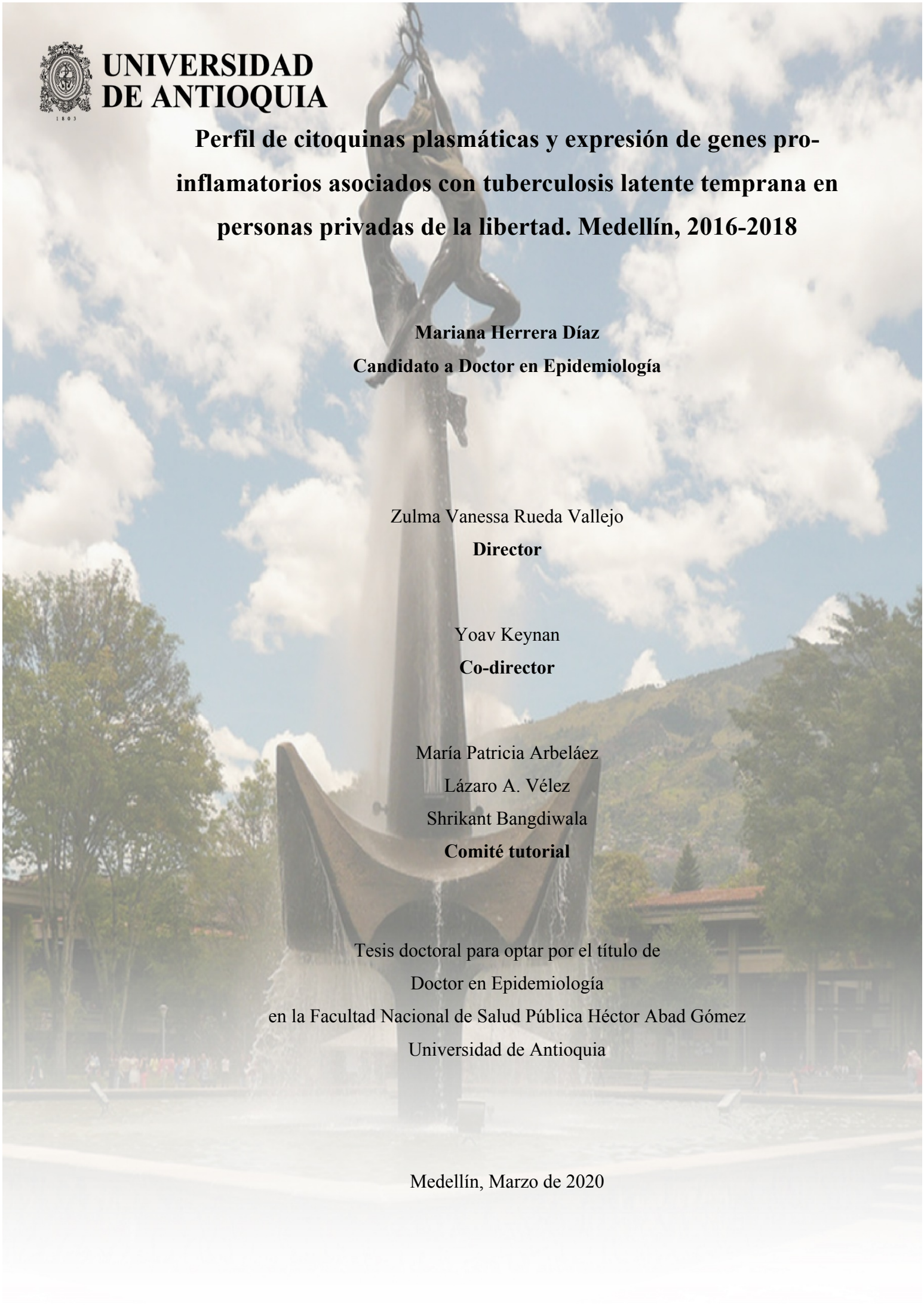

UNIVERSIDAD DE ANTIOQUIA

Perfil de citoquinas plasmáticas y expresión de genes pro-inflamatorios asociados con tuberculosis latente temprana en personas privadas de la libertad. Medellín, 2016-2018

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DESCRIPCIÓN DEL CONTENIDO DEL INFORME

El presente documento de tesis está dividido en siete capítulos:

El primer capítulo (Introducción) contiene el marco teórico, la justificación, los objetivos y la hipótesis de esta investigación.

Los capítulos dos, tres, cuatro y cinco son artículos derivados de la investigación, y cada uno incluye introducción, metodología, resultados, discusión, referencias bibliográficas y material suplementario (anexos) cuando la información lo ameritaba.

En el **capítulo dos** se indagó sobre la densidad de incidencia de tuberculosis latente en personas recién ingresadas a la prisión, y en aquellas con más de un año de reclusión; además, se determinaron los factores de riesgo que tiene una persona privada de la libertad para convertir en la prueba de tuberculina (pasar de negativo a positivo durante los seguimientos), y aquellos asociados con tener tuberculosis (TB) activa.

El **capítulo tres** es una revisión sistemática llevada a cabo con el fin de identificar qué parámetros inmunes se encuentran incrementados exclusivamente en personas con infección por tuberculosis (denominada TB latente), y algunos vacíos en el conocimiento y en el diseño de trabajos publicados anteriormente, que permitieran reconocer y controlar posibles sesgos al momento de realizar la medición de sustancias inmunes en humanos. La pregunta que nos planteamos fue: ¿cuáles son las citoquinas asociadas con TB latente, en comparación con las citoquinas expresadas en individuos con TB activa y aquellos no infectados por *Mycobacterium tuberculosis*?. Esta revisión fue previamente inscrita en la plataforma pública PROSPERO, documento que se encuentra en los anexos de la revisión.

El objetivo del **capítulo cuatro** fue determinar la concentración plasmática de 18 citoquinas/quimioquinas asociadas con la presencia de infección reciente por *M. tuberculosis*, y compararla con la concentración en personas con TB pulmonar y personas expuestas a *M. tuberculosis*, pero no infectadas. En el análisis multivariado se usó la

variable tiempo de reclusión para ajustar los modelos, debido a la observación de que la incidencia de TB latente varía según el tiempo de encarcelamiento y a que la concentración de citoquinas/quimioquinas variaba en el grupo de personas privadas de la libertad no infectadas, y entre los individuos con infección latente, de acuerdo con si tenía más o menos de un año de reclusión al momento de ingresar a la cohorte.

En el **capítulo cinco** se determinó la expresión genética diferencial de personas con TB latente, TB activa, y sin infección por *M. tuberculosis*, usando la técnica de RNA *sequencing*. Con este capítulo se pretendía entender la dinámica de la infección reciente por *M. tuberculosis* y los perfiles de expresión diferencial entre los tres grupos de interés. Además, se comparó la expresión de RNA con la concentración plasmática de las 18 sustancias inmunes evaluadas en el capítulo cuatro.

El **capítulo seis** contiene las concusiones generales de este documento de tesis, el aporte a la epidemiología y a las investigaciones en tuberculosis que tuvo esta investigación.

Por ultimo, el **capítulo siete** (actividades de divulgación del conocimiento) contiene los nombres de los artículos derivados de esta investigación que han sido sometidos, las presentaciones nacionales e internaciones realizadas, y otras actividades de difusión de la investigación que se llevaron a cabo.

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RESUMEN

Objetivo: Determinar el perfil de citoquinas plasmáticas y expresión de genes pro-inflamatorios de las personas privadas de la libertad asociado con TB latente de adquisición temprana (primer dos años post-exposición) comparado con personas con TB activa, TB latente de adquisición tardía (después de dos años post-exposición) y personas expuestas a *M. tuberculosis* sin infección.

Metodología: se realizó un estudio de cohorte prospectiva en dos establecimientos penitenciario de Medellín e Itaguí. Desde septiembre de 2016 se incluyeron 164 personas privadas de la libertad, tuberculina de dos pasos negativa, las cuales fueron evaluadas hasta diciembre de 2018 para el diagnóstico de TB latente o TB activa; además, se incluyeron 51 personas con diagnóstico nuevo de TB activa. En cada una de las 6 visitas de seguimiento se diligenció un formulario de recolección de datos, se aplicó la prueba de tuberculina, cada seis meses para las personas sin infección y hasta que fuera positiva, y se tomaron muestras de sangre periférica cada tres meses.

Se evaluó la prevalencia e incidencia de infección latente, y los factores asociados al desarrollo de TB latente entre las personas recién ingresadas a la cárcel, y las que tienen mas de un año de reclusión, además, los factores asociados a TB activa. Se evaluó la expresión genética (en PBMCs) y el perfil de 18 citoquinas/quimioquinas (en plasma). El análisis se llevó a cabo en los paquetes STATA y R®, teniendo en cuenta otras variables de ajuste como el consumo de sustancia psicoactivas, presencia de cicatriz BCG, índice de masa corporal, centro de reclusión, sexo, entre otras.

Resultados: La densidad de incidencia fue de 28.8 casos por 100 personas-año en personas recientemente encarceladas, y fue de 7.1 casos por 100 personas-año en personas que han estado encarceladas durante más de 1 año. La vacunación con BGC reduce el riesgo de adquirir LTBI en las personas con infección reciente por *M. tuberculosis*. El VIH, la diabetes, el consumo de drogas, el cigarrillo y el encarcelamiento por más de 1 año se asociaron con un mayor riesgo de tuberculosis.

Con respecto a la búsqueda de biomarcadores encontramos que la bibliografía existente (36 artículos revisados de forma sistemática) tiene varias limitaciones que han impedido concluir con respecto al uso de citoquinas para diagnóstico de infección latente, entre las

que se destacan la heterogeneidad entre las poblaciones estudiadas, los métodos de laboratorio y los protocolos de estimulación celular utilizados, entre otros. Luego de evaluar las citoquinas/quimioquinas plasmáticas observamos que una combinación de concentraciones de MIP-3α, MIP-1β, MCP-1, y CD14 soluble fue capaz de distinguir a las personas con infección reciente por MTB de TB pulmonar y controles no infectados. Por su parte, el perfil especifico de las personas con TB activa mostró 22 genes sobre expresados, el de las personas con infección reciente por *M. tuberculosis* 210 genes, y el de las personas no infectadas 129. Estos genes estuvieron involucrados en vías relacionadas con el ciclo celular, la interacción entre citoquinas y sus receptores, señalización inmune, y fosforilación, entre otros.

La concentración de citoquinas y de expresión de genes varía de acuerdo con el tiempo de reclusión.

Conclusiones: Este estudio muestra la importancia de detectar LTBI al ingresar a la prisión y seguir a aquellos que son negativos cada año, ofreciendo tratamiento LTBI a los nuevos convertidores. Las concentraciones de citoquinas y quimiocinas pueden usarse para comprender la dinámica de transmisión e identificar potencialmente a las personas con alto riesgo de desarrollar TB activa. La expresión de genes permite estudiar los eventos ocurridos luego de una infección reciente por *M. tuberculosis*; además al relacionada principalmente con vías de expresión inmunes y de ciclo celular podría abrir nuevas oportunidades de investigación para la búsqueda de biomarcadores o blancos terapéuticos.

Palabras claves: tuberculosis activa, tuberculosis latente, prisioneros, biomarcadores, citoquinas, RNA sequensing

ABSTRACT

Objective: To determine the plasma cytokines and gene expression profile of prisoners associated with early latent TB infection (first two years post-exposure) compared to people with active TB, late LTBI (after one year post-exposure) and people exposed to M. tuberculosis without infection.

Methodology: a prospective cohort study was carried out in two prisons in Medellín and Itaguí. Since September 2016, we include 164 inmates, TST two-step negative, who were evaluated until December 2018 for the diagnosis of latent TB or active TB; besides, 51 people with a new diagnosis of active TB were included. At each of the 6 follow-up visits, a data collection form was completed, the tuberculin test was applied every six months for people without infection, and until it was positive. Peripheral blood samples were taken every three months.

The prevalence and incidence of latent tuberculosis infection, and the factors associated with latent TB among people newly admitted to prison, and those with more than one year of confinement, were evaluated, as well as the factors associated with active TB. We determine the gene expression (in PBMCs) and the profile of 18 cytokines/chemokines (in plasma). The analysis was carried out on the STATA and R® packages, taking into account other variables such as the consumption of psychoactive substances, presence of BCG scar, body mass index, prison, and sex.

Results: The incidence density was 28.8 cases per 100 person-years in people recently incarcerated, and it was 7.1 cases per 100 person-years in people who have been incarcerated for more than 1 year. Vaccination with BGC reduces the risk of acquiring LTBI in people with recent M. tuberculosis infection. HIV, diabetes, drug use, smoking, and incarceration for more than 1 year were associated with an increased risk of tuberculosis.

Concerning new biomarkers, we found that the existing bibliography (36 systematically reviewed articles) has several limitations that have prevented the conclusion regarding the use of cytokines for the diagnosis of latent infection. Some limitations are the heterogeneity among the populations investigated, the laboratory methods, and the cellular stimulation protocols used. After evaluating plasma cytokines/chemokines we observed that

a combination of concentrations of MIP-3α, MIP-1β, MCP-1, soluble CD14 and IL-17A was able to distinguish people with recent MTB infection from pulmonary TB and not infected controls. For its part, the specific profile of people with active TB showed 22 over-expressed genes, that of people with recent infection with M. tuberculosis 210 genes, and that of uninfected people 129. These genes were involved in pathways related to the cell cycle, the interaction between cytokines and their receptors, immune signalling, and phosphorylation, among others.
The concentration of cytokines and gene expression varies according to the time of confinement.
Conclusions: This study shows the importance of detecting LTBI upon entering the prison and following those who are negative each year, offering LTBI treatment to new converters. Cytokine and chemokine concentrations can be used to understand transmission dynamics and potentially identify people at high risk for developing active TB. Gene expression allows us to study the events that occurred after recent infection with M. tuberculosis; in addition to that related mainly to immune expression and cell cycle pathways, it could open new research opportunities for the search for biomarkers or therapeutic targets.

Keywords: active tuberculosis, latent tuberculosis infection, RNA sequensing, prisoners, biomarkers

1 CAPITULO 1

INTRODUCCIÓN

1.1 PLANTEAMIENTO DEL PROBLEMA

La tuberculosis (TB) es una enfermedad causada por la bacteria *Mycobacterium tuberculosis,* y es uno de los principales problemas de salud a nivel mundial, siendo la enfermedad transmisible con mayor mortalidad y ubicándose dentro de las diez enfermedades mas mortíferas en todo el mundo. Aunque se considera que la TB está disminuyendo año tras año a nivel mundial y en todas las regiones, en el 2018 se calculó que 10,0 millones de personas contrajeron la enfermedad y 1,2 millones fallecieron por esta causa[1,2]. Además, se estima que un 23% de la población, es decir, 1.7 billones de personas están infectadas (pero no enfermas) por *M. tuberculosis*, lo que se conoce como TB latente[3].

Entre el 5-10% de las personas recientemente infectadas con la micobacteria desarrollan TB activa en los 2-5 años subsecuentes a la exposición[4]; el porcentaje restante que queda con TB latente tiene el 5-10% de probabilidad de desarrollar TB activa a lo largo de su vida; este porcentaje puede aumentar al 10% por cada año de vida en las personas que tienen algún tipo de inmunosupresión. El resto de las personas quedarán infectados por el bacilo tuberculoso, sin exhibir enfermedad activa, pero con el riesgo de una reactivación en los años posteriores[2,5], lo que los convertirá en nuevas fuentes de infección para otros hospederos susceptibles.

Las personas que tienen mayor riesgo de adquirir y desarrollar TB son las personas con sistemas inmunes debilitados, por ejemplo aquellas con el virus de la inmunodeficiencia humana (VIH/SIDA) (RR*=20,0), diabetes (RR=3,10), silicosis, cáncer, las que se encuentran en tratamiento con corticoesteroides, los fumadores (RR=2,00), y las personas que han tenido contacto reciente con la micobacteria, es decir, aquellas que trabajan o residen con pacientes con TB (RR=12,40), o en instituciones como hospitales, albergues

* RR= Riesgo relativo calculado teniendo como base el riesgo de la población general.

para desamparados, centros correccionales o prisiones (RR=23,00), asilos para ancianos y residencias para personas con VIH[6–8].

Entre los grupos con mayor prevalencia de TB latente y activa, y con mayor riesgo de contagio (al menos un tercio de ellas se infectan en los dos primeros años de post-exposición) se encuentran las personas privadas de la libertad (ppl), quizás, por las condiciones en las que habitan: consumo de narcóticos y de cigarrillo, hacinamiento, alta incidencia de TB activa y latente, instalaciones con insuficiente ventilación, higiene y saneamiento; comida nutricionalmente deficiente; servicios de salud inexistentes o precarios que permite largos periodos de infección o enfermedad sin diagnóstico y tratamiento inadecuado, entre otros[9].

La prevalencia de TB activa en las prisiones es de 3 a 100 veces más alta que lo reportado en la población general[9], lo que conlleva a una frecuente y prolongada exposición de las ppl a la micobacteria. En estudios realizados alrededor del mundo las prevalencias de TB activa en cárceles son mucho más altas que las reportadas en la población general. En ppl en la antigua Unión Soviética la prevalencia de TB activa fue de 5995/100.000[10] prisioneros, en China de 1556/100.000[11], y en Etiopia de 2139/100,000[12]. En Brasil se han reportado prevalencias de 917/100,000 ppl[13] y 1,236 cases/100,000[14]; y en Colombia tres estudios muestran una situación de TB activa similar a otros países: 1. En Medellín en dos centros penitenciarios, la incidencia de TB en la prisión uno fue entre 353 y 500/100.000 prisioneros, mientras que en la prisión dos fue de 517/100.000 prisioneros[15]; 2. En un Centro penitenciario en Guaduas la prevalencia de TB activa fue de 1026 /100.000 prisioneros[16]; y 3. La evaluación de 10 centros penitenciarios en el departamento de Tolima en el 2013 mostró una incidencia de 244,2/100.000 reclusos[17]. Todas las tasas en prisiones en Colombia son más altas que las de la población general (25-30/100.000).

En el caso de la TB latente, los porcentajes varían de acuerdo al país: 3% en Suiza, 40,3%[18]-54.6%[19] en España, 17,2% en Italia[20], 6.3%-18%[21] en Estados Unidos, 54,2% en Nigeria[20] y 81% en Malasia[22]. Para el caso de Brasil, la prevalencia de TB latente varía entre 11,7%-22,5% en los reclusos antiguos y 8,6% en los reclusos nuevos, y se incrementó en un 5% por cada año de reclusión en la cárcel[13]. En un centro penitenciario de Medellín

se encontró una prevalencia de TB latente de 77,6%[15], y en Guaduas de 67,6%, ambas superiores cuando se comparan con la población originaria de los casos de TB (42,7%) y con convivientes de casos con TB (65,9%) de la ciudad de Medellín[23]. Así mismo, la incidencia de TB latente reportada en un centro penitenciario de Medellín fue de 29.2% a los dos años de seguimiento, lo cual indica que un tercio de las ppl que eran negativas para tuberculina se infectaron dentro de la cárcel[24].

Debido a la presentación asintomática de la TB latente, los hospederos infectados por *M. tuberculosis* pasan desapercibidos, y una vez desarrollan TB activa, se convierten en una fuente importante de infección para otros hospederos susceptibles[3]. Actualmente existen dos métodos validados para el diagnóstico de TB latente: la prueba de Tuberculina y los IGRAs (por su nombre en inglés *Interferon-Gamma Release Assays*), sin embargo, ambas pruebas son imperfectas al momento de realizar el diagnóstico de infección tuberculosa y pueden dar resultados discordantes entre sí[25]. Teniendo en cuenta esto, es necesario desarrollar nuevas y mejores metodologías diagnósticas, pero para ello hacen falta investigaciones que permitan reconocer nuevos blancos moleculares o biomarcadores para diseñarlas[26].

La adquisición de *M. tuberculosis* luego de la exposición incrementa el riesgo de desarrollar TB activa en los siguientes dos años post-infección, por tanto, es fundamental poder establecer los factores asociados con la infección reciente y quienes tienen mayor riesgo de enfermar entre las personas previa y recientemente infectadas. A pesar de que se han realizado estudios al respecto, su principal limitación es no poder establecer el momento o el tiempo de infección que tiene un individuo, que al ser positivo en la tuberculina o el IGRA, la infección pudo haberse adquirido días, meses o incluso años antes.

Uno de los retos de las investigaciones en diagnóstico de TB latente es entender la dinámica molecular e inmunológica de la infección y la enfermedad tuberculosa y la identificación de biomarcadores (del hospedero y la micobacteria) indicativos de los diferentes estados de la enfermedad[27]. Dada la complejidad de la patogenia de *M.*

tuberculosis en el interior del huésped humano, se han investigado diferentes citoquinas del hospedero que influyen en la progresión de la infección tuberculosa. Inicialmente el INF fue la citoquina estudiada por excelencia, pero al comprender su incapacidad para diferenciar TB activa de latente, empezaron a estudiarse otras citoquinas como la IL-2, la proteína IP-10, la IL-17, entre otras[28,29]. Hasta la fecha varias citoquinas muestran resultados alentadores para diagnosticar la infección latente; sin embargo, estudiarlas de manera individual parece no tener los mejores resultados. Por este motivo, se han empezado a estudiar grupos de citoquinas tratando de establecer cuales de ellas serían los mejores biomarcadores; algunas citoquinas evaluadas son el INF-γ, el TNF-α, y las interleucinas IL-2, IL-10, IL-36, IL-1, IL-4, IL-13, IL-17, IL-22[30–38]. El conocimiento actual permite conocer que muchas citoquinas pro y anti inflamatorias, producidas por diversos tipos celulares, están involucrados en el control de la infección tuberculosa [35], de esta manera, encontrar un patrón de disminución y aumento entre dichas sustancias, puede ayudar a establecer un perfil que indique de forma discriminatoria la presencia de infección latente, progresión a enfermedad o enfermedad activa establecida.

Se sugiere que la infección reciente por *M. tuberculosis* tiene una señal inmune plasmática y un patrón de expresión genético único que permite identificar las personas recientemente infectadas y por ende con mayor riesgo de desarrollar TB activa. La posibilidad de incluir en el estudio individuos en todo el rango de la historia natural de la enfermedad, es una de las principales fortalezas de esta investigación. Poder focalizar los esfuerzos en aquellas personas con mayor riesgo de adquirir la infección por *M. tuberculosis* y desarrollar la enfermedad disminuiría la transmisión en los centros penitenciarios, y esto podría extrapolarse a otros lugares con alta incidencia de infección.

Para finalizar, y teniendo en cuenta el panorama anteriormente planteado, nos preguntamos: si estudiamos personas no infectadas por *M. tuberculosis* (tuberculina negativa), las cuales ingresan a un centro penitenciario donde se presentan todas las condiciones para que se dé la trasmisión de la bacteria, y que además, este lugar tiene alta incidencia y prevalencia de TB activa y latente, ¿será posible detectar diferencias en la expresión de citoquinas plasmáticas y en los genes pro inflamatorios en los conversores en la prueba de la tuberculina, los cuales puedan ser potencialmente utilizados como biomarcadores

diagnósticos, factores protectores o de riesgo, comparado con personas que no convierten la tuberculina (permanecen negativos) y aquellos con tuberculosis activa?.

1.2 JUSTIFICACIÓN

La TB es una enfermedad vigente en todo el mundo, y a pesar de que ha disminuido siguen apareciendo nuevos casos principalmente en las poblaciones vulnerables: los pobres, los migrantes y las personas recluidas[39], en quienes el control de la enfermedad puede ser más complejo dada la dificultad para acceder a los servicios de salud.

Debido a esto, organizaciones gubernamentales nacionales e internacionales, invitan a los países y la comunidad a continuar trabajando con el fin de disminuir la carga mundial de TB. La OMS y la OPS en su estrategia "Fin de la Tuberculosis" y la Organización de las Naciones Unidas en el objetivo 3 de Desarrollo Sostenible, proponen acabar con la epidemia mundial de TB, es decir, reducir las muertes en un 95% y los nuevos casos en un 90% entre 2015 y 2035[40–42]; de igual forma, el Plan Decenal de Salud Pública colombiano tienen como meta controlar la TB en todo el territorio nacional, reduciendo progresivamente la mortalidad a menos de 1,59 casos por 100.000 habitantes para el 2021[43].

Según la OMS, en el 2018 habían 1.7 billones de personas infectadas, pero no enfermas por la micobacteria[44]. Los individuos con TB latente tienen entre el 5-10% de riesgo de desarrollar TB activa durante el trascurso de su vida, pero el mayor riesgo de enfermar se presenta durante los dos primeros años luego de la infección. Así, identificar a las personas recientemente infectadas, es fundamental para el control y la eliminación de la TB, pues se previene que nuevos individuos desarrollen la enfermedad y de esta manera, la mortalidad y la trasmisión, controlando la diseminación de la infección a otros individuos susceptibles[45].

Poder individualizar a cada persona bajo un perfil de riesgo de infección o de enfermedad, e identificar los conversores recientes en la prueba de tuberculina sería de gran utilidad, especialmente en lugares como los centros penitenciarios, los cuales tiene presencia de

hacinamiento, alta prevalencia de TB, retraso en el diagnóstico y otros factores de riesgo importantes para la infección y la enfermedad tuberculosa[46]. En los centros penitenciarios los conversores tempranos tienen el mayor riesgo de desarrollar la enfermedad activa, y una vez enfermos, de propagar la enfermedad no sólo a los compañeros, sino también a la comunidad[47,48].

Los individuos infectados por *M. tuberculosis* pasan desapercibidos ante las pruebas diagnósticas microbiológicas convencionales[49], pero pueden dar un resultado positivo en la prueba de IGRAs y en la tuberculina. Sin embargo, ambas pruebas son imperfectas al momento de realizar el diagnóstico de infección tuberculosa[50], siendo su principal limitación la incapacidad para diferenciar entre TB latente y TB activa, ya que se obtiene un resultado positivo en ambas presentaciones clínicas[51]. Es por esto que se hace necesario buscar nuevos biomarcadores que ayuden a diferenciar las personas infectadas de las enfermas; así, al mejorar el diagnóstico se puede establecer un control efectivo de la epidemia de TB[51]. Los investigadores han enfocado sus esfuerzos en el descubrimiento de biomarcadores procedentes del hospedero que permitan entender la patogénesis de la infección, diagnosticar la infección y enfermedad tuberculosa, monitorear el tratamiento y mirar desenlaces como curación o recaída[52,53].

Las nuevas tecnologías bioinformáticas permiten conocer posibles biomarcadores de infección o enfermedad, pero adicionalmente, pueden ayudar a entender porqué dos personas en igual condición de exposición a *M. tuberculosis* tienen diferentes tiempos de infección. Poder identificar cómo difieren los trascriptos (proteínas y mRNA) entre las personas sanas, y las que tienen TB latente o TB activa, puede mejorar el diagnóstico, el entendimiento de la respuesta inmune protectora frente a antígenos micobacterianos específicos, y esto a su vez, permite el estudio de nuevos blancos moleculares candidatos para vacunas[54].

Las investigaciones recientes que involucran la expresión de RNA sin duda ayudan a entender la infección tuberculosa. Las nuevas investigaciones en este campo deben estar encaminadas a la identificación de una señal inmune protectora o de riesgo frente a la

infección por *M. tuberculosis*; el diseño de investigaciones prospectivas, en las cuales se realice un seguimiento a individuos expuestos a la bacteria permitiría: 1. Comparar individuos infectados versus individuos que permanecen libres de infección; 2. Sujetos que se enferman versus aquellos con infección latente, tratando de identificar la respuesta de susceptibilidad o resistencia a la enfermedad; y 3. individuos infectados por largos periodos de tiempo versus aquellos que desarrollan de forma temprana la enfermedad (en los dos primeros años) con el fin de identificar la respuesta necesaria para contener la infección[54].

Pero a pesar de los grandes avances al respecto, algunas dificultades se han detectado en los estudios que asocian la expresión génica y de citoquinas/quimioquinas con el estado de infección tuberculosa. Recientemente algunos artículos[55,56] mencionan las heterogeneidades entre los estudios publicados que hacen más difícil la comparación entre ellos, y lo más importante, no permiten extrapolar sus conclusiones. Teniendo en cuenta que la TB puede ser albergada durante muchos años, y que ésta es heterogénea en su presentación, su gravedad y duración, las mayores dificultades y discordancias encontradas entre los estudios son:

- La incapacidad para estimar la exposición a la micobacteria en los controles. Se han utilizado controles positivos en la tuberculina, controles positivos para la producción de interferón gamma, convivientes no afectados y controles de la población, estos últimos, aunque no tienen nexo epidemiológico evidente, pueden vivir en lugares de alta prevalencia, haber estado expuestos a la micobacteria y tener un perfil protector a la infección.
- El diagnóstico de la TB. Se han utilizado cultivos en diferentes medios sólidos y líquidos; coloración para bacilos ácido-alcohol resistentes (BAAR), respuesta al tratamiento y/o evidencia de enfermedad en la radiografía de tórax.
- Clasificación del órgano afectado. No existen diferencias al evaluar pacientes con TB pulmonar o extrapulmonar.
- La cepa específica de *M. tuberculosis* que infecta a una persona pueden influir en la gravedad de la enfermedad[57].

- La edad de inicio de la enfermedad puede tener un impacto en la heterogeneidad entre los estudios publicados. Se ha publicado que la susceptibilidad genética difiere entre la población pediátrica y la población adulta, o entre la TB primaria y la reactivación de la enfermedad[58]
- El tiempo desde la adquisición de la infección latente hasta la conversión a TB activa puede ser variable entre los individuos. Por lo tanto, agrupar a todos los infectados en el mismo grupo de TB latente puede eliminar los tiempos más interesantes de la infección: la adquisición temprana y la transición a la enfermedad activa.
- Al realizar la inclusión de participantes y el análisis de los resultados, en la mayoría de los reportes no se realizan ajustes (o al menos no se mencionan) por factores que pueden comportarse como confusores, por ejemplo el sexo, la edad, la etnia, algún tipo de inmunocompromiso o alteraciones inflamatorias inespecíficas[59], el tiempo de infección o enfermedad, entre otros, situación que puede alterar los resultados obtenidos.
- En los estudios de expresión génica para búsqueda de señales exclusivas de TB activa, se han observado diferentes perfiles transcripcionales, debido posiblemente a la heterogeneidad en el tipo de diseño del estudio, en las características demográficas de los participantes, en el sitio y duración de la enfermedad, el tiempo de tratamiento y las técnicas utilizadas para detectar el perfil transcripcional[60].

Los aspectos antes mencionados y la iniciativa de instituciones como la OMS que incentivan a las comunidades académicas a realizar nuevas investigaciones para el desarrollo de nuevas pruebas diagnósticas para infección tuberculosa, basadas en biomarcadores que identifiquen las bacterias en estado latente[61], justifican en gran medida el desarrollo de este proyecto.

1.3 MARCO TEÓRICO Y CONCEPTUAL

1.3.1 Tuberculosis

El término tuberculosis (TB) es usado para describir un amplio espectro de enfermedades causadas por la bacteria *M. tuberculosis,* la cual afecta principalmente el pulmón, causando

lo que se denomina TB pulmonar. *M. tuberculosis* es un bacilo aerobio, de crecimiento lento (tiempo de generación 15-20h), no formador de esporas, inmóvil y con un alto contenido lipídico en la pared celular. El bacilo de la TB es transmitido de persona a persona por medio de aerosoles que salen de las personas enfermas e ingresan a hospederos susceptibles[52].

1.3.2 Determinantes de la infección por *M. tuberculosis*

Existen personas que progresan rápidamente a infección tuberculosa, y otros, que a pesar de la exposición no se infectan, y si se infectan no se enferman. Se ha demostrado que existen factores microbianos y no microbianos, que determinan el proceso de exposición-infección-enfermedad. Dentro de los factores microbianos se han descrito: la virulencia, la viabilidad, la dosis infectante y el perfil de resistencia a medicamentos de la cepa infectante[56]. Entre los factores no microbianos se describen: la duración e intensidad de la exposición[62], el sexo[63], el estado inmune y la presencia de comorbilidades en los hospederos, tales como la desnutrición y la diabetes, y algunos factores medioambientales como la ventilación y el hacinamiento[64].

1.3.3 Patogénesis de la enfermedad

Una vez se presenta el contacto entre una persona enferma y un hospedero susceptible, ingresan al organismo los aerosoles conteniendo las micobacterias, ubicándose usualmente en la zona media del pulmón y en el espacio subpleural. Ya instaladas en el pulmón, las bacterias son ingeridas por los macrófagos alveolares, los cuales eliminan un pequeño número de los bacilos; sin embargo, la multiplicación bacteriana puede, en algunos casos, superar la respuesta de los macrófagos alveolares[65].

En el microambiente pulmonar, *M. tuberculosis* interactúa con células del epitelio respiratorio, macrófagos alveolares, células dendríticas, linfocitos y neutrófilos[66]. La interacción de la bacteria con estas células estimula la producción de citoquinas/quimioquinas proinflamatorias como el factor de necrosis tumoral alpha (TNF-α) y el interferón gamma (IFN-γ), IL-10, IL-18, IL-1β, IL-12 (figura 1). Estas células también aumentan la producción de moléculas antimicrobianas como las beta-defensinas, la sustancia surfactante, el oxido nítrico sintasa 2 (NOS_2) y otras moléculas que ayudan a

eliminar las micobacterias o a aumentar la función antimicrobiana de los macrófagos infectados. Adicionalmente, una vez internalizadas en el fagosoma las micobacterias bloquean la maduración, la fusión y la acidificación lisosomal[67].

Una vez establecida la infección pueden ocurrir tres circunstancias mediadas por la producción de citoquinas y similares: 1. Que los individuos queden con una **infección latente**, es decir, que sean capaces de contener la infección durante largos períodos de tiempo y permanezcan libres de síntomas[65]. 2. Que la respuesta inmune del huésped no sea suficiente (días, semanas o meses después de la infección) y ocurra la **TB activa** causando necrosis, cavitaciones y esparcimiento de la infección a los bronquios. Las razones de la disminución en la capacidad para contener la infección incluyen, la infección por el VIH, la malnutrición, el uso de medicamentos inmunosupresores, incluyendo los esteroides[68], la alta carga bacteriana, el incremento en la virulencia de la bacteria, y la susceptibilidad genética[67]. Por último, 3. Que las personas hagan una **infección abortiva**, es decir, que realicen una respuesta inmune innata exitosa y quede libres de la infección.

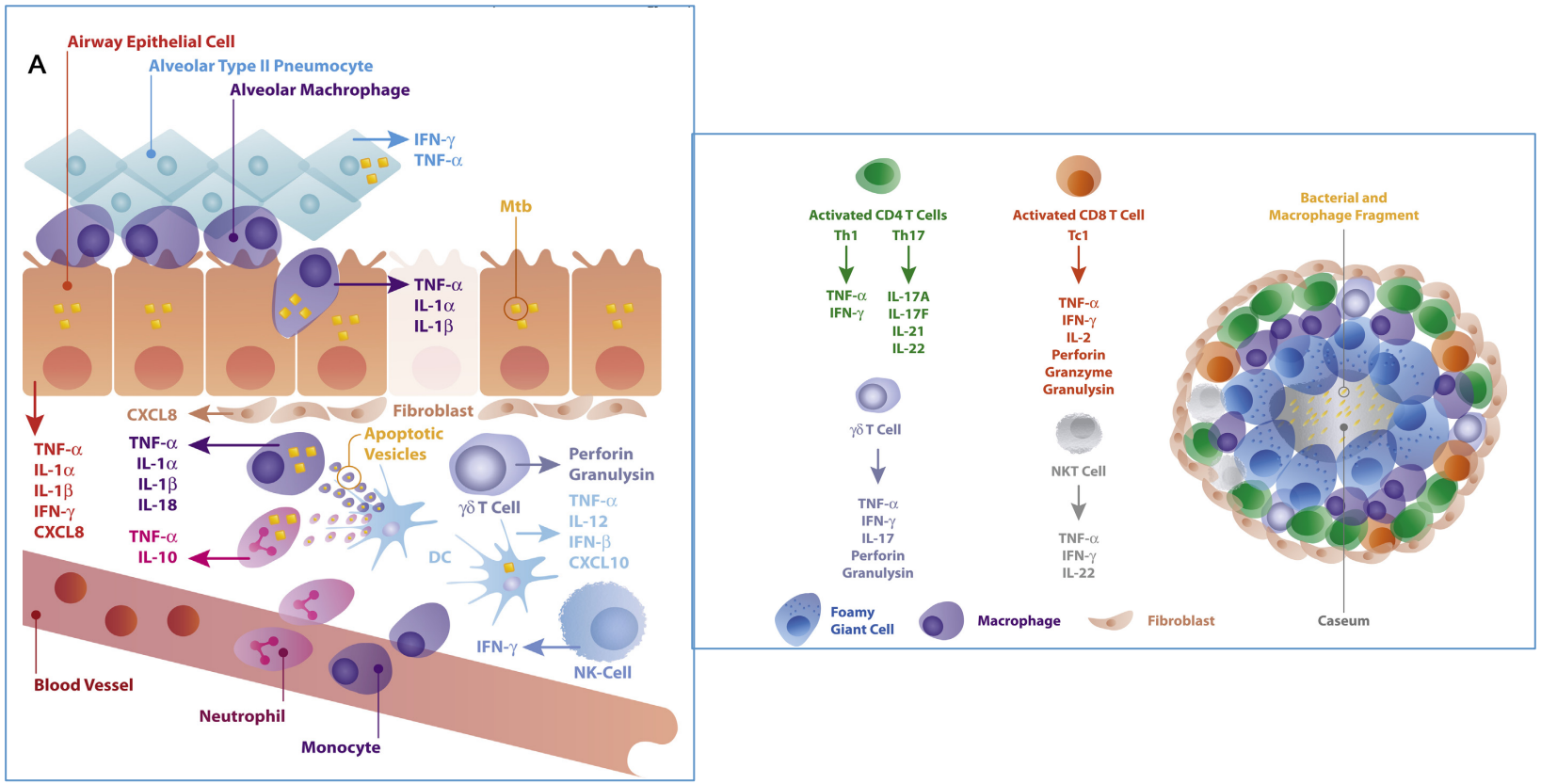

Figura 1-1. Citoquinas/quimioquinas involucradas en la respuesta inmune frente a la infección tuberculosa inicial y tardía. Tomado de[66]

1.3.4 Diagnóstico de TB latente.

Debido a la presentación asintomática de la TB latente, realizar el diagnóstico oportuno de infección latente se convierte en un reto para todos los programas de salud pública enfocados en la disminución y el control de la enfermedad.

