Universitat Autònoma de Barcelona

New approaches for the future of tuberculosis diagnosis
Interpreting conversations between the host and Mycobacterium tuberculosis

Patricia Comella del Barrio

Ph. D. Thesis

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Interpreting conversations between the host and *Mycobacterium tuberculosis*

Thesis presented by Patricia Comella del Barrio in order to obtain the Ph.D. in Microbiology by the Genetics and Microbiology Department of the Universitat Autònoma de Barcelona

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quien me inculcó el amor por el trabajo bien hecho.

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Mèsi

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Without Knowledge, Skill cannot be focused.
Without Skill, Strength cannot be brought to bear
and without Strength, Knowledge may not be applied.

Attributed to Alexander the Great

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Summary

Summary

Summary

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis*, which is mainly transmitted when sick people expel the bacteria into the air. It is estimated that a quarter of the population is infected with *M. tuberculosis* and that approximately 5-10% of these people will progress to the disease. In 2020, an estimated 9.9 million people worldwide were ill with TB. Although TB is a curable disease that usually affects the lungs (but can affect other sites), it is the second leading cause of death globally from an infectious agent after COVID-19. Diagnosis is the first step to good clinical care and proper disease management. However, easy access to TB diagnosis is far from guaranteed. In 2020, about 41.4% of TB patients were not diagnosed or reported, contributing to the estimated 1.5 million annual deaths caused by TB worldwide. In addition, the disruptions to diagnostic and treatment services caused by COVID-19 have had a major impact on health systems, affecting TB control programmes. This fact was reflected in a reduction in the number of people who die from TB with the first year-on-year increase (of 5.6%) since 2005 and the total number of deaths in 2020 returning to the 2017 level (annex 1).

Bacteriological confirmation by culture or sputum-based molecular methods are the gold standard for TB diagnosis. However, the incubation time, infrastructure, and biosafety level of cultures, as well as the cost and operational requirements of rapid molecular methods, limit their integration and use in primary care settings, where the patient first seeks care. In addition, current diagnostic methods have low sensitivity in patients with difficulties in obtaining a representative sample from the site of infection (patients with extrapulmonary TB, children, HIV co-infected patients or individuals in early stages of disease). To improve TB diagnosis, more accurate and accessible non-respiratory diagnostic methods need to be developed, especially in low- and middle-income countries, where the greatest burden of disease resides.

In recent years, novel non-sputum-based biomarkers have been identified for the diagnosis of paediatric TB, however, there is still no test with sufficient sensitivity to develop a diagnostic test for TB in children (annex 2). The first article of this thesis describes a set of cytokines and a series of individual factors (ferritin, 25-hydroxyvitamin D [25(OH)D], parasitic infections and nutritional status) identified from serum and plasma samples by immunoassays to assess different discriminant patterns between *M. tuberculosis* infection and disease in children (article 1 and annex 5). As a result, the combination of IP-10, IFN-γ, ferritin and 25(OH)D showed the best diagnostic performance, achieving optimal sensitivity and specificity for the development of a promising early TB diagnostic test in children (93.2 and 90.0%, respectively).

Urine is a readily available and non-invasive sample, which would be especially useful in patients unable to expectorate or obtain a representative respiratory specimen, such as children, the elderly and adults without a productive cough, or individuals with disseminated, extrapulmonary, non-cavitary disease. In article 2, the performance of the Fujifilm SILVAMP TB LAM assay (FujiLAM), a new urine-based test for TB diagnosis, is evaluated in HIV-positive and negative adults with presumptive TB from three district hospitals in Nigeria, showing that FujiLAM has a good diagnostic accuracy to consider its application in HIV-positive and -negative TB patients. With regard to TB risk groups, article 3 evaluates the use of FujiLAM in children with presumptive TB in a paediatric hospital in Haiti and shows high specificity of the assay which, together with its characteristics as a point-of-care test, indicates that the test would have potential for TB diagnosis in children.

Currently, the development of omics, has allowed the generation of knowledge on the underlying mechanisms of the disease. In this regard, article 4 identifies and characterises a metabolic profile of TB in urine using high-field nuclear magnetic resonance (NMR) spectrometry by developing mathematical models capable of discriminating TB patients from pneumococcal pneumonia patients, latently TB infected individuals, or healthy controls. Towards the development of a more accessible test, this article also evaluates whether the metabolic profile of TB can be detected with a low-field portable NMR spectrometer by demonstrating its ability to discriminate TB patients from all other groups without being influenced by anti-TB treatment or TB location (annex 4). With a focus on the diagnosis of paediatric TB, we explore whether urine NMR-based metabolomics could be used to identify differences in the metabolic response of children with different TB diagnostic certainty, showing differences in the metabolic fingerprint of children with bacteriologically confirmed and unconfirmed TB compared to children with unlikely TB, as well as between children with unconfirmed TB with TB-compatible radiographs and non-pathological radiographs (article 5 and annex 4) This finding would assist in a more accurate characterisation of TB in the paediatric population.

The last study included in this thesis describes the urinary metabolic fingerprint of TB patients monitored during treatment to successful completion (article 6). This article shows differences in the metabolic fingerprint throughout treatment, such that, at the end of treatment, patients have a similar metabolic fingerprint to that of healthy controls. Given the emerging acquisition of resistance to anti-TB drugs and the increase in treatment failures, this proof of concept could be considered for monitoring the response to treatment of patients with drug-resistant TB. Advances in systems biology in relation to TB would address more precisely clinically relevant questions for the future, such as the early diagnosis of TB, the individualisation of preventive therapies in terms of the choice of anti-TB drugs and host-directed

therapies administered, and the prediction of the outcome of a treatment regimen (annex 3).

