoprophylaxis with potential morbidity, adverse side effects and consumption of health care resources; 2) there is not risk of false positive results due to booster effect; 3) the IFN-γ assays include controls to identify anergyc patients; and 4) on the light of the preliminary results in immunossuppresed patients, the IFN-γ tests seem to be more sensitive than the TST.

Therefore, in order to be cautious, the utilization of the IFN-γ tests in this special population, with a really high risk of progression to active TB, should be recommended after a negative TST result in order to increase the sensitivity of detecting latent TB infection cases.

In BCG vaccinated patients, a positive TST result could be confirmed by IFN-γ tests to identify unspecific TST result. However, it is already not clear how safe is it, in patients undergoing anti-TNF-α therapies, to not treat patients with positive TST but negative IFN-γ test.

Although careful studies comparing the IFN-γ tests to the TST are needed to validate their exact role, the IFN-γ tests seem to be an useful method in combination with TST for screening and monitoring of TB infection in patients receiving anti-TNF-α therapy.

Competing interests

In 2005, JD participated in Oxford Immunotec (manufacturer of T-SPOT.TB) advisory board meetings. Authors are members of the European Tuberculosis Network (TB-NET) Group.

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