Actualmente existen dos métodos para el diagnóstico de TB latente: 1. Los IGRASs, de los cuales existen dos pruebas comercialmente disponibles: el QuantiFERON-TB Gold In-Tube test (QFT-GIT) y el QuantiFERON-TB Gold (QFT-G), que miden la cantidad de INF-γ expresado por las células T, comparado con un control, luego de la exposición a los antígenos específicos de *M. tuberculosis:* ESAT-6 (early secreating antigen 6), CFP 10 (culture filtrate protein 10), y la proteína TB7.7 (también conocida como Rv2654c (104). 2. La prueba de Tuberculina está basada en la respuesta de hipersensibilidad retardada a *M. tuberculosis*[69]. Se inyectan intradérmicamente 0,1 ml (5 UI) de tuberculina (purified protein derivative - PPD) en la superficie de flexión de la parte superior del antebrazo y se examina, una vez transcurridas 48-72 horas, se considera positiva si alrededor del punto de inyección se observa una zona de induración de más de 10 mm de diámetro en personas inmunocompetentes y más de 5 mm de diámetro en inmunosuprimidos[70]. Un resultado negativo se debe confirmar entre 1 a 8 semanas después (Figura 2).

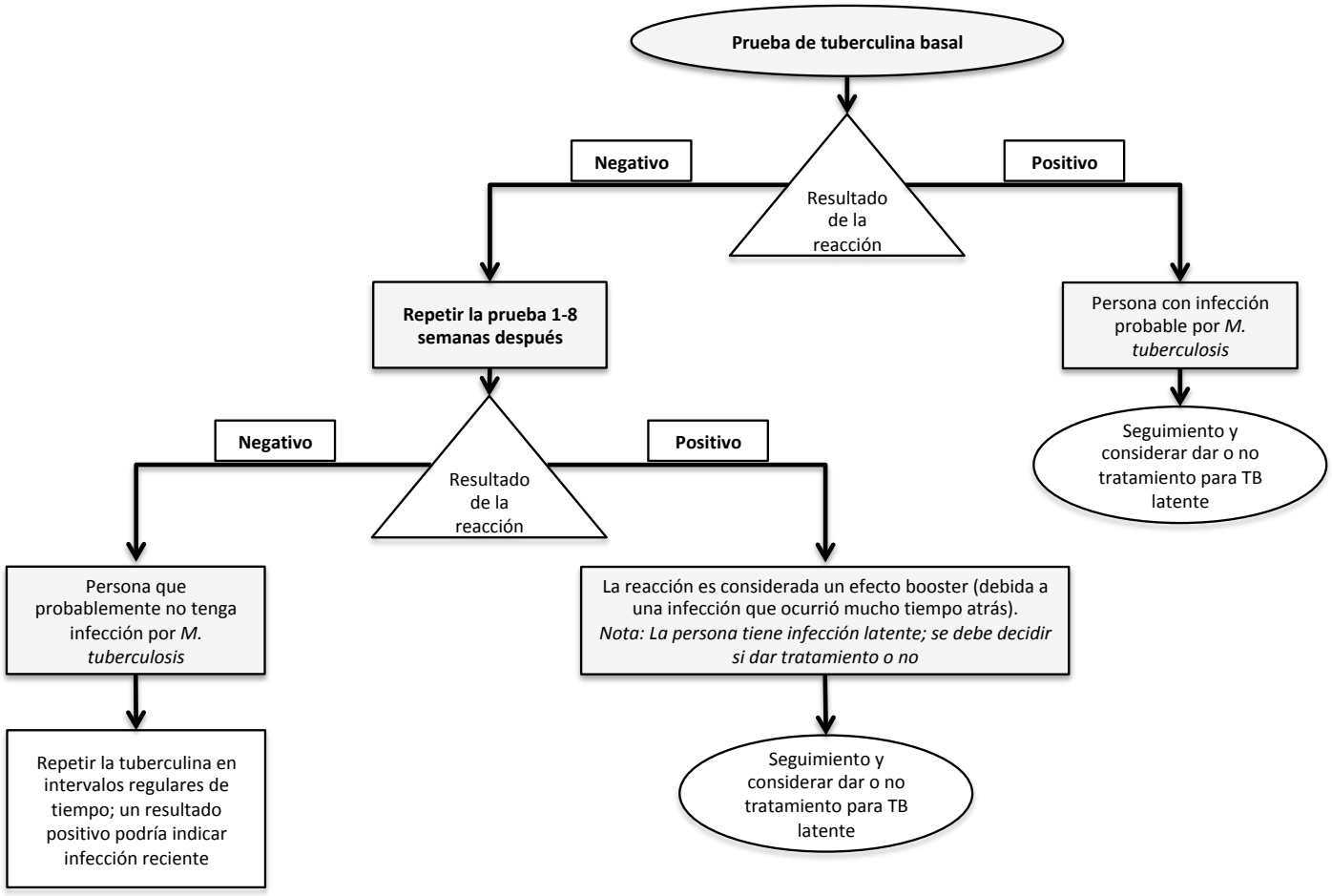

Figura 1-2. Flujograma de diagnóstico de TB latente utilizando la prueba de tuberculina. Adaptado de los CDC[71]

Sin embargo, la tuberculina y los IGRAs son imperfectos al momento de diagnóstico de infección tuberculosa, pues no distinguen entre infección enfermedad activa, y además, pueden dar resultados discordantes entr tuberculina puede dar resultados falsos positivos cuando la vacuna BCG ha sid o cuando existe una infección por micobacterias no tuberculosas[73]; y pued falsos negativos en al menos el 20% de las personas con tuberculosis activa en pacientes con tratamiento con corticoides, sarcoidiosis, enfermedad reticul inmunosuprimidos y en aquellos recientemente vacunados con virus v sarampión o varicela[50]. Los IGRAs por su parte, presentan baja reprodu variable sensibilidad[74,75]; reacción cruzada con micobacterias ambientales *kansasii, M. szulgai, y M. marinum*[76]; un resultado que indica conversión no es con infección o exposición reciente y existe una inexplicable variabilidad realizan pruebas consecutivas en ciertos grupos poblacionales[50,77]; además, la s es más baja en comparación con la tuberculina en pacientes con diabetes mell tanto, aunque un resultado positivo es muy importante para hacer el diag resultado negativo en ambas pruebas, no excluye la infección por *M. tuberc* tabla 1.

Tabla 1-1Ventajas y desventajas de la prueba de tuberculina y los IGRAs. Adaptado de[71,79–81].

Característica	TST	IGRAs	Comenta
Discrimina TB latente de TB activa	No	No	Ambas dan resul positivos
Estudio	In vivo	Ex vivo	N/A
Infraestructura	Poca	Laboratorio y equipos	La tuberculina n ningún equipo especializado y p realizada en trab campo.

Interpretación del resultado	Subjetivo	Objetivo	Aunque ambas pruebas requieren experiencia para llevarlas a cabo, la lectura de la tuberculina puede verse afectada por la experiencia del lector
Disponibilidad del resultado	48-72 h, y hasta 3 semanas	24 horas	Hasta tres semanas en la tuberculina cuando es necesaria la aplicación del booster.
Visitas requeridas	2	1	El IGRAs es recomendado en poblaciones con poca tasa de retorno a la visita de lectura.
Influenciado por vacunación previa con BGC	Si	No	La tuberculina se ve afectada si se aplica cercana a la vacuna. En Colombia se aplica en el momento del nacimiento y múltiples estudios sugieren que después de 10 años de la vacunación no se afecta el resultado de la tuberculina.
Recomendación para niños menores de 5 años.	Alta	Baja	No hay suficientes datos para la recomendación de los IGRAs.
Recomendaciones para realizar pruebas seriadas	Alta	Baja	IGRAs con baja reproducibilidad, conversiones y reversiones al realizar pruebas seriadas,

			por tanto, a la fecha no se recomienda su aplicación para hacer seguimiento.
Reacción cruzada con micobacterias ambientales	Si	Si	N/A
Identificación de progreso de la infección a enfermedad	No	No	N/A
Costos	Bajo	Alto	Comparando ambas pruebas.

Cabe mencionar que a pesar de las limitaciones antes mencionadas y de que están fase de prueba otras metodologías para el diagnóstico de TB latente, como por ejemplo el C-Tb[82,83] y el Diaskintest[84] (ambas, pruebas cutáneas basadas en la respuesta a los antígenos ESAT-6 y CFP-10), la tuberculina y los IGRAs continúan siendo las pruebas diagnósticas de referencia para infección latente, pues las nuevas pruebas aún están en fase de investigación, no han sido validadas, y se desconoce su desempeño en la población general y en los grupos de alto riesgo para TB.

1.3.5 Investigaciones recientes usando las sustancias inmunes como biomarcadores

La complejidad del proceso de exposición-infección-enfermedad no está suficientemente explicada por los factores genéticos del hospedero, pues existen interacciones genes-genes, genes-ambiente, que también están involucrados en el proceso de expresión[56]. Aparecen entonces la proteómica y la transcriptómica como respuesta a estas dificultades. Estas técnicas han sido utilizadas para el estudio de las micobacterias y su interacción con los hospederos con resultados prometedores[85]. Las conclusiones de estas investigaciones sugieren que las ciencias ómicas pueden ser de gran importancia para entender la micobacteria, su patogénesis, su virulencia y su interacción con las células del hospedero,

pero además, puede tener gran impacto para el desarrollo de nuevos medicamentos, vacunas y biomarcadores, pues con su uso se podrían encontrar nuevas moléculas blanco[85].

En la búsqueda de nuevos biomarcadores, citoquinas antiinflamatorias, proinflamatorias, y otras proteínas (como las quimioquinas), se han estudiado de forma individual o conjunta, en suero, plasma y sobrenadante de células estimuladas o no de pacientes con TB activa, TB latente y en pacientes curados, entre las que se destacan el INF-γ, el TNF-α, las interleucinas IL-2, IL-10, IL-36, IL-1, IL-4, IL13, IL-17, IL-22, factores de crecimiento, y la Granzima A[30–32 86–88].

En Colombia, teniendo en cuenta que es importante estudiar el papel que juegan las citoquinas o los componentes inmunes, para luego asociarlas con perfiles inmunes de susceptibilidad o protección frente a la TB, y su implicación en el diagnóstico, Marin DN y colaboradores estudiaron la expresión de marcadores celulares y de citoquinas en pacientes con TB activa, TB latente y personas no infectadas; evidenciando que la expresión de los marcadores CD27 y CD45RO es diferencial en las células CD4+ y CD8+. Además, los resultados mostraron que células CD4+ provenientes de personas con infección latente, tenían una mayor expresión de IFN-γ^{+}, comparada con las personas sanas y una mayor expresión de IL-17 en las células provenientes de personas con TB activa, comparada con pacientes con TB latente[89].

Adicionalmente Niño V. y col., evidenciando que el estudio de la respuesta inmune en individuos infectados y no infectados con la micobacteria puede ser critica para identificar biomarcadores o perfiles de protección y riesgo para la enfermedad, evaluaron la expresión in vitro de 4 citoquinas (IL-2, IL-12p70, IL-15 e IL-10) a partir de sobrenadantes de cultivos de celulares estimulados con antígenos micobacterianos; encontrando que los niveles de IL-2 y IL-12p70 eran mayores en los cultivos procedentes de personas con TB activa y latente que en individuos sanos[90].

Por otro lado, se ha propuesto el uso de citoquinas como moléculas para el diagnóstico de infección o enfermedad tuberculosa. Una de las citoquinas más evaluada como posible biomarcador para identificar la infección latente es la proteína IP-10 (Proteína 10 inducida

por interferón gamma). El estudio de esta quimioquina se ha realizado en pacientes (adultos[91,92] y niños[93]) con TB activa, TB latente, y personas sin infección, con diferentes estados de inmunosupresión, luego de estimulación celular con antígenos micobacterianos o directamente de sangre y orina; y aunque los resultados parecen prometedores no superan a los alcanzados utilizando la medición de INF, es decir, no pueden diferenciar enfermedad de infección, incluso intentando medir la expresión del ácido ribonucleico mensajero (mRNA)[94].

De forma similar, la IL-2 ha sido una citoquina bastante estudiada[95–97], cuyos resultados fueron resumidos en una revisión sistemática con meta análisis conducida por S Mamish y col., en la cual reportaron que esta citoquina es un muy buen biomarcador para distinguir infección latente, que cuenta con una sensibilidad del 81% y una especificidad del 95%, y que además, puede mejorar el rendimiento del INF cuando se usan en conjunto[28]. Cuando se evaluó el uso en trabajadores de la salud, una población altamente expuesta a la infección, la IL-2 mostró tener un buen resultado, incluso para potencialmente clasificar los individuos con resultados discordantes entre la tuberculina y los IGRAs[98]. A pesar de esto, se requieren estudios de validación en diferentes poblaciones para su implementación.

Teniendo en cuenta que la búsqueda de una sola citoquina como biomarcador ha sido infructuosa, investigar múltiples citoquinas se ha convertido en la opción a tener en cuenta. Dentro de los grupos de citoquinas estudiadas, el INF-γ aparece de forma repetitiva, pues su función para el control de la infección tuberculosa ha sido ampliamente estudiado. Kim y col. estudiaron en pacientes con TB activa, tuberculina positiva y personas sin infección seis citoquinas, encontrando que el IFN-γ, IL-2, IL-10 y la IL-13 eran potenciales biomarcadores para distinguir infección tuberculosa, y aunque su objetivo inicial era distinguir TB activa de TB latente, este objetivo no se pudo cumplir[30].

Así mismo, Stherlan y col. evaluaron en personas con TB activa, y contactos infectados y no infectados, la producción de siete citoquinas luego de la estimulación de células sanguíneas con tres tipos de antígenos micobacterianos. Los autores concluyen, que el INF-α, la IL-12(p40) y la IL-17 pueden discriminar entre personas con TB activa y TB

latente, cuando las células son estimuladas por siete días con el antígeno TB10.4 (un antígeno esencial para la virulencia de *M. tuberculosis*)[99].

A pesar de estas y otras publicaciones[29,100,101] y sus resultados prometedores, aún no se han podido establecer conclusiones al respecto, y en su mayoría los resultados son inconsistentes, quizás, por la variabilidad entre las metodologías y las poblaciones evaluadas (ver justificación, limitaciones de los estudios y capítulo 3). Además, cabe resaltar que la mayoría de las investigaciones buscan la identificación de biomarcadores que ayuden a diagnosticar la enfermedad y no la infección tuberculosa, y además, los autores evidencian la necesidad de realizar estudios de cohorte para ver la evolución de la enfermedad.

Intentando diagnosticar la infección tuberculosa, y elucidar los factores del hospedero que están involucrados en el proceso y el progreso de exposición-infección-enfermedad, aparecen las nuevas técnicas de expresión génica. Han sido varias las metodologías empleadas para evaluar la expresión de RNA de personas con tuberculosis: citometría de flujo de RNA[102], PCR transcriptasa reversa (RT-PCR)[103], RT-PCR en tiempo real[104], microensayos[105], y la secuenciación del RNA (RNAseq)[106]. Evaluar la expresión génica no solo ayuda a buscar novedosos blancos moleculares diagnósticos, sino también a entender la compleja respuesta inmune frente a *M. tuberculosis*, entendiendo el papel de los diferentes tipos de RNAs.

1.3.6 La expresión genética como herramienta diagnóstica

El transcriptoma se define como la totalidad de los transcriptos (RNA) de una célula y su cantidad en un momento dado del desarrollo o condición fisiológica. Entre las principales razones para conocer el transcriptoma se encuentran: interpretar la función del genoma, revelar las moléculas que participan en la construcción de células y tejidos, la regulación celular, y entender el desarrollo de las enfermedades entre otras. La variación de la expresión de genes (transcriptoma) durante los procesos de enfermedad es aprovechada como herramienta diagnóstica[107].

Los métodos para evaluar la expresión genética se pueden subdividir en dos grupos: el primero grupo se refiere a técnicas que hacen medición de genes individuales, como la PCR transcriptasa reversa (RT-PCR y RT-PCR cuantitativa [qPCR]) y el *northern hybridisation*. En el segundo grupo se encuentran las técnicas que realizan detección masiva de genes, como por ejemplo los microarreglos, que evalúan entre cientos a miles de genes pre-seleccionados usando la tecnología de hibridación por sondas, y el RNA sequencing (RNA-seq) que evalúa la expresión celular global y es capaz de identificar miles de genes simultáneamente al realizar la secuenciación del RNA total o el RNA mensajero (mRNA)[108].

Dentro del primer grupo, los estudios más básicos han intentado detectar la expresión de unas pocas moléculas[59,104] y diferenciar pacientes con TB activa de individuos con TB latente e individuos "sanos", infectados o no por el virus de la inmunodeficiencia humana[109]. Kim et al, luego de medir la expresión de mRNA del IFN-γ, expandieron su experiencia midiendo la expresión de ciertas citoquinas/quimioquinas utilizando RT-PCR a partir de células sanguíneas incubadas con antígenos micobacterianos. Una combinación del factor de necrosis tumoral alpha (TNF-α) con IL-2R, CXCL9, y CXCL10 fue capaz de identificar entre TB activa y latente; la sensibilidad del TNF-α, el IL-2R, y la IP-10 en personas con TB activa fue de 96,43%, 96,43%, y 100%, respectivamente, y en personas con infección latente fue de 86,36% y 81,82% para la IL-2R y la IP-10, respectivamente. Los autores sugieren realizar nuevos estudios para considerar, entre otros aspectos técnicos, la estabilidad del RNA durante el transporte de la muestra y los criterios de inclusión de la población de estudio[59].

En otro estudio se evaluó la expresión de 85 genes usando PCR y 27 sustancias inmunes con análisis de citoquinas basado en perlas, en células sanguíneas, en presencia y ausencia de los antígenos ESAT6 y CFP-10, entre dos grupos de pacientes: uno con enfermedad tuberculosa pulmonar y otro con enfermedad por otras micobacterias no tuberculosas. Los hallazgos mostraron que los dos grupos tenían diferente perfil de expresión, incluso luego de ajustar por otras variables como la edad, el sexo y la etnia. En las muestras no estimuladas los niveles plasmáticos de IL-12p70, IL12A y TLR9 fueron significativamente más altos en pacientes infectados por micobacterias no tuberculosas, mientras que los

niveles de IL-15, IL-8 y MIP-1α fueron más altos en aquellos con tuberculosis. En las células estimuladas las concentraciones de IFN-γ, IL-2, GM-CSF, y TNF-α, y la expresión de CCL4, CXCL10, IL-1B y TLR4 fue superior en los pacientes con TB[110].

Por otra parte, los micro RNAs se han comparado en personas sanas y con TB activa[111], adultos con TB latente y población pediátrica sana, con TB latente y TB activa[112]. En adultos, al realizar la validación de resultados previos obtenidos con microarreglos, se identificaron 27 micro RNAs en células T CD4(+) que se encontraban diferencialmente expresados en las personas con TB latente y activa, y correlacionados de forma inversa con la expresión de INF-g. Los autores evaluaron los 27 miRNAs y su participación en las vías de señalización. Entre las tantas vías de señalización, se identificaron la vía de la proteína quinasa activadora de mitógeno (MAPK), previamente asociada con la respuesta a la infección tuberculosa, así como la de receptores de matriz extracelular y la vía de señalización de GnRH[113]. Estos estudios muestran la existencia de vías específicas e interacciones complejas que son fundamentales en la patogénesis de la TB.

Con respecto a los ***métodos del segundo grupo***, aquellos que realizan secuenciación masiva, la literatura reporta ventajas que tiene el RNA-seq sobre los microarreglos. La principal ventaja radica en su alta reproducibilidad disminuyendo así la necesidad de replicas técnicas en los experimentos; además, no está limitado a un número especifico de transcriptos pues la detección no está sujeta a la presencia de una sonda; y tiene buen rendimiento para realizar la identificación y cuantificación de isoformas (genes iguales o relacionados que pueden provocar distintas formas de una misma proteína), los RNAs no codificantes y los trascriptos desconocidos[114,115]. La mayor desventaja del RNA-seq radica en la baja detección de transcriptos largos, pues dentro de la técnica existe un paso de fragmentación del RNA; ambas técnicas idealmente deben ser validadas usando qPCR o métodos de proteómica[115]. La elección de una u otra metodología dependerá del objetivo del estudio.

Varios estudios han usado microarreglos como técnica de detección de la expresión de RNA, en muestras (principalmente PBMCs y sangre periférica total) procedentes de niños[116] y adultos[117] con TB activa, TB latente, e individuos no infectados; en ambos casos se han usado los individuos infectados y sanos como controles. En pacientes con TB

activa se han reportado expresión diferencial (aumentada o disminuida) en vías de señalización del interferón[117,118], procesos biosintéticos de nucleótido trifosfato, diferenciación de osteoblastos[119], señalización de apoptosis (HSP90AA1, LRRK2, TGFBR2, FASLG, CASP8), modulación de transcripción (FOS y DDIT3), (TUBA1A y TUBB4B), y autofagia (CASP8 y TNFRSF10B)[117].

Por su parte, individuos infectados por *M. tuberculosis* tenían micros RNAs diferencialmente expresados involucrados en la vía de señalización celular fosfoinositiol-3 quinasa (PI3K-Akt)[120,121], interacción de matriz-receptor, desarrollo del sistema cardiovascular, procesos metabólicos relacionados con componentes del nitrógeno, cáncer pulmonar de células pequeñas[121], diferenciación de células hematopoyéticas, entre otras[122].

Con relación al RNA-seq, el proceso de secuenciación inicia con la extracción o liberación de RNA de las células, seguido por la selección o enriquecimiento del RNA mensajero, y la depleción del RNA ribosomal; posteriormente se realiza la formación del cDNA y la preparación de una librería. La librería es secuenciada, realizando millones de *reads* por muestras (10-50 millones) y el paso final es el análisis computacional que consiste en el alineamiento, ensamble de las secuencias, la cuantificación, filtro y normalización entre las muestras, y por ultimo, el análisis estadístico para establecer los cambios en la expresión de los individuos entre los grupos de estudio[123].

La secuenciación del RNA ha sido aplicada para dilucidar nuevos blancos moleculares para el diagnóstico de la enfermedad tuberculosa[106,124–126]y para predecir el riesgo de reactivación o enfermedad pulmonar[127,128]. La utilidad del RNA como biomarcador para el diagnóstico de TB activa fue evaluada frente a individuos sanos, los cuales también incluían individuos con TB latente, y encontraron que 24 microRNAs estaban sobre regulados y 6 disminuidos en los pacientes con TB, comparados con los tres grupos controles. Luego de validar sus resultados en un pequeño grupo de pacientes, los investigadores proponen a hsa-miR-196b y hsa-miR-376c, sustancias involucradas en la inflamación, como posibles biomarcadores para TB activa[106]. De forma similar, establecer el riesgo de TB activa por medio de una señal de mRNA exclusiva (utilizando RNA seq y RT-PCR), fue el objetivo de Daniel E Zak y col. Inicialmente establecieron una señal única de RNA, teniendo en cuenta la expresión de múltiples moléculas, con el fin de predecir el desarrollo de TB activa en los individuos infectados, y luego validaron la señal mediante el

seguimiento de personas infectadas con la micobacteria; se obtuvo una sensibilidad de 53,7% (IC95% 42,6-64,3) y una especificidad del 82,8% (IC95% 76,7-86). Una vez detectada la señal utilizando una qRT-PCR, los individuos con el mayor riesgo recibieron tratamiento profiláctico para evitar el posterior desarrollo de TB activa[128]. Los autores son los primeros en mostrar que una señal de RNA tiene potencial predictor, lo cual estimula la realización de estudios similares para evaluar la probabilidad de infección, y posterior progresión a TB entre individuos expuestos.

Particularmente, para la búsqueda de biomarcadores para el diagnóstico de TB latente, el RNAseq se ha usado para evaluar la expresión de genes de células T CD4(+) procedente de personas infectadas con *M. tuberculosis* y personas sin infección; 74 genes fueron diferencialmente expresados entre los dos grupos y los genes ABCB1, c-KIT, y GPA33 fueron los que mayores diferencias en la expresión tenían, e incluso esta expresión fue evidente a nivel de proteínas[129].

Para finalizar y teniendo en cuentas los resultados reportados en la literatura, se puede decir que el estudio de la expresión de genes y los perfiles de sustancias inmunes plasmáticas son prometedores para potencialmente establecer el riesgo individual de infectarse y enfermarse, y adicionalmente, su estudio abre la puerta a nuevas investigaciones sobre la respuesta inmune protectora o de riesgo, biomarcadores diagnóstico de infección latente y blancos moleculares terapéuticos.

1.3.7 Introduciendo el concepto de Exposoma

El exposoma es la sumatoria de todas las exposiciones que tiene un sujeto desde su nacimiento hasta su muerte[130]; complementa el genoma y provee una descripción de toda la historia de exposición de un individuo durante su vida. Comprende procesos internos como el metabolismo, la morfofisiología, la actividad física, la inflamación, el microbioma y el estrés; además, factores externos generales, como los ambientales (la exposición a químicos y polución) y sociales como la educación, el estado financiero, el clima y el tipo de ambiente (rural o urbano); y factores externos específicos como son el estilo de vida

(por ejemplo el consumo de alcohol y consumo de sustancias psicoactivas), e intervenciones médicas[130,131].

El exposoma se convierte en una herramienta para poder estudiar y entender una condición biológica individual (salud, enfermedad, infección y/o riesgo). En el contexto del exposoma interno, las ciencias ómicas (proteómica, transcriptómica, metabolómica, etc.) se convierten en una herramienta para explorarlo. La integración de la información proporcionada por las ciencias ómicas permite realizar un perfil individual, permitiendo así una mejor atención en salud que incluye: evaluación del riesgo, diagnóstico oportuno y preciso, monitoreo de la progresión de una enfermedad, tratamientos dirigidos, entendimiento de las bases biológicas de los procesos de enfermedad, y por ultimo prevención de la enfermedad[132].

La investigación de asociaciones complejas entre el exposoma y los desenlaces en salud es un desafío al realizar estudios epidemiológicos. Los retos vienen desde diferentes aproximaciones: primero, la medida de las exposiciones, las cuales son múltiples, internas y externas, frecuentemente correlacionadas, y cambiantes en el tiempo; segundo, el estudio del desenlace, el cual dependerá de la pregunta de investigación y que se convierte usualmente en un fenotipo, que en el caso de tuberculosis es bastante complejo dado el espectro de estados de salud que se pueden presentar; tercero, el análisis, que involucra la presencia de numerosas variables, correlacionadas muchas de ellas, una estructura jerárquica de los datos, la presencia medidas repetidas, entre otros, que necesitan ser abordados con técnicas de reducción de datos, análisis multinivel con variación espacial y temporal, y teniendo en cuenta las correlaciones y las múltiples comparaciones[133,134].

A la fecha se ha hablado sobre el exposoma de sangre[135], tracto gastrointestinal[136] y orina[137], y su relación con enfermedades como asma[138], alergias, enfermedades cardiovasculares, y cáncer[139]; desde nuestro conocimiento no hay estudios que relacionen el exposoma con *M. tuberculosis* y entendemos las dificultades adyacentes al estudio de una enfermedad infecciosa usando este abordaje[140].

Con los resultados previos del grupo de investigación, y entendiendo el transcriptoma, la idea es poder tener una aproximación más amplia a la complejidad del proceso exposición-infección-enfermedad en TB. En nuestro caso, entender el exposoma puede ayudar a

identificar biomarcadores de infección o enfermedad, y como determinadas variables y sus interacciones con el genoma funcionan como modificadores del riesgo individual.

1.3.8 Resultados previos del grupo de investigación

Nuestro grupo de investigación ha demostrado que la TB activa y latente constituyen un problema de salud pública importante en las cárceles de Colombia, de acuerdo con los siguientes hallazgos:

- La incidencia anual de TB activa en el 2010-2011 en dos prisiones fue de 505/100000 prisioneros (Medellin:515/100000; Bucaramanga: 443/100000), mucho más alta que la registrada para la población general en este fecha (25/100000)[141,142].
- Existen factores de riesgo para la adquisición y desarrollo de la enfermedad, tales como un alto porcentaje de hacinamiento (>200%) y ventilación inadecuada (bajo recambio de aire por hora), lo que facilita la transmisión de la micobacteria entre las ppl.
- De acuerdo con el análisis de genotipificación de cepas de *M. tuberculosis* de pacientes con TB pulmonar activa, al menos el 19% de las ppl que se enferman, adquieren la infección dentro de los centros penitenciarios.
- Los resultados también demuestran que la mediana de negativización de la baciloscopia es de 33 (IQR: 31-60) días y del cultivo 55 (IQR: 32-68) días, pero este tiempo se puede extender hasta mas de 100 días[143].
- La prevalencia de TB latente fue de 77,6%[144]. Además, existe una mayor conversión a TB latente dentro de los primeros 2 años (incidencia acumulada: 29,5%[24]), en comparación con otras poblaciones de alto riesgo como convivientes de casos de TB (1,8%)[23], y los trabajadores de la salud (prevalencia del 41,6%, y proporción de conversión anual del 3,7%)[145]. Los principales factores de riesgo asociados con la infección latente fueron la reclusión previa y por periodos mayores a un año[15].
- Los guardias constituyen otro grupo de riesgo para tuberculosis latente: La prevalencia de TB latente es diferencial entre las prisiones de Medellín e Itaguí, 55,8% y 39,1%, respectivamente. La incidencia acumulada de conversión en la prueba de tuberculina a los seis meses fue de 3,2%[146].

1.4 OBJETIVOS

1.4.1 Objetivo general

Determinar el perfil de citoquinas/quimioquinas plasmáticas y de expresión de genes pro-inflamatorios de las personas privadas de la libertad asociado con TB latente comparado con personas con TB activa, y personas expuestas a *M. tuberculosis* sin infección.

1.4.2 Objetivos específicos

- Identificar el perfil de citoquinas/quimioquinas plasmáticas de las ppl con TB latente.
- Identificar el perfil de expresión de genes pro-inflamatorios de las ppl con TB latente.
- Comparar el perfil de citoquinas/quimioquinas plasmáticas y de expresión de genes pro-inflamatorios asociados con TB latente, con el perfil de personas con TB activa, y personas expuestas a *M. tuberculosis* sin infección.

1.5 HIPÓTESIS

1.5.1 Objetivo 1

Existe un perfil de citoquinas/quimioquinas plasmáticas expresadas de forma exclusiva en personas con infección latente. Este perfil de citoquinas corresponde principalmente a citoquinas proinflamatorias y surgen como respuesta a la infección por la micobacteria. Poder identificar no sólo una citoquina, sino un grupo de ellas y entender cómo la respuesta inmune difiere entre los individuos saludables y los infectados o enfermos, puede mejorar el entendimiento de la respuesta inmune protectora.

Dado que las ppl serán captadas en la fase aguda de la infección o la enfermedad, y puesto que serán seguidas cada 3 meses, en plasma se podrá ver reflejada la reacción inmune localizada en el pulmón, por medio de la medición de citoquinas, y por tanto, la estimulación de células mononucleares con antígenos micobacterianos y la posterior medición de citoquinas en sus sobrenadantes de cultivo, no será necesaria.

Este aspecto de la estimulación celular, en particular, es realizado por la mayoría de los investigadores, dada la incapacidad para establecer el momento de la infección tuberculosa, y con el fin de "despertar" las células de memoria.

1.5.2 Objetivo 2

Los perfiles de expresión génica son diferentes entre las personas infectadas y las enfermas por *M. tuberculosis*, entender este perfil de expresión y encontrar diferencias entre los grupos ayudará a entender cómo se comporta el RNA mensajero y el sistema inmune cuando es capaz de realizar una respuesta protectora contra la infección por la micobacteria.

1.5.3 Objetivo 3

Los patrones de citoquinas/quimioquinas y de expresión de genes pro inflamatorios se correlacionan diferencialmente entre las personas con TB latente temprana, tardía y activa, permitiendo tener una señal inmune (genética y plasmática) exclusiva que pueda proponerse como biomarcador diagnóstico o como una señal predictora de riesgo para TB latente temprana.

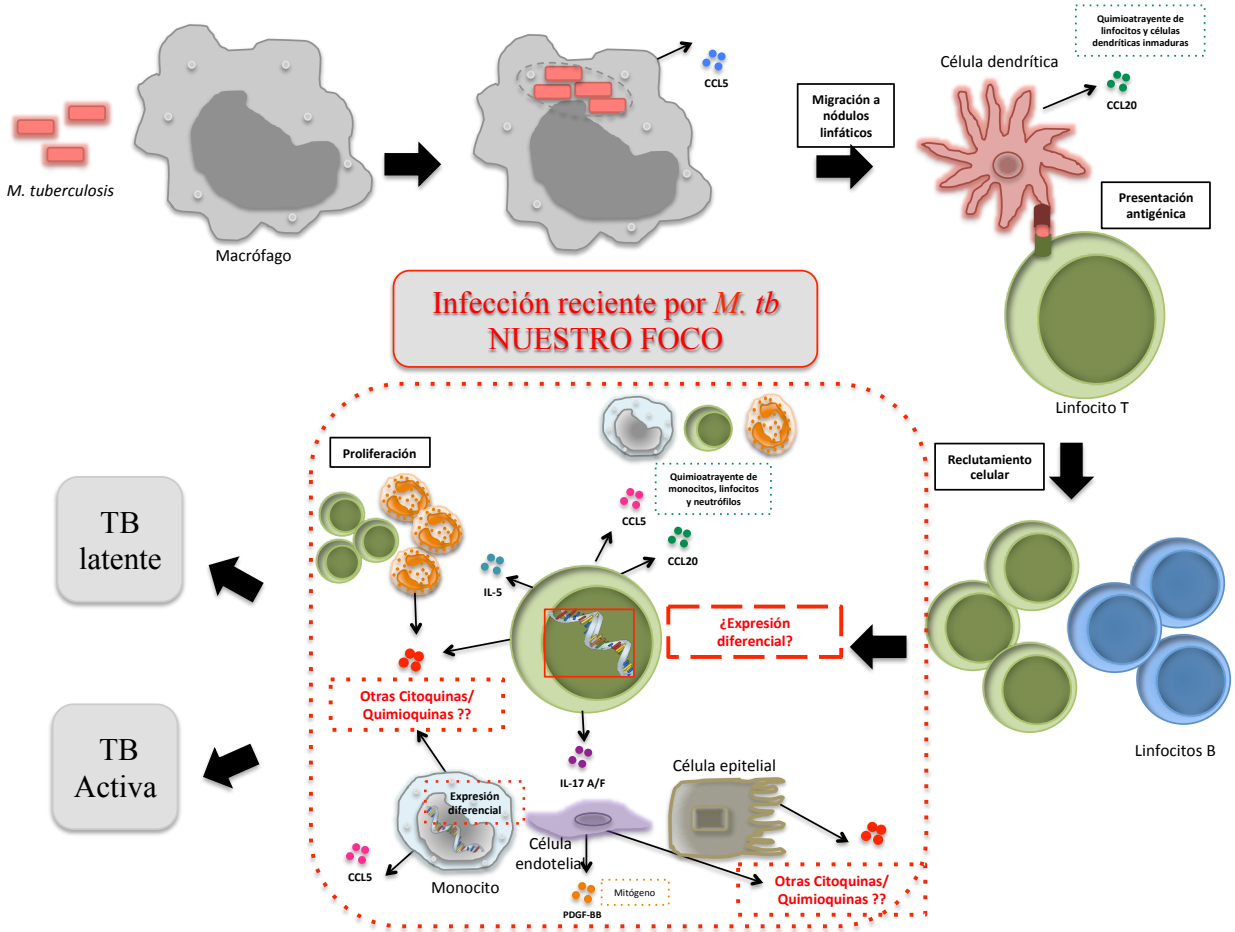

Figura 1-3 Descripción gráfica de la hipótesis de investigación, relacionando los tres objetivos específicos

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1.7 MATERIAL SUPLEMENTARIO

Ver Anexo. Prevalencia e incidencia de TB activa y TB latente en prisioneros: una revisión de la literatura

2 CAPITULO 2

TIME TO IMPLEMENT MANDATORY SCREENING AND FOLLOW UP FOR SUSCEPTIBLE PEOPLE IN PRISONS: LATENT TUBERCULOSIS INFECTION AND TUBERCULOSIS

Abstract

Prisoners are at high risk of acquiring *M. tuberculosis* infections (LTBI) and progressing to tuberculosis (TB). We sought to determine the incidence density of LTBI and TB and the factors associated with tuberculin skin test (TST+) conversion and TB in Colombian prisons.