Overall, this thesis focuses on describing and evaluating new approaches in the search for potential non-sputum-based biomarkers contributing to improve future TB diagnosis and the assessment of response to anti-TB treatment.

Resumen

La tuberculosis (TB) es una enfermedad causada por *Mycobacterium tuberculosis*, que se transmite cuando las personas enfermas exhalan las bacterias al aire. Se calcula que una cuarta parte de la población mundial está infectada con *M. tuberculosis* y que aproximadamente un 5-10% de estas personas desarrollan la TB. Se estima que, en 2020, 9.9 millones de personas en todo el mundo enfermaron de TB. Aunque la TB es una enfermedad curable que suele afectar a los pulmones (pero puede afectar a otros lugares), es la segunda causa de muerte mundial por un agente infeccioso por detrás de la COVID-19. El diagnóstico es una fase clave para el manejo clínico y para lograr el control de la enfermedad. Sin embargo, el fácil acceso al diagnóstico de la TB está lejos de estar garantizado. En 2020, se estima que el 41.4% de los pacientes con TB no se diagnosticaron ni se notificaron, contribuyendo a los 1.5 millones de muertes anuales estimadas, causadas por TB en todo el mundo. Además, las interrupciones de los servicios de diagnóstico y tratamiento causadas por la COVID-19, han tenido un gran impacto en los sistemas de salud, afectando a los programas de control de la TB. Este hecho ha repercutido con la reducción del número de personas que mueren de TB con el primer aumento interanual (del 5,6%) desde 2005 y el número total de muertes en 2020 volviendo al nivel de 2017 (anexo 1).

La confirmación bacteriológica mediante cultivo o métodos moleculares basados en el esputo son los métodos de referencia para el diagnóstico de la TB. Sin embargo, el tiempo de incubación, la infraestructura y el nivel de bioseguridad de los cultivos, así como el coste y los requisitos operativos de los métodos moleculares rápidos, limitan su integración y utilización en los centros de atención primaria, donde el paciente busca atención por primera vez. Además, los métodos de diagnóstico actuales tienen una baja sensibilidad en pacientes con dificultades para obtener una muestra representativa del lugar de la infección (pacientes con tuberculosis extrapulmonar, niños, pacientes coinfectados por el VIH o individuos en fases iniciales de la enfermedad). Para mejorar el diagnóstico de la TB, se necesitan desarrollar métodos de diagnóstico alternativos a los que requieren muestra biológica respiratoria que sean más precisos y accesibles, especialmente en los países de ingresos bajos y medios, donde reside la mayor carga de la enfermedad.

En los últimos años, se han identificado nuevos biomarcadores no basados en muestras respiratorias para el diagnóstico de la TB pediátrica; sin embargo, todavía no existe ninguna prueba con la sensibilidad suficiente para desarrollar una prueba de diagnóstico de la TB en niños (anexo 2). El primer artículo de esta tesis describe un conjunto de citocinas y una serie de factores individuales (ferritina, 25-hidroxivitamina D [25(OH)D], infecciones parasitarias y estado nutricional) identificados a partir de muestras de en suero y plasma mediante inmunoensayos para evaluar diferentes patrones discriminantes entre infección y la enfermedad por *M. tuberculosis* en niños (artículo 1)y anexo 5). Como resultado, la combinación de IP-10, IFN-γ, ferritina y

25(OH)D mostró el mejor rendimiento diagnóstico, logrando una sensibilidad y especificidad óptimas para el desarrollo de una prometedora prueba de diagnóstico temprano de la TB en niños (93.2% y 90.0%, respectivamente).

La orina es una muestra fácilmente disponible y no invasiva, que sería especialmente útil en pacientes incapaces de expectorar, como los niños, los ancianos y los adultos sin tos productiva, o los individuos con enfermedad diseminada, extrapulmonar y no cavitaria. El artículo 2, evalúa el rendimiento del ensayo Fujifilm SILVAMP TB LAM (FujiLAM), una nueva prueba basada en muestras de orina para el diagnóstico de la TB, en adultos VIH-positivos y negativos con presunta TB de tres hospitales de distrito en Nigeria, mostrando que FujiLAM tiene una buena precisión diagnóstica para considerar su aplicación en pacientes seropositivos y negativos con TB. Con respecto a los grupos de riesgo al desarrollo de TB, el artículo 3 evalúa el uso de FujiLAM en niños con presunta TB de un hospital pediátrico en Haití y muestra elevada especificidad del ensayo que, junto a sus características como prueba en el punto de atención indican que la prueba tendría potencial para el diagnóstico de la TB en niños.

En la actualidad, el desarrollo de las tecnologías ómicas ha permitido generar conocimiento sobre los mecanismos subyacentes de la enfermedad. En este sentido, el artículo 4 identifica y caracteriza un perfil metabólico de la TB en la orina mediante espectrometría de resonancia magnética nuclear (RMN) de alto campo desarrollando modelos matemáticos capaces de discriminar a pacientes con TB de pacientes con neumonía neumocócica, individuos con infección de TB latente y los controles sanos. Hacia el desarrollo de una prueba más accesible, este artículo también evalúa si el perfil metabólico de la TB se puede detectar con un espectrómetro de RMN portátil de bajo campo demostrando su capacidad para discriminar a los pacientes con TB del resto de los grupos sin verse influida por el tratamiento antituberculoso o la localización de la TB (anexo 4). Con el foco puesto en el diagnóstico de la TB pediátrica, se explora si la metabolómica basada en la RMN de la orina podría utilizarse para identificar las diferencias en la respuesta metabólica de los niños con diferente grado de certeza diagnóstica de la TB, mostrando diferencias en la huella metabólica de los niños con TB confirmada y no confirmada bacteriológicamente en comparación con los niños con TB poco probable, así como entre los niños con TB no confirmada con radiografías compatibles con TB y con radiografías no patológicas (artículo 5 y anexo 4). Este hallazgo puede ayudar a una caracterización más precisa de la TB en la población pediátrica.