We included 240 TST negative prisoners and followed them to evaluate TST conversion. We also included 2185 prisoners that were investigated to rule out active TB.

Latent TB incidence density was 28.8 cases per 100 person-year in recently incarcerated individuals at baseline, and 7.1 cases per 100 person-year in individuals that had been incarcerated for >1 year at baseline. TB incidence was 3.1/100 person-year. BCG vaccination reduced the risk of acquiring LTBI in both groups. HIV, diabetes, drug consumption, smoking, and incarceration > 1 year were associated with higher risk of TB. This study indicates that LTBI screening and treatment should become mandatory rather than recommended.

Keywords: Latent tuberculosis infection, pulmonary tuberculosis, risk factors, prisons

2.1 BACKGROUND

Tuberculosis (TB) remains a significant public health problem, mainly in low- and middle-income countries, where more than 80% of the global TB burden resides[1]. Prisoners are one of the high-risk populations to progress to TB, and the prevalence of active TB among prisoners is between 4 to 100 times higher than that seen in the general population[2–4], with latent tuberculosis infection (LTBI) rates between 7.1 to 88.8%[5,6]. In Colombian prisons, high rates of exposure translate to high risk of infection and progression to active TB in this population[7–10].

Inmate demographics (intravenous drug use, poverty, homelessness, etc.) linked to overcrowded environmental conditions, poor ventilation, late case detection, and inadequate isolation and treatment of patients, increase the risk of active TB and LTBI[2,3,11], and it may have a substantial effect in the general population[3]. The interaction between the community and the institutional amplifiers (visitors, guards and administrative staff), results in generalized endemic TB[12].

The End TB Strategy has set goals for decreasing TB incidence[13]. However, their goal of a 2% decrease per year in 2015 to 10% per year by 2025 is currently not being met, with a 1.6% reduction reported in Latin America[1]. In the prison system, TB remains the primary cause of infectious disease[14], however, World Health Organization (WHO) guidelines suggest that LTBI screening and treatment in prisoners are optional and not mandatory as it is suggested for other high-risk populations (household contacts of those with TB, people living with HIV, etc.). Identifying the period in which individuals have the greatest risk of infection or of developing active TB in prisoners after entry to prison (i.e. identifying susceptible individuals at the time of their initial exposure to high burden settings within the prison environment) would allow us to establish preventive strategies such as screening and treatment for people with LTBI, and diagnostic algorithms of all forms of TB to improve case finding[2,4,7,14]. A major limitation of previous studies was that inmates were recruited at different time points throughout their incarceration. As the time spent in prison is thought to play an important role in the risk of TB acquisition, separating prisoners into those that have recently been incarcerated allows us to prospectively identify risk factors associated with new LTBI infections.

The objectives of this study were to determine the incidence density of LTBI as well as the factors associated with tuberculin skin test (TST+) conversion and the development of active TB in Colombian prisons.

2.2 METODOLOGY

2.2.1 Latent tuberculosis infection

To determine the prevalence of LTBI at baseline, 155 individuals that had recently entered prison and 231 prisoners with more than one year of incarceration were recruited and screened between September 2016 to September 2018.

To determine the incidence density, 240 prisoners that had a negative two-step TST result were included. Among these, 179 individuals had more than one year of incarceration (of which 129 were recruited between November 2012 to December 2013[7,15], and 50 between September 2016 and December 2018). The remaining 61 individuals were people who had recently entered prison (less than three months), and were recruited and followed between September 2016 and December 2018. All prisoners are from two male prisons in Medellin and Itagui.

Inclusion criteria: Individuals who had a negative two-step TST at baseline, who volunteered to participate in the study and signed a written consent form.

Exclusion criteria were: (1) administration of live vaccines (measles, mumps and rubella, varicella or the live attenuated influenza vaccine) in the last 4 weeks before TST administration, (2) severe adverse events with a previous TST administration, and (3) previous active TB. For the cohort that started in September 2016, an additional exclusion criterion was included: people who were no longer incarcerated in prison (released or moved into house detention) because of the difficulties associated with follow-ups.

Follow-up: Due to budget constraints between November 2012 to December 2013, we administered the TST at two years of follow-up. For people recruited between September 2016 and December 2018, including individuals that were evaluated in 2012 and 2013 that

continued to have a negative TST, we administered a TST every six months for up two years.

Diagnosis of LTBI: LTBI diagnosis was attained by administering a TST with tuberculin PPD RT-23, 2 TU/0,1 ml, Statens Serum Institut®), according to the CDC guidelines[16]. Reading was performed within 48–72 h of administration and an experienced nurse recorded the induration measurements in mm. A second TST was administered to evaluate the booster effect. Follow-ups started after the two-step TST applications were negative. Subsequent TST administrations were used to identify new LTBI. All procedures were done according to[15].

2.2.2 Active tuberculosis

To determine the incidence density of active TB, we had access to the data of several cohort studies conducted in two periods- 2010 to 2015[8,15], and 2016 to 2018. All of the individuals who had cough of any duration and/or expectoration, and those with abnormal breathing sounds on lung auscultation were studied to rule out active TB.

The information in the database that was used for this analysis was collected from five prisons across Colombia:

1. Between April 30, 2010 and April 30, 2012 in two female and two male prisons in Medellin and Bucaramanga (n= 1305).

2. From 26 November 2012 to 10 December 2013 in two male prisons in Medellin and Itagui (n= 829).

3. From September 2016 to December 2018, in two male prisons in Medellin and Itagui (n= 51). The same prisons from item 2.

One male prison from item 1, 2 and 3 has been always included in the studies because of the high TB and LTBI incidence.

Diagnosis: all sputum samples were processed using the conventional sodium hydroxide-N-acetyl-L-cysteine method, with standard decontamination, and concentration methods. A smear was prepared for auramine-rhodamine staining to visualize acid-fast bacilli (AFB).

The sputum sample from each inmate was inoculated into: a) Lowenstein-Jensen (LJ) medium; b) a mycobacterial growth indicator tube (MGIT) which was incubated in a MGIT 960 BACTEC instrument (BD Diagnostics, Sparks, MD, USA); and c) thin-layer agar (TLA) for the detection of resistance to rifampicin and isoniazid [8]. *Mycobacterium tuberculosis* was identified by standard biochemical tests.

2.2.3 Variables

Outcomes:

1. New LTBI: convertors: A TST test with ≥10 mm diameter with a difference of at least ≥6mm between the second and subsequent administrations. The new LTBI group was divided into two subgroups: "early LTBI" for those TST converters who started follow-up within the first three months of incarceration, and "late LTBI" which signified converters who had began their follow-up after more than one year of incarceration.
2. Microbiologically confirmed pulmonary TB.

Predictor Variables: Socio-demographic and behavioral variables that have previously been reported as risk factors for active TB and LTBI were considered[7,15,17–22]. The following information was collected for all individuals: age; history and time of prior incarceration; use of drugs (inhaled, injected, or smoked) or alcohol; comorbidities (chronic obstructive pulmonary disease, diabetes, chronic kidney disease, HIV, and any other immunosuppressive disease); previous contact with a TB case (outside and/or inside the prison); history of prior TB, including date of last episode, and outcome; weight and height; city and neighborhood where the person lived prior to incarceration. To determine if the prisoners had previously been vaccinated with BCG, they were visually inspected by an experienced nurse for the presence of a BCG scar.

2.2.4 Ethics statements

All studies were approved by the Ethics Committees at each of the participating academic institutions (Facultad Nacional de Salud Pública, Universidad de Antioquia, Universidad

Pontificia Bolivariana), as well as by the Instituto Nacional Penitenciario y Carcelario (INPEC), and the director of each prison. Written consent forms were explained and signed by each prisoner in the presence of two witnesses (i.e. other prisoners). All prisoners were treated with anti-TB treatment as soon as smear and/or culture results were received. New converters were reported to the prison health authority in all prisons, and prisoners from one prison were offered LTBI treatment. As LTBI treatment in prisoners is not mandatory according to the international and Colombian guidelines, the healthcare personnel from two prisons opted not to offer LTBI treatment.

2.2.5 Analysis

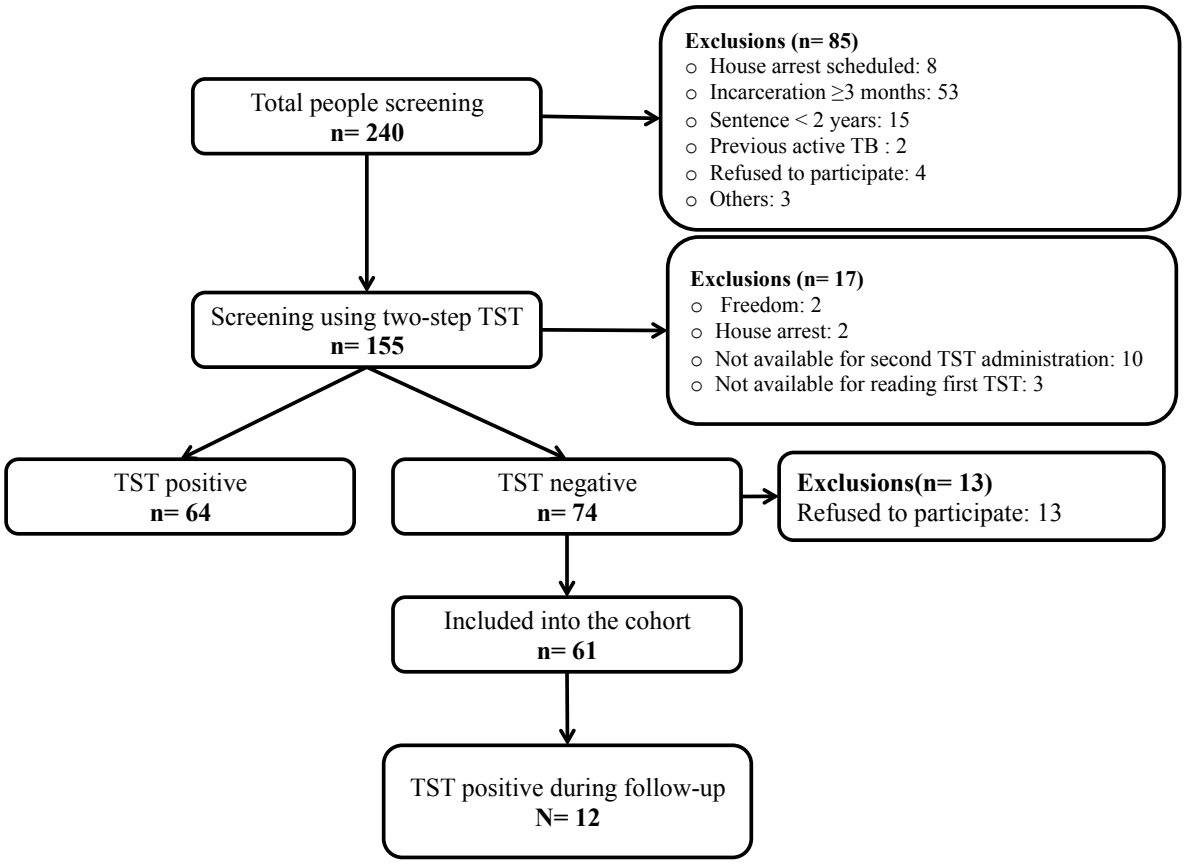
All the analysis was performed using STATA v.14 (Stata Corp, College Station, TX, USA). Descriptive statistics (median [IQR] and n [%]) were used to report the variables of interest. LTBI and active TB incidence density were estimated. Chi-squared, Fishers Exact, or Mann-Whitney U tests were used to evaluate the factors associated with the following groups: early LTBI or late LTBI, compared to Non-LTBI; and to evaluate factors associated with active TB compared to non-active TB. A two-tailed p-value <0.05 was considered significant.

For multivariable analyses, Poisson regression was used to determine the effect (risk ratios) of the variables on LTBI outcomes (early LTBI and late LTBI), or active TB. Variables included in the final model were selected using a manual backward stepwise approach. We evaluated socio-demographic, and behavioral variables to adjust the model. The standard errors in Bivariate and multivariable models were adjusted by cluster (i.e. courtyards and prisons for the LTBI and active TB models, respectively). Courtyards were chosen for the LTBI models because the prisons that were evaluated are located in the same state (Antioquia) and TB is affected by clustering in the courtyards. In contrast, we included entire prisons for the active TB model because the prisons that were evaluated are located in different states (Antioquia and Santander).

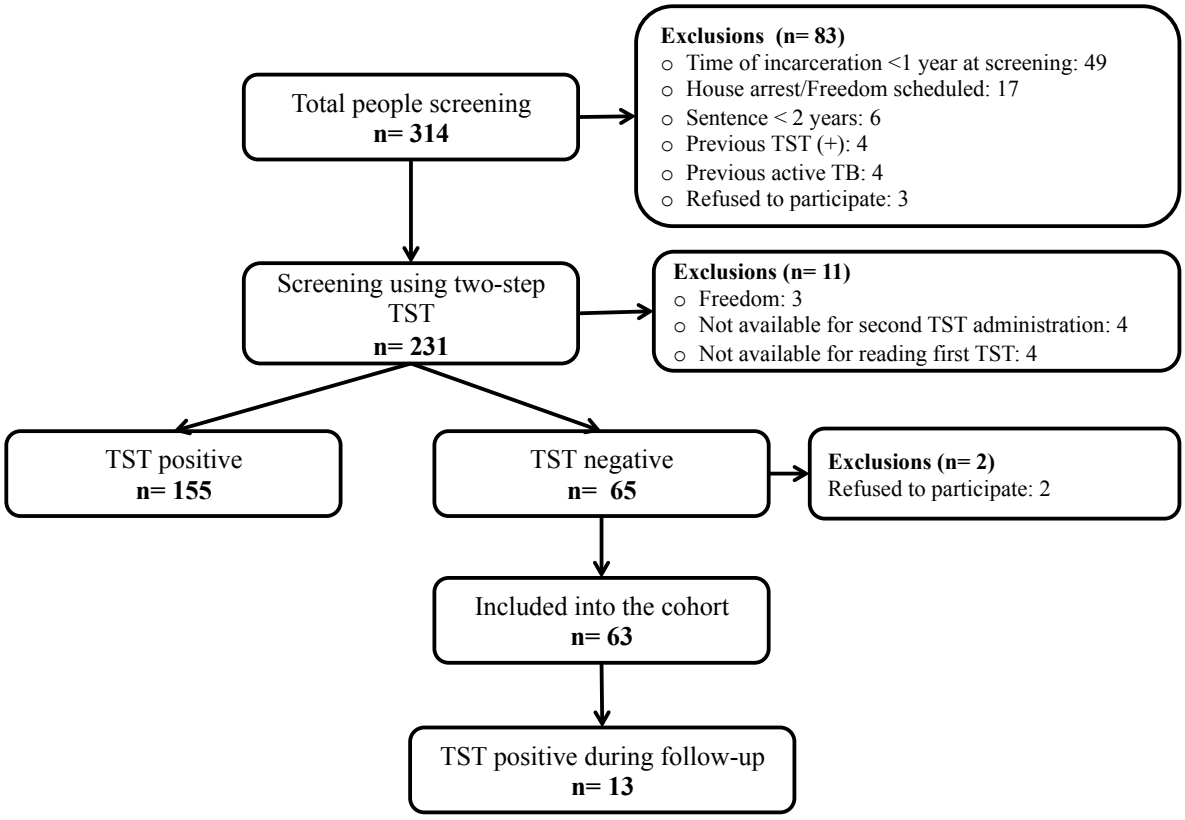
2.3 RESULTS

2.3.1 Prevalence of latent tuberculosis infection

In the cohort that started in September 2016, LTBI prevalence at admission was 46.4% (64/138) (Figure S1), and in those that had spent more than one year of incarceration it was 70.4% (155/220) (Figure S2). Figure S1 and S2 show the flowchart of people screened and included between September 2016 and December 2018.



Anexo 2-1. Flow chart of people with less than three months of incarceration screened and included in the study between 2016 to 2018. TST: tuberculin skin test



Anexo 2-2**.** Flow chart of persons with one or more years of incarceration screened and included in the study between 2016 to 2018. TST: Tuberculin skin test

2.3.2 Incidence of latent tuberculosis infection

Among the 240 negative two-step TST prisoners, the cumulative incidence of LTBI was 27.1% and the median duration of follow-up was 33.9 months [IQR 14.9-52.5]. Early LTBI incidence density was 28.8 cases per 100 person-year, and late LTBI was 7.1 cases per 100 person-year.

Figure 1 illustrates that the majority of new LTBI infections occurred between 9.3-15.4 months (8/12 conversions) in individuals that at baseline had recently been incarcerated (early LTBI group) compared to 28.0-34.1 months (22/53 conversions) in those that at baseline had been incarcerated for ≥one year (late LTBI group).

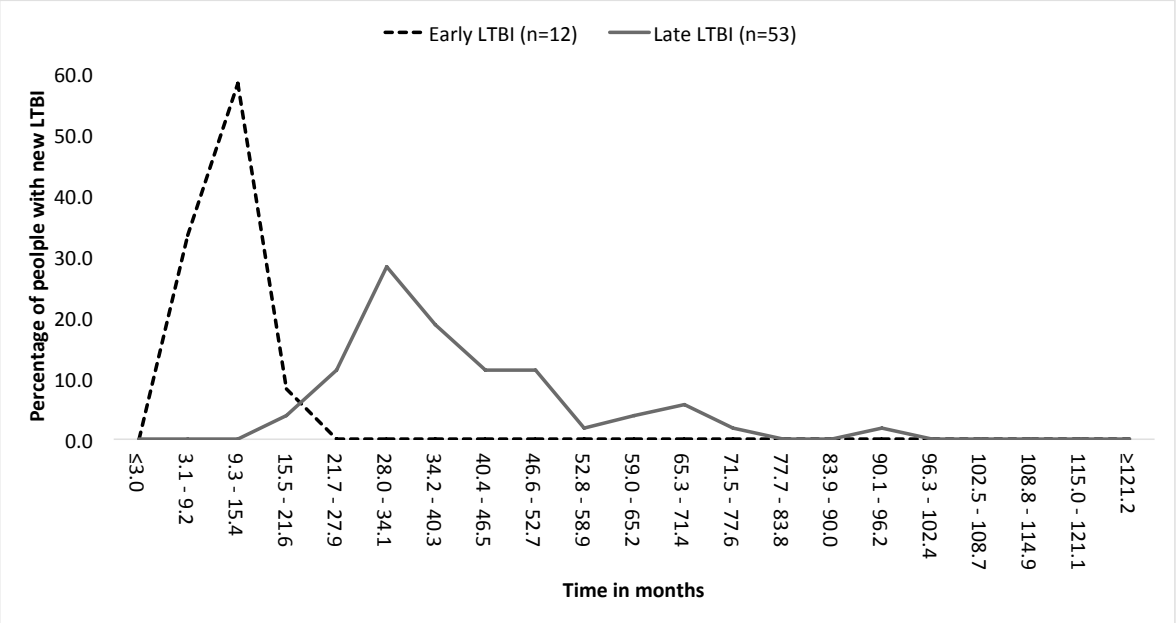


Figura 2-1 Percentage of new LTBI cases during incarceration (time in months). Early LTBI (dashed black line) was defined for those TST converters who started follow-up within the first three months of incarceration while late LTBI (gray line) were those converters who had began their follow-up after more than one year of incarceration. Time zero was the date of incarceration.

In general, most of the new infections occurred in one prison (89.2%) and were in young individuals (median age of 32 [IQR 25-47] years). Individuals of early LTBI group had a low prevalence of comorbidities, and none of them had diabetes, neutropenia or cancer. People with late LTBI had more comorbidities, however, those comorbidities have not been described as risk factors for LTBI (e.g. gastritis, high blood pressure). The table 1 shows general characteristics at baseline of new infected and non-infected people by time into the prison.

Tabla 2-1. Baseline characteristics of prisoners evaluated for latent tuberculosis infection, 2012 to 2018, Colombia

Group of individuals who had began their follow-up after more than one year of incarceration

Variables	Group of individuals who started follow-up within the first three months of incarceration		Group of individuals who had began their follow-up after more than one year of incarceration	
	Early LTBI n = 12 (%)	**Non-infected n = 49 (%)**	**Late LTBI n = 53 (%)**	**Non-infected n = 126 (%)**
Age, years, median [IQR]	32 [25-36.5]	31 [23-39]	32 [25-56]	33 [28-46]
Time in prison, months, median [IQR]	12.1 [8-12.7]	6.6 [2.6-13]	36.4 [30.7-47.5]	43.9 [32.8-67.9]
Comorbidities	2 (16.7)	7 (14.3)	17 (32.1)	49 (38.9)
COPD	1 (8.3)	4 (8.2)	7 (13.2)	8 (6.3)
HIV	1 (8.3)	0	0	1 (0.8)
Diabetes	0	0	2 (3.8)	3 (2.4)
Other	0	3 (6.1)	8 (15.1)	37 (29.4)
Current smoke drug use	4 (33.3)	14 (28.7)	14 (26.4)	36 (28.6)
Current inhaled drug use	1 (8.3)	7 (14.3)	8 (15.1)	20 (15.9)
Current cigarette smoking	6 (50.0)	25 (51.0)	25 (47.2)	49 (38.9)
Current alcohol consumption	2 (16.7)	14 (28.7)	7 (13.2)	18 (14.3)
BCG scar	9 (75.0)	46 (93.9)	29 (59.2)	95 (77.9)
Contact with a TB case	0	4 (8.2)	13 (24.5)	41 (32.5)
Prison				
Prison 1	6 (50.0)	14 (28.6)	52 (98.1)	85 (67.5)
Prison 3	6 (50.0)	35 (71.4)	1 (1.9)	41 (32.5)
BMI<18.5 kg/m^2	0	1 (2.0)	2 (3.8)	3 (2.4)
Active TB during follow-up*	1 (8.3)	0	10 (18.9)	0

LTBI: latent tuberculosis infection; IQR: interquartile range; COPD: Chronic obstructive pulmonary disease; HIV: human immunodeficiency virus; BCG: bacillus Calmette-Guerin; BMI: body mass index. *Two persons that dropped out of the study were subsequently diagnosed with active TB, 16 and four months later. During our follow-up they were TST negative. We do not know if these persons were new converters or TST negative between their dropped out and the TB diagnosis. In addition, there were five people diagnosed with active TB among 799 people with a TST positive at baseline (prevalent LTBI cases) that we could trace their TB status.

2.3.3 Factors associated with early LTBI

After adjusting by cluster effect, BCG vaccination reduced the risk of acquiring early LTBI (aRR 0.33; 95% CI 0.12-0.86).

2.3.4 Factors associated with late LTBI

In the multivariable analysis, after five years of incarceration the risk of late LTBI decreased by 70%. Similarly, BCG vaccination decreased the risk of late LTBI by 39%, while the presence of COPD increased the risk of late LTBI (Table 2).

Tabla 2-2**.** Factors associated with late latent tuberculosis infection in prison inmates

Variable	Late LTBI	
	Crude RR^{+} [95% CI]	**aRR*$^{+}$ [95% CI]**
Prison >60 months	0.32 [0.12-0.82]	0.30 [0.11-0.82]
BCG scar	0.55 [0.36-0.84]	0.61 [0.40-0.93]
COPD	1.66 [0.93-2.96]	1.90 [1.04-3.48]

BCG: bacillus Calmette-Guerin; LTBI: Latent tuberculosis infection; COPD: chronic obstructive pulmonary disease; CI: confidence interval; aRR: adjusted risk ratio; *aRR are based on Poisson regression. $^{+}$The standard errors or CI are adjusted for cluster effect (courtyard) in the bivariable and multivariable models.

2.3.5 Active TB in prisoners

Among 2185 prisoners studied for TB, 127 (5.8%) had a microbiological diagnosis of pulmonary TB; 12 of them were prevalent cases. The incidence density was 3.1 cases per 100 person-year. Similar to LTBI, one prison had the highest number of cases (82.7%), and people were young with a median of 31 [IQR 25-42] years old. Individuals with active TB had spent a median of 23.2 months [IQR 11.1-42.2] in the prison at the time of diagnosis (Table 3).

Tabla 2-3. Baseline characteristics of prisoners with and without active TB, 2010 to 2018, Colombia

Variable	Active TB n = 127 (%)	Without TB n = 2058 (%)
Age, years, median [IQR]	31 [25-37]	31 [25-42]
Time incarcerated, months, median [IQR]	23.2 [11-42.2]	13.3 [6-30.6]
Sex		
Male	118 (92.9)	1953 (94.9)
At least one comorbidity	34 (26.8)	627 (30.5)
COPD	11 (8.7)	149 (7.2)
Diabetes	4 (3.15)	34 (1.6)
HIV	5 (3.94)	13 (0.63)
Smoke drugs		
Never	26 (20.5)	738 (35.9)
Past	34 (26.8)	439 (21.3)
Currently	67 (52.8)	880 (42.8)
Inhaled drugs		
Never	52 (40.9)	1070 (52.0)
Past	44 (34.6)	601 (29.2)
Currently	31 (24.2)	387 (29.2)
Cigarettes		
Never	33 (26.0)	630 (30.6)
Past	22 (17.3)	437 (21.2)
Currently	72 (56.7)	991 (48.1)
Liquor		
Never	28 (22.0)	297 (14.4)
Past	64 (50.4)	1008 (49.0)
Currently	35 (27.6)	753 (36.6)
BCG scar	107 (84.9)	1577 (78.6)
Contact with a TB case		
No contact	81 (63.8)	1473 (71.6)
External	12 (9.4)	248 (12.0)
Internal	29 (22.8)	220 (10.7)
Both	1 (0.8)	5 (0.2)
Unknow	4 (3.1)	112 (5.44)
Prison 1	105 (82.8)	1446 (70.3)
Prison 2	8 (6.3)	129 (6.3)
Prison 3	11 (8.7)	450 (21.9)
Prison 4	3 (2.4)	33 (1.6)
BMI<18.5 kg/m^2	32/123 (26.0)	108/1193 (9.0)

TB: active tuberculosis; IQR: interquartile range; COPD: Chronic obstructive pulmonary disease; HIV: human immunodeficiency virus; BCG: bacillus Calmette-Guerin; BMI: body mass index.

Majority of TB cases were in people who had been incarcerated between one to seven years (figure 2).

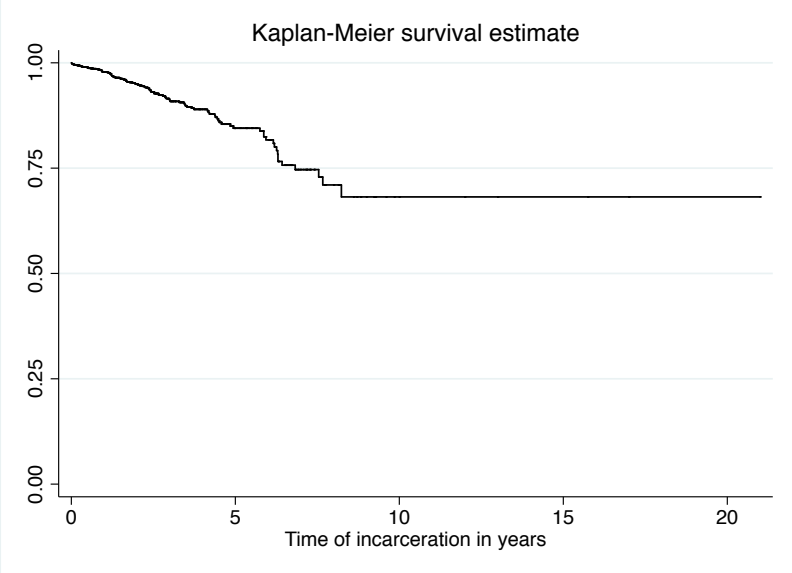


Figura 2-2**.** Tuberculosis cases during incarceration (time in years). Time zero is the time of prison entry. 12 TB cases were diagnosed within three months of incarceration (prevalent cases).

2.3.6 Factors associated with active TB

Persons living with HIV, diabetes, inhaled drugs consumption smoked drugs, cigarette smoking, presence of BCG scar and incarceration for more than one year were all associated with higher risk of developing active TB (Table 4). Since the model had been adjusted by cluster (prison), sex was not included in the multivariable model since the prisons were either exclusively for men or women.

Tabla 2-4**.** Factors associated with active tuberculosis in prisons in Colombia.

Variable	Active TB	
	Crude RR[+] [95% CI]	**aRR*[+] [95% CI]**
HIV	4.93 [2.70-9.01]	3.91 [1.77-8.64]
Diabetes	1.84 [1.06-3.19]	3.14 [2.21-4.47]
Incarceration for >1 year	1.92 [1.77-2.10]	1.98 [1.86-2.12]
Inhaled drugs [*Ref Never*]	1.0	1.0
Past	1.47 [1.07-2.03]	1.10 [1.06-1.55]
Current	1.60 [1.25-2.04]	1.36 [1.21-1.52]
Smoked drugs [*Ref Never*]	1.0	1.0
Past	2.11 [1.45-3.08]	2.31 [1.83-2.91]
Current	2.08 [1.77-2.44]	1.91 [1.59-2.29]
Cigarette smoking [*Ref Never*]	1.0	1.0
Past	0.96 [0.70-1.32]	0.97 [0.52-1.80]
Current	1.36 [1.19-1.55]	1.19 [1.05-1.34]

aRR: adjusted risk ratio; TB: tuberculosis; *aRR are based on based on Poisson regression; CI: confidence interval. [+]The standard errors or CI are adjusted for cluster effect (prisons) in the bivariable and multivariable models. The multivariable model was adjusted by BCG vaccination. Body mass index was not included because there were a high number of participants (40%) with missing data.

2.4 DISCUSSION

The main finding of this study was that individuals that were recently incarcerated had higher risk of acquiring new latent tuberculosis infections compared to people that had been in prison for more than one year. Identifying a group, which has increased risk of TB acquisition, provides an opportunity for intervention, through screening incoming inmates for LTBI upon prison entry, and by following those who are negative every year, offering LTBI treatment to new converters. As previously reported by our group[15], the number of prisoners that would need to screened to identify a single case of LTBI is low (1 in every

3.4 people that had a negative TST initially would have LTBI) and should be evaluated in further studies.

The timing of infection after incarceration is an important factor when it comes to understanding *Mycobacterium tuberculosis* (MTB) disease dynamics in prisons. The main reason that could explain the increased risk of infection in newly incarcerated individuals, compared to those who have been in prison for more than one year is the changes related to the new events of incarceration (stress, depression, changes in the eating habits, etc.)[23], which may produce changes in the immune system predisposing the individual to LTBI.

In our cohort of TST negative prisoners, the cumulative percentage of new LTBI (i.e. recently acquired MTB infection) was 27.1% in a median of 2.7 years. This percentage was similar to reports from low- and middle-income countries like Brazil, where the incidence was 25.7% at one year of incarceration in 12 prisons[24], and 29% at six months in a female penitentiary[25]. These values were higher than other studies which reported 7.6% of incident cases at six months in Iran[26], 0.23% between 2004 and 2009 in US[27], and 0.4% during two years in Japan[28].

The prevalence of LTBI was high in prisoners with more than one year of incarceration (70.4%) compared to the prevalence at the time of prison entry (46.4%). The prevalence at entry was similar to that reported by del Corral *et al*. (42.7%) in 2009 which studied general population in Medellin, Colombia[29], highlighting the importance of infection transmission inside prisons. Prevalence in people with more than one year in the prison was similar to previous results from the same prisons reported by Rueda *et al*.[7], and to the results reported by Guerra *et al*. in Guaduas, another Colombian prison, (67.6%)[10]. Authors from other countries also reported high LTBI prevalence in inmates in Iran (62.6%)[26], Brazil (73%)[30], and Malaysia (87.6-88.8%)[5,31].

Presence of a BCG scar was the only protective factor for early and late LTBI in both multivariable models. The presence of a BCG scar has been reported as a protective factor of conversion in QuantiFERON-*TB* Gold In-Tube (QFT-GIF) (aOR 0.53, 95% CI 0.28-1.00) by He G. *et al*., among physicians in China[32], in adult contacts of TB in a UK cohort

(aOR 0.70; 0.56–0.87)[33], and in HIV negative inmates from Taiwan, where QFT-IT results inversely correlated with the percentage of BCG scar, and the authors suggested that BCG vaccine seems to have a protective effect against LTBI[34]. BCG have also been associated with long-term active TB protection in a retrospective population-based cohort study in Norway[35], and in Colombian general population[36].

An important consideration is that most people incarcerated in these prisons are young individuals, who have other risk factors such as poor nutrition, and drug and alcohol abuse[37], which predisposes them to development of active TB. Additionally, from a broader perspective, infected young prisoners become part of the LTBI reservoir and may later in life reactivate, contributing to TB transmission during their lifetime[38]. Prior incarceration has been shown, in other cohorts, such as the study from Estonia, to increase the risk of TB (OR 12.42 [3.57–43.22])[39]. This observation suggests that decreasing new infections among young prisoners presents an opportunity to decrease the overall burden of TB[40].

Our study shows that 25% of all TB cases develop active TB within 11 months of incarceration, and 75% of all TB cases had 42.2 months or less of incarceration, with an incidence density of 31/1000 person years. This finding draws attention to the increased risk of progressing to active TB in people in prison. A systematic review published by Campbell JR *et al*., shows that the incidence rate of TB in prisoners with a positive TST was 45/1000 person years, compared to silicosis (36.9/1000 person-years), people living with human immunodeficiency virus (PLHIV) (27.1/1000 person-years) and previous contact with a known TB case (9.4 to 23.4/1000 person-years). This finding demonstrates that prisoners are one of the groups at highest risk of TB. In addition, Campbell JR *et al* evaluated the incidence ratio rate (IRR) for TB after having a positive LTBI test (TST, IGRAs, or both) in populations at risk according to the WHO. Prisoners had an IRR (TST positive ≥10 mm / TST negative) of 31.0 [95% CI 4.1-233.9], which was higher than the IRR of PLHIV (11.1, [6.2-19.9]), those that had close and casual TB contacts (6.9, [3.3–14.4]), those that were recent immigrants (4.0 [2.1-7.9]), people with silicosis (1.7, [0.5-5.5]), and people had were on dialysis (2.6, [1.4-4.8])[41]. The authors suggest that diagnostic tests for LTBI are useful in discriminating between people at higher and lower risk of TB[41].

We agree that people in prisons must be screened for LTBI and that priority should be given to screen those at time of entry to prison. However, in the prisons we studied, almost half of the population had a positive TST at the time of incarceration, with an additional 25% becoming positive during the first two years of incarceration. We therefore propose, based on a previous study[15] and on the incidence during the first year of incarceration, mandatory screening of prisoners at the time of incarceration using a two-step TST, with follow-ups conducted each year in individuals that had a negative result. When a positive TST conversion is detected, LTBI treatment should be offered in order to decrease the incidence of active TB and new infections. In addition, follow-ups should be implemented for individuals that had an initial positive TST to ensure a rapid diagnosis of active TB in the presence of any respiratory symptoms, of any duration.