El último estudio incluido en esta tesis describe la huella metabólica urinaria de los pacientes con TB monitorizados durante el tratamiento hasta finalizarlo con éxito (artículo 6). Este artículo muestra diferencias en la huella metabólica a lo largo del tratamiento, de tal forma que, al finalizar el tratamiento los pacientes tienen una huella

similar a la de los controles sanos. Teniendo en cuenta la incipiente adquisición de resistencia a los fármacos antituberculosos y el aumento de fracasos terapéuticos, esta prueba de concepto podría considerarse para monitorizar la respuesta al tratamiento de pacientes con TB resistente a los medicamentos. El avance de la biología de sistemas permitiría abordar con mayor precisión cuestiones clínicamente relevantes para el futuro de la TB, como el diagnóstico precoz, la individualización de las terapias preventivas (la elección de los fármacos antituberculosos y la administración de terapias dirigidas al huésped) o la predicción del resultado del tratamiento antituberculoso (anexo 3).

En conjunto, esta tesis se centra en describir y evaluar nuevos enfoques en la búsqueda de posibles biomarcadores no basados en muestras respiratorias contribuyendo a mejorar el futuro diagnóstico de la TB y la evaluación de la respuesta al tratamiento antituberculoso.

Rezime

Tibèkiloz (TB) se yon maladi ki soti lan *Mycobacterium tuberculosis*, ki kapab transmèt lè yon moun ki trape maladi sila a pwopaje bakteri a nan lè a. Daprè yon etid, yon moun sou kat lan popilasyon an enfekte ak mycobacterium, e maladi sila a ap devlope apeprè nan 5-10% moun sa yo. Nan lane 2020, yo ta estime ke 9.9 milyon moun atravè lemonn ta trape maladi TB la. Malgre ke TB se yon maladi ki kapab geri e ki an jeneral afekte poumon yo (men ki kapab afekte lòt pati nan kò a tou), li se dezyèm kòz lanmò a travè lemonn e li jwenn sous li nan yon ajan enfektye menm jan ak maladi COVID-19. Dyagnostik se premye etap lan pwosesis laswenyay la. Aloske, aksè pou fe dyagnostik TB la pa garanti. Nan lane 2020, anviwon 41.4% nan pasyan TB yo pat dyagnostike osinon ka yo pat rapòte, sa ki lakòz ke 1.5 milyon moun mouri de maladi TB chak ane atravè lemonn. Mete sou sa, maladi COVID-19 la lakòz anpil pètibasyon lan sèvis dyagnostik ak tretman, ki lakòz ke anpil moum ki soufri maladi TB afekte, e ki lakòz yon ogmantasyon nan kantite moun kap mouri, yon reyalite ki direkteman afekte e ranvèse rediksyon nan kantite moun kap mouri nan TB avèk yon premye ogmantasyon ane an ane (swa 5.6%) depi lane 2005, ak kantite total lanmò nan lane 2020 ki retounen nan menm nivo ak kantite lanmò lan lane 2017 (anèks 1).

Konfimasyon bakteriolojik a pati kilti osinon krache se metòd molekilè ki pi efikas pou dyagnostike TB. Aloske, peryod enkibasyon, enfrastrikti, ak nivo biyosekirite nan eksplwatasyon kilti yo, ajoute sou pri ak egzijans operasyonèl pou fè metòd rapid molekilè a, limite entegrasyon ak itilizasyon yo nan mizanplas premye swen, kote se laswenyay pasyan an premye chèche. Anplis de sa, metòd dyagnostik aktyèl yo pa gen gran efè sou pasyan ki gen difikilte pou jwenn yon echantiyon reprezantatif nan sit enfeksyon yo (pasyan ki gen TB ekstrapulmonè, timoun, pasyan ki ko-enfekte ak VIH osinon moun ki fenk trape maladi an). Pou amelyore dyagnostik TB la, yap bezwen devlope dyagnostik ki pa baze sou metòd krache, ki pi egzak e pi aksesib, espesyalman nan peyi ki gen revni ki ba ak revni mwayen, kote maladi a andemik.

Nan dènye ane sa yo, yo ta idantifye nouvo indikatè ki pa baze sou krache pou dyagnostike TB kay timoun, aloske, toujou pa gen okenn tès ki ase reyaktif pou devlope yon tès dyagnostik pou TB kay timoun (anèks 2). Premye atik tèz sa a dekri yon seri sitokin ak yon seri de faktè endividyèl (ferritin, 25-hydroxyvitamin D [25(OH)D], enfeksyon parazit ak kondisyon nitrisyonèl) ke yo idantifye nan echantiyon seròm ak plasma apati analiz iminolojik pou evalye diferan modèl selektif ant enfeksyon *M. tuberculosis* ak maladi lakay timoun yo (atik 1 ak anèks 5). Kòm rezilta, konbinezon IP-10, IFN-γ, feritin ak 25(OH)D demontre pi bon pèfòmans nan dyagnostik la e reyisi atenn pi bon reyaksyon ak espesifisite pou devlopman yon bon jan tès dyagnostik TB byen bonè lakay timoun yo (93.2 ak 90.0% ,pou chak ka).

Pipi se yon echantiyon ki disponib fasilman epi ki paka pwopaje, ki kapab itil sitou pou

pasyan ki pa kapab krache, tankou timoun, granmoun aje ak granmoun ki gen yon latous sèk, osinon moun ki gen yon maladi ki pwopaje lan kò yo , maladi ekstrapilmonè, ak sa ki gen yon maladi ki pa kavitè. Nan atik 2 a, pèfòmans nan tès Fujifilm SILVAMP TB LAM (FujiLAM), yon nouvo tès ki baze sou pipi pou dyagnostike TB, ke yo eseye nan twa lopital distrik Nijerya sou granmoun ki seropozitif ak granmoun ki seronegatif e ki pwobableman soufri TB, demontre ke FujiLAM gen yon bon jan dyagnostik ak presizyon pou konsidere aplikasyon li sou pasyan TB seropozitif ak negatif. Konsènan gwoup a risk TB yo, atik 3 evalye itilizasyon FujiLAM nan on gwoup timoun ki ta samble gen TB nan yon lopital pedyatrik lan peyi DAyiti e li demontre bon jan espesifisite segondè nan tès sila a ki, ansanm ak karakteristik li yo kòm yon tès ki pi prè pasyan an e endike ke tès sila a ta gen yon gwo potansyèl pou dyagnostike TB lakay timoun yo.

Kounye a, devlopman kap fèt lan etid jenom yo pèmèt jenerasyon save yo gen konesans sou mekanis ki kache nan maladi sila a. Nan objektif sa a, atik 4 la idantifye e karakterize yon pwofil metabolik maladi TB la nan pipi avèk itilizasyon gwo frekans sonorite mayetik nikleyè (NMR) spektrometri, pandan lap devlope modèl matematik ki kapab diferansye pasyan TB, soti nan pasyan nemokok nemoni, moun ki gen enfeksyon TB kache, oswa moun ki an sante. Sou wout pou devlope yon tès ki pi aksesib, atik sa a evalye tou si yo kapab detekte pwofil metabolik TB avèk yon spektomèt NMR a ti frekans pandan lap demontre kapasite li nan diferansyasyon pasyan TB pa rapò ak tout lòt gwoup san yo pa enfliyanse pa tretman anti-TB oswa kote yap trete TB (anèks 4). Pandan yap mete yon anfaz sou dyagnostik TB lakay timoun, nou fe etid sou pipi ki baze sou metabolis ke NMR la ta ka itilize pou jwenn diferans ki genyen nan repons metabolik lakay timoun ki gen diferan TB dyagnostik sètifye, ki montre diferans ki genyen nan anprent metabolik lakay timoun ki lan nivo bakteriolojik ki positif ak negatif TB, konpare ak timoun ki gen TB fasil, osi byen ke ant timoun ki gen TB ki pa konfime ak radyografi, TB-konpatib ak radyografi ki pa patolojik (atik 5 ak anèks 4) . Rezilta sa a ap ede fe yon karakterizasyon ki pi egzak nan mitan popilasyon timoun ki soufri TB.

Dènye etid ki enkli nan tèz sa a dekri anprent metabolik ki baze sou metòd pipi a nan pasyan ki soufri TB et ki sou kontwol pandan tretman jiskaske trètman an fonksyone (atik 6). Atik sa a montre diferans ki genyen nan anprent metabolik la pandan tout tretman an, tankou, nan fen tretman an, pasyan yo gen menm anprent metabolik ak sa yo ki nan bon kondisyon lasante yo. Etandone rezilta kwasan nan rezistans fas a medikaman anti-TB ak ogmantasyon nan echèk tretman yo, yo ta ka konsidere prèv sa a nan objektif pou kwontole repons tretman an nan pasyan ki devlope rezistans ak medikaman TB yo. Pwogrè nan sistèm biyoloji sou kesyon TB ap mete plis limyè sou kesyon klinik ki enpòtan yo nan tan kap vini yo, tankou dyagnostike maladi TB la byen bonè, endividyalizasyon nan terapi prevantif an tèm de chwa nan medikaman anti-TB, terapi ki dirije pa moun kap resevwal la , ak prediksyon nan rezilta yo lan zafè pwogram tretman an (anèks 3).

Pou fini, tèz sa a mete aksan sou deskripsyon ak evalyasyon yon nouvo apwòch nan rechèch sou potansyèl indikatè ki pa baze sou krache kap kapab kontribye nan amelyorasyon dyagnostik TB la nan tan kap vini yo ak analiz sou zafè repons a tretman anti-TB a.

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List of publications

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Articles

1. **Comella-del-Barrio, P.**; Abellana, R.; Villar-Hernández, R.; Coute, M. D. J.; Mingels, B. S.; Aliaga, L. C.; Narcisse, M.; Gautier, J.; Ascaso, C.; Latorre, I.; Dominguez, J.; Perez-Porcuna, T. M. **A Model Based on the Combination of IFN-γ, IP-10, Ferritin and 25-Hydroxyvitamin D for Discriminating Latent From Active Tuberculosis in Children.** *Front. Microbiol.* **2019**, 10 (AUG), 1–15. https://doi.org/10.3389/fmicb.2019.01855.