The active TB model is consistent with previous studies which looked at risk factors associated with pulmonary TB that were conducted in prisoners in the Democratic Republic of Congo[42], Cameroon[43], Brazil[44], Thailand[45], and Tajikistan[19]. Those studies showed that HIV infection[19,45], diabetes, malnutrition (BMI lower than 18.5 kg/m^2)[42,43], and the use of psychoactive substances[19,44], increased the risk of active TB. In addition, as was seen previously[42,45], duration of incarceration equal to or greater than 12 months was a significant risk factor for developing active TB, and this association was maintained even after adjusting for additional co-variables, similar to other studies. Incarceration may be associated with rapid changes in nutrition and weight, increased stress, and consequently alter the immune status of inmates[46], increasing the risk for active TB[47]. The time spent in a high burden environment provides multiple exposure events that may contribute to the association of duration of incarceration with the development of active TB.

Additional factors reported in the literature that protect against or predispose individuals to MTB infection and active TB, such as overcrowding[42], sex[48], and race[44], were not included in our models due to the fact that these variables were constant in our population as all prisoners live in overcrowded conditions (the mean living area per person was 1.28 ± 3.05 m^2)(8), 96 and 100% of the people included in the TB and LTBI studies, respectively, were male, and in Colombia most people have a mixed genetic background[49].

Prisoners are frequently in contact with internal (other prisoners, guards) and external (family, visitors) TB cases. Previous contact with a known TB case occurred in 18.4 and 22.9% of individuals with LTBI and active TB, respectively. Although previous contact was not significant in the model, we consider it an important and difficult evaluation point given the prison´s conditions. Being exposed to a TB case can occur frequently, and go unnoticed in the context of crowded environment such as prisons. Continued contact with the community through prison staff, the frequent re-entry and short stay and transfer, and the high prevalence of TB, make prisons a reservoir of tuberculosis, that may result in the amplification of infection and disease in the community[50]. Undoubtedly, the management of TB within prisons would benefit TB control in the community.

Our results confirm that prisoners, particularly those who had been recently incarcerated, are at increased risk for LTBI and for developing active TB.

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3 CAPÍTULO 3

GAPS IN STUDY DESIGN FOR IMMUNE PARAMETERS RESEARCH FOR LATENT TUBERCULOSIS INFECTION. A SYSTEMATIC REVIEW

Abstract

Background: Immune parameters (IP) have been extensively studied to distinguish between latent tuberculosis (LTBI) and active tuberculosis (TB). Objective: To determine the IP associated with LTBI, compared to active TB and individuals not infected by *M. tuberculosis* published in literature.

Methods: We conducted a systematic search using Google Scholar and PubMed databases, combining the MesH terms: latent tuberculosis, *Mycobacterium tuberculosis*, cytokines, and biological markers; with the free terms, biomarkers and cytokines. Spanish, English and Portuguese articles comparing the concentration of IP associated with LTBI, either in plasma/serum or in vitro, in adults, non-immunocompromised, versus individuals with TB or without *M. tuberculosis* infection between 2006-july/2018-july were included. Two blinded reviewers carried out the searches, read the abstracts and selected the articles for analysis. Participant's information, diagnostic criteria, IP, detection methods, and biases were collected.

Results: we analyzed 36 articles (of 637 abstracts) with 93 different biomarkers in different samples. We found 24 parameters that were increased only in active TB (TGF-α, CSF3, CSF2, CCL1 [I-309], IL-7, TGF-β1, CCL3 [MIP-1α], sIL-2R, TNF-β, CCL7 [MCP-3], IFN-α, fractalkine, I-TAG, CCL8 [MCP-2], CCL21 [6Ckine], PDGF, IL-22, VEGF-A, LXA4, PGE2, PGF2α, sCD163, sCD14, 15 Epi LXA4), five were elevated in LTBI (IL-5, IL-17F, IL-1, CCL20 [MIP-3α], and ICAM-1) and two substances were increased among uninfected individuals (IL-23 and basic FGF). We found high heterogeneity between studies including failure to account for the time/illness of the individuals studied; varied samples and protocols; different clinical classification of TB; different laboratory methods

for IP detection, which in turn leads to variable units of measurement and assay sensitivities; selection bias regarding TST and booster effect. None of the studies adjusted the analysis for the effect of ethnicity.
Conclusions: It is mandatory to harmonize the study of immune parameters for LTBI diagnosis. PROSPERO registration number: CRD42017073289.

3.1 BACKGROUND

Latent tuberculosis (LTBI) is defined as the presence of positive tuberculin skin test (TST) or interferon-γ (IFNγ) release assays (IGRAs) in the absence of clinical or radiographic signs of disease. These accepted tests are imperfect for LTBI diagnosis for several reasons: 1) the sensitivity and specificity are between 71-82% for TST and 81-86% for IGRAs [1], 2) the sensitivity is reduced in immunocompromised patients, 3) inability to differentiate between LTBI and active tuberculosis (TB), 4) a positive TST or IGRAs result does not automatically imply LTBI, as individuals who eliminate the infection successfully might still be TST or IGRAs positive because of memory T cell responses, and partly explains the low predictive value of TST and IGRAs [1], 5) genetic factors may impact test sensitivity as well as the susceptibility for acquisition of mycobacterial infection [2–4]. To date there is no available diagnostic tool that allows to diagnose LTBI and differentiate clearly between LTBI and active TB.

For the above mentioned reasons, World Health Organization, governments and non-governmental organization and private sector established as one of the priorities to identify "What biomarkers or combinations of markers will help distinguish the various stages of the spectrum of LTBI (from sterilizing immunity to subclinical active disease)" [5].

The improvement in high throughput cytokine measurement platforms has sparked enthusiasm for identification of novel pathways involved in the pathogenesis of TB that can inform development of assays for LTBI determination. In tuberculous infection some important immune molecules are known to play a pivotal role in the protective response

against the bacteria. Among the main ones described are IFN-γ, produced by T CD4+, CD8+ and NK cells; IL-1 and TNF-α, secreted by macrophages and lymphocytes, known to prevent the growth and multiplication of mycobacteria in host cells [6,7]. However, additional biomarkers such as IL-2, IL-5, IL-10, IL-1RA and MCP have been studied for their ability to differentiate between the LTBI and active TB [8] and it is believed that the cellular and immune profile expressed during tuberculous infection depends to a great extent on the stage of disease i.e LTBI or active, where immune biomarkers present in blood could have the ability to differentiate with greater precision between both stages [9].

Despite advances in the study of immune parameters, there are pervasive limitations in the analysis and conclusions of many of these studies. Cytokine / chemokines expression is affected by ethnicity [2,10], cell simulation protocols (or no stimulation) [11–13], time of LTBI (which in most cases is impossible to quantify), and if the comparison group is people with TB, the clinical manifestations of disease (pulmonary vs extrapulmonary TB) [14].

In order to identify which immune parameters are increased exclusively in LTBI, in addition to find gaps in knowledge and study design of previous published papers, we performed a systematic review. The question posed is: what are the cytokines associated with LTBI, compared to cytokines expressed among individuals with active TB and those not infected by *M. tuberculosis*?

3.2 METHODS

According to the Preferred Reporting Items for Systematic reviews and Meta-Analysis protocols (PRISMA-P), this systematic review was registered with the International Prospective Register of Systematic Reviews (PROSPERO) on August 31, 2017 (registration number CRD42017073289).

3.2.1 Eligibility criteria

Studies were selected according to the following criteria:

Study designs: We included clinical trials, prospective and retrospective comparative cohort, case-control and cross-sectional studies. We excluded descriptive studies, case reports and series, and reviews.

Participants: Articles published between January 2006 and July 2018, that compared people with LTBI with 18 years or older, or adults and children, without any immunocompromising medical conditions, versus individuals with active TB or without *M. tuberculosis* infection under the same conditions. We excluded manuscripts assessing the production of IFN-γ as part of the evaluation of IGRA's, that were performed in animal models, immunocompromised individuals, and studies exclusively conducted in children.

Exposure: Articles that evaluated the expression of cytokines associated with LTBI, either in plasma or in vitro, with or without stimulation of mycobacterial antigens. The antigens used to perform cell stimulation were not restricted.

Comparators: The expression of cytokines associated with active TB confirmed by clinical, epidemiological contact, X-rays, and/or laboratory, and/or subjects with no evidence of *M. tuberculosis* infection, evidenced by negative results of tuberculin skin test or Interferon-Gamma Release Assays.

Outcome: People with LTBI compared to active TB or with no evidence of *M. tuberculosis*.

Timing: There was no restriction on the length of follow-up for clinical trials or cohort studies.

Setting: There was no restriction by type of setting.

Language: Articles in English, Spanish or Portuguese.

3.2.2 Information sources

Search for original articles utilized two electronic databases: Google Scholar and PubMed.

To identify additional literature, the reference list of all papers was reviewed, and we followed the same process for abstract reviewing and data extraction as we did for papers identified by electronic search. Articles suggested by the reviewers, not detected in the previous searches, were also included.

3.2.3 Search strategy

Papers published between 2006 to July 2018 were included. We used the following MeSH terms in English, Spanish and Portuguese languages: latent tuberculosis, *Mycobacterium tuberculosis*, cytokines, and biological markers. In addition, we used the free terms: biomarkers and cytokines. Additional file 1 contains the search strategies used.

3.2.4 Study selection, data collection process and data items

Once the articles were identified using each of the search strategies, we proceeded with the elimination of duplicate items. Subsequently, the titles and abstracts of all manuscripts identified by two independent evaluators were reviewed according to the selection criteria. All disagreements between the two reviewers were resolved with a third evaluator by consensus. Articles that met the selection criteria were read completely by the same reviewers, blinded and independently.

The data extracted and typed in Excel file from the selected articles were the following: consecutive number of the article (whole number assigned by investigators), article title, year, first author, journal, study country of origin, outcome or result reported in the article, type of study population (special feature), number of patients in the intervention or comparison group, follow-up in each group, type of control or unexposed population, number of patients in the control or unexposed group, follow-up in the control or non-exposed group (months), age, sex (female percentage), active TB diagnostic method, LTBI diagnostic method, LTBI time, immune parameters studied, increased IP (with and without statistical differences) - the group in which the IP was increased is reported first-, IP that

remained normal, decreased IP (with or without statistical differences), IP concentration values, the level of confidence they used in their statistical analyses (90%, 95%, 99%), method of detection of IP, was ethnicity reported? what is the study populations? type of study, quality of the study (see below), bias (types of bias), proportion of prior BCG vaccination, conflict of interest statement and other important findings such as the cell stimulation used (times and antigens used).

We conducted a pilot study for the search strategies, abstract reviewing, and data extraction of full-text articles to standardize all process and concepts before to start each step. A third reviewer was in charge of comparing the files to identify disagreements at each step of the process. A fourth reviewer participated in the validation of the biological findings, only at the end of the full-data extraction for included papers to avoid investigator bias.

3.2.5 Risk of bias in individual studies

Selection bias were controlled through the application of inclusion and exclusion criteria to eligible titles and/or summaries; likewise, possible information biases were controlled by the independent revision of two observers, where at the end of the review, a third reviewer compared their findings. The risk of bias of the studies were assessed using the Newcastle - Ottawa scales for case - control and cohort studies [15], and the National Institutes of Health (NIH) evaluation scale for observational studies [16]. Jadad scale was applied to evaluation of clinical trials [17] (Additional file 3 and 4).

The Newcastle-Ottawa scale evaluates four main points: population, that is, the choice of cases or exposed people, and controls or not exposed; the measurement of the outcome and exposure; and the comparability between groups [15]. Similarly, the NIH scale is based on 14 questions that include the clear definition of the objective, the population (including the sample size), the measurement of dependent and independent variables, and the control of the confounders [16].

For both scales one or two points are given when a study complies with the evaluated requirements, (comparability for Newcastle-Ottawa). This final score determines the risk of bias: high risk (0-2 points), moderate (between 3-6 points), low risk of bias (≥7 points).

3.2.6 Summary measures

Due to the clinical heterogeneity of the population, the samples and the stimulation protocol used, the multiple techniques used for immune parameters detection, the different units reported for the substances, and the differences in the diagnosis of LTBI and active TB, it is was deemed inadequate to perform a meta-analysis [18,19]. Therefore, we report the systematic review with a qualitative synthesis of the papers.

3.3 RESULTS

3.3.1 Articles:

Upon searching according to the keywords, 637 relevant articles were retrieved, among them, 58 met the selection criteria and were read in full text. At the end, 36 met all criteria and were included in the systematic review (Figure 1). The excluded articles and the reasons for exclusion are provided in Additional file 2.

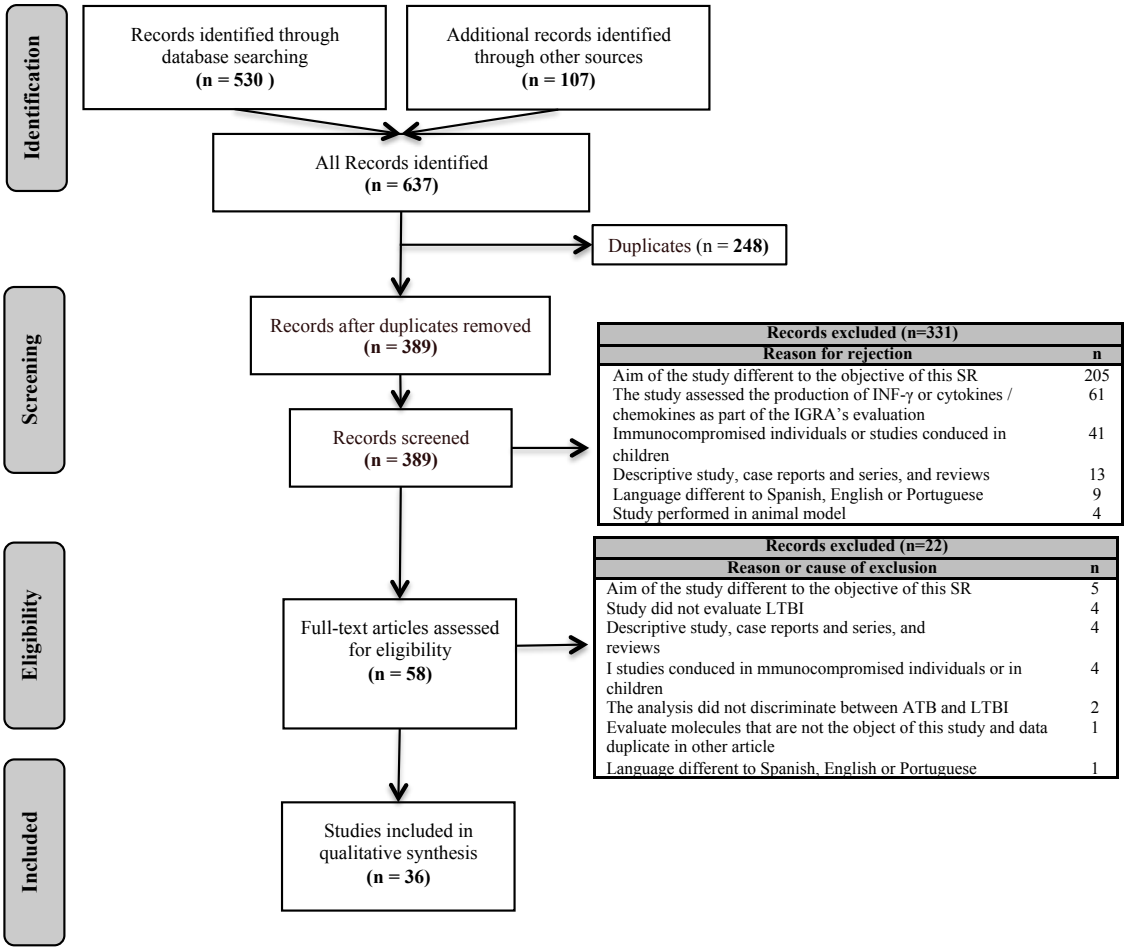


Figura 3-1 PRISMA diagram showing the results of systematic searches and articles analyzed. Legend: ATB: Active tuberculosis. LTBI: Latent tuberculosis infection

Publications included 34 cross-sectional and 2 cohort studies, the later with follow-up for 6 and 24 months after baseline sampling.

3.3.2 Participants:

Most of the studies evaluated individuals with active TB treatment or within hospital programs. Their community or family contacts, or voluntary hospital or community-based controls with or without TB infection served as controls. Five studies were conducted in healthcare-workers, four in places endemics for TB and one from a region with a high rate

of malnutrition. The minimum and maximum number of subjects included in the studies were 7 and 148 in the LTBI group, 10 and 147 in the active TB group and 8 and 168 in the non-infected group. Table 1 describes the characteristics of the population included in each study.

3.3.3 BCG vaccination status

Among the 36 included articles, 22 reported BCG vaccination (21-29, 31-35, 37-40, 43, 44, 48, 49, 50) (Table 1). The proportion of BCG vaccination was similar among the groups with LTBI, active TB and non-infected individuals. Seven papers (27,29,39,43,50,53,55) compared the inflammatory mediators between those with and without vaccination, and only two reported significant differences (27,53), having lower percentage of BCG vaccination in the active TB group compared to healthy controls (healthy persons with no history of close contact with a TB case) (27,53), people with history of close contact with a TB case with QFT negative results (53), or people with LTBI [household contact TST+ (27), or QFT positive (53)]. Only one article included BCG status and other sociodemographic characteristics in the multivariable regression model to adjust the association of QFT/TST results with cytokine response, and found that positivity (cytokine level greater than the normal detection limit) of TNF-α and IL-6 were associated with higher risk of having QFT+/TST+ results (39).

3.3.4 Evaluation of conversion to LTBI and progression to active TB

The majority of the studies did not evaluate progression to active TB and conversion to LTBI. Most articles reviewed were cross-sectional studies that only reported the prevalence or frequency of LTBI, active TB, and those who are TST-negative or IGRAs-negative. We found two cohort studies (33,43), and one article did not report which of those contacts progress to TB, nor did the authors discriminate the results at entry, 6 and 12 months by QFT positive and negative results (43). Therefore, it was not possible to evaluate the immune parameters that represent protection or risk to progress to TB disease. The other

study evaluated spouses of TB patients and followed them for two years, but it reported that they had low number of convertors (2/101 individuals) and none of the spouses without TB at baseline developed TB during the two-years of follow-up (33). Therefore, it was impossible to identify cytokines that are associated with progression to either, LTBI and TB.

Tabla 3-1 Characteristics of studies included in the systematic review.

First author, Year of publication	Country where the study was conducted	Outcome of interest	Special feature of the population under study	Number of people with LTBI	Number of people in the control group	Age	Sex (% women)	Proportion with BCG vaccination (%)	Active TB diagnosis method	LTBI diagnosis method	Immune parameters evaluated	Increased immune parameters (with statistical differences)*	Samples	Antigen and times used for stimulation	Method of detection of cytokines, commercial kit
Zeev T. Handzel, 2007 [20]	East European, Ethiopian and Israel	LTBI	Immigrant patients from Eastern Europe and Ethiopia and their contacts in Israel	39	PTB = 39 NI = 21	Not reported	Not reported	Not reported	Culture, Clinical diagnosis, X-ray, TST>15	TST	INF-γ, IL-2R, IL-10, IL-6, IL-12p70	**Unstimulated** **LTBI vs NI**: IL-10 and IL-6 **Stimulated** **LTBI vs NI**: INF-γ **TB vs LTBI:** sIL-2R, INF-γ and IL-10 **Serum** **TB vs LTBI**: sIL-2R, IL-10	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated) and Serum	PPD Time: 48 hours	ELISA, (R & D Systems, Minneapolis, MN, USA)
Novel N. Chegou, 2009 [21]	South Africa	LTBI	Contacts of people with TB and patients with TB from an endemic area	34	PTB = 23	Mean+-SD TB: 30.3 ± 13.6 LTBI/NI: 31.8 ± 14.2	TB: 26 LTBI/NI: 58.8	Not reported	Smear (ZN)	QuantiFERON-TB Gold In-Tube Test, TST	(IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, CXCL8, IL-1ra, sCD40L, CCL11, fractakline, G-CSF, GM-CSF, IFN-γ, CXCL10, CCL2, CCL3, CCL4, TGF-α, TNF-α, VEGF	**Unstimulated** **TB vs LTBI:** EGF, TGF-α, TNF-α and sCD40L **Stimulated** **LTBI vs TB**: sCD40L, VEGF. **TB vs LTBI**: IL-1α	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6, CFP-10 and TB7.7 Time: not specifically referred	Microbead-based method, LINCO-plex ® kits (Millipore, St. Charles, Missouri, USA)

R. Biselli, 2010 [22]	Italy	LTBI	Laboratory personnel without *M. tuberculosis* infection and TB cases of Infectious Diseases L. Spallanzani, and The Infectious Diseases Department of Sapienza Universita` di Roma	20	PTB = 20 NI = 20	Median LTBI: 42.3 TB: 35.7 NI: 31.4	LTBI: 40 TB: 45 NI: 30	LTBI: 0 TB: 35 NI: 0	Culture	QuantiFERON-TB Gold In-Tube Test, TST	INF-γ, IL-2	**Stimulated** **LTBI and TB vs NI**: INF-γ **LTBI vs TB and NI**: IL-2	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6, CFP-10 and TB7.7 Time: 18 and 72 hours	ELISA, ELISA assay (DRG GmbH, Germany)
Jayne S. Sutherland, 2010 [23]	South Africa	LTBI	TB case contacts and TB cases	20	TB/NRCF= 36 NI =19	Median (IQR) LTBI: 27 (19–39) TB: 25 (20–37) NI: 22 (18–31)	TB: 27 LTBI: 74 NI: 65	Not reported	Smear (ZN, auramine-rodhamine), Culture	TST	TNF-α, IFN-γ, IL-10, IL-12(p40), IL-13, IL-17, IL-18	**Stimulated** **LTBI vs NI**: IFN-γ, IL-13 e IL-17 **TB vs NI**: IL-10, IL-12(p40), IL-13, IL-17, IFN-γ and TNF-α. **TB vs LTBI:** TNF-α and IL-12(p40)	Blood Culture supernatant unstimulated or antigen stimulated	ESAT-6/CFP-10, PPD or TB10.4 Time: 7 days	Microbead-based method, 7-plex kit, BioRad.
Subash Babu, 2010 [24]	India	LTBI	Adult population with and without *M. tuberculosis* exposure	25	NI = 25	Median (range) LTBI: 32 (19-50) NI: 30 (15-48)	LTBI: 40 NI: 40	All participants	N/A	TST	IL-2, IFN-γ, TNF-α, IL-12, IL-4, IL-5, IL-10, IL-13, IL-17, IL-23, IL-6, IL-1β, IL-23	**Stimulated** **NI vs LTBI**: IL-17, IL-23	Non-stimulated and antigen-stimulated PBMCs culture supernatants	PPD or Mtb CFA Time: 24 hours	Microbead-based method and ELISA for IL-23, BioRad
Marc Frahm, 2011 [25]	Not reported	LTBI	Adult population with and without TB from two previous cohorts	32	PTB = 9 EPTB: 3 NI = 26	Median (range) LTBI: 50 (2–66) TB: 43.5 (4–93) NI: 46.5 (26–62)	LTBI: 47 TB: 33 NI: 27	LTBI: 31 TB: 33 NI: 0	Culture from a clinical specimen or Clinical diagnosis	QuantiFERON-TB Gold In-Tube Test, TST	IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40/70, IL-13, IL-15, IL-17, TNF-α, IFN- α, IFN-γ, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, Eotaxin, RANTES, MCP.	**Stimulated** **LTBI and TB vs NI**: INF-γ, IP-10, MIG, IL-2, MCP-1, IL-15, IL-RA. **TB vs LTBI:** IL-15. With a more flexible cut-off point: MCP-1, IL-1RA, IFN-α and IL-4	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6, CFP-10 and TB7.7 Time: 16–24 hours	Microbead-based method, Human Cytokine 25-plex (Biosource, Camarillo, CA)

Ji Young Hong, 2012 [26]	Korea	LTBI	Contacts of patients with confirmed TB. Cases of TB were hospitalized patients with comorbidities.	22	PTB = 46 NI = 32 Combined EPTB lesion: 2 (4.3%)	Median (range) LTBI: 37.5 (22–53) TB: 30 (22–74) NI: 28 (22–57)	LTBI: 18 TB: 25 NI: 18	LTBI: 90.9 TB: 54.3 NI: 75.0	Culture	QuantiFERON-TB Gold In-Tube Test, TST	IP-10, INF-γ	**Unstimulated plasma** **TB vs LTBI and NI**: IP-10 **Stimulated plasma** **LTBI and TB vs NI**: IP-10, INF-γ **Serum** **TB vs LTBI and NI**: IP-10	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated) and serum	ESAT-6, CFP-10 and TB7.7 Time: 20 hours	ELISA, (R&D Systems, Minneapolis, MN, USA)
S. Y. Kim, 2012 [27]	Not reported	LTBI	TB case partners with and without LTBI	19	PTB = 32 NI = 30	Median (range) LTBI: 47 (23-60) TB: 31 (20-77) NI: 28 (22-57)	LTBI: 68.4 TB: 46.8 NI: 53.3	LTBI: 94.7 TB: 64.5 NI: 76.7	Smear (ZN), culture and/or pathology	QuantiFERON-TB Gold In-Tube Test, TST	IFN-γ, IL-2, IL-10, IL-13, IL-17, TNF-α	**Stimulated** **LTBI and TB vs NI**: IFN-γ, IL-2, IL-10 and IL-13	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6, CFP-10 and TB7.7 Time: 20 hours	Microbead-based method, MILLIPLEX® MAP human cytokine/chemo-kine kit (Millipore, Billercia, MA, USA)
Pierre-Alain Rubbo, 2012 [28]	France	LTBI	Healthcare workers with high risk of *M. tuberculosis* exposure	41	NI = 29	Median (IQ): 44 (36–50)	All participants: 84.3	All participants	N/A	QuantiFERON-TB Gold In-Tube Test	IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40/70, IL-13, IL-15, IL-17, TNF-α, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, Eotaxin, RANTES, MCP, IFN-γ	**Stimulated** **LTBI vs NI:** IL-2, IL-15, IP-10 and CXCL9	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6, CFP-10 and TB7.7 Time: 24 hours	Microbead-based method, Cytokine human panel; Invitrogen, Villebon sur Yvette, France) and
Sen Wang, 2012 [29]	China	LTBI	Adults living in an endemic area to TB	73	PTB = 66 NI = 76	Median (range) LTBI: 41(18–83) TB: 45 (16–86) NI: 38 (18–50)	LTBI: 52.1 TB: 40.9 NI: 45.2	LTBI: 74.0 TB: 78.9 NI: 89.5	TB contact history, smear (ZN), Culture, Clinical diagnosis and R-rays	QuantiFERON-TB Gold In-Tube Test, TST	IP-10, IL-2, TNF-α, INF-γ	**Unstimulated** **LTBI vs TB**: IP-10 **LTBI and TB vs NI**: IP-10, IL-2, TNF-α, INF-γ **TB vs LTBI:** TNF-α. **Stimulated** **TB vs LTBI:** IFN-γ,	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6, CFP-10 and TB7.7 Time: 20 hours	Duoset ELISA, The Duoset ELISA Development kit (R&D Systems Inc, MN, USA)

												IP-10 and IL-2			
Yang Yu, 2012 [30]	China	LTBI	Individuals exposed to *M. tuberculosis*, healthy volunteers without infection and hospitalized patients with TB	20	PTB = 12 NI = 12	Mean LTBI 1: 40.7 LTBI 2: 46.1 TB: 38.5 NI: 30.7	LTBI 1: 60 LTBI 2: 50 TB: 58.3 NI: 41.6	Not reported	Culture, Clinical diagnosis, X-ray and/or HRCT	T–SPOT®, TST	CCL1, CCL2 CCL3 CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL17, CCL20, CCL21, CCL24, CCL26, CCL27, CXCL5, CXCL, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL1, IL-2, IL-15, IL-4, IL-13, IL-7, IL-9, IL-5, GM-CSF, IL-6, IL-12, G-CSF, TNF-α, IL-10, IFN-γ, IL-1RA, IL-1β, IL-17	**Stimulated PBMCs** **LTBI 1 vs NI**: IP-10, CXCL11 and CXCL12 **LTBI 2 vs LTBI 1**: IL- 2, CXCL10, CXCL11 and CXCL12 **TB vs NI:** IL- 2, IP-10, CXCL11, IL-6, IL-9, IL-10, CCL-8, CXCL13, CXCL12, CCL1, CCL21 **Plasma**: **TB vs NI**: IL-6, CCL1, IL-9 and CXCL9	Non-stimulated and antigen-stimulated PBMCs culture supernatants and plasma	Lysed bacteria proteins and ESAT-6 Time: 72 hours	Microbead-based method, Human Cytokine/Chemokine Panel (MPXHCYTO-60K, MPXHCYP2-62K and MPXHCYP3-63K, Millipore, USA).
Novel N. Chegou, 2012 [31]	South Africa	LTBI	TB case contacts and TB cases from a high TB-endemic community	23	PTB: 15	Mean (SD) 31.5 (15.9)	All participants : 39.5	Not reported	ZN	TST	EGF, fractalkine, IFN-a2, IFN-c, IL-4, IL-10, IL-12(p40), TGF-a, TNF-a, VEGF, IP-10, RANTES.	**Unstimulated** **TB vs Contact**: EGF, IFN-a2 and IL-4. **Stimulated ESAT-6/CFP-10 TB vs contacts** : EGF, TGF-a and TNF-a. **Stimulated Rv0081** **Contacs vs TB**: IFN-g, IFN-a2, IL-12(p40), IP- 10, TNF-a, VEGF, IL-10 and RANTES.	Plasma samples from whole blood (unstimulated or antigen stimulated)	Resucitation promoting factors (Rv0867c,Rv2389c) and DosR regulon-encoded antigens (Rv2032, Rv0081, Rv1737c) Time: 7 days	Microbead-based method, Milliplex kits (Merck Millipore, St. Charles, Missouri, USA)

												Stimulated Rv2032 **TB vs contacts:** fractalkine, IL-12(p40), TGF-a, TNF-a, VEGF, IL-10, RANTES. **Stimulated Rv1737c** **TB vs contacts:** IL-10, TGF-a, TNF-a, IL-12(p40) and EGF			
D. Anbarasu, 2013 [32]	India	LTBI	Family of TB cases from an endemic area to *M. tuberculosis*	7	PTB =10	Range LTBI: 28-55 TB: 26-52	LTBI: 28.6 TB: 30	Not reported	Smear (ZN) and culture	TST	IL-1β, IL-1RA, IL-2, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, Eotaxin, FGF basic, G-CSF, GM-CSF, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, RANTES and VEGF	**Stimulated CFP-10, Rv3716c and TrxC** **LTBI vs TB:** IL-6 **Stimulated FbpB/Rv2626c** **TB vs LTBI**: G-CSF, IL-7, IL-8, IL-9 and PDGF. **LTBI vs TB:** IL-6	Blood Culture supernatant unstimulated or antigen stimulated	Protein fraction 11_24 (Rv2626c and FbpB) Time: 6 days	Microbead-based method, Bioplex multiplex cytokine assay system (Bio-Rad Laboratories, Hercules, CA, USA)
Yun-Gyoung Hur, 2013 [33]	Malawi	LTBI	TB cases from a cohort with their contacts	It is not clear (143 in LTBI and NI)	PTB = 15	Mean: TB: 41 LTBI: 40	LTBI: 67 TB: 60	Not reported	Smear (ZN)	TST	IL-10, IL-13, IL-17, CXCL10, TNF-α.	**Stimulated** **LTBI and TB vs NI**: IFN-γ, CXCL10, IL-10, TNF-α and IL-17. **LTBI vs TB and NI**: IL-10 **LTBI vs TB**: IL-17 **TB vs LTBI**: IL-17 and IL-10, in the followings.	Blood Culture supernatant unstimulated or antigen stimulated	PPD or ESAT-6 Time: 6 days	Duoset ELISA, R & D Systems
Mayer-Barber, 2014 [34]	China and India (Cohort reported by Andrade BB, 2013)	LTBI	TB cases from a Chinese cohort and healthy community controls	China: 14 *India*: 39	PTB = 94 Healthy controls = 11 *India*: PTB: 97	Median (IQR) PTB: 27 (23-44.7) Healthy controls: 33 (23-40)	PTB: 38.3 Healthy controls: 54.5 LTBI: 85.7	Not reported	Smear (ZN) *India* Smear and culture	QuantiFERON-TB Gold In-Tube Test *India* QuantiFERO	IL-1α, IL-1β, IL-10, IL-1Ra, IL1R1, IL1R2, IFN-γ, IFN-α, IFN-β, TNF-α, PGF2α, PGE2, LXA4, 15-Epi-	**LTBI vs NI and TB** IFN-α **NI vs LTBI and TB:** IL-1α, IL-1β, TNF-α, IL1Ra **TB vs LTBI and NI**:	Plasma samples	Not apply	ELISA kits (R&D Systems) and Flowcytomix Multiplex Arrays (eBioscience, San Diego, CA)

			India TB cases (pulmonary and extra pulmonary), LTBI and healthy donors recruited as part of a TB cohort study		EPTB: 35 Healthy controls: 40	LTBI: 38.5 (34.2-43.5) *India* Median (IQR) Healthy control: 29 (21-59) LTBI: 25 (21-49) EPTB: 33 (18-65) PTB: 40 (19-70)	*India* PTB: 33 EPTB: 84 Healthy controls: 75 LTBI: 77			N-TB Gold In-Tube Test and TST, absence of Chest radiograph or pulmonary symptoms	LXA4 *India* IL-1α, IL-1β, IL-10, IL-1Ra, IFN-γ, IFN-α, IFN-β, TNF-α, PGF2α, PGE2 LXA4, 15-Epi-LXA4, IL-1R1, IL-1R2	IL-10, IL-1RI, IFN-γ, PGF2α, PGE2 *India* **LTBI vs NI and TB:** IL1Ra, PGF2α **NI vs LTBI and TB:** IL-1α, sIL-1R1 **TB vs LTBI and NI**: IL-1 β, PGE2, TNF-α, IFN-γ, IFN-α, IL-10, LXA4, 15-Epi-LXA4			and Oxford Biomedical Research, (Oxford, MI) *India* ELISA kits (R&D Systems) and enzyme immunoassay (EIA) kits (Cayman Chemical, Ann Harbour, MI) and Oxford Biomedical Research, (Oxford, MI)
Ikaria Sauzullo, 2014 [35]	Italy	LTBI	Healthcare workers studied for LTBI	TST+/QFT − = 34 TST+/QFT + = 29 Total 63	PNI = 126	Mean (range) 43 (25–60)	All participants : 50.5	All participants: 3.1	N/A	QuantiFERO N-TB Gold In-Tube Test or TST, and had one of the following risk factors: chest X-ray suggestive of prior TB infection, a history of exposure to a case of active TB, or coming from an area with a high prevalence of TB infection.	IFN-γ, IL-2	**Stimulated** **LTBI vs NI**: IL-2, INF-γ	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6, CFP-10 and TB7.7 Time: 72 hours	ELISA, (DRG GmbH, Germany)
K. Kim, 2014 [36]	Australia	LTBI	Patients of the Western Australian Tuberculosis	30	PTB = 23 EPTB: ~8 (25%)	Median (IQR) LTBI: 32 (25-39) TB: 35 (29-	LTBI: 50 TB: 32.3	Not reported	Culture	IGRAs, TST	IFN-γ, TNF-α, IL-10	**Stimulated** **LTBI vs TB**: IFN-γ **TB vs LTBI:** TNF-α	Non-stimulated and antigen-stimulated PBMCs culture	PPD, ESAT-6 or CFP-10 Time: 6 hours	ELISA, BD OptEIATM Sets (BD Biosciences, USA)