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Annexes

1. **Comella-del-Barrio, P.**; Souza-Galvão, M. L. De; Prat-Aymerich, C.; Domínguez, J. **Impact of COVID-19 on Tuberculosis Control.** *Arch. Bronconeumol.* **2020.** https://doi.org/10.1016/j.arbres.2020.11.016.

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Patents

4. Izquierdo-Garcia, J.L.; Dominguez, J.; **Comella-del-Barrio, P.**; Prat-Aymerich, C. **Identification of Metabolomic Signatures in Urine Samples for Tuberculosis Diagnosis.** *European Patent Office*, **2019**, WO2019243347A1. https://worldwide.espacenet.com/patent/search?q=pn%3DWO2019243347A1.

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3

General Introduction

Introduction

3.1. Epidemiology of tuberculosis

Tuberculosis (TB) is one of the top ten causes of death worldwide with an estimated 1.5 million deaths each year and the second leading cause of death from a single infectious agent (behind COVID-19)[1].

TB is caused by bacteria from the *Mycobacterium tuberculosis* complex (MTBC), and can affect anyone, anywhere[2]. Consequently, a heterogeneous distribution of the disease prevails in the global population. About a quarter of the population is estimated to be infected by MTBC, however, only one in ten people will develop the active form of the disease[1].

In 2020, there were an estimated 9.9 million new TB cases worldwide, 56% were men, 33% women, and 11% children[1,3]. Of the total number of estimated new TB cases, 7.1 million were diagnosed and reported[1]. Closing the gap between reported and diagnosed cases is key to curbing disease transmission[2].

Geographically, 30 low- and middle-income countries accounted for 86% of new TB cases worldwide. India (26%), China (8.5%), Indonesia (8.4%), Philippines (6.0%), Pakistan (5.8%), Nigeria (4.6%), Bangladesh (3.6%), and South Africa (3.3%) accounted for two thirds of global TB incidence[1]. In contrast, only 2.3% of global TB cases were reported in the WHO European region and 3% in the WHO Americas region. Figure 1 illustrates the geographical distribution of the incidence of new TB cases in 2020.

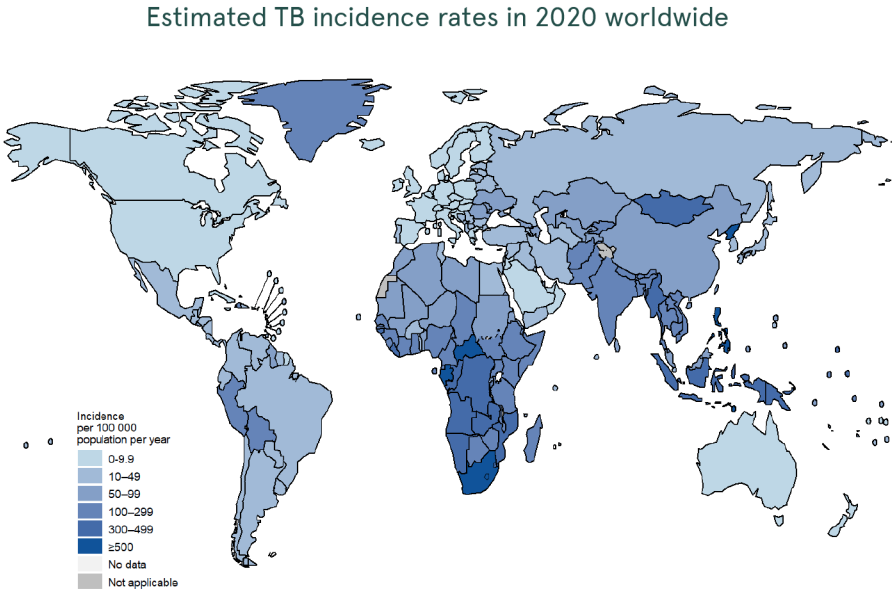

Figure 1. Estimated TB incidence rates in 2020 worldwide. From Global tuberculosis report 2021. Geneva: World Health Organization; 2021. License: CC BY-NC-SA 3.0 IGO. Copyright © World Health Organization 2021.

In 2020, it was estimated that globally 8% of TB cases occurred in people co-infected with HIV[1]. In the WHO African region, 85% of newly reported TB cases had HIV co-infection. With a risk 18 times higher than in the rest of the world's population, HIV infection is one of the major susceptibility factors for disease progression. Malnutrition, alcoholism, smoking, and diabetes are other risk factors for the development of TB[2,4].

The irruption of COVID-19 at the end of 2019 has left nearly 240 million confirmed cases and more than 4.8 million deaths worldwide[5]. The response to the current COVID-19 pandemic has compromised routine health services in all countries and threatened to reverse recent advances in reducing global TB burden[6]. In particular, the containment measures and reallocation of human, financial, and equipment resources from TB to the COVID-19 response, have negatively impacted TB prevention and care programmes[7-10], affecting first those with TB. Consequently, the pandemic has reversed years of global progress in reducing the number of people who die from TB, with the first year-on-year increase (of 5.6%) since 2005 and the total number of deaths in 2020 returning to the level of 2017[1]. This impact has particularly affected low- and middle-income countries, with a decline in new TB case diagnosis and treatment follow-up[11]. In India, Indonesia, the Philippines, and South Africa, four countries that account for 44% of the global TB burden, the first six months of 2020 experienced a 25-30% reduction in TB cases diagnosed compared to the same period in 2019[12]. Unfortunately, these countries with one of the highest incidences of TB have the least resources to vaccinate against COVID-19. We commented this in an editorial published in *Archivos de Bronconeumología* (See Annex 1).