			Control Program			42.5)							supernatants		
Yun Hee Jeong, 2015 [37]	South Korea	LTBI	Patients with Active TB, and contacts with LTBI	20	PTB: 33 NI: 26	Median (range) LTBI: 44 (22– 60) TB: 30 (20–63) NI: 25 (22–54)	LTBI: 80 TB: 38.7 NI: 53.8	LTBI: 90 TB: 63.6 NI: 53.8	Clinical, radiological, microbiological, and/or pathological results	TST	IL-2, IL-6, IL-8, IL-10, IL-13, TNF-α, IFN-γ, MIG, IP-10, I-TAG, and MCP-1.	**Unstimulated** **LTBI vs TB**: IL-2, IL-10, IL-13, IL-8, and IFN-γ **Stimulated** **TB vs NI**: IL-2, IL-6, IL-13, MIG, IP-10, I-TAG, MCP-1, and IL-8. **TB vs LTBI**: IL-2, IL-6, IL-10, IL-13, TNF-α, MIG, IP-10, and I-TAG, INF-γ **LTBI vs NI**: IL-8.	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6, CFP-10, and TB7.7 Time: 24 hours	Microbead-based method, BD Biosciences, San Jose, CA, USA
Babak Pourakbari, 2015 [38]	Iran	LTBI	Individuals vaccinated and without previous exposure to *M. tuberculosis* and patients infected with *M. tuberculosis*, taken at the hospital	30	PTB = 30 NI = 30	Mean+-SD LTBI: 40.2 ± 15.8 TB: 35.3 ± 18.8 NI: 45.3 ± 5.6	LTBI: 27 TB: 13 NI: 73	Not reported	Culture	QuantiFERON-TB Gold In-Tube Test, TST	IL-2	**Stimulated** **LTBI vs TB and NI**: IL-2	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	PE35 (Rv3872) and PPE68 (Rv3873) Time: 3 days	ELISA, ELISA kit (Mabtech AB, Sweden)
Prachi R. Bapat, 2015 [39]	India	LTBI	Individuals living with TB cases and individuals in the community. Malnourished population	QFT+/TST + = 26 QFT+/TST − = 12 QFT−/TST + = 1 Total 39	NI = 35 Community = 16	Mean (range) 34.4 (12-65)	All participants: 45.9	All participants: 30	N/A	QuantiFERON-TB Gold In-Tube Test, TST	IL-6, IL-10, IL-2, TNF-αR, INF-γ	**Stimulated** **LTBI vs NI**: IL-6 **LTBI and NI vs community**: IL-6, IL-10 **NI vs LTBI:** IL-10	Plasma samples from whole blood (unstimulated or antigen stimulated)	ESAT-6, CFP-10 and/or TB7.7 Time: 20 – 24 hours	Microbead-based method. IMMULITE-1000 Immunoassay System (Siemens Healthcare Global)

Yun-Gyoung Hur, 2015 [40]	Korea	LTBI	Adults with TB, individuals recently exposed to *M. tuberculosis*, healthy participants without *M. tuberculosis* exposure and patients with Non-TB mycobacteria infections	51	PTB = 86 NI = 133 EPTB: 1 (1.7%)	Median (range) LTBI: 44 (18-82) TB: 32 (20-76) NI: 31 (20-61) MNT: (43-84)	LTBI: 74.5 TB: 49 NI: 51 MNT: 76.1	LTBI: 84.6 TB: 56.9 NI: 63.6 MNT: 60.5	Smear/culture or R-rays	QuantiFERON-TB Gold In-Tube Test, TST	IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-22, IFN-γ, TNF-α, IFN-α, sCD40L, CXCL10, VEGF-A	**Stimulated** **TB and LTBI vs Controls**: IFN-γ, IL-2, CXCL10 **Serum** **TB vs NI**: IL-22, CXCL10, and VEGF-A. **TB vs LTBI:** VEGF-A **TB vs MNT:** IL-2, IL-9, IL-13, IL-17 and TNF-α **MNT vs TB:** sCD40L	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated) and serum	ESAT-6, CFP-10 and TB7.7 Time: 24 hours	Microbead-based method, BD FACSVerse" (BD Biosciences, San Jose, CA, USA)
M. Wei, 2015 [41]	China	LTBI	Controls hospitalized for other causes without radiological signs of TB and patients hospitalized for TB	40	PTB = 40 NI = 40	Mean+-SD LTBI: 18.0±10.35 TB: 18.47±12.68 NI: 16±9.06	LTBI: 55 TB: 47.5 NI: 50	Not reported	Clinical diagnosis	T–SPOT®, TST	CCL1, CXCL9, IL-6, IL-10, CSF3, CSF2, IL-1α, IL-8, IL-7, IL-2, TGF-β1, CCL2, TNF-α	**Unstimulated** **TB vs LTBI and NI**: CCL1, CXCL9, IL6, IL-10, CSF3, CSF2, IL-1-α, IL-8, IL-7, IL-2, TGF-β1, CCL2, TNF-α. **Stimulated** **TB vs LTBI**: CCL1 (I-309), CXCL9 (MIG), IL-10, IL-6, CSF2, CSF3, IL-8, IL-1α, IL-7, TGF-β1, CCL2, IL-2, and IL-13	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6 and CFP-10 Time: 20 hours	Quantitative immuno-microarray, (Quantibody Human Cytokine Array 1, RayBiotech, Inc., Norcross, GA)
Ji Yeon Lee, 2015 [42]	Korea	LTBI	Healthy and TB patients from the National Medical Center and Community Health Center of Korea	25	PTB = 24	Mean (range) LTBI: 48 (23-59) TB: 48 (28-75)	LTBI: 44 TB: 37.5	Not reported	Smear (ZN) and/or cultures, and X-rays	TST	IL-1, IL-6, IL-10, TNF-α, IL-17, GM-CSF, IL-4, IL-1β, INF-γ, LXA4 and PGE2	**Monocytes** **Stimulated MTSA LTBI vs TB** IL-10 **MTSA + INF-γ**: IL-1, IL-6, IL-10 **Stimulated - CD4+ T cells and monocytes**	Non-stimulated and antigen-stimulated PBMCs culture supernatants and plasma samples	H37Rv soluble antigens Time: 5 days	Microbead-based method (Bio-Rad Laboratories, Hercules, CA ELISA for IL-1β, ELISA kit (R&D Systems) EIA for LXA4, (Oxford Biomedical

												with PPD: TNF-α. **Plasma** **TB vs LTBI**: LXA4 and PGE2			Research, Oxford, MI) EIA for PGE2, (Cayman Chemical, Ann Arbor, MI) Bio-Plex Multiplex Immunoassay Systems (Bio-Rad Laboratories, Hercules, CA)
Mulugeta Belay, 2015[43]	Ethiopia	LTBI	Individuals from health centers in an endemic area to TB	148	PTB = 147 NI = 68	Mean LTBI: 32 TB: 29.4 NI: 32.4	LTBI: 55.5 TB: 41.5 NI: 52.9	LTBI: 37 TB: 28.1 NI: 35.3	Smear (ZN)	QuantiFERON-TB Gold In-Tube Test	IFN-γ, TNF-α, IL-10	**Stimulated** Basal: **NI vs LTBI and TB**: IFN-γ, TNF-α, IL-10 **NI and TB vs LTBI:** IFN-γ, TNF-α and IL-10 Six months: **TB and LTBI vs NI**: INF-γ. **TB and LTBI** TNF-α and IL-10: baseline <6 months <12 months.	Blood Culture supernatant unstimulated or antigen stimulated	E6C10 and Rv2031 Time: 48 hours	ELISA, Ready-Set-Go! cytokine ELISA kits (eBioscience, USA)
Sunghyun Kim, 2015 [44]	Korea	LTBI	Adult population, contacts of TB cases with and without *M. tuberculosis* infection	22	PTB = 28 NI = 29	Mean (range) LTBI: 46.5 (22-69) TB: 32.1 (21-69) NI: 30.1 (22-44)	LTBI: 86.3 TB: 71.4 NI: 79.3	LTBI: 95.5 TB: 32.1 NI: 79.3	Culture	QuantiFERON-TB Gold In-Tube Test, TST	IFN-γ, TNF-α, IL-2R, IL-4, IL- 10, CXCL9, CXCL10, CXCL11	**Stimulated** **LTBI vs NI**: IFN-γ, TNF-α, IL-2R, CXCL9, CXCL10 **LTBI vs TB**: IL-17 **TB vs NI**: INF-γ TNF-α, IL-2R, CXCL9, CXCL10 **TB vs LTBI:** TNF-α, CXCL11	RNA from Antigen-stimulated whole blood cell pellets	ESAT-6, CFP-10 and TB7.7 Time: 24 hours	Real-time RT-PCR, TaqMan probe assay and the ABI 7500 FAST instrument system (Applied Biosystems, Foster City, CA) ELISA

Ida Wergeland, 2016 [45]	Norway	LTBI	TB case and people with LTBI from a hospital	48	PTB =14 EPTB: 4 NI = 16	Median (range) TB: 32 (18–62) LTBI: 40 (13–67) LTBI borderline: 40 (25–53) NI: 47 (16–68)	TB: 66.6 LTBI: 63.8 LTBI borderline: 63.6 NI: 75	Not reported	Culture or Clinical diagnosis and X-ray	QuantiFERON-TB Gold In-Tube Test	IL-1β, IL-1, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF	**Unstimulated** **LTBI vs TB**: IL-1β, IL-1ra, IL-9 and IL-17A. **LTBI and TB vs NI**: RANTES **NI vs TB and LTBI**: IL-15, eotaxin and basic FGF **NI vs TB**: IL-2, IL-4, IL-13, IL-17A and IFN-γ. **Stimulated** **TB and LTBI vs NI**: IL-1ra, IL-2, IL-13, IL-15, IFN-γ, IP-10 and MCP-1. **LTBI vs LTBI bordeline and NI**: IL-1ra, IL-2, IFN-y **LTBI vs NI**: IP-10, IL-13, IL-15, IL-17A, MCP-1	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6, CFP-10 and TB 7.7 Time: 16 – 24 hours	Microbead-based method, Bio-Plex Pro Human Cytokine Group 27-Plex Panel (Bio-Rad Laboratories Inc., Hercules, CA)
Tao Chen, 2016 [46]	China	LTBI	TB case and LTBI medical staff who worked at the institute for TB prevention. People with cancer and pneumonia	21	It is not clear	Mean ± SEM NI: 25.5 ± 9.1 LTBI: 38.0 ± 10.4 TB: 32.5 ± 12.7 others: 48.6 ± 22.1	21	Not reported	Cough with blood-tinged sputum; Fever; Chest X-rays positive; 4. Microbiological test, IGRA positive	T–SPOT®, TST	IL-8, MIG, I- 309, Eotaxin-2 and ICAM-1	**TB vs NI and LTBI**: IL-8, MIG, and I-309 **LTBI vs NI and others:** Eotaxin-2, ICAM-1, and MIG	Serum	N/A	Microarray and quantitative ELISA, Quantibody Human Cytokine Array 1, RayBiotech, Inc., Norcross, GA
Fabiana A. Zambuz, 2016 [47]	Brazil	LTBI	TB case and people with LTBI from a hospital	14	PTB = 17 NI =16	Mean LTBI: 31.4 TB: 39.6 NI: 27	LTBI: 78.6% TB: 17.6 NI: 81.2	Not reported	Microbiology confirmed and Clinical diagnosis or X-ray	TST	IL-1b, IL-4, IL-5, IL-6, IL-10, IL-12p70, IFN-a2, TNF-a, IFN-γ, IP-10, RANTES, MCP-1, GM-CSF, IL-17, MIP- 1a, MIP-1b, sCD163 and sCD14	**TB vs NI and LTBI**: IL-6, IP-10, TNF-a, sCD163 and sCD14. **LTBI vs TB and NI**: RANTES **TB vs LTBI**: GMCSF	Plasma	N/A	Duoset ELISA for sCD163 and sCD14, and Microbead-based method, 16-plex, EMD Millipore Corporation, Billerica, Massachusetts,

															USA
Miguel Santin, 2016 [48]	Spain	LTBI	Adult population recruited at eight TB centers	43	PTB = 37 EPTB: 32 (46.4%) NI = 28	Median LTBI: 54 (46-64) TB: 41 (31-52) NI: 57 (44.5-77.3) Discordant: 49 (44.5-54)	Not reported	LTBI: 100 TB: 36.8 NI: 33.3 Discordant: 85.7	Microbiology confirmed; or compatible when clinical, radiological and/or ADA and/or histology positive, and cure was achieved after therapy	QuantiFERON-TB Gold In-Tube Test, TST	IFN-γ, IL-2	**Stimulated** **LTBI vs NI**: IL-2, INF-γ	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6, CFP-10 and TB7.7 Time: 72 hours	Quantitative ELISA, Quantikine® ELISA Human IL-2 Immunoassay (R&D Systems Inc, Minneapolis, MN, USA)
Xiangyang Yao, 2017 [49]	China	LTBI	Two cohorts each one with healthcare workers with LTBI and TB case	10 and 15	PTB = 40 and 20 NI = 9 and 15	Median (range) TB: 34.5 (20-78) and 29 (16-67) LTBI: 38.5 (20-48) and 38 (20-67) NI: 33 (18-56) and 48 (18-68)	TB: 60 and 45 LTBI: 60 and 60 NI: 44 and 35	TB: 35 and 25.8 LTBI: 100 and 100 NI: 100 and 86.7	Clinical, radiological, microbiological and histopathological	QuantiFERON-TB Gold In-Tube Test	sCD40L, EGF, Eotaxin, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, TGF-α, TNF-α, TNF-β, VEGF 6Ckine, BCA-1, CTACK, ENA-78, Eotaxin-2, Eotaxin-3, I-309, IL-16, IL-20, IL-21, IL-23,	**Unstimulated** **TB vs NI and LTBI**: sIL-2Ra, IP-10 and MIP-1a **TB and NI vs LTBI:** IL-8 **Stimulated** **TB and LTBI vs NI**: G-CSF, GM-CSF, IFN-γ, IL-1a, IL-2, IP-10, BCA-1 and Eotaxin-2. **TB vs LTBI:** G-CSF. **TB vs LTBI and NI**: IL-8, VEGF, MCP-3	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6 and CFP-10 Time: 22 ± 2 hours	Microbead-based method, Millipore Milliplex map system (EMD Millipore Corporation, Billerica, MA, USA

											IL-28A, IL-33, LIF, MCP-2, MCP-4, MIP-1d, SCF,SDF-1A+β, TARC, TPO, TRAIL, TSLP GCP2, I-TAC, IL-11, IL-29, Lymphotactin, M-CSF, MIG, MIP-3α, MIP-3β				
Jing Wu, 2017 [50]	Not reported	LTBI	Contacts of TB cases with and without LTBI	36	PTB = 25 NI = 31	Mean (range) LTBI: 48 (7-76) TB: 51(22-85) NI: 42 (5-80)	LTBI: 66.9 TB: 28 NI: 65.5	LTBI: 86.1 TB: 68 NI: 77.4	TB contact history, Smear (ZN), Culture, Clinical diagnosis	T–SPOT®, TST	IL-1β, IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin, FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, PDGF-BB, MIP-1β, RANTES, TNF-α, VEGF	**Unstimulated** **LTBI vs TB**: IP-10, PDGF-BB and RANTES. **TB vs LTBI:** VEGF **Stimulated** **LTBI vs TB**: IL-2, IL-10, IFN-γ, IP-10 and TNF-α. **TB vs NI:** IL-2, IL-10, IP-10	Non-stimulated and antigen-stimulated PBMCs culture supernatants	PPD Time: 24 hours	Microbead-based method, Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad, CA, USA)
R. Kamakia, 2017 [51]	Kenia	LTBI	Patients with suspected active TB and patients with active TB from Mbagathi District Hospital, Kenya, as well as contacts of people with TB	16	PTB = 19 NI = 8	Mean (IQR) LTBI: 35.6 (27-39.8) TB: 36.8 (25.8-5.15) NI: 33.5 (23.3-45.3)	LTBI: 50 TB: 21.1 NI: 75	Not reported	ZN, X-ray	QuantiFERON-TB Gold In-Tube Test	IL-17F, IFN-γ, GM-CSF, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-22, IL-9, IL-1b, IL-33, IL-2, IL-4, IL-21, IL-23, IL-5, IL-6, IL-17E/IL- 25, IL-27, IL-31, MIP-3α, TNF-α, TNF-β, IL-28A	**Stimulated** **LTBI vs TB:** IL-17F, MIP-3α, IL-13, IL-17A, IL-5, INF-γ, IL-9, IL-2. **LTBI vs NI**: INF-γ, IL-9, and IL-2	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6, CFP-10 and TB7.7 Time: 2 hours	Microbead-based method, Milliplex w MAP Human Th17 Magnetic Bead Kit (Millipore, St Louis, MO, USA)
Eun-Jeong Won, 2017 [52]	Korea	LTBI	Patients with LTBI, individuals without infection and cases of TB from a university	15	PTB = 48 NI = 13	Median (range) LTBI: 52.0 (36-75) NI: 28.9 (16-74) TB QFT+: 73.0 (15-86)	LTBI: 46.7 TB: 58.3 NI: 53.8	Not reported	Culture	QuantiFERON-TB Gold In-Tube Test	EGF, eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13,	**Unstimulated** **TB vs LTBI:** TNF-α and VEGF. **TB vs LTBI and NI**: IL-8, IL-13, INF-γ, IL-2, IP-10 and VEGF. **Stimulated**	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen	ESAT-6, CFP-10 and TB7.7 Time: 16 - 24 hours	Microbead-based method, Milliplex MAP Human Cytokine/Chemokine 29-plex kits (Millipore, Billerica, CA)

			hospital			TB QFT-: 73.5 (25-89)					IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, VEGF	**LTBI and TB vs NI**: GM-CSF, IFN-γ, IL-1RA, IL-2, IL- 3, IL-13, IP-10, and MIP-1β. **LTBI vs TB:** EGF, GM-CSF, IL-5, IL-10, and VEGF	stimulated)		
Ditthawat Nonghanphithak, 2017 [53]	Thailand	LTBI	All individuals were from Srinagarind Hospital, Khon Kaen. Healthy persons with a history of TB contact and healthy individuals with no known TB exposure.	38	PTB: 48 Early clearance: 162 NI: 39	Mean ±SD LTBI: 45±12 TBA: 52±15 EC: 37±16 HC: 40±14	TB: 35.4 LTBI: 81.6 EC: 66 HC: 82.1	Not reported	Smear (ZN), Culture or a molecular test (Xpert MTB/RIF, Clinical diagnosis	QuantiFERON-TB Gold In-Tube Test	CCL2, CXCL10, IFN-γ	**Unstimulated** **NI vs TB and LTBI:** CCL2 **TB vs NI, EC and LTBI:** CXCL10 **LTBI vs HC and EC:** CXCL10 **Stimulated** **LTBI vs TB**: INF- γ. **TB vs EC and HC**: INF- γ, CXCL10. **TB and LTBI vs NI**: CXCL10.	Non-stimulated and antigen-stimulated PBMCs culture supernatants	ESAT-6, CFP-10 and TB7.7 Time: 24 hours	ELISA, Biolegend, (California, USA).
Marco Pio La Manna, 2018 [54]	Italy	LTBI	Patients with Active TB, health workers and people with LTBI in a hospital.	32	PTB: 27 NI: 20 Others NON-TB pulmonary infections: 20	Range LTBI: 17- 84 TB: 17-82 NON-TB: 24-76 NI: 21-68	LTBI: 25 TB: 22 NON-TB: 40 NI: 30	Not reported	Culture or genexpert MTB/RIF from biopsy specimens and/or biological fluids.	QuantiFERON-TB Gold In-Tube Test, TST	IL-1α, IL-1β, IL-1ra, IL-2 IL-2Ra, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-16, IL-17, IL-18, IFN-α2 IFN-γ, TNF-α, TNF-β, TRAIL, CXCL1 (GRO-α), CXCL9 (MIG), CXCL10 (IP-10), CXCL12 (SDF-1α), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (MCP-3),	**Unstimulated** **LTBI vs NI and NON-TB**: IL- 1β, IL12p70 and VEGF **TB vs NI and NON-TB:** PDGF-BB, IL-1β, IL-2, IL-8, IL12p70, MCP-1 and LIF. **Stimulated** **TB/LTBI vs NON-TB**: IL12-p40, IL-2ra, SCF, TRAIL, IL-2, IFN-γ, IP-10, b-NGF, LIF and MIG. **TB vs NON-TB**: IFNα2, IL-3, and TNF-β. **LTBI vs NON-TB**:	Plasma samples from whole blood (unstimulated, antigen stimulated or mitogen stimulated)	ESAT-6/ CFP-10 Time: 16 – 24 hours	Microbead-based method, there is not information

											CCL11 (Eotaxin), CCL27 (CTACK), G-CSF, M-CSF, GM-CSF, SCF, SCGF-β, LIF, MIF, FGF-β, b-NGF, PDGF-BB, VEGF, HGF	IL-13. **LTBI and NON-TB vs TB**: MIF			
Leonar Arroyo, 2018 [55]	Colombia	LTBI	TB case contacts and TB cases	20	PTB: 21	Median (IQR) LTBI: 38.5 (26.75-52.75) TB: 28 (24-41)	LTBI: 45 TB: not reported	Not reported	Smear (ZN)	Positive response (≥22 pg/ ml) to the CFP10 antigen of Mtb and the absence of clinical symptoms compatible with clinical TB	IFN-γ	**Stimulated** **LTBI vs TB**: IFNγ in response to all antigens	Non-stimulated and antigen-stimulated PBMCs culture supernatants	Mtb DosR (Rv1737c, Rv2029c and Rv2628) and Rpf (Rv0867c and Rv2389c) antigens Time: 7 days	Microbead-based method, Millipore (Millipore, Billerica, MA, USA)

EPTB: Extrapulmonary TB, PTB: pulmonary TB. TB/NRCF: The article does not report the clinical form of TB

***Table 1, an example of paper 1 for the row Increased immune parameters interpretation**: *Unstimulated, TB vs NI and LTBI: IL-10 and IL-6*, means that in an Unstimulated sample, IL-10 and IL-6 were increased in TB compared to not infected individuals and persons with LTBI.

3.3.5 Diagnostic methods for LTBI and active TB

The methods used for LTBI diagnosis were: 18 studies used TST and IGRAs, nine relied on TST alone, one TST or IGRAs plus clinical criteria, and eight utilized IGRAs alone. In studies where the two tests were used, the discordant results between the two tests are evident.

In order to establish the diagnosis of active TB, researchers used one or a combination of the following criteria: history of contact with a TB case, smear (Ziehl-Neelsen or auramine rhodamine stain), culture, clinical diagnosis, molecular test, pathology and/or X-rays.

3.3.6 Measurement of immune parameters

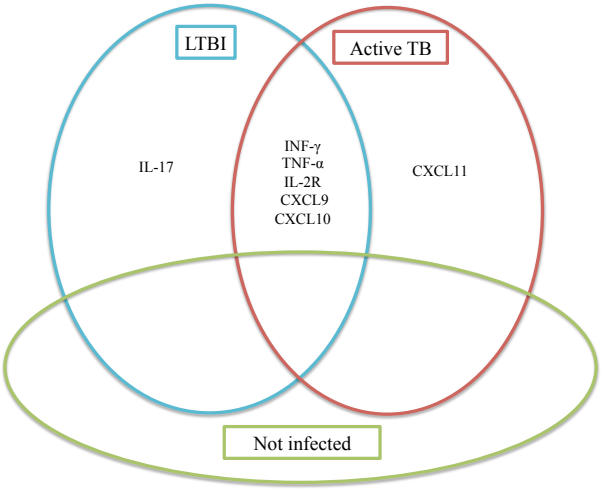
In total, 93 substances (Table 2) were studied, including: growth factors, interferons, receptors, tumor necrosis factors, alpha, beta and delta chemokines, interleukins and others like sCD40L, MIF, and sCD14.

Tabla 3-2 Substances evaluated for ability to differentiate people with active, latent tuberculosis and uninfected with TB.

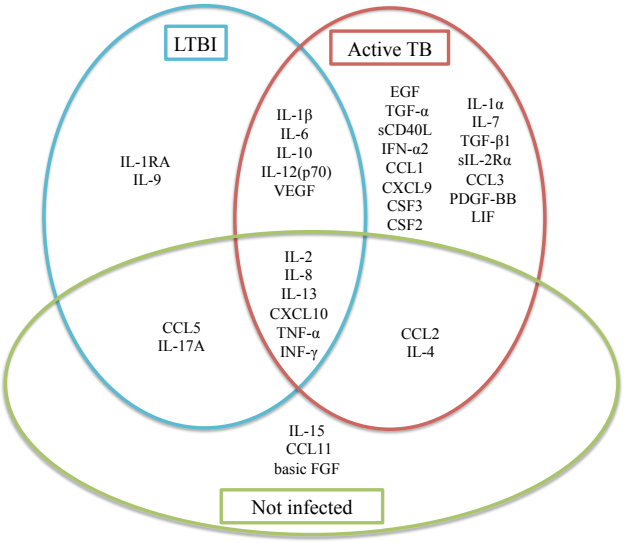
Interleukins	IL-1, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-12 (p40/70), IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-17A, IL-17E, IL-17F, IL-18, IL-21, IL-22, IL-23, IL- 25, IL-27, IL-28A, IL-31, IL-33
Growth factors	PDGF, PDGF-BB, EGF, PGF2α, FGF, TGF-α, TGF-β1, G-CSF, CSF2, CSF3, GM-CSF, VEGF, VEGF-A, SFC, β-NGF, basic FGF
Interferon	INF-γ, IFN-α, IFN-α2
Receptors	IL-1RA, IL-2R, sIL-2Rα, TNF-αR, IL-1R1, IL-1R2
Tumor necrosis factors	TNF-α, TNF-β, TNF-SF10 (TRAIL)
Alpha chemokines	CXCL5 (ENA-78), CXCL6 (GCP-2/LIX), CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC), CXCL12 (SDF-1α+β), CXCL13 (BCA-1)

Beta chemokines	CCL1 (I-309), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL11 (Eotaxin), CCL13 (MCP-4), CCL15 (MIP-1δ), CCL17 (TARC), CCL20 (MIP-3α), CCL21 (6Ckine), CCL24 (Eotaxin-2), CCL26 (Eotaxin-3), CCL27 (CTACK)
Delta chemokines	CX3CL1 (Fractalkine)
Others	ICAM-1 (CD54), sCD163, sCD14, sCD40L, I-TAG, MIF, LIF, LXA4, 15-Epi-LXA4

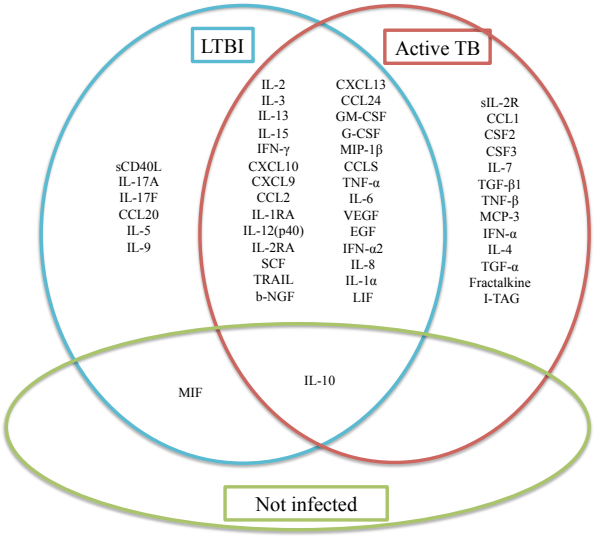
Of these, 24 substances were increased only in active TB, five increased only in the LTBI group, and two in uninfected individuals, regardless of the sample analyzed (Figure 2).



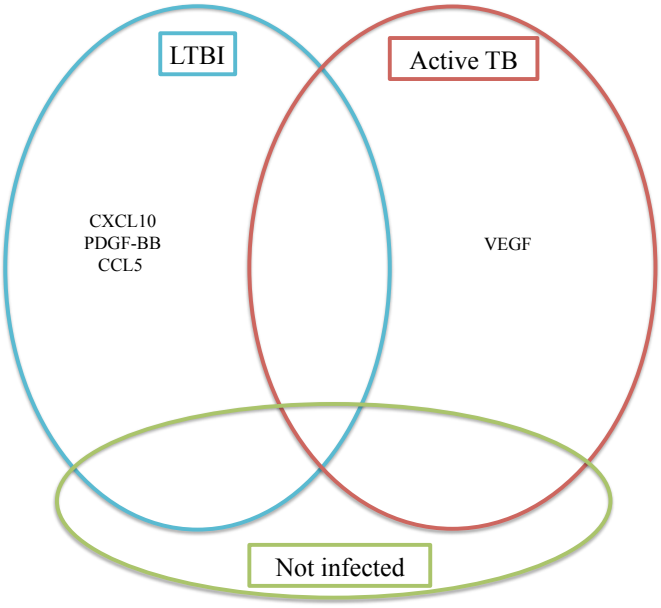
A. RNA from antigen-stimulated from whole blood cell pellets



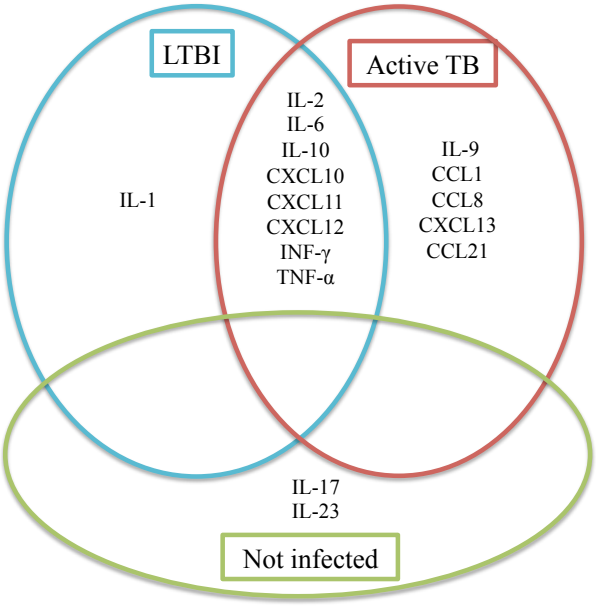
B. Plasma samples from whole blood – Unstimulated



C. Plasma samples from whole blood - Stimulated



D. PBMCs culture supernatant – Unstimulated



E. PBMCs culture supernatant – Stimulated

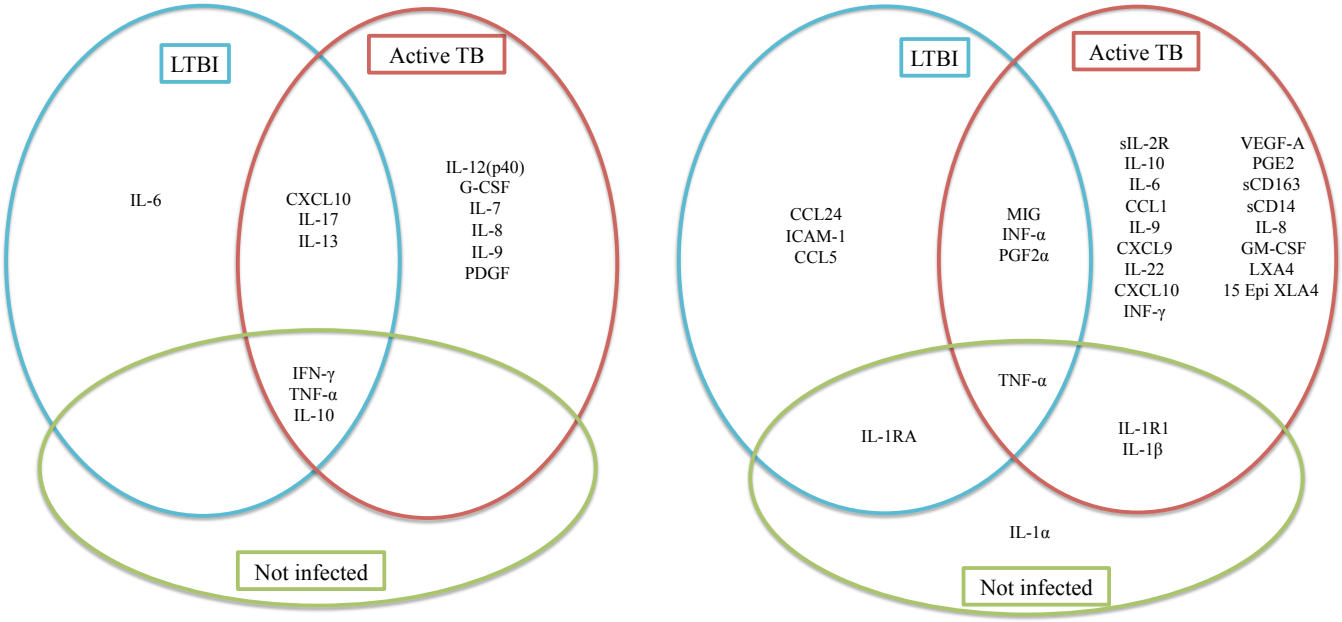


Figura 3-2 A-G. Immune cytokine/chemokine mediators statistically different reported in active TB, latent tuberculosis infection and non-infected individuals in each sample type

Table 1 shows all the antigens and times used for *ex-vivo* stimulation and cytokines whose concentration was statistically different, by each group. The most frequently measured mediators were IL-6, IL-10, IL-2, TNF-α, INF-γ and IP-10.

Most of the studies reported mediators after *ex-vivo* stimulation, with most using supernatants of interferon gamma release assays.

Only eight papers evaluated serum or plasma samples without stimulation. There were no differences between them, only MIG was increased in plasma samples in TB patients, but elevated in serum samples in both, LTBI and active TB.

Among evaluated immune mediators, most were measured in plasma samples from stimulated or unstimulated (as controls) whole blood (Figure 2A-2G). Other samples utilized were supernatants from PBMCs cultures, whole blood culture and RNA from blood cells.

The stimulation antigens used were: ESAT-6, PPD, CFP-10, TB7.7 (Rv2654), TB10.4, PE35 (Rv3872), PPE68 (Rv3873), Rv262, FbpB, E6C10, Rv2031, Protein fraction 11_24 (Rv2626c), H37Rv soluble antigens, DosR Rv1737c, Rv2029c, Rv2628, Rpf Rv0867c Rv2389c, and inactivated bacteria (Table 1).

The concentration of biomarkers involved in the immune response is dependent on the type of protocol used for *in-vitro* stimulation and the sample evaluated, and has high variability between studies. Among the five substances exclusively elevated in LTBI and the two elevated in uninfected individuals, there was inconsistency in the samples processed throughout the studies. For example, IL-5 was evaluated in plasma (1 article), plasma samples from stimulated whole blood (11 articles), PBMCs culture supernatant (3 articles), blood culture supernatant (1 article), but was only increased in two papers that used plasma samples from stimulated whole blood (2/11 articles). In the case of active TB, one substance (CCL1/ I-309) was elevated in four different samples types; one (IL-7) in three samples types, and the rest in two (usually plasma samples from whole blood unstimulated and stimulated) or one sample types.

ELISA (n=17) and microbead-based method (n=20) were the most frequently used methods for IP measurement (Table 1). Some of the articles used both methods. Table 1 describes details regarding to laboratory measurements.

3.3.7 Risk of bias of included articles

Of the 36 articles reviewed using the Newcastle-Ottawa scales and the Quality Assessment Scale for NIH observational studies, 7 articles had low risk of bias, 29 moderate, and none had high risk of bias. Only one study out of the 36 performed calculation of the sample size and took into account the statistical power of their results (Additional file 3 and 4).

3.3.8 Biases

The main biases identified in the articles reviewed were: absence of a second administration of tuberculin skin test to detect a possible booster effect, thus leading to the potential inclusion of individuals with false negative results of the TST. Another bias was the analysis of patients with pulmonary and extrapulmonary TB in the same group of active TB as the underlying immune competence and immune response may vary between localized or disseminated disease. In addition, children and adults were included in some studies, however the analysis was not stratified for each population. Finally, patients with pulmonary TB were included in different phases of treatment; some studies included individuals that completed treatment at the time of IP measurement. The declining microbial burden during or at the end of therapy may contribute to false negative results (Additional file 3 and 4).

Only 4 of the reports took into account the study origin and population's ethnicity as a confounding factor, and these were evaluated by self-reported ethnicity [25,38,41,46].

Of the included manuscripts, 27 articles provided a declaration of conflicts of interest (S3 and S4).

3.4 DISCUSSION

The immune response against infection and disease caused by *M. tuberculosis* is mainly mediated by the recruitment and activation of T cells and macrophages, which in turn are regulated by multiple immune mediators such as interleukins and chemokines, possessing a diverse pro and anti-inflammatory properties. The success of the immune response in halting the acquisition of *M. tuberculosis* is influenced by a myriad of environmental, microbial and host factors. The host response is measured in order to determine *M. tuberculosis* infection in the form of skin tests or IGRAs, but this approach is limited by the inability to differentiate LTBI from active TB infection. The ability to refine

diagnostics by using assays that incorporate measurement of multiple biomarkers will be critical in order to stride towards TB control and eventual elimination.

Most of the studies analyzed in this review focused on the main pro and anti-inflammatory interleukins involved in the immune response, mediated mainly by Th1 and Th2 lymphocytes; a few others expanded the markers measured to include chemokine-like substances, growth factors and receptors as part of the search for new diagnostic biomarkers that can discriminate between LTBI and active TB.

Several immune mediators in addition to INF-γ, have been identified. The most frequently evaluated markers are the cytokines IL-6, IL-10, IL-2, TNF-α, and IP-10. Although the response to TB is reliant on Th1 (TNF-α, INF-γ and IL-2), these have been expanded by addition of Th2 signature cytokine profile such as IL-6 and IL-10.

Elevated immune mediators and markers that where only detected in active TB share a chemoattractant functions involved in trafficking of cells involved in the immune response, among which are: T lymphocytes (CD4 + and CD8 +), macrophages, dendritic cells, basophils and eosinophils. These cytokines affect cell growth, maturation and differentiation (Additional file 5). In LTBI, only interleukins IL17F, and IL-5, associated with effector T cell profiles, are overexpressed. The effect of the cytokines found overexpressed in LTBI is related to the increased production of immune substances, chemoattraction, multiplication and activation of lymphoid cells[56]. Of the cytokines identified in the systematic review, three (IL-12 and TGF-β for active TB, and IL-23 for uninfected individuals) are well established markers involved in immune response to mycobacterial infection (Figure 3: Available at: http://www.genome.jp/kegg/pathway.html) [57].

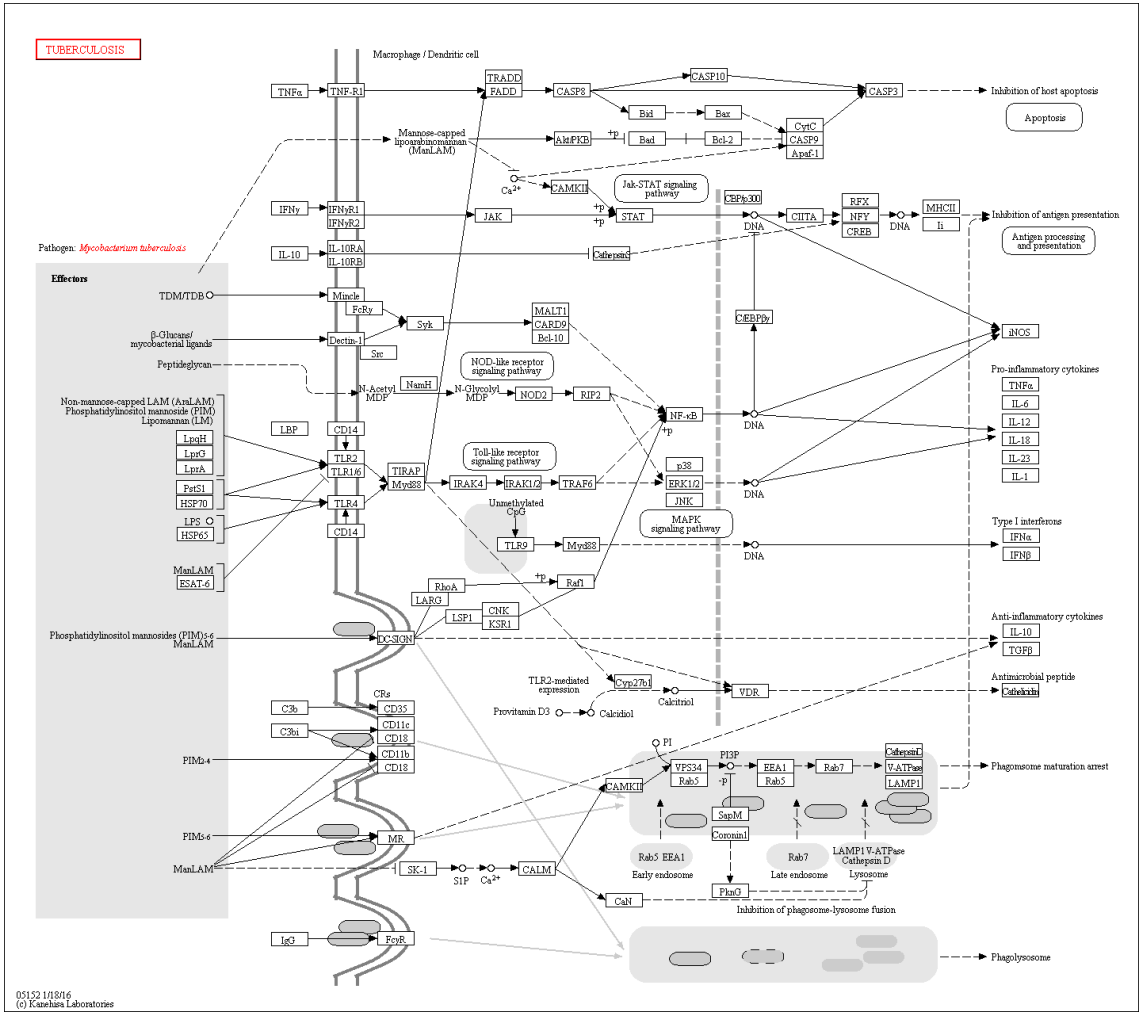


Figura 3-3 KEGG pathway, highlighting some of the pathways and mediators identified in the reviewed studies. Available at: http://www.genome.jp/kegg/pathway.html).

While most attention has been directed to immune cells, some of the immune substances that participate in the response to *M. tuberculosis* are produced by epithelial cells, which play a fundamental role in the initiation and expansion of host defense mechanisms in the lung, providing protection against mycobacteria. Epithelial cell participate in activation of innate immunity, as well as adaptive immunity, inducing the recruitment and activation of dendritic cells, T and B lymphocytes, which in turn increase antigen recognition, production of antibodies and other immune substances [58,59]. These markers merit further investigation for the ability to distinguish early and late mycobacterial infection.

Despite some signals suggesting that the biomarker expression differences between LTBI and active TB can be used for diagnostics, choosing a panel of reproducible, discriminatory markers based on the results of the studies analyzed is quite difficult due to:

1. Failure to account for the time / illness of the individuals studied. Not surprisingly the biology of TB is much more complex than previously thought, and therefore classification in LTBI and active TB is insufficient. What is considered LTBI actually corresponds to a range of infection status, which may have been recently acquired or present for decades. Recently acquired TB is associated with higher progression rate to active disease pointing to distinct biological properties. The study of pulmonary immune substances in animal model reveals changes in the expression of cytokines / chemokines in the cells that make up the granuloma. The diversity of granulomas (diverse functions and architectures and microenvironments) have consequences on the bacterial control [60,61]. It is suggested that after the in vitro stimulation, changes in the cellular expression due to the phase of infection or tuberculosis disease can lead to a varied response that is evidenced in the analyzed studies. Among the papers included, there were 22 different antigens used for in vitro stimulation, with concentrations that widely varied within the same immune factors and within the same group of patients, for example, INF-γ ranges from 0 to 2640 pg/ml, with overlapping concentrations between the uninfected individuals, LTBI and active TB group, independent of the sample used (Additional file 6).

Given the heterogeneity of the biology of disease associated with LTBI, the ability to

identify the duration of infection remains a challenge for future research. Inability to determine the duration and type of LTBI (ie, what type of granuloma), might modify the observed response to a mycobacterial antigen leading to blurring of the ability to interpret differences between study groups [60,61].

2. Different samples and varied cell stimulation protocols. The samples used for the studies were predominantly plasma, however, culture supernatant, serum and RNA were used introducing variability in measured concentration caused by the matrix used (Table 1 and Additional file 6). Several potential reasons for the variation in immune substance concentrations in plasma and serum from whole blood include: inhibition of detection for specific cytokines (e.g., EGF, GM-CSF, IL-3, and IL-4) in the serum [62]; delay in processing of serum or plasma, sample hemolysis, presence of debris, or freeze-thaw cycles all of which can adversely affect cytokine detection[63]; and the release of several mediators by platelets which can increase cytokine serum levels, especially CCL5 and CD40L[64].

In addition, the wide variety of antigens (ESAT-6, CFP-10, TB7.7, PPD or Mtb CFA, among others) used to stimulate cells and different incubation times, leads to the increases or decreases the time of cellular exposure to the stimulus, and therefore, the concentration of the detected immune mediators and other substances. In addition, cellular stimulation adds complexity to the diagnostic utility of detecting biomarkers, especially in areas with limited laboratory infrastructure or access, as is the situation in many of the countries or settings where TB is endemic. In addition, reviewed articles show variations in the results due to the antigen used for stimulation [11–13]. In experiments with whole bacteria, it has been demonstrated that the strain used to carry out stimulation modifies the type of immune response in vitro; for example, the most recent strains in the *M. tuberculosis* lineage show a lower inflammatory response in macrophages when compared to the older strains [65]. Likewise, Leyten et al. evaluated 25 antigens of latency related to the DosR regulator of *M. tuberculosis*, it was observed that different antigens can give different cellular responses (measured by the production of INF-γ) after in vitro stimulation, and in addition, this can vary between healthy people, LTBI and active TB cases [11]. This limitation can be overcome

in longitudinal studies applying the same measurement at different times along the natural history of *M. tuberculosis* infection.

3. The variety of laboratory methods used for detection of substances, which in turn leads to the variable units of measurement and assays sensitivity. The ability to compare the heterogeneous samples is further compounded by use of ELISA, microbead assays, EIA and real time PCR- in the absence of endogenous standard, yield variable dynamic ranges [66]. The intra individual variability cannot be assessed, as only 2 studies were longitudinal. This variability results in difficulty to compare and quantify studies.

4. Another significant limitation of the studies analyzed is the presence of a selection bias for non-application of the booster when individuals are screened using the tuberculin skin test. It is known that the booster effect can occur in individuals and is only detected when a second TST is applied to negative individuals between 1 to 4 weeks after the first administration. The increase in the frequency of positive individuals is notable in the population without any underlying diseases (in prisoners an increase in positivity was reported from 66% to 77.6% [67]), or in those with other disorders such as rheumatoid arthritis (where the booster positivity changed from 31.3% and 21.7% to 46.5% and 28.8% in early and late rheumatoid arthritis, respectively) [68]. The lack of application of two-step TST may lead to erroneous classification to the uninfected group, resulting in false negative results [67–70]. Equally important, LTBI diagnoses were done using TST and/or IGRAs, which can introduce heterogeneity within the results. Indeed, in many studies when both methods were used, the results demonstrated inconsistent findings, a common theme discussed in literature [71–73]. Additionally, although some articles used TST for LTBI diagnosis, they did not consider the rate of BCG vaccination within children under 10 years old in their analyses [74].

5. None of the studies adjusted the analysis for the effect of ethnicity on the association between IP concentrations and the different stages of TB. A study published by Coussens et al. reported that the inflammatory profile differs according to ancestry. Individuals of African descent with TB, despite having similar mycobacterial strains, similar

sociodemographic and clinical characteristics, have a different inflammatory profile compared to Eurasian patients with the same disease [75]. Similarly, Mwantembe O et al. reported ethnic variation of cytokines (IL-1RA, IL-12) and chemokines (CCL2, CCL5, CCL11, CXCL8) in South African patients with inflammatory bowel disease [76]. The concentrations of these chemokines and cytokines are determined by allelic frequency and have been involved in the response to *M. tuberculosis* infection. Likewise, genes coding for proteins such as CCL2 [77], IL-17F, IL-17A [78], IL-12(40) [79], have been described as polymorphic, variation in allele frequency is affected by ethnic variation, affecting the antimycobacterial response and thus, may be driving the higher risk for development of TB among different populations. The examples emphasize the importance to adjust by ethnicity of the population at the time of reporting the results as these clearly impact biomarker concentrations.

Ethnicity is also related to the response to current *M. tuberculosis* infection screening tests. Genetic variants associated with the reaction to TST and IGRAs have been described. The TST1 locus associated with a TST positivity per se (TST1 on 11p14), and the TST2 locus associated with the intensity of TST reactivity (TST2 on 5p15) [3]. On the other hand, the production of INF-γ has been associated with genetic factors such as the locus located in chromosomal regions 8q12-22n and 3q13-22 [2]. The ethnicity must be considered when performing the immunological analysis in further research.

6. The heterogeneity of the populations studied. First, the comparison group of active TB included patients with pulmonary TB and extrapulmonary TB together. In these two groups of patients the presentation of the disease is different, and the main factors of innate immunity, cytokines and chemokines, which play a role in cell-mediated immunity, involved in the dissemination of *M. tuberculosis*, differ. Mutations have been reported in genes encoding the INF-γ receptor, the IL-12 receptor and the transcription-activating signal1 (STAT-1) in patients with extrapulmonary TB. Likewise, Dong Yang et al. reported that there are differences in the immunopatogenicity of pulmonary and extrapulmonary infections. The production of CCL2, CXCL9 and CXCL8 modifies the type of tuberculous disease that a patient has, and they play a special role in the formation of granuloma [80]. Patients with pulmonary TB showed lower levels of the cytokines studied than those with

extrapulmonary TB. CXCL8 concentration was found to be elevated in fatal TB, increases in CCL2 were observed with disseminated and meningeal TB [81], and TGF-β increased in extrapulmonary TB in children compared to pulmonary TB [14].

Secondly, patients with active TB included in the studies were at different phases of treatment (before, during and after completion of antituberculous therapy). Several studies have been carried out with the aim to evaluate new biomarkers that allow monitoring the patient's condition after initiating antituberculous therapy. Several of the studies were longitudinal, making it evident that the immune substances changed during the administration of TB treatment [82,83]. Changes in lung bacterial load related to treatment administration would appear to influence the concentration of cytokines detected in non-stimulated cells, with 17 out of the 27 cytokines / chemokines analyzed (IL-1β , IL-2, IL-4, IL-5, IL-6, IL-9, IL-13, IL-17, Eotaxin, IFN-γ , IP-10, MCP-1, MIP-1α , MIP-1β , PDGF, RANTES, VEGF) being significantly lower in patients with higher bacterial load, and levels of IL10, IL15 and TNF-α being higher in the same patients [84].

One final aspect of the study of immune substances to differentiate LTBI compared to TB and non-infected individuals is the effect of BCG vaccine. BCG vaccination has been associated with the modulation of the host's immune system with some suggested protection against MTB infection and disease [85,86]. BCG vaccination could potentially modify the concentration of immune substances in vaccinated adults, based on the changes in the concentrations of some immune parameters in children and adolescents[87]. Further studies should evaluate the BCG vaccination effect and duration on the immune markers concentrations.

Finally, it is important to note that our review did not include HIV infected populations or individuals with other type of immunosuppression, nor children, since those populations have several confounders and particular characteristics that need to be analyzed separately and are beyond the scope of the present review. In addition, as the main goal of our paper was to identify immune markers associated with LTBI, we did not included articles that consider biomarkers for TB before and after the treatment.

3.5 CONCLUSIONS

Identification of biomarkers that individually or in combination can differentiate LTBI and active TB have been a research priority, however, a constellation of markers that differentiates between infection and disease is not yet available. The advances in high throughput technologies for biomarker measurement are promising but the variability of studies and potential biases that we have highlighted undermine the ability to identify reproducible markers. Although five parameters were exclusively increased in LTBI and 24 in active TB, only a single substance was consistently differential. These substances were not measured in all studies and results are inconsistent between study groups, prohibiting the desired classification. Undoubtedly, the study of multiple immune substances seems to give better results than the study of a single biomarker, consequently, the search for immune profiles with multiple immune substances, should be the goal of future research.

In order to the results obtained with different immune markers future research should 'harmonize' the methodological conditions to evaluate immune markers as the first step to draw any conclusion about LTBI parameter(s) to use as a diagnostic test. Those aspects include the presence of the booster effect, clinical classification of TB, the ethnicity of participants, sample size estimation. In addition, cohort studies will allow to identify immune substances associated with conversion to LTBI and progression to active TB, and variations in the immune response due to the individual's stage of TB, measurement variation for cytokine/chemokines, and hormonal influences [88,89].

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3.7 Material suplementario Capítulo 3 (Ver archivo de Excel)

Anexo 3-1Additional file S1 – Search strategies
Anexo 3-2Additional file S2 – Summary of excluded studies
Anexo 3-3Additional file S3 – Quality of cohort studies
Anexo 3-4Additional file S4 – Quality of cross-sectional studies
Anexo 3-5Additional file S5 – Immune factors, production cell, diana cell and effect in different tuberculosis stages
Anexo 3-6Additional file S6 – Concentration of immune factors in persons with different tuberculosis stages.

4 CAPITULO 4

CYTOKINE/CHEMOKINE IN PEOPLE IN PRISON WITH EARLY INFECTION BY *Mycobacterium tuberculosis*

Abstract

We hypothesize that people with early latent tuberculosis infection (LTBI) but without active TB have a unique cytokine/chemokine profile that could be used as a potential biomarker.

Methods: We evaluated socio-demographic variables and levels of 18 cytokines/chemokines in plasma samples from 47 patients with pulmonary TB, 24 people with new LTBI [people with negative two-step TST that became positive during follow-up, were divided in early LTBI (they had ≤3 months of incarceration at enrolment) and late LTBI (they had ≥1 year of incarceration at enrolment)], and 47 people without infection after two-years of follow-up, from two Colombian prisons. We performed a multinomial regression to identify the immune parameters capable to differentiate between the groups.

Results: The concentration of immune parameters changed over time. The concentration of sCD14 and IL-18 were increased in early LTBI individuals compared to those with late LTBI. IP-10 was increased in late LTBI compared to early LTBI.

We identified that high concentration of chemokine MIP-3α increases the risk of early LTBI (adjusted relative risk ratio – aRRR= 10.59; 95% CI [2.84–39.59]). High concentrations of MCP-1 are associated with an increased risk of late LTBI. We found that high concentrations of sCD14 (aRRR= 26.30; 95% CI [11.83-58.44); INF-γ (aRRR= 8.67; 95% CI [1.35-55.89]), and TNF-α (aRRR= 5.41; 95% CI [1.09-26.89]) are associated with an increased risk of active TB, while high concentrations of IL-18 (aRRR= 0.06; 95%CI [0.01-0.40]), and MCP-1 (aRRR= 0.16; 95%CI [0.05-0.53]) and BCG vaccination (aRRR= 0.11; 95%CI [0.03-0.43]) decrease the risk of having active TB compared to non-infected individuals.

Conclusions: There are immune markers associated with new LTBI, and active TB. However, the concentration of immune mediators varies among newly infected individuals in relation to the time of incarceration (early and late LTBI), potentially serving as a proxy

of duration and intensity of the exposure in a high-risk setting. Further studies are needed to evaluate new biomarkers using 'omics' approach to identify new LTBI, that take into account different concentrations between groups by the time of exposure, to understand the changes in the immune response to the mycobacterium.

Keywords
Latent tuberculosis infection; *Mycobacterium tuberculosis*; cytokines; chemokines; tuberculosis; biomarkers.

4.1 BACKGROUND

Diagnosis and treatment of latent *Mycobacterium tuberculosis* infection (LTBI) presents one of the major challenges in the path towards tuberculosis (TB) elimination. A systematic review published in 2019 found that the global prevalence of LTBI is 24.8% (95% CI: 19.7-30.0%) and 21.2% (95% CI: 17.9-24.4%) based on the results of the Interferon-gamma Release Assays (IGRAs) and Tuberculin Skin Test (TST), respectively[1]. Prevalence among high-risk populations, including incarcerated people, are generally higher and can be as high as 88.8%[2].

There is an urgent need to improve techniques for the diagnosis of LTBI, since the TST has a sensitivity of 77% (CI, 71-82%) and IGRAs between 70-90%, with a specificity of 98% in non-BCG vaccinated populations for both tests, and the TST having lower specificity in BCG-vaccinated populations[3]. Test results may be affected by multiple circumstances, such as recent vaccination with live vaccines or BCG, immunosuppression states, and non-tuberculous mycobacterial infection[4–7]. Moreover, these tests are unable to determine if an individual has a persistent and controlled infection, or a cleared up infection long after contact, and are unable to distinguish between infection and disease[8–11].

Despite numerous articles reporting immune markers associated with LTBI and TB, there are high level of heterogeneity in the design, samples processing and analysis that make difficult to compare study results between them due to: inclusion of people with different

clinical presentation (pulmonary and extrapulmonary TB)[12–14], or with different phases of anti-TB treatment, individuals with LTBI in whom the time of infection is not known, the inclusion of "healthy" individuals where the presence of infection was not ruled out, discordant results between IGRAs and TST[15], the administration of a single dose of TST, ignoring the existence of a booster effect in those with negative results[16–18], and the use of different stimulation protocols (antigens, incubation times and sample variables)[19–21].

Measurement of cytokines/chemokines in stimulated samples, whether from isolated PBMCs or whole blood, have the advantage of detecting a specific immune response, induced by TB. Also, using PBMCs has the advantage that the number of cells that are producing the substance is known, and there is no interference by other cellular components. However, isolating or stimulating cells are associated with cost and infrastructure requirements, with some assays requiring freshly obtained sample and large blood volumes making these more complicated to administer, especially in resource limited settings[22,23].

In this study, we focus on an incarcerated population from two prisons where we have previously shown one of the highest rates of infection and disease[24]. Active TB occurs most frequently in newly infected people, within the first 2 years of acquiring mycobacterial infection, thus, identifying this group of people and prioritizing them to offer LTBI treatment could be an effective measure to decrease TB transmission. The objective of this study was to determine the plasma concentration of 18 cytokines/chemokines associated with the presence of new (recent) *Mycobacterium tuberculosis* (MTB) infection, and compare it with the concentration in people with pulmonary TB and exposed but uninfected people.

4.2 METHODS

4.2.1 Ethics statement

Approval for the study was obtained from the Ethics Committees of the Universidad Pontifica Bolivariana and the University of Manitoba. The Instituto Nacional Penitenciario

y Carcelario (INPEC), and the director of each prison approved the project. Written consent forms were explained and signed in the presence of two witnesses (prisoners). All inmates diagnosed with active TB received treatment and follow-up whether or not they agreed to participate in the study. New converters were reported to prison health authority in both prisons, and prisoners from prison 1 were offered LTBI treatment. LTBI treatment in prisoners is not considered mandatory in the international and Colombian guidelines, the healthcare personnel from prison 2 opted not to offer LTBI treatment, and therefore prisoners incarcerated there did not receive it. Only one patient with LTBI progressed to active TB at prison 1, and was treated accordingly; the patient was diagnosed with active TB before LTBI treatment initiation.

4.2.2 Study design and settings

This was a cohort study conducted between September 2016 and December 2018 in two male prisons in Colombia.

4.2.3 Participants

We included 62 prisoners with negative two-step TST and one year or more of incarceration (Cohort 1), 62 prisoners with negative two-step TST and less than three months of incarceration (Cohort 2), and all pulmonary TB cases diagnosed in prison (n= 51).

4.2.4 Eligibility criteria

Cohorts 1 and 2:

Inclusion criteria: Individuals who had two-step TST administration at baseline, who voluntarily agreed to participate in the study.

Exclusion criteria were: (1) administration of live vaccines (measles, mumps and rubella, varicella or the live attenuated influenza vaccine) in the 4 weeks before TST administration, (2) severe adverse event with a previous TST administration, (3) previous active TB, and (4) people who had a sentence of less than 2 years of incarceration in prison (released or moved into house detention) because of the challenges for follow up.

Active TB group:

People diagnosed with pulmonary TB with less than 15 days of anti-tuberculosis treatment who voluntarily agreed to participate in the study and signed the written consent form.

4.2.5 Procedures

Entry and follow up

A nurse visited each prison from Monday through Friday during the study period and identified individuals who met the inclusion criteria as described above and reported by Rueda et al.[25]. At baseline, a blood sample, a TST application (see below) and socio-demographic data were collected. Follow-up was carried out every three months, to collect socio-demographic and clinical information as well as a blood sample; TST was performed every six months or until a positive result was obtained.

Data source

The following socio-demographic biometric information was collected from all individuals: age; history and time of prior incarceration; use of drugs (inhaled, injected, or smoked) or alcohol; comorbidities (chronic obstructive pulmonary disease, diabetes, chronic kidney disease, HIV, and any other immunosuppressive disease); contact with a TB case (outside and/or inside the prison); history of prior TB, including date of last episode, and outcome; city and neighborhood of residence before imprisonment; weight and height. To determine previous exposure to the BCG vaccine, the filed team checked all participants for the presence of a scar.

Sample collection

All prisoners included in the study provided blood samples at baseline and during follow-up. The samples were collected in sodium heparin tubes, the plasma samples were separated and stored at -80°C until processing.

Diagnosis of LTBI

LTBI diagnosis was based on TST administration with tuberculin PPD RT-23, 2 TU/0,1 ml, Statens Serum Institute®), according to CDC recommendations[26]. Reading was performed within 48–72 h of administration and measured in mm of induration across the forearm. A second TST was applied in the case of a negative result in the first administration (to detect the booster effect) and the case definitions (converter and negative) were applied as in our previous work[24]. Conversion was defined as a new reaction (after booster application) ≥10 mm and an increase of at least 6 mm[27].

Diagnosis of pulmonary TB

Individuals with active TB provided a spontaneous or induced sputum sample. All the sputum samples were processed using the conventional sodium hydroxide-N-acetyl-L-cysteine method, with standard decontamination, and concentration methods. A smear was prepared for auramine-rhodamine staining to visualize acid-fast bacilli (AFB). Sputum sample was inoculated in Lowenstein-Jensen (LJ) medium, in a mycobacterial growth indicator tube (MGIT) incubated in MGIT 960 BACTEC instrument (BD Diagnostics, Sparks, MD, USA), and in thin-layer agar (TLA) for the detection of resistance to rifampicin and isoniazid as previously reported[25]. *M. tuberculosis* was identified by standard biochemical tests.

Cytokines and chemokine inclusion and detection

The cytokines/chemokines quantified in the study were selected based on: 1) a systematic review conducted to identify the relevant cytokines/chemokines associated with LTBI[28] [Chapter 3] (protocol number: PROSPERO 2017 CRD42017073289). 2) published reviews about the pathophysiology of *M. tuberculosis* infection[29,29–31], 3) results from animal models (guinea pig, macaques and mice)[29,30,32–34], 4) immune response to intracellular bacteria[35,36], 5) *M. tuberculosis* pathway[37] (available at: http://www.genome.jp/kegg/pathway.html) and 6) our previous results in patients with active TB[38]. Table S1 has information about the selection criteria.

Commercial multiplex and singleplex bead-based fluorescent assays kits were used to quantify 18 cytokines/chemokines of interest from plasma samples as follows: Macrophage Inflammatory Protein 3α (MIP-3α/CCL20), Human Cytokine/Chemokine magnetic Panel

III, Milliplex® Map kit); Interleukin 18 (IL-18), Human IL-18 Singleplex Magnetic Bead kit, Milliplex® Map kit; soluble CD14 (sCD14), Human Cardiovascular Disease (CVD) Panel 6 Magnetic Bead kit, Milliplex® Map kit; Eotaxin 1 (CCL11), Interferon gamma (INF-γ), Interleukin 5 (IL-5), Interleukin 6 (IL-6), Interleukin 10 (IL-10), Interleukin IL-12 p40 homodimer (IL-12[p40]), Interleukin 13 (IL-13), Interleukin 15 (IL-15), Interleukin (IL-17), Interleukin-1 receptor antagonist (IL-1RA), human interferon-inducible protein 10 (CXCL10/ IP-10), monocyte chemoattractant protein-1 (CCL2/MCP-1), macrophage inflammatory protein 1α (CCL3/MIP-1α), macrophage inflammatory protein 1 β (CCL4/MIP-1β), Tumor necrosis factor alpha (TNF-α), Human Cytokine/Chemokine magnetic Bead Panel, Milliplex® Map kit, Millipore Corporation, Billeria, MA, USA. The limit of detection for each substance is in the supplementary material S2.

The assays were performed according to the manufacturer's instructions, using 25µl of plasma per sample (5 µl to run sCD14 assay) and overnight incubation. Standards were reconstituted and serially diluted to generate standard curves. Two controls with low and high concentration, provided by the commercial kit, were included in each assay and considered as positive controls for the experiment. Results were analyzed in the BioPlex-200 instrument (Bio-Rad, Mississauga, Canada) and reported as mean fluorescence intensity and converted to pg/ml or ng/ml using the BioPlex® Manager version 6.0 (Bio-Rad, Mississauga, ON).

To control for potential biases, the samples were processed using standardized protocols, with two thawing steps (aliquots from the main sample and processing), and the bead assays and follow up were performed by the same people.

4.2.6 Analysis

The primary outcome for this study was new LTBI (converters) during follow-up. We divided the converters according to the time of incarceration at baseline: early LTBI those people with ≤3 months of incarceration (group 1), and late LTBI those people with 1 year or more of incarceration (group 2).

We measured plasma concentration of 18 cytokines/chemokines at baseline for active TB cases, at baseline, the time point of TST conversion, and 3 months before to TST

conversion for new LTBI cases, and at the last follow-up for non-infected (TST negative) people. We compared new LTBI and late LTBI to non-infected (group 3, exposed people who are negative TST at the end of the follow up) and to pulmonary active TB cases (group 4).

Pre-treatment of the data was performed as follow: 1) samples with levels of cytokines/chemokines below the lower limit of detection, were assigned the value of limit of detection divided by two, and 2) Cytokine/chemokine concentrations were categorized for the multinomial analysis because the immune markers did not meet the assumption of monotonicity for quantitative variables.

We used descriptive statistics (median [IQR] and n[%]) to report the variables of interest (cytokines/chemokines and socio-demographic variables), and chi-squared and Mann-Whitney tests were used to evaluate differences between groups. Wilcoxon test was used to compare the changes in cytokines/chemokines over time between early LTBI and late LTBI groups. A two-tailed p-value <0.05 was considered significant.

We evaluated the BCG scar as a potential interaction term with the cytokine/chemokine concentrations and the presence of early LTBI, late LTBI and active TB compared to non-infected individuals.

For multivariable analysis, we used multinomial logistic regression to determine the effect (relative risk ratio) of each cytokine/chemokine on the outcome (early LTBI, late LTBI, active TB, non-infected). Variables included in the final regression model were selected using biological plausibility criterion, and, a manual backward elimination method, looking for the effect of each cytokine on the coefficients and confidence intervals. Moreover, we evaluated socio-demographic variables to adjust the model and we retained variables that changed the coefficients and confidence interval. All models were adjusted by geographic cluster, using 15 different courtyards in the two prisons. All analysis was done in STATA® version 14.

4.3 RESULTS

4.3.1 Study participants

At the end of the follow-up, there were 25 converters (new LTBI) among 124 people who had a negative two-step TST at the time of enrolment. 13 out of 25 were early LTBI (they had 3 months or less of incarceration at enrolment); and 12 late LTBI (they had more than 1 year of incarceration at enrolment). We had 21 samples available at baseline, 18 at pre-conversion (3 months before TST conversion), and 19 at the time of TST conversion (Fig 1). At the end of the follow-up one converter developed active TB.

Ninety-nine individuals remained TST negative after the follow-up, and we evaluated the cytokines/chemokines in 43 out of 99 prisoners with the longest follow-up.
Additionally, we included 51 cases of pulmonary TB and we had plasma samples at baseline from 50 of them (Fig 1). We excluded 3 participants with HIV infection, thus 47 were included in analysis.

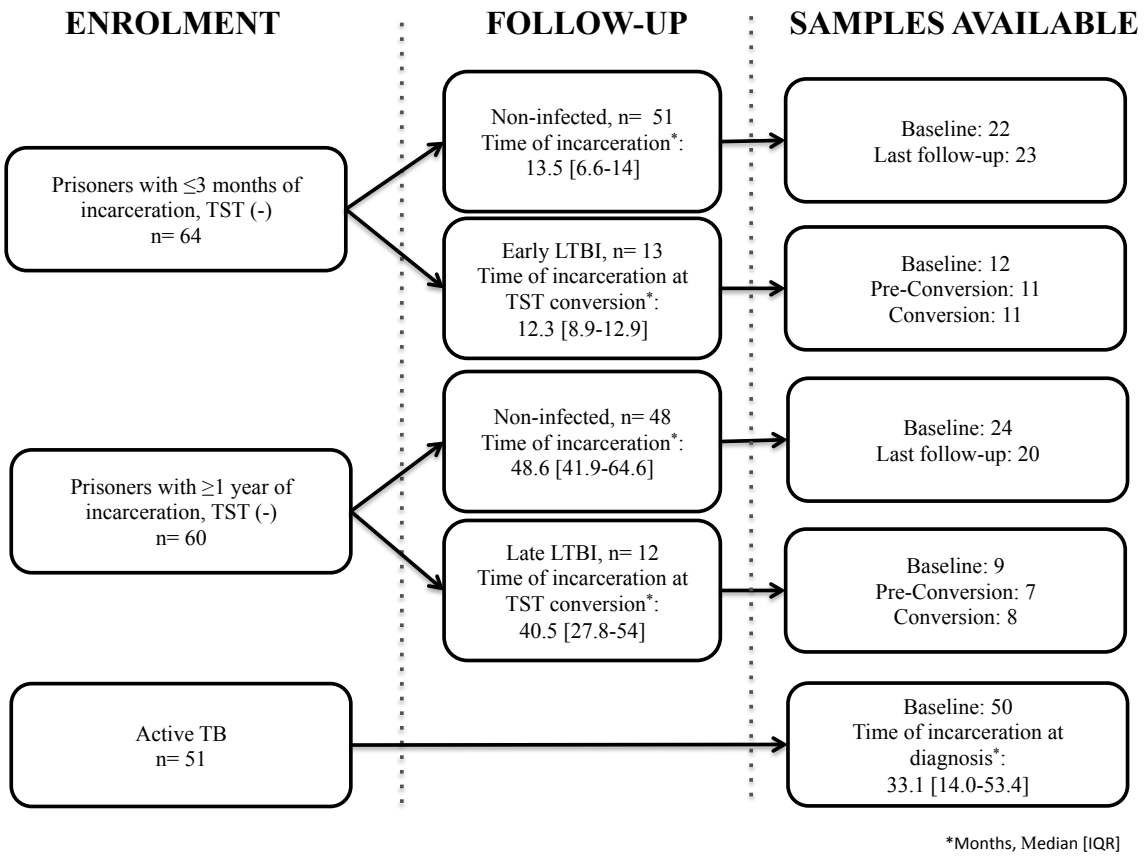


Figura 4-1. TB: tuberculosis; TST: tuberculin skin test. New LTBI [people with negative two-step TST that became positive during follow-up, were divided in early LTBI (they had ≤3 months of incarceration at enrolment) and late LTBI (they had ≥1 year of incarceration at enrolment)].

The median age was 34 [30-44] years for the non-infected group, 33 [IQR 30-39] in early LTBI, 31 [IQR 25-56] in late LTBI, and 32 [IQR 26-37] in the active TB group. The median time of TST conversion in the early LTBI group was 12.3 months, and in the late LTBI group was 40.5 months (Fig 1). Ten individuals in the study had body mass index ≤18.5 kg/m^2 and 9 of them were TB cases. Individuals diagnosed with active TB reported higher consumption of inhaled drugs (34%), smoked drugs (61.7%), alcohol (46.8%), and cigarette (55%) (Table 1).

The majority of LTBI were in prison one (75%). The history of having contact with an

active TB case was reported in 8.3% of LTBI, 34% in active TB cases, and 19.2% in the non-infected group. Table 1 reports socio-demographic information at baseline about the participants included in the analysis.

Tabla 4-1 Baseline characteristics of study participants with active TB, converter (early and late), and non-infected people.