Vaccine development has so far proven to be the key tool in the battle against COVID-19. To date, at least seven different vaccines have been deployed and more than 200 vaccine candidates are in development. However, its allocation is being carried out unevenly, neglecting countries with fewer financial resources to vaccinate against COVID-19 (Figure 2)[13,14]. In terms of diagnostics, testing is essential at every stage of the pandemic response to help limit the spread of COVID-19 and to provide reliable information for life-saving decisions. In addition, diagnostics are needed to assess vaccine efficacy and inform vaccine deployment in the face of likely supply and funding constraints. In a collaborative framework and with the goal of ending the pandemic as quickly as possible through the accelerated development, equitable allocation and expanded delivery of tests, treatments, and vaccines, emerged the COVID-19 Access to Tools Accelerator[15,16]. This multidisciplinary support structure allows partners to share resources and expertise. While the COVAX initiative (proposed by the CEPI, Gavi, WHO, and UNICEF) aims to promote equitable global access to COVID-19 vaccines, the diagnostics pillar (co-convened by FIND and the Global Fund) has set an ambitious agenda to advance universal and equitable access to simple, accurate, and affordable diagnostic tests. Under the slogan "No one is safe until everyone is safe" this

initiative is currently the best effort we have to ensure a global response to COVID-19 and to ensure that vaccines, diagnosis, and treatment reach people all over the world[17]. By contrast, BCG is the only vaccine licensed for prevention of TB. Developed more than 100 years ago, BCG prevents severe forms of TB in children, but is not effective in preventing the disease in adults, and has little effect against the pulmonary forms of the disease, which are responsible for transmission. Despite 14 candidate vaccines in clinical trials in 2021 and efforts to accelerate the development of new TB vaccines, the overall level of investment (US$ 0.9 billion in 2019) falls far short of the global target (US$ 2 billion per year)[1].

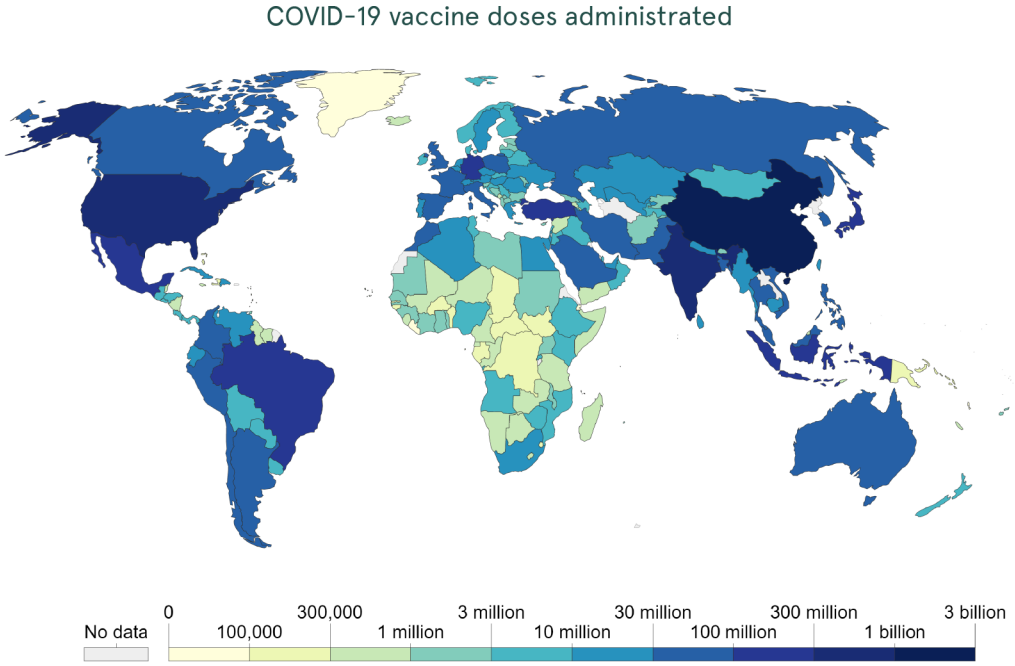

Figure 2. COVID-19 vaccine doses administrated, Oct 19, 2021. For vaccines that require multiple doses, each individual dose is counted. As the same person may receive more than one dose, the number of doses can be higher than the number of people in the population. Published online at Our World in Data – Last updated 19 October 2021, 09:00 (London time). Retrieved from ourworldindata.org/coronavirus | CC-BY.

3.2. General characteristics of *Mycobacterium tuberculosis*

As mentioned previously, TB is an infectious disease caused by bacteria from the genus *Mycobacterium* and grouped in the MTBC. The complex consists of: *M. tuberculosis, M. africanum, M. canettii, M. microti, M. bovis, M. bovis bacillus Calmette-Guérin (BCG), M. caprae, M. mungi, M. orygis,* and *M. pinnipedii*[18]. All MTBC bacteria derive from a common ancestor, however, despite their genetic similarities, they differ widely in terms of phenotypes, geographic distribution, epidemiology, and host preference. Humans are the definitive host for *M. tuberculosis* and *M. africanum, although M. bovis, M. caprae, M. microti, M. canettii,* and *M. orygis* have also been detected in humans[19].