Variable	Not infected (n=47) n (%)	Early LTBI (n=11) n (%)	Late LTBI (n=13) n (%)	Active TB (n=47) n (%)	*p* value
Age, years, median [IQR]	34 [30-44]	33 [30-39]	31 [25-56]	32 [26-37]	0.201
Time of current incarceration, months, median [IQR]	11.2 [1.6-39.4]	12.3 [8.9-12.9]	40.5 [27.8-54]	33.1 [14-53.4]	**0.001**
BMI ≤18.5	1 (2.2)	0	0	9 (19.1)	**0.013**
Prison					**<0.001**
Prison One	22 (46.8)	7 (63.6)	11 (84.6)	47 (100.0)	
Prison Two	25 (53.2)	4 (36.7)	2 (15.4)	0	
Comorbidities	9 (19.1)	0	4 (30.7)	9 (19.1)	0.284
COPD	3 (6.4)	0	1 (7.7)	3 (6.4)	0.849
Diabetes mellitus	1 (2.1)	0	2 (15.4)	2 (4.3)	0.173
Psychiatric illness	3 (6.4)	0	1 (7.7)	0	0.256
Others	3 (6.4)	1 (9.1)	0	4 (8.5)	0.736
Inhaled drug use					**0.001**
Never	32 (68.1)	6 (54.5)	9 (69.2)	14 (30.0)	
Past	11 (23.4)	5 (45.5)	3 (23.1)	16 (34.0)	
Current	4 (8.51)	0	1 (4.17)	17 (34.0)	
Smoked drug use					**<0.001**
Never	28 (59.6)	7 (63.6)	7 (53.8)	7 (14.9)	
Past	11 (23.4)	2 (18.2)	3 (23.1)	11 (23.4)	
Current	8 (17.0)	2 (18.2)	3 (23.1)	29 (61.7)	
Tobacco consume					0.294
Never	18 (38.3)	6 (54.5)	3 (23.1)	11 (23.4)	
Past	12 (25.5)	1 (9.1)	4 (30.7)	10 (21.3)	
Current	17 (36.2)	4 (36.4)	6 (46.1)	26 (55.3)	
Alcohol use					0.051
Never	12 (25.5)	3 (27.4)	1 (7.7)	8 (17.0)	

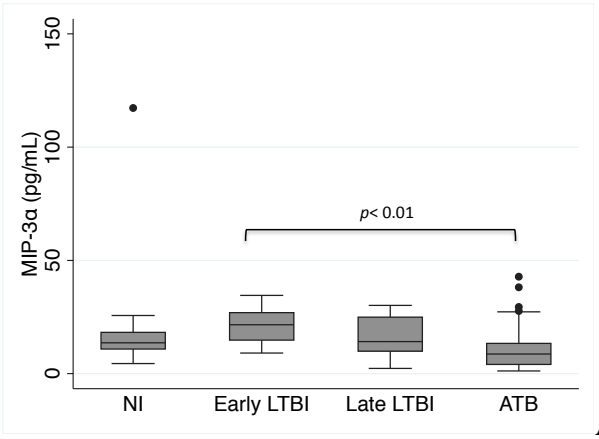
Past	22 (46.8)	7 (63.6)	8 (61.5)	17 (36.1)	
Current	12 (25.5)	0	3 (23.1)	22 (46.8)	
Occasional	1 (2.13)	1 (9.1)	1 (7.7)	0	
History of contact with a TB case	9 (19.1)	1 (9.1)	1 (7.7)	16 (34.0)	0.087
Contact with prisoner	7 (14.9)	1 (9.1)	1 (7.7)	14 (29.8)	
Contact with relative	2 (4.3)	0	0	2 (4.1)	
BCG Scar	45 (95.7)	8 (72.2)	8 (61.5)	41 (87.2)	**0.007**

IQR: interquartile range; TB: tuberculosis; HIV: human immunodeficiency virus; COPD: Chronic obstructive pulmonary disease; BCG: Bacillus Calmette-Guerin.

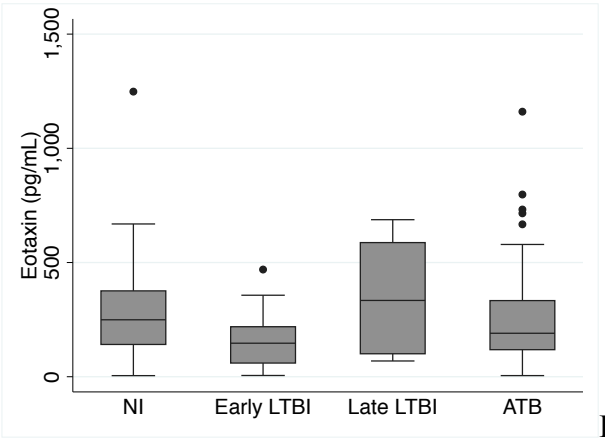
4.3.2 Comparison of concentration of cytokines/chemokines between groups

The cytokine/chemokines IL-10, IL-12, IL-13, Il-15, IL-17A, IL-1RA, IL-5, IL-6 and MIP-1α had more than 30% of the results below the lower limit of detection. Therefore, these were excluded from analysis. The graphs that show the concentration and fluorescence intensity for each cytokine are shown in supplementary material S3.

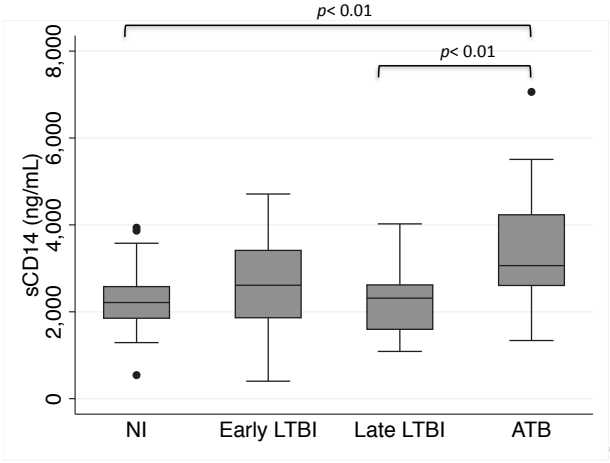
There were differences in the median plasma concentrations of sCD14, MIP-3α, IL-18, TNF-α, and MCP-1 between groups (Table 2). The concentrations of sCD14 was higher in the active TB group compared to non-infected individuals and late LTBI group (Fig 3C). TNF-α was higher in the active TB group compared to non-infected individuals (Fig 3I). MIP-3α was higher in the early LTBI compared to active TB group (Fig 3A). MCP-1 was higher in the late LTBI compared to active TB group (Fig 3G). IL-18 (Fig 3E) and MCP-1 (Fig 3G) have lower concentrations in active TB group compared to non-infected individuals.


MIP-3α (pg/mL)
0
50
100
150
p< 0.01
NI
Early LTBI
Late LTBI
ATB

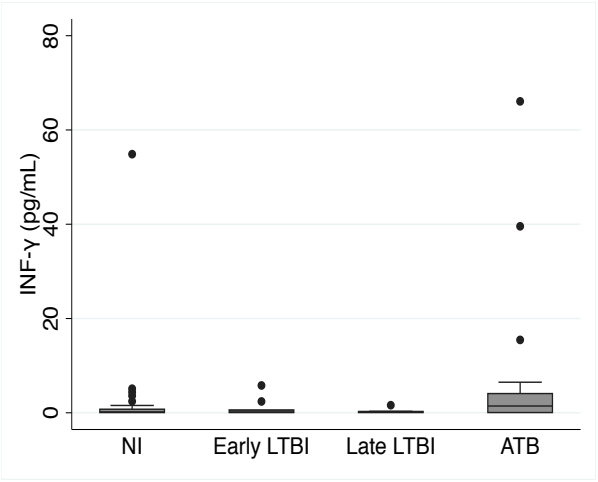
A


Eotaxin (pg/mL)
0
500
1,000
1,500
NI
Early LTBI
Late LTBI
ATB

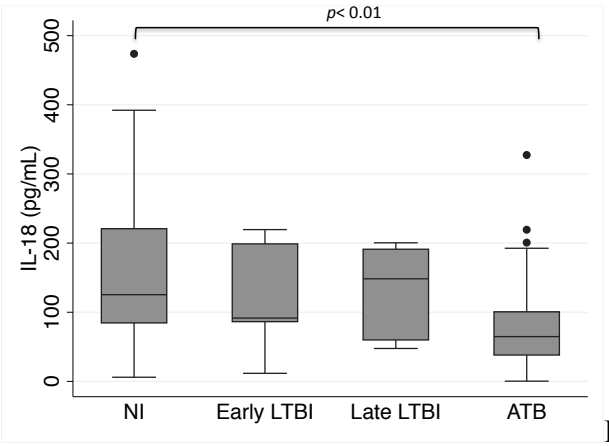
B


sCD14 (ng/mL)
0
2,000
4,000
6,000
8,000
p< 0.01
p< 0.01
NI
Early LTBI
Late LTBI
ATB

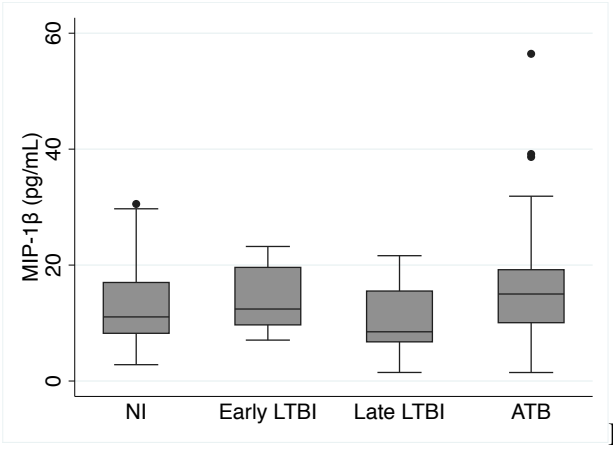
C


INF-γ (pg/mL)
0
20
40
60
80
NI
Early LTBI
Late LTBI
ATB

D


IL-18 (pg/mL)
0
100
200
300
400
500
p< 0.01
NI
Early LTBI
Late LTBI
ATB

E


MIP-1β (pg/mL)
0
20
40
60
NI
Early LTBI
Late LTBI
ATB

F

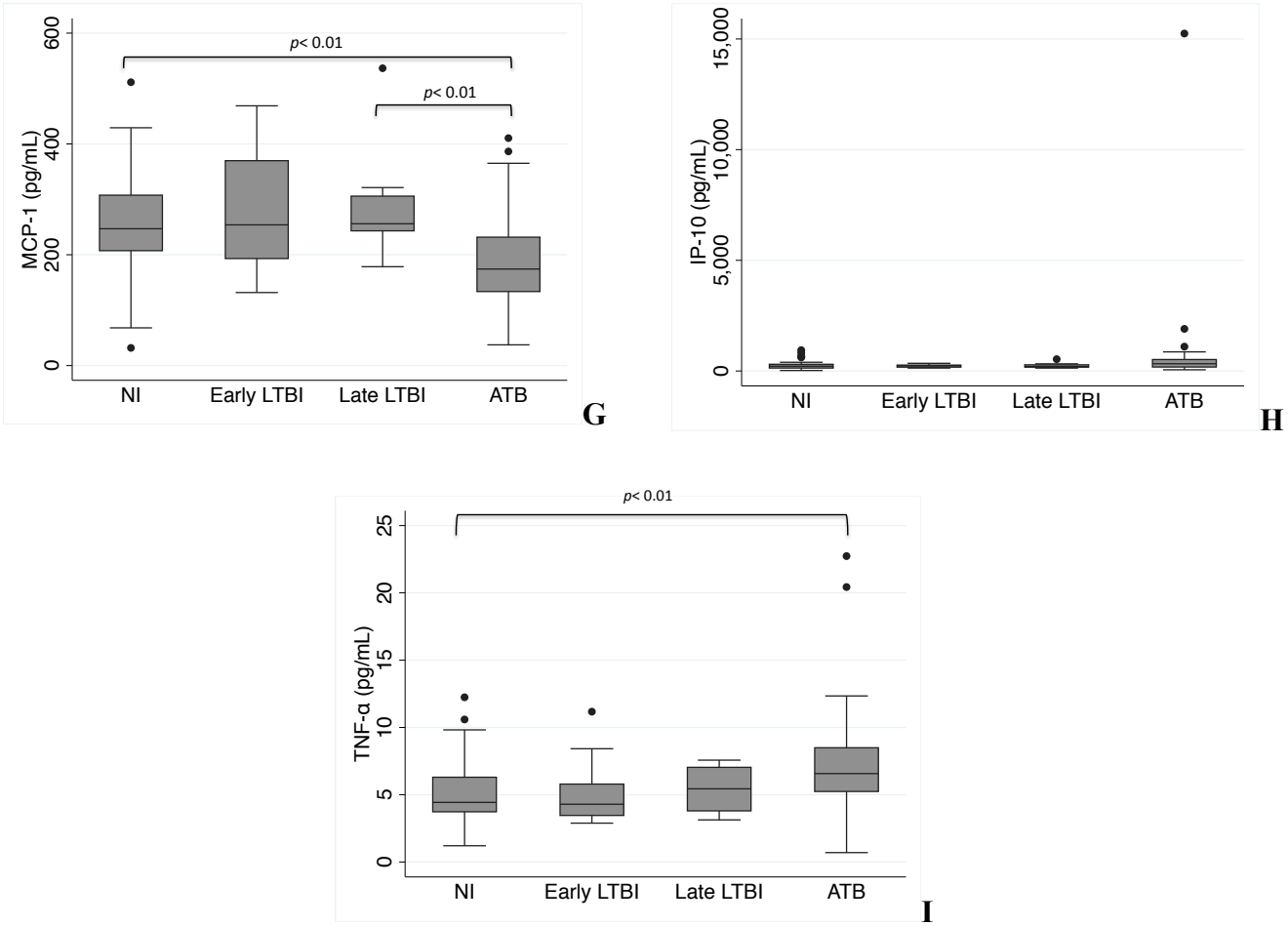


Figura 4-2 Concentration of immune parameters in converters (early LTBI [n= 11] and late LTBI [n= 13]), people with active TB (n=47), and non-infected people (n=47). New LTBI [people with negative two-step TST that became positive during follow-up, were divided in early LTBI (they had ≤3 months of incarceration at enrolment) and late LTBI (they had ≥1 year of incarceration at enrolment)]. **A.** MIP-3α; **B**. Eotaxin; **C**. sCD14 (ng/ml); **D.** INF-γ; **E.** IL-18; **F.** MIP-1β; **G.** MCP-1; **H.** IP-10; **I.** TNF-α. Values reported in pg/ml for all cytokines, except for sCD14 reported in ng/ml. Boxplots are median and interquartile range. *p* value using Mann-Whitney U test and adjusted by multiples comparisons.

Tabla 4-2 Concentration of cytokine/chemokine and differences between the groups (active TB, early and late converter, and non-infected)

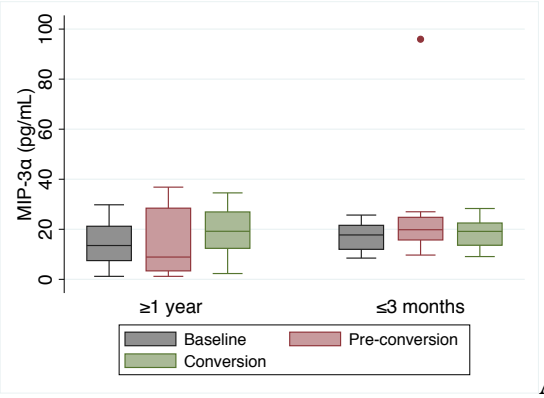
Cytokines/ Chemokines	**Non-infected pg/ml (IQR)**	**Early LTBI pg/ml (IQR)**	**Late LTBI pg/ml (IQR)**	**Active TB pg/ml (IQR)**	***p value****
sCD14 (ng/ml)	2213.8 (1851.9-2579.9)	2610.8 (1863.6-3413.0)	2314.9 (1596.8-2619.5)	3063.4 (2605.7-4233.1)	**0.0001**
MIP-3α	13.6 (10.9-18.2)	21.6 (14.8-26.9)	14.2 (9.9-24.9)	8.7 (4.1-13.4)	**0.0001**
IL-18	125.4 (84.5-220.8)	91.6 (86.3-198.9)	148.4 (59.9-191.1)	64.8 (38.1-100.5)	**0.0001**
Eotaxin	249.3 (141.3-375.9)	146.8 (59.8-218.8)	334.0 (100.4-587.2)	190.4 (118.3-333.4)	0.2421
INF-γ	0.19 (0.05-0.8)	0.4 (0.05-0.6)	0.12 (0.05-0.3)	1.44 (0.05-4.1)	0.1714
MIP-1β	11.1 (8.2-17.0)	12.4 (9,7-19.6)	8.5 (6.7-15.5)	15.0 (10.1-19.2)	0.1532
TNF-α	4.4 (3.7-6.3)	4.3 (3.5-5.8)	5.4 (3.8-7.0)	6.6 (5.2-8.5)	**0.0466**
IP-10	217.4 (133.2-303.6)	233.7 (177.1-267.5)	199.7 (174.8-280.2)	328.7 (179.7-518.6)	0.0842
MCP-1	246.9 (207.3-307.5)	253.9 (193.1-369.8)	256.0 (243.1-305.8)	174.2 (133.6-231.7)	**0.0006**

IQR: interquartile range; TB: tuberculosis; LTBI: Latent tuberculosis infection **p* value using Kruskal-Wallis test. New LTBI [people with negative two-step TST that became positive during follow-up, were divided in early LTBI (they had ≤3 months of incarceration at enrolment) and late LTBI (they had ≥1 year of incarceration at enrolment)].

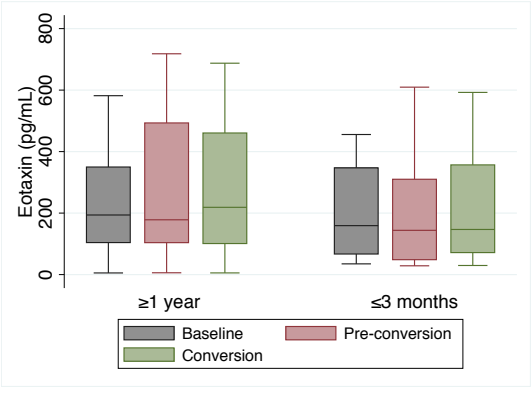
4.3.3 The concentration of cytokines/chemokines is affected by the incarceration ti

Twenty-five people changed TST status from negative to positive during follow-up. We compared the concentration of the immune mediators at baseline, TST conversion point a three months before the TST conversion (pre-conversion point) in converters individuals. Fig 1 shows the number of samples available in each group and used in the analysis, and table S4 shows the concentration of the cytokine/chemokines at each time point.

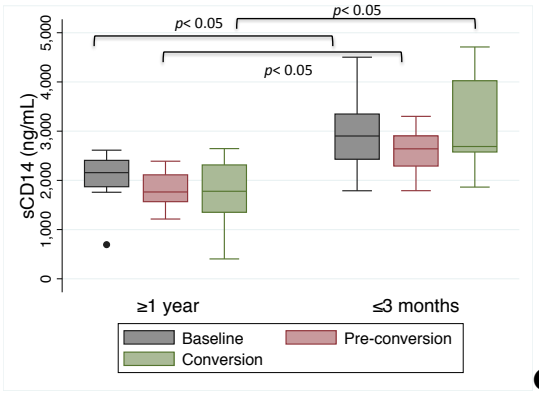
We hypothesized that markers of immune activation and inflammation at the time of infection could be different between individuals newly admitted to prison and those that remain TST negative but have been in prison for longer periods of incarceration. The results show that the concentration of the immune parameters changed over time (baselin pre-conversion and conversion) (Fig 2). In addition, the levels of sCD14 (Fig 2C), and IL 18 (Fig 2E) were increased in people newly incarcerated compared to those who had sper one year or more of incarceration ($p< 0.05$). The concentration of IP-10 (Fig 2H) was higher in people who had one or more years of incarceration. The figures 2C, 2E, and 2H show statistical differences between groups.



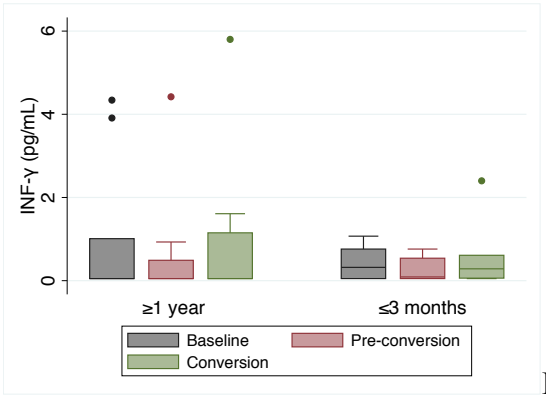
A



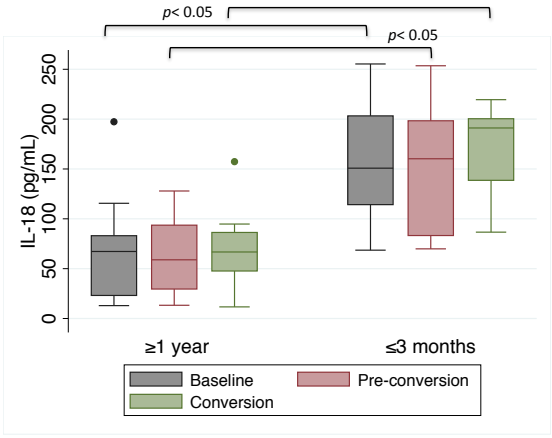
B


p< 0.05
p< 0.05
p< 0.05
sCD14 (ng/mL)
0
1,000
2,000
3,000
4,000
5,000
≥1 year
≤3 months
Baseline
Pre-conversion
Conversion

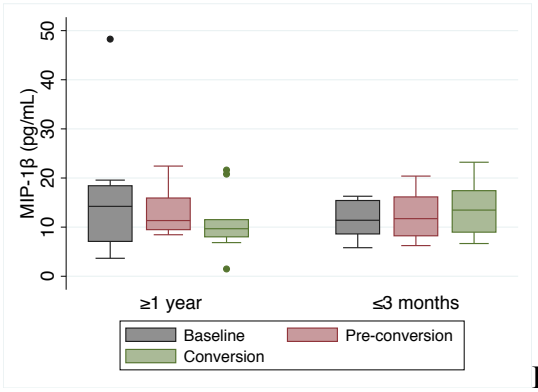
C


INF-γ (pg/mL)
0
2
4
6
≥1 year
≤3 months
Baseline
Pre-conversion
Conversion

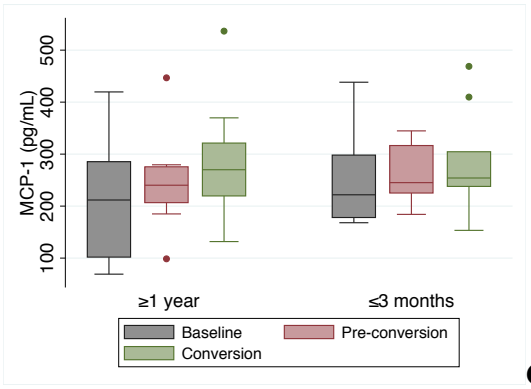
D


p< 0.05
p< 0.05
IL-18 (pg/mL)
0
50
100
150
200
250
≥1 year
≤3 months
Baseline
Pre-conversion
Conversion

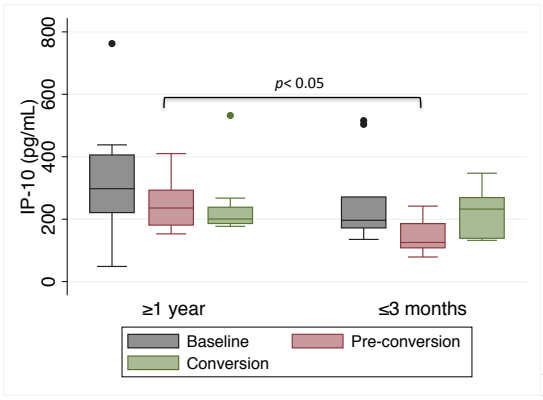
E


MIP-1β (pg/mL)
0
10
20
30
40
50
≥1 year
≤3 months
Baseline
Pre-conversion
Conversion

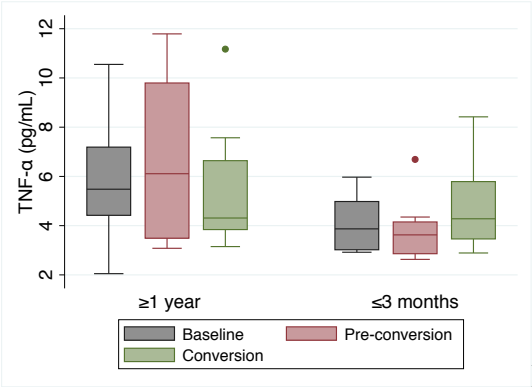
F


MCP-1 (pg/mL)
100
200
300
400
500
≥1 year
≤3 months
Baseline
Pre-conversion
Conversion

G


p< 0.05
IP-10 (pg/mL)
0
200
400
600
800
≥1 year
≤3 months
Baseline
Pre-conversion
Conversion

H



I

Figura 4-3 Concentration of cytokines/chemokines in people with new latent tuberculosis infection at baseline, pre-conversion, and TST conversion time, by the time of incarceration. New LTBI [people with negative two-step TST that became positive during follow-up, were divided in early LTBI (they had ≤3 months of incarceration at enrolment) and late LTBI (they had ≥1 year of incarceration at enrolment)]. Values are reported in pg/ml for all cytokines/chemokines except for sCD14 were results are reported in ng/ml. Boxplots are median and interquartile range. **A.** MIP-3α; **B**. Eotaxin; **C**. sCD14; (ng/ml) **D.** INF-γ; **E.** IL-18; **F.** MIP-1β; **G.** MCP-1; **H.** IP-10; **I.** TNF-α**.**

4.3.4 Multivariate model

In the bivariable analyses, a list of socio-demographic and cytokines/chemokines were associated with active TB and new LTBI (early and late LTBI) (Tables 1 and 3). In the multivariable analysis, only having a BCG scar was associated with early LTBI and active TB. The other socio-demographic variables (age, history of contact with a TB case, and drugs consumption) were not associated with early LTBI, late LTBI nor active TB. We did not find interaction between BCG and any of the cytokines/chemokines. Although to have a BMI less than 18 kg/m^2 and liquor consumption were significant factors, the multinomial model did not converge when we included those variables due to the low number of outcomes in the 'yes' category.

In the final multinomial model, we identified that high concentration of chemokine MIP-3α was associated with increased risk of early LTBI and BCG scar decreases the risk of early LTBI compared to non-infected individuals.

In addition, we documented that high concentrations of MCP-1 increase the risk of late LTBI.

High concentrations of sCD14; INF-γ, and TNF-α were associated with an increased risk of active TB. Finally, we found that high concentrations of IL-18 and MCP-1 and BCG scar were associated with a decreased risk of having active TB, compared to non-infected individuals.

Tabla 4-3. Adjusted RRR by early LTBI, and late LTBI and active TB *vs* non-infected people in a multinomial model.

Cytokines/ Chemokines	Early LTBI		Late LTBI		Active TB	
	cRRR^{+} [95% CI]	**aRRR*$^{+}$ [95% CI]**	**cRRR^{+} [95% CI]**	**aRRR*$^{+}$ [95% CI]**	**cRRR^{+} [95% CI]**	**aRRR*$^{+}$ [95% CI]**
Non-infected	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
sCD14 (≥2605.8 ng/ml)	3.00 [0.68-13.28]	3.05 [0.74-12.56]	1.80 [0.56-5.76]	3.67 [0.68-19.69]	9.00 [5.56-14.58]	**26.30 [11.83-58.44]**
MIP-3α (≥14.9 pg/ml)	4.85 [1.19-19.79]	**10.59 [2.84-39.59]**	1.25 [0.28-5.53]	1.04 [0.24-4.44]	0.46 [0.23-0.93]	0.38 [0.11-1.33]
IL-18 (≥100.51 pg/ml)	0.4 [0.07-2.43]	0.12 [0.01-2.02]	1.00 [0.29-3.45]	0.49 [0.08-3.11]	0.18 [0.06-0.55]	**0.06 [0.01-0.40]**
Eotaxin (≥200.1 pg/ml)	0.26 [0.05-1.31]	0.26 [0.06-1.06]	1.03 [0.15-6.88]	2.18 [0.40-11.77]	0.47 [0.18-1.20]	0.86 [0.26-2.88]
INF-γ (≥0.81 pg/ml)	1.06 [0.15-7.71]	4.77 [0.50-45.12]	0.61 [0.05-6.79]	0.31 [0.004-21.23]	4.25 [1.45-12.44]	**8.67 [1.35-55.89]**
MIP-1β (≥10.2 pg/ml)	1.59 [0.37-6.80]	7.10 [0.20-249.45]	0.41 [0.07-2.27]	0.34 [0.07-1.56]	1.81 [1.13-2.91]	1.63 [0.76-3.53]
TNF-α (≥6.01 pg/ml)	0.56 [0.11-2.83]	0.08 [0.003-1.68]	2.23 [0.57-8.71]	2.19 [0.33-14.37]	3.90 [1.28-11.82]	**5.41 [1.09-26.89]**
IP-10 (≥217.41 pg/ml)	2.33 [0.57-9.59]	3.93 [0.52-29.91]	0.60 [0.15-2.46]	0.47 [0.06-3.93]	1.93 [0.89-4.19]	0.42 [0.09-2.01]
MCP-1 (≥231.71 pg/ml)	0.83 [0.22-3.17]	0.88 [0.16-4.88]	3.89 [0.54-27.96]	**6.21 [1.61-23.96]**	0.18 [0.10-0.34]	**0.16 [0.05-0.53]**
BCG scar	0.12 [0.01-1.04]	**0.05 [0.004-0.78]**	0.07 [0.01-0.57]	0.07 [0.002-2.44]	0.29 [0.05-1.52]	**0.11 [0.03-0.43]**
Age in years	0.98 [0.95-1.02]	1.01 [0.97-1.05]	1.02 [0.99-1.04]	1.03 [0.96-1.12]	0.98 [0.95-1.00]	0.98 [0.93-1.03]

*aRRR: adjusted relative risk ratio based on multinomial logistic regression analysis; TB: tuberculosis. New LTBI [people with negative two-step TST that became positive during follow-up, were divided in early LTBI (they had ≤3 months of incarceration at enrolment) and late LTBI (they had ≥1 year of incarceration at enrolment)]. All variables included in the multinomial regression model were selected through a manual backward elimination method. $^{+}$The bivariable and multivariate models are adjusted by cluster effect (15 courtyards). In all models, none of the sociodemographic characteristics were confounders, and were non-significant.

4.4 DISCUSSION

The main results of our study were: 1) The concentration of immune mediators varies according to the time of exposure and infection; 2) there are individuals who, despite being highly exposed to mycobacteria, do not convert to a positive TST; and 3) there are cytokines/chemokines associated with active TB, early TBLI, and late LTBI.

The most important distinguishing feature of our study is the study design (cohort). In cross-sectional studies, the duration that an individual has had LTBI cannot be quantified. The main limitation of a cross-sectional approach is that human and primates studies have demonstrated there is a spectrum of LTBI stages[9,10,39,40], on which there is little published work[8,10], and that potentially could alter the concentration of the immune parameters according to the stage of the infection. The cohort design allowed us to quantify the concentration of cytokines closest to the time of infection (new conversion), and identified individuals in whom conversion occurred recently. Once MTB infection is acquired, the immune mechanisms that allow some individuals to eliminate infection, control it in latent state or subclinical TB, or progress to active TB are unknown[41]. The mechanisms that underlie reactivation in certain individuals are also unknown. The transition between different states of TB infection is mediated by interaction of factors related to mycobacteria, host and environment[41,42]. It has been postulated that after infection, the creation of granuloma is part of the life cycle of MTB, allowing the organism to persist and subsequently spread to new infectious foci[43], or other susceptible individuals. The concentration of cytokines/chemokines is dynamic, and different expression profiles are observed at the onset of infection compared with a well-established granuloma[29], these variations, presented mainly in lung, can be reflected in plasma.

Our findings suggest that among prisoners, who, given the conditions inside the prisons, are continually exposed to MTB, infections occur at variable times after entering the prison (Fig 1), and immune response at the time of infection was different between those newly admitted to prison compared to those with longer incarceration (Fig 3). There are several plausible reasons that could explain the differences in acquisition of infection in people

who share the same environment. 1. The existence of a genetic component that predisposes to early MTB infection. 2. A protective and sustained immune response over time, which allows a sterilizing immune response that prevents productive infection; and 3. Modulation of the pathogenicity of the bacterium and resistance by the host, a characteristic of co-evolution, which allow adaptation and resistance to infection[44]. An additional mechanism of protection, may be related to lung microbiome with variability in risk of infection depending on microbiome diversity[45,46].

In this study, the concentration of immune mediators varies among newly infected individuals according to the time since incarceration. The levels of sCD14 and IL-18 were increased in individuals incarcerated for ≤3 months compared to those with ≥1 year of incarceration. IP-10 was increased in those incarcerated for one year or more compared to those recently incarcerated. These variations among individuals could occur for several reasons: first, individuals who are continuously exposed (≥1 year in prison) could eliminate the bacteria repeatedly, owing to effective immune response. Eventually, mycobacterium manages to breakthrough and establish infection, and in response, plasma levels of IP-10 become increased. Another potential explanation: the presence of factors unrelated to the infection, such as stress. Several studies document a change in the production of pro-inflammatory cytokines when an individual is under a chronic stressful situation[47], performs physical activity/exercise[48], or as reported by Sribanditmongkol *et al*.[49], when a stress-generating situation is compounded by an infectious process, such as influenza vaccination. Study participants are exposed to acute and chronic stressors, given the social and safety conditions that prevail in prisons in which they live, or the changes associated with deprivation of freedom[50]. Another situation of interest in our results is that 19% of the individuals had prior history of contact with a TB case, yet continued to be uninfected. Well established genetic and immune factors that allow some individuals to be completely resistant to MTB infection or to eliminate the infection and remain negative TST can underlie this observation[31]. In addition, there is the possibility of genetic variability in the response to TST, due to genetically determined polymorphism that has been reported in the Colombian population[51].

In the multivariate model, elevated concentration of MIP-3α was associated with a higher probability of having early LTBI. The MIP-3α/CCL20, is T cell, B cell or monocyte chemoattractant, and has function on cell proliferation and activation[35,52,53]. Rivero-Lezcano *et al*. reported that the expression of MIP-3α increased up to 39-fold when monocytes from healthy donors were infected with *M. tuberculosis*[54]. On the other hand, MIP-3α was up-regulated in PBMC and bronchoalveolar lavage fluids, from TB patients compare to healthy controls after *in vitro* stimulation with the 30 kDa antigen (Ag) of *M. tuberculosis*[55]. MIP-3α from serum samples appears relevant to be evaluated in further studies of early MTB infection.

After adjusting for the other co-variables, individuals incarcerated for over one year with elevated concentrations of MCP-1 had a higher risk of conversion. In a model of macaques previously vaccinated with BCG, the production of β-chemokine MCP-1 increased in lung lesions of animals after 5 weeks of infection with MTB[56]. These immune molecules are mainly associated with recruitment of monocytes/macrophages, in keeping with the observation that animals presented more macrophages (CD14+ CD68+) in peripheral blood at three weeks post infection with MTB[56]. In this case, the increase in cells and β-chemokines may be a sign of the recruitment of these cells from peripheral blood and to the site of infection in the lung.

We did not find associations between IP-10, IL-17, IL-10, and LTBI, (the first one was not different between groups, and the latter two had concentrations below the lower limit of detection) contrary to other publications[57,58]. An explanation to this discrepancy might be that measurement of immune mediators in prior studies, were performed after stimulation with antigens, including mycobacterial antigens, in contrast to plasma, unstimulated measurement in our study. Similar discrepancy occurs with other published articles[12,13,16,20,21,59–66]. Another reason for different results may stems from the fact that individuals included in other longitudinal studies were evaluated with only one administration of TST, and in those with a negative result may represent a false negative due to missing of the booster effect because a two-step TST was not performed[27]. In our

previous studies, a second administration of TST identified an additional 11.6% positive TST individuals[67].

Regarding the results of the model for active TB, we demonstrate herein, that increases in the concentrations of sCD14, INF-γ and TNF-α are associated with a higher probability of having TB. As in the case of LTBI, these immune substances are highly involved in monocyte/macrophage activation and trafficking pathways[68,69]. Macrophages are the main niche for the growth and survival of *M. tuberculosis*[70]; likewise, macrophages are the most important cells for infection control in both animal and human models, and they are the cornerstone of host response during active disease[70–72]. Soluble CD14, whose membrane-bound portion is highly expressed in these cells, functions as a mediator of macrophage activation, and serves as a receptor for mycobacterial lipoarabinomannan[69], was present in higher concentrations in people with active TB, compared to people exposed to MTB, not infected. Increased levels of sCD14 have been reported in patients with active TB[73,74], infected or not by HIV[75,76], and individuals with diabetes mellitus[77], due to the host's response against MTB trying to limit the systemic spread of mycobacteria[75]. Likewise, high concentrations of sCD14 were detected between six and 18 months before the second episode of TB in a cohort examining the recurrence of pulmonary TB in HIV (+) patients after the initiation of antiretroviral therapy[78]. On the other hand, Magdalena D. *et al*., reported lack of association between IGRAs and sCD14 concentrations in medical personnel and in patients living with TB patients[79].

A published article by Lawn SD *et al*., sCD14 may increase the concentration of TNF-α due to the high load of mycobacterial antigens in HIV infected patients with active TB[75]. Similar to our study, TNF-α is increased in adults[60,63,74,80,81] and children[82] with active TB, but with discordant results in healthy individuals, or those latently infected with MTB[20,81]. TNF-α is a potent macrophage activator, promoting cell migration and mediation of the production and interaction of different cytokines, among which are CCL5 (RANTES), CXCL9 (MIG) and CXCL10 (IP-10)[83]. TNF-α has been demonstrated to be protective against tuberculous infection and that monoclonal antibodies that block TNFs interaction with its receptor increase the likelihood of disease progression in people with LTBI[84]. In

our study, no association was observed between TNF-α concentration and LTBI, but once the active disease was established, there was an increase in plasma concentrations of TNF-α, presumably contributing to cell recruitment, the production of other pro-inflammatory cytokines, and apoptosis of MTB-infected cells[83].