Main responsible of the high TB incidence worldwide, *M. tuberculosis* is an aerobic, non-motile, non-spore-forming, non-capsule-forming, and slow-growing bacillus. Similar to other bacteria of the genus *Mycobacterium, M. tuberculosis* has a unique and complex lipid-rich cell envelope that confers acid-alcohol resistance to Gram staining, low permeability, and intrinsic resistance to the entry of many hydrophobic antibiotics, as well as resistance in different hostile microenvironments[20–22]. The wall of *M. tuberculosis* consists of a cellular skeleton composed of a thin layer of peptidoglycans and arabinogalactans bound to a thick peripheral lipid layer of long-chain mycolic acids alternated with waxes and glycolipids[23]. Glycolipids, such as lipoarabinomannan (LAM), lipomannan, and phosphatidylinositol mannosides, are bound together with the cell skeleton to the plasma membrane. These molecules play a key role in the infection process and pathogenesis of *M. tuberculosis* (Figure 3)[24,25].

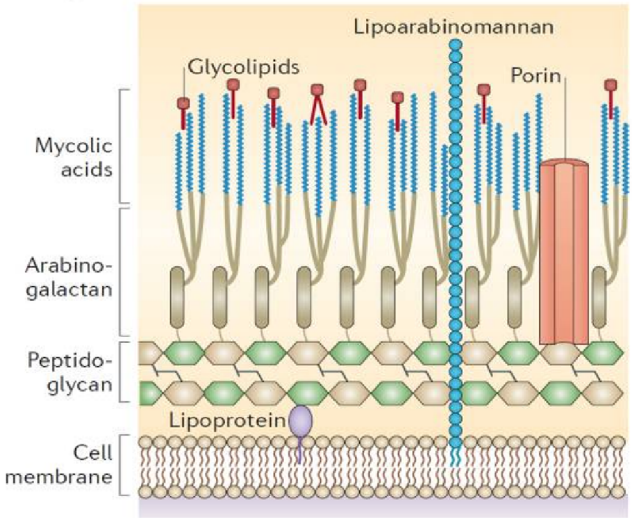

Figure 3. Cell wall structure of mycobacteria. It consists of thin layers of peptidoglycan and arabinogalactan, and a thick layer of mycolic acids. Glycolipids and porins are also found in these cell walls, as is lipoarabinomannan, which is anchored to the cell membrane by diacylglycerol. This cell wall surrounds a single lipid membrane. Adapted from Brown, L. et al. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria, and fungi. Nat Rev Microbiol. 13(10): 620–630 (2015). Copyright © 2015, Nature Publishing Group, a division of Macmillan Publishers Limited. Reprinted with permission from Springer Nature.

3.3. Transmission and immunopathology of tuberculosis

TB is mainly an airborne disease that can be spread through droplets when people with pulmonary TB (the transmissible form of the disease) cough or sneeze (for example). These infectious droplets can then be inhaled by another person during close and/or continuous contact[1,2]. When this occurs, *M. tuberculosis* bacilli enter the respiratory tract and colonise the pulmonary alveoli where they will be phagocytized by alveolar macrophages and arrested in phagosomes[26]. From here, a dynamic confrontation between antimicrobial defences (antimicrobial peptides, nitrosative stress,

phagolysosome fusion and autophagy) and *M. tuberculosis* immune evasion will be initiated[27]. Infected alveolar macrophages will induce the secretion of proinflammatory cytokines and chemokines generating a non-specific response involving the recruitment of macrophages, neutrophils, monocytes, and dendritic cells to the site of infection. Due to its intracellular nature, *M. tuberculosis* will prevent the fusion of the phagosome with the lysosome ensuring its survival and replication within the phagosome. It will inhibit host cell apoptosis to promote the accumulation of more bacteria in the cell before they are released and promote necrotic death and macrophage recruitment by virulence mechanisms (such as the ESX-1 secretion system)[28]. This will expand the bacterial population through the uptake of mycobacteria by adjacent, newly recruited phagocytes. Failure of the first line of host defence to eliminate the bacteria will result in *M. tuberculosis* invading the pulmonary interstitial tissue, either by directly infecting the alveolar epithelium or by infected alveolar macrophages migrating into the lung parenchyma (Figure 4a)[2].