Changes in the expression of TNF-α also modulate the expression pattern of other cytokines such as MIP-1β[68], and whose increase in plasma increases the probability of active TB (point estimator was increased although the confidence intervals were non-significant). MIP-1β is chemoattractant of leukocytes and NK cells and therefore increases the local inflammatory response. MIP-1β potential as a biomarker of TB has been extensively studied with little encouraging result[15,74,85–87]; but conversely, Eon-Jeong Won *et al*., reported high concentrations of the chemokine in whole blood stimulated with ESAT-6, CFP-10 and TB7.7 among people with LTBI and active TB, compared to healthy individuals; in addition, combinations of MIP-1β with IL-2, IP-10, IL-1RA, IL-3 and INF-γ could be good discriminators of infection and tuberculosis disease[60].
On the contrary, in the final model, higher concentrations of IL-18 and MCP-1 showed a protective effect in NI individuals. IL-18 has an important function on TB as a pro-inflammatory cytokine[88] but the studies are not conclusive. Two studies published by Wawroscki S *et al*.[89], and Yamada G *et al*.[90] showed that increased IL-18 serum concentrations were associated with active TB patients compared to healthy individuals (including TST converters). However, in concordance with our finding Vankayalapati RR *et al*. showed that *M. tuberculosis*-stimulated culture supernatants from patients with tuberculosis have lower concentrations of IL-18 compared to those from healthy TST converters[91].

In the same way, the MCP-1, a potent chemotactic factor for monocytes[92], was increased in late LTBI, and decreased in TB cases, however most reports about this chemokine show increased concentration in active TB patients[93,94]. Variable results were published whether whole blood was subjected to stimulation with mycobacterial antigens or in the absence of stimulation. Increased concentrations of MCP-1 were observed in stimulated samples from individuals with active TB, and increased concentrations in samples without stimulation

from non-infected people[95]. Some explanations for those differences between the studies are: the ancestry of population; the kind of samples (serum, whole blood, or supernatants of cultured PBMC); the high exposure to *M. tuberculosi*s in NI individuals inside the prison; and the presence of genetic polymorphisms in the IL-18 or MCP-1[96] in the populations, which could alter the final structure, the concentration, or the function of the protein.

Our study has strengths: the ability to identify new converters close to the time of infection, compare with cases of TB and uninfected people from the same population. Rigorous selection criteria among cases of TB including only those with microbiologically confirmed infection and with less than 15 days of treatment, application of booster, rigorous monitoring of uninfected persons and administration of TST every six months to all negative individuals.
The main limitation of this study is that some bacterial factors may modify the response to cytokines such as the infectious bacterial strain, however, in people with LTBI it is not feasible to isolate the mycobacterium, and therefore the role of MTB strain cannot be assessed.

4.5 CONCLUSIONS

Early MTB infection is associated with high concentrations of MIP-3α, in prisoners recently incarcerated, and this parameter varies according to the time of incarceration. In addition, when evaluating the results in the light of the previously reported literature, the results show that the study of only one cytokine is not effective in differentiating MTB infection, including the infection that has recently occurred, from non-infected individuals and active TB. Early LTBI is a transient stage after exposure, which is related to the life cycle of mycobacteria, and markers of early infection should be studied using other techniques like RNA sequencing to identify potential biomarkers for early LTBI detection and therefore treatment prioritization among the vast numbers of individuals with LTBI globally.

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4.7 Material suplementario

Ver anexo CAPÍTULO 4: DATA PRE-TREATMENT OVERCOMING THE CHALLENGE OF CYTOKINE/CHEMOKINES ANALYSIS - NORMAL DISTRIBUTION OR NEEDING TRANSFORMATION?.

Este capítulo se llevó a cabo con el objetivo de utilizar los datos transformados en el modelo multinomial; el modelo no cumplió con los supuestos estadísticos y los datos transformados no fueron utilizados en el capítulo 4, pero se incluyó la información de pre tratamiento de datos como un anexo para resaltar la necesidad de tener en cuenta la variabilidad de los datos biológicos y la necesidad de realizar pre-tratamiento antes de llevar a cabo los análisis y emitir las conclusiones.

Anexo 4-1 Criteria for cytokine/chemokine selection according to a systematic review, the previous results from the research group, the pathophysiology of TB, and the KEGG immune pathway.

LTBI (SR)	**LTBI and active TB (SR)**	**Pathway *M. tb* H37Rv**	**Pathophysiology**	**Previous results**	**Chosen**
-	IL-1α / IL-1RA	IL-1	IL-1 (α y β)	-	IL-1RA
-	IL-2 / IL-2R	-	IL-2	-	-
IL-5	-	-	-	-	IL-5
-	-	IL-6	IL-6	-	IL-6
-	-	-	IL-8	-	-
-	IL-9	-	-	-	-
-	-	IL-10	IL-10	IL-10	IL-10
-	-	IL-12	IL-12	IL-12	IL-12 (p40)
-	IL-13	-	-	IL-13	IL-13
-	IL-15	-	IL-15	-	IL-15
IL-17A/IL-17F	-	-	IL-17	1L-17	IL-17A
-	-	IL-18	IL-18	-	IL-18
-	-	-	IL-21	-	-
-	-	IL-23	IL-23	-	-
-	-	-	IL-26	-	-
-	-	INF-α/INF- β	INF-γ	-	INF-γ
-	-	TNF-α	TNF-α	-	TNF-α

-	VEGF	-	-	-	-
-	sCD40L	-	-	-	-
-	-	-	CCL2	-	CCL2 (MCP-1)
-	-	-	CCL3 (MIP-1α)	CCL3 (MIP-1α)	CCL3 (MIP-1α)
-	CCL4 (MIP-1β)	-	-	CCL4 (MIP-1β)	CCL4 (MIP-1β)
CCL5 (RANTES)	-	-	CCL5 (RANTES)	-	-
	-	-	CCL19	-	-
CCL20 (MIP-3α)	-	-	-	-	CCL20 (MIP-3α)
-	-	-	CXCL2	-	-
-	-	-	CXCL3	-	-
-	-	-	CXCL-5/CXCR5	-	-
-	-	-	CXCL8	-	-
-	CXCL9 (MIG)	-	-	-	-
-	CXCL10 (IP-10)	-	CXCL10	-	CXCL10 (IP-10)
-	CXCL11 (I-TAC)	-	-	-	-
-	CXCL12 (SDF-1α+β)	-	CXCL12	-	-
-	-	-	CXCL13	-	-
-	-	-	-	CCL11(Eotaxin)	CCL11(Eotaxin)
-	-	-	-	sCD14	sCD14

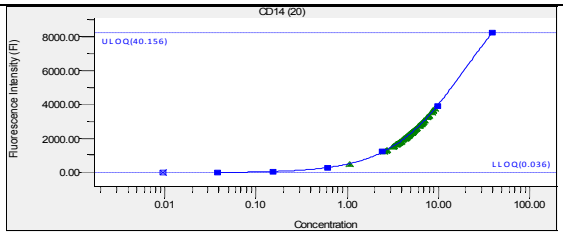
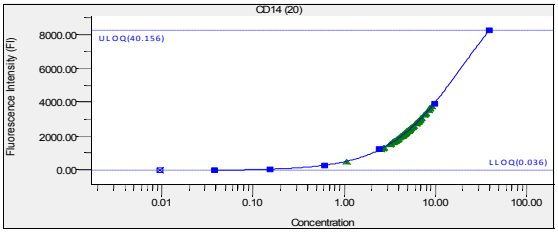
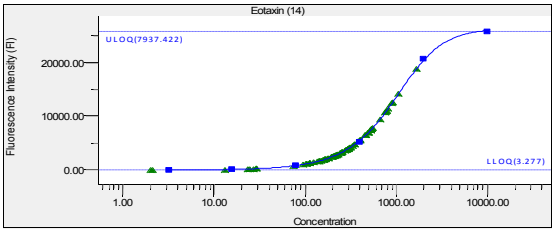
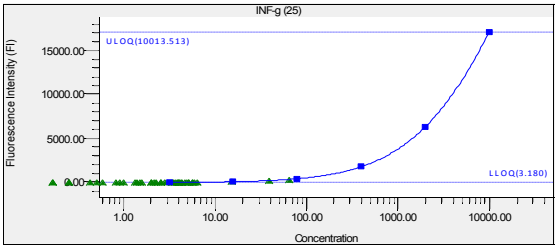
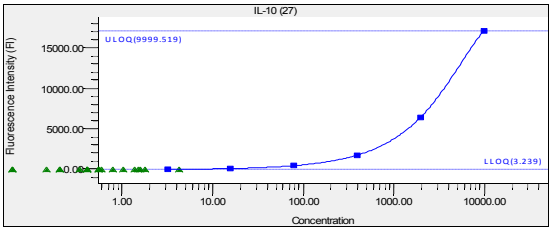
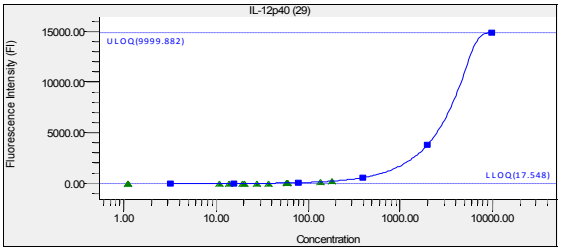
Latent tuberculosis infection; TB: tuberculosis; SR: systematic review; M. tb: *Mycobacterium tuberculosis*

Anexo 4-2 Limit of detection and where all the samples fall in the range for each cytokine/chemokine commercial kits

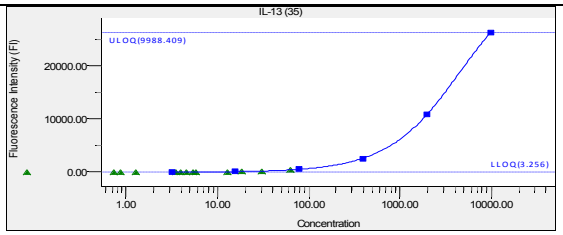
Cytokines/ Chemokines	**Minimum detectable concentration* (pg/mL)**
sCD14 (ng/ml)	0.008
MIP-3α	2.0
IL-18	1.48
Eotaxin	4.0
INF-γ	0.8
IL-10	1.1
IL-12 (p40)	7.4
IL-13	1.3
IL-15	1.2
IL-17A	0.7
IL-1RA	8.3
IL-5	0.5
IL-6	0.9
MIP-1α	2.9
MIP-1β	3.0
TNF-α	0.7
IP-10	8.6
MCP-1	1.9

*****Information available in the commercial kits

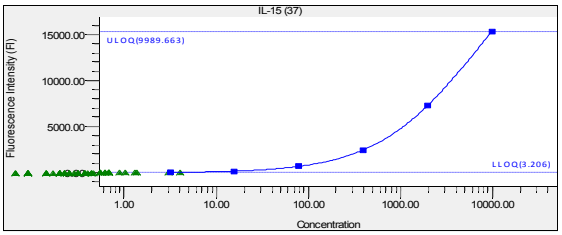
Cytokines/Chemokines	**Graph of samples in the range**

sCD14	
MIP-3α	Not graph available
IL-18	
Eotaxin	
INF-γ	
IL-10	
IL-12 (p40)	

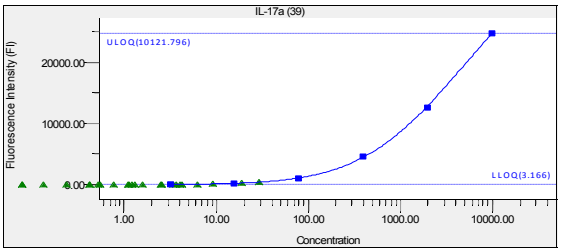
IL-13



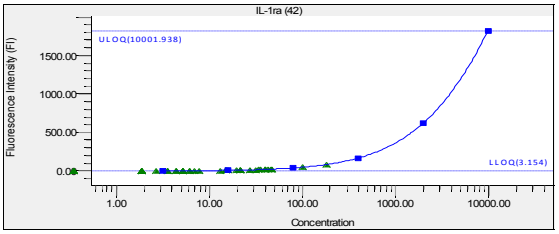
IL-15



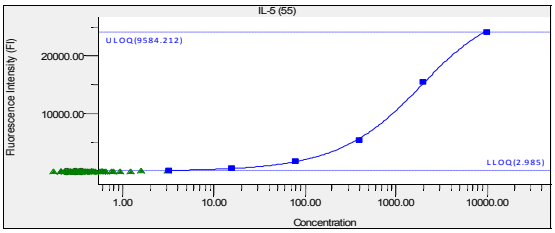
IL-17A



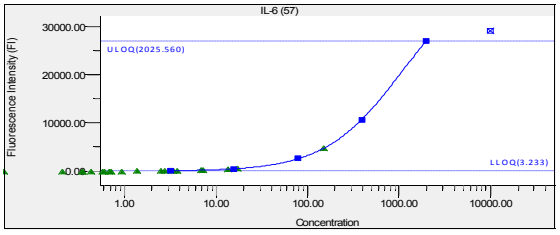
IL-1RA



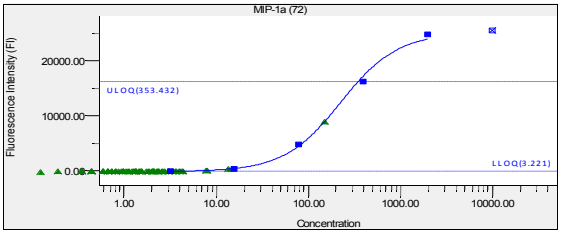
IL-5

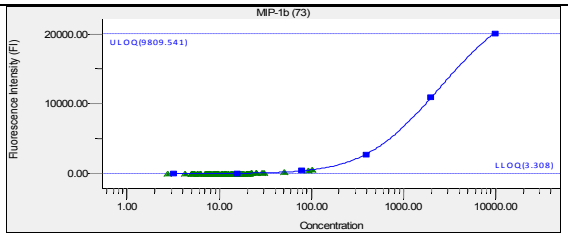
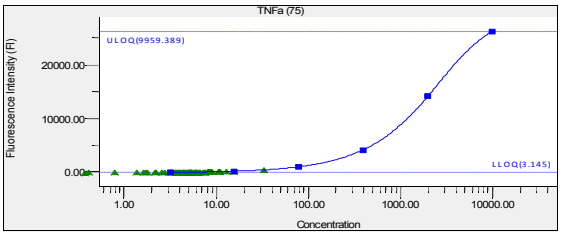
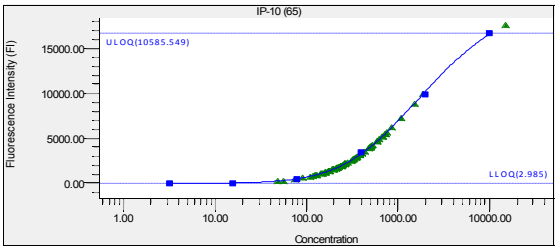


IL-6



MIP-1α



MIP-1β	
TNF-α	
IP-10	
MCP-1	Not graph available

Anexo 4-3**.** Evaluation of BCG as an interaction term with cytokines/chemokines and early LTBI, late LTBI and active TB compared to non-infected individuals

Cytokines/ Chemokines	***p* value of interaction**		
	Early LTBI	***Late LTBI***	***Active TB***
sCD14	0.142	0.218	0.234
MIP-3α	0.326	0.387	0.445
IL-18	0.798	0.222	0.310
Eotaxin	0.854	0.187	0.354
INF-γ	The model does not converge		
MIP-1α	0.787	0.667	0.186
MIP-1β	0.297	0.212	0.235
TNF-α	0.877	0.747	0.161

IP-10	0.415	0.696	0.740
MCP-1	0.263	0.177	0.559

5 CAPITULO 5

GENE EXPRESSION PROFILING IDENTIFIES CANDIDATE BIOMARKERS FOR NEW LATENT TUBERCULOSIS INFECTIONS

Abstract

Introduction: Tuberculosis (TB) remains a public health leading infectious disease worldwide, and about a third of the population is infected with *Mycobacterium tuberculosis*. n recent years, several published studies have reported the use of RNA sequencing as a tool to evaluate LTBI and active TB. The objective of this research was to determine the gene expression profile in individuals with early infection with MTB, and to compare them with people with active TB and those exposed to mycobacteria but not infected. In addition, the concentrations of 18 cytokines/chemokines were compared with the RNA expression of the genes that regulate those immune factors.

Methodology: We evaluated 10 people with pulmonary TB, 15 people with recent infection with *M. tuberculosis*, and 11 people exposed, but not infected. RNA extraction was performed from PBMCs, and we did RNA sequencing. We determined the differential gene expression using the Bioconductor package in R, and the exclusive genes in each group were selected. We carried out an analysis of metabolic and immune pathways to evaluate the participation of the specific genes of each group (TB, latent TB and not infected). The time of incarceration was taken into account as a fundamental variable in the analysis.

Results: we found several different gene expressions between LTBI and non-infected individuals that have different time of exposure (recently incarcerated and with one or more year of incarceration at the time of starting the follow-up) compared to active TB

Keywords

Latent tuberculosis infection, tuberculosis, RNAseq, conversion, biomarkers.

5.1 INTRODUCTION

Tuberculosis (TB) remains a public health leading infectious disease worldwide, and about a third of the population is infected with *Mycobacterium tuberculosis*[1] (MTB). Individuals with latent TB infection (LTBI) are a reservoir for the bacterium, are at risk to progress to active TB, and therefore pose a risk for spread of infection to their families and the community. In order to control the TB epidemic, it is essential to prevent new infections and new cases of TB by offering treatment for LTBI[2,3].

Given the high number of people infected by the MTB, offering massive treatment, although a highly desirable option, is not feasible for low- and middle-income countries where the majority of those infected reside[4]. Therefore, diagnosing and treating patients with new MTB infection, within the first five years after the infection, could be a promising strategy to prevent progression to active TB in people with LTBI, because they are at the highest risk for progression to active TB [1].

Identification of those recently infected is one of the main challenges because existing tests for the diagnosis of LTBI (Tuberculin skin test [TST] and Interferon-γ release assays [IGRAs]) are unable to predict the time of infection, have low sensitivity in some populations, and cannot differentiate between LTBI and active TB, among other previously published disadvantages[1 5 6 7 8,9 10]. For this reason, it is important to identify new targets for diagnostic tests or to improve the available tests for the diagnosis of LTBI.

RNA sequencing is a tool to measure gene expression and identify different patterns of expression between cells, tissues, disease states and treatments[11]. Hence, RNA seq may be useful to identify potential biomarkers, based on host response to MTB infection.

In recent years, several published studies have reported the use of RNA sequencing in different types of samples, with the aim to predict the progression of infected individuals to active TB[12,13], the outcome at time of completion of anti-TB treatment[14], to identify different gene expression profiles between active TB, LTBI, and non-infected people[15,16], among healthy individuals compared to LTBI[17], and among people with pulmonary TB, extrapulmonary TB and other lung infectious diseases[18]. Few studies have been conducted

to understand MTB infection exclusively, and these studies have focused on the detection of micro RNAs[17,19].

Studying individuals with new or early LTBI not only provides a better understanding of the host's response to mycobacteria at the closest time of infection, it might allow to identify a specific gene expression profile that can provide candidates for biomarkers. Therefore, the objective of this research was to determine the gene expression profile in individuals with early infection with MTB, and to compare them with people with active TB and those exposed to mycobacteria but not infected. In addition, the concentrations of 18 cytokines/chemokines were compared with the RNA expression of the genes that regulate those immune factors

5.2 METHODS

5.2.1 Ethics statement

Approval for the study was obtained from the Ethics Committees of the Universidad Pontifica Bolivariana and the University of Manitoba. The Instituto Nacional Penitenciario y Carcelario (INPEC), and the director of each prison approved the project. Written consent forms were explained and signed in the presence of two witnesses (prisoners). All inmates diagnosed with active TB received treatment according the National guidelines. New converters were reported to prison health authority in both prisons, and prisoners from prison 1 were offered LTBI treatment. LTBI treatment in prisoners is not considered mandatory in the international and Colombian guidelines, the healthcare personnel from prison 2 opted not to offer LTBI treatment, and therefore prisoners incarcerated in that facility did not receive therapy.

5.2.2 Study design and Population

A cohort study conducted between September 2016 and December 2018 in two male prisons in Colombia. Non-immunosuppressed individuals meeting the following criteria for each group:

Given that the time of incarceration is a variable that affects the risk of becoming infected and sick, and the concentration of some immune substances change by this variable, according to our previous results (data in the process of publication), we divided individuals with and without infection according to whether they were newly incarcerated (they started the follow-up with less than three months of incarceration) or they already had more than one year in prison when started the follow-up. Therefore, we divided them into early (E) or late (L) incarceration, for non-infected and new TST converters:

- 11 non-infected people: Prisoners that at baseline had two-step negative TST, and never converted their TST during follow-up (time of follow-up, median [IQR]; 15.3 [13.0-43.9] months). Individuals were categorized to two groups, early incarceration non-infected (ENI), late incarceration non-infected people (LNI).

- 15 new TST converters, prisoners that at baseline of the cohort recruitment had two-step negative TST and then converted their TST during every 6-months follow-up according to the CDC recommendations[20]. A converter was defined as TST≥10 with an increase of at least 6 mm between the measure of TST at baseline and a new TST at follow-up[21]. Among the converters there were 8 converters with early incarceration and new LTBI (ELTBI), and 7 with late incarceration and new LTBI (LLTBI). Times of incarceration until TST conversion were median [IQR] 12.1 months [10-12.5] for ELTBI, and 39.4 months [24.2-60.3] for LLTBI.

- 10 patients with pulmonary TB microbiologically confirmed (culture and auramine-rhodamine stain), with less than 5 days of treatment and HIV negative. Time of incarceration until the development of active TB: median [IQR]; 34.1 [15-69] months.

5.2.3 Sociodemographic variables

We collected from all individuals: age; history and time of prior incarceration; use of drugs (inhaled, injected, or smoked) or alcohol; comorbidities (chronic obstructive pulmonary disease, diabetes, chronic kidney disease), contact with active TB case (outside and/or inside the prison); weight and height to calculate de body mass index (BMI); and BCG vaccine checked by the presence of a BCG scar.

5.2.4 Procedures

Sample: Blood samples were collected at baseline from all the participants, and every three months until to end the follow-up in people with LTBI and non-infected people. PBMCs were separated by density gradient with Ficoll hystopague (Sigma-Aldrich, Misuri, US), and preserved at -121ºC in DMSO solution, bovine fetal serum and RNA later® (Ambion, Texas, US), until the RNA extraction. At the same time, plasma samples were separated and stored at -80°C until processing.

RNA extraction: Samples were thawed at 37ºC for 2 min and separated by centrifugation at 5000g/5min. Total RNA extraction was done from the PBMCs using the commercial kit RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany), with the following modifications: 10 min of incubation into the lysis buffer and treatment on column with the RNase-Free DNase Set (Qiagen, Hilden, Germany) during 15 min. The total RNA was suspended in 30ul of RNAse free water (Qiagen, Hilden, Germany). RNA quality was evaluated using the TapeStation (Agilent Technologies, Inc, California, US), RNA concentration was evaluated with Quibit 2.0 (Thermo Fisher, Massachusetts, US), and the purity using Nanodrop™ 2000c (Thermo Fisher, Massachusetts, US). Samples with a RNA Integrity Number (RIN) ≥7, ratio of 260/280 ≥1.8 and 28S/18S ratio ≥1.5 were selected for sequencing. The evaluations and subsequent steps, followed published established protocols by Sheng Q *et al*.[22]s, and Conesa A *et al*.[23].

Library preparation and RNA sequencing: The library was prepared using TruSeq stranded mRNA library kit (Illumina, California, US), and the sequencing was done by

Macrogen Inc, (Seoul, Korea), using NovaSeq system (Illumina, California, US). The cDNA libraries generated paired end reads.

Cytokines and chemokine detection: Commercial multiplex and singleplex bead-based fluorescent assays kits were used to quantify 18 cytokines/chemokines of interest from plasma samples of 112 people (50 active TB cases, 11 ELTBI, 7 LLTBI, 23 ENI and 20 LNI), as previously described (article submitted to Cytokine): Macrophage Inflammatory Protein 3α (MIP-3α/CCL20), Human Cytokine/Chemokine magnetic Panel III, Milliplex® Map kit); Interleukin 18 (IL-18), Human IL-18 Singleplex Magnetic Bead kit, Milliplex® Map kit; soluble CD14 (sCD14), Human Cardiovascular Disease (CVD) Panel 6 Magnetic Bead kit, Milliplex® Map kit; Eotaxin 1 (CCL11), Interferon gamma (INF-γ), Interleukin 5 (IL-5), Interleukin 6 (IL-6), Interleukin 10 (IL-10), Interleukin IL-12 p40 homodimer (IL-12[p40]), Interleukin 13 (IL-13), Interleukin 15 (IL-15), Interleukin (IL-17), Interleukin-1 receptor antagonist (IL-1RA), human interferon-inducible protein 10 (CXCL10/ IP-10), monocyte chemoattractant protein-1 (CCL2/MCP-1), macrophage inflammatory protein 1α (CCL3/MIP-1α), macrophage inflammatory protein 1 β (CCL4/MIP-1β), Tumor necrosis factor alpha (TNF-α), Human Cytokine/Chemokine magnetic Bead Panel, Milliplex® Map kit, Millipore Corporation, Billeria, MA, USA.

5.2.5 RNA-seq data analysis

The sequencing data were uploaded to the Galaxy web platform, and we used the public server at usegalaxy.org to analyze the data[24]. We performed quality control using the program FastQC (Babraham Bioinformatics)[25], and the adapters and low-quality sequences were trimmed by Trimmomatic program v14(0.36)[26]. When all sequences met the quality criteria established for RNA in the FAStQC program, the filtered reads were aligned to the reference Homo sapiens genome GRCh38[27] using STAR aligner version 2.7.3a[28]. Then, aligned reads were quantified to obtain the gene-level counts using HTseq-Counts[29].

Our main outcome was new LTBI, both in early and late incarceration. We evaluated the changes in gene expression of PBMC among Early Latent Tuberculosis Infection (ELTBI) and Late Latent Tuberculosis Infection (LLTBI) compared to Active TB (ATB), Early non-infected (ENI), and Late non-Infected (LNI).

Differential expression was assessed using DESeq2 package in Bioconductor[30], yielding a result based on a negative binomial model[31]. The differential expression was defined as a change in the log-fold change (logFC). Only genes with |logFC| >1.0 and an adjusted p-value < 0.1 were selected as DEGs. We analyzed the differences in the enrichment of Gene Ontology terms and KEGG pathways using InterMiner (v.1.4.1)[32–34].

In a previous paper we found that the concentration of cytokine/chemokines varies between individuals with active TB, LTBI, and those non-infected, and the variation in concentration was affected by the time of incarceration. We compared the plasma concentration and the levels of RNA expression of the 18 immune substances.

5.3 RESULTS

5.3.1 Participants

All participants were males with current consumption of smoked drugs (28.3%), cigarettes (45.2%) and alcohol (27.5%). Only one non-infected individual had underweight (BIM<18.5 kg/m^2). Table 1 summarizes demographic information of people with active TB, LTBI and non-infected people.

Tabla 5-1**.** Baseline characteristics of prisoners with active TB, latent tuberculosis infection and non-infected people according to the duration of incarceration

Variable	**ATB** **n= 10**	**ELTBI** **n= 8**	**LLTBI** **n= 7**	**ENI** **n= 6**	**LNI** **n= 5**
Age, years, median [IQR]	33 [24-4]	34.5 [30.5-39]	56 [26-62]	24.5 [22-27]	39 [35-41]
BMI, median [IQR]	20.8 [20.1-23.4]	24.0 [19.2-25.7]	22.9 [20.4-26.7]	21.3 [18.7-25.6]	23.7 [21.5-26.3]
Time into the prison, months, median [IQR]	34.1 [15-69]	12.1 [9.9-12.5]	39.4 [24.2-60.3]	13.4 [12.7-14.7]	43.8 [28.7-56.8]
At least one comorbidity	2	1	2	1	1
COPD	0	1	0	1	0
Diabetes	1	0	2	0	0
Current smoke drugs use	6	1	0	2	0
Current inhaled drugs use	2	0	0	1	0
Current cigarettes use	5	4	2	4	1
Current alcohol use	6	2	**0**	0	**1**
Presence of BCG scar	10	6	5	5	4
Contact with a TB case	5	0	0	1	1
Prison					
Prison 1	10	2	7	0	5
Prison 2	0	6	0	6	0

LLTBI: Late Latent tuberculosis infection; ELTBI: Early Latent tuberculosis infection; ATB: active tuberculosis; ENI: Early non-infected; LNI: Late non-infected; IQR: interquartile range; BMI: Body mass index (kg/m^2); COPD: Chronic obstructive pulmonary disease; BCG: bacille Calmette-Guérin

5.3.2 RNA sequencing data:

On average, the cDNA libraries generated a read count median 52,724,802; IQR [48,707,999 – 56,210,532], of 151 nucleotides in length, with Q30 median of 93.6; IQR [93.42-93.85]. After quality trimming, the reads were mapped to GRCh38. A principal component analysis (PCA) from normalized read count revealed that exist an intragroup heterogeneity (figure S1).

5.3.3 Differential expression among the five groups

In the search of group-specific differentially expressed genes, group comparisons were undertaken and figure 3 depicts the Venn diagrams for up and down-regulated genes.

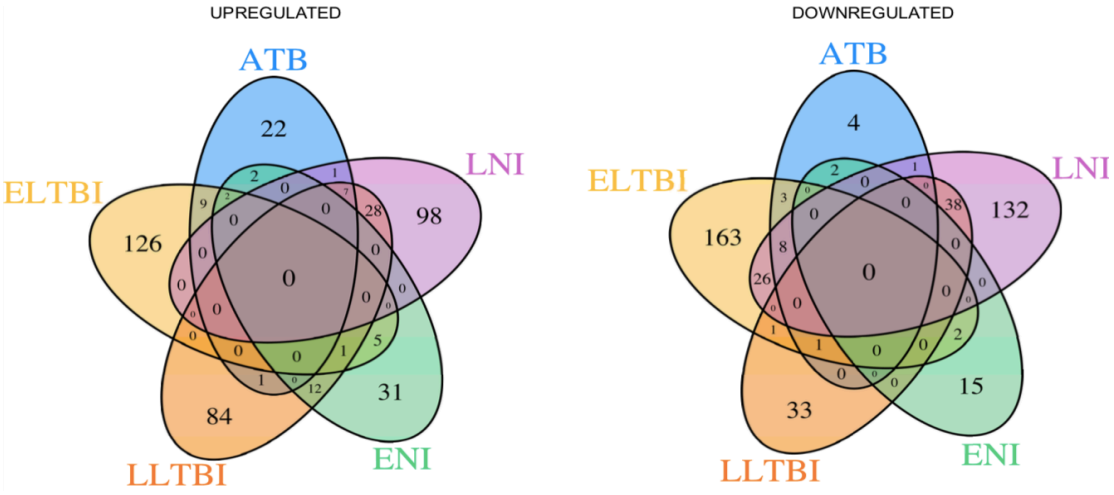


Figura 5-1 Number of specific differentially expressed genes between new LTBI, active TB and non-infected individuals.

Legend: Inmates were separated according to the time of incarceration as early and late. Early means that the individual started follow-up within the first 3 months of incarceration, and late follow-up began after more than one year of incarceration. ELTBI: early latent tuberculosis infection. LLTBI: late latent tuberculosis infection. ENI: early non-infected. LNI: late non-infected. ATB: Active TB.

Supplementary file 1 reports the upregulated and downregulated genes exclusively found in each group with the ID number, symbol, functional description, and if that gene has been found in previous publications.

There are three pathways that involve the largest number of up-regulated genes among individuals early LTBI: cytokine signaling, signal transduction, and the immune system, and there are two pathways involving a larger number of down-regulated genes: neutrophil degranulation and the innate immune system. In individuals with late LTBI, the only detected pathway was Emi1 phosphorylation.

The expression of genes related to the cell cycle are increased in early non-infected individuals, and decreased in late non-infected people (tables 2 and 3).

No pathway was detected among specific differentially expressed genes in people with active TB.

The GO enrichment analysis is summarized in table 2 for upregulated and downregulates genes.

Tabla 5-2. Reactome pathways of group-specific upregulated and downregulated genes in people with new LTBI, active TB and non-infected

Group	Reactome pathway	Adjusted p-value	Number of upregulated genes	Reactome pathway	Adjusted p-value	Number of downregulated genes
TBA	N/A	N/A	N/A	N/A	N/A	N/A
ENI	Cell Cycle, Mitotic	4.46E-10	12	N/A	N/A	N/A
	Cell Cycle	1.87E-09	12			
	M Phase	4.26E-04	7			
	G1/S-Specific Transcription	0.004769504	3			
	G1/S Transition	0.013668196	4			
	Mitotic G1-G1/S phases	0.015873093	4			
	Cell Cycle Checkpoints	0.016116383	5			
	S Phase	0.019864498	4			
	Mitotic Metaphase and Anaphase	0.030274743	4			
	Mitotic Prometaphase	0.030274743	4			
	Orc1 removal from chromatin	0.030731979	3			
	Separation of Sister Chromatids	0.03236454	4			

	Mitotic Anaphase	1 0.032387762	4			
	DNA Damage/Telomere Stress Induced Senescence	0.032661066	3			
	Polo-like kinase mediated events	0.037632916	2			
	Switching of origins to a post-replicative state	0.039193071	3			
LNI	N/A	N/A	N/A	Interleukin-10 signaling	1.75E-06	8
				Cell Cycle, Mitotic	1.09E-05	18
				Cytokine Signaling in Immune system	6.64E-05	21
				Cytokine-cytokine receptor interaction	8.31E-05	12
				Cell Cycle	8.40E-05	18
				Signaling by Interleukins	1.11E-04	15
				G1/S-Specific Transcription	3.65E-04	5
				Rheumatoid arthritis	7.30E-04	7
				G alpha (i) signaling events	0.003807673	12
				Cell cycle	0.004142511	7
				Cyclin A/B1/B2 associated events during G2/M transition	0.004431131	4

				Malaria	0.004509032	5
				NOD-like receptor signaling pathway	0.007828307	5
				Condensation of Prometaphase Chromosomes	0.008137635	3
				M Phase	0.008690615	11
				Phosphorylation of proteins involved in the G2/M transition by Cyclin A:Cdc2 complexes	0.019337922	2
				Cell Cycle Checkpoints	0.020199346	9
				Polo-like kinase mediated events	0.020969303	3
				Resolution of Sister Chromatid Cohesion	0.023064917	6
				G1/S Transition	0.02589064	6
				Osteoclast differentiation	0.026057756	6
				Immune System	0.034098438	27
				Mitotic Prometaphase	0.037753482	7
				G2/M DNA replication checkpoint	0.041781373	2
				RAF-independent MAPK1/3 activation	0.042017212	3
				Mitotic G1-G1/S phases	0.042377925	6
ELTBI	G alpha (i) signalling events	1.55E-04	14	Neutrophil degranulation	1.24E-05	19

	Cytokine Signaling in Immune system	1.82E-04	20	Innate Immune System	4.67E-04	25
	Chemokine receptors bind chemokines	1.99E-04	7	Phosphorylation of Emi1	0.008179412	3
	Interleukin-10 signaling	2.85E-04	6	Immune System	0.010287755	34
	Class A/1 (Rhodopsin-like receptors)	0.002936423	11	Class A/1 (Rhodopsin-like receptors)	0.03461297	11
	Cytokine-cytokine receptor interaction	0.002995896	10	Chemokine receptors bind chemokines	0.044200545	5
	Immune System	0.004273297	29			
	RUNX2 regulates osteoblast differentiation	0.00445326	4			
	Signaling by Interleukins	0.006434591	12			
	Chemokine signaling pathway	0.006730335	8			
	GPCR ligand binding	0.007242515	12			
	Peptide ligand-binding receptors	0.007306545	8			
	RUNX2 regulates bone development	0.00892581	4			
	Signaling by GPCR	0.016702907	19			
	Signal Transduction	0.04398543	31			

		4				
LLTBI	Phosphorylation of Emi1	0.001048194	3	N/A	N/A	N/A

Legend: Participants are separated according to the time of incarceration as early and late. Early means that the individual started the follow-up within 3 months of incarceration, and late were follow-up began after more than one year of incarceration. ELTBI: early latent tuberculosis infection. LLTBI: late latent tuberculosis infection. ENI: early non-infected. LNI: late non-infected. ATB: Active TB.