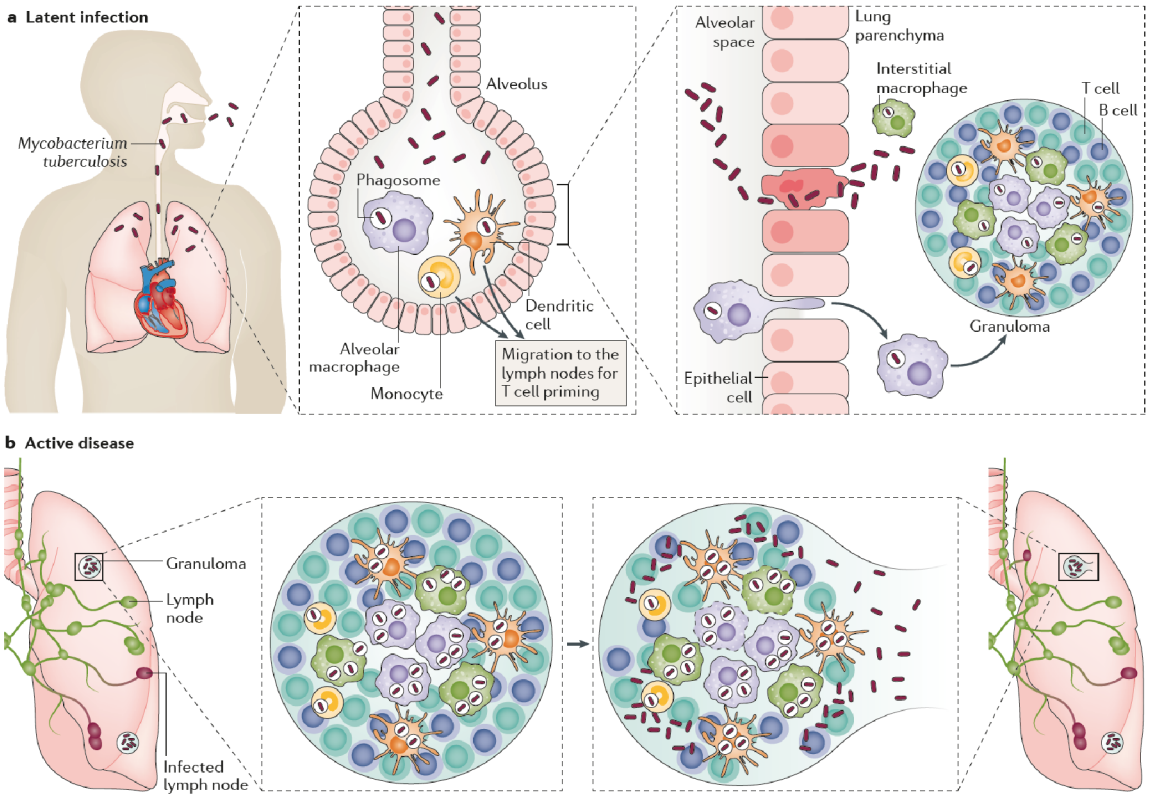

Figure 4. *Mycobacterium tuberculosis* infection. **(a)** Infection begins when *Mycobacterium tuberculosis* enters the lungs via inhalation, reaches the alveolar space and encounters the resident alveolar macrophages. **(b)** The bacteria replicate within the growing granuloma. From Pai, M. et al. Tuberculosis. Nat. Rev. Dis. Prim. 2, (2016). Copyright © 2016 Macmillan Publishers Limited, part of Springer Nature. Reprinted with permission from Springer Nature.

Subsequently, antigen-presenting cells (APCs) will migrate to the mediastinal or intrathoracic lymph nodes and present the processed *M. tuberculosis* fragments to naive T-cells, triggering an adaptive immune response. Briefly, peptide and cytosolic antigens will be presented by major histocompatibility complex (MHC) class I and II to

CD4+ and CD8+ T-cells respectively. This binding will trigger cell differentiation: activated CD4+ T-cells will divide, according to their cytokine secretion profiles, into Th1, Th2, Th17, T regulatory (Treg), non-conventional T delta gamma (gy), and T CD1 subpopulations. Activated CD8+ T-cells will become cytotoxic T lymphocytes (CTL) that trigger infected cell death by granule exocytosis and programmed cell death[29,30]. Protective immunity against *M. tuberculosis* relies mostly on CD4+ T-cells that differentiate into Th1. These cells mainly secrete interferon (IFN)-gamma(γ) and tumour necrosis factor (TNF)-alpha(α) to recruit and activate innate immune cells, as well as interleukin (IL)-2 to activate T-cells[28,31].

The clustering of inflammatory cells around the site of infection (usually infected or uninfected macrophages surrounded by lymphocytes in the periphery) will lead to granuloma formation in the lung parenchyma. This formation will control *M. tuberculosis* infection by limiting the progressive growth of the bacterial population and preventing its spread. However, for reasons that are not fully understood, the granuloma will not be able to contain the infection and the bacteria will spread to other organs. At this stage, *M. tuberculosis* may enter the bloodstream and replicate in other parts of the body causing extrapulmonary or disseminated forms of the disease or re-enter the respiratory tract where it can be released and, therefore, allow disease transmission (Figure 4b)[2].

In about 90% of the population, the immune system is able to control the infection in granulomas for a prolonged period of time (traditionally considered as latency stages)[32]. However, for reasons that are not fully understood, leading to reactivation of the bacteria and uncontrolled multiplication causing active disease and TB transmission[28,33,34].

3.4. Spectrum of tuberculosis

To facilitate the classification of TB in clinical settings, the binary model of latent TB infection (LTBI) as opposed to active TB, has been traditionally used. This binary model classifies LTBI as an asymptomatic, non-communicable state, and active TB, as the communicable state of the disease with the appearance of signs and symptoms[35]. Insights into the pathophysiology of TB have enabled progress towards an understanding of the outcome of *M. tuberculosis* infection as a continuous spectrum of conditions that may or may not lead to the development of active TB[36–38]. This broad spectrum has been categorised into five states defined as: (a) cleared TB infection, which corresponds to the state in which after initial exposure, the infection has been completely cleared by the host immune response; (b) LTBI, in which the infection is contained without significant immune compromise or clinical, radiological or microbiological evidence; (c) incipient TB infection, in which there is infection with

metabolically active *M. tuberculosis* bacteria that are actively replicating and likely to progress to active disease but have not yet manifested clinical, radiological or microbiological symptoms of active TB; (d) subclinical TB, in which there is transmissible but asymptomatic disease with the appearance of disease-compatible radiological or microbiological abnormalities; and (e) active TB, in which there is transmissible disease with clinical symptoms such as fever, persistent cough (which may be accompained by blood), fatigue, and weight loss together with radiological and/or microbiological evidence of *M. tuberculosis*[36].

The broad spectrum of conditions is marked by bacterial and metabolic activity and immune responses that will promote bacterial replication, persistence, or death[38]. Thus, individuals infected with *M. tuberculosis* may progress rapidly or slowly to active TB or move back and forth through the different stages of the spectrum before developing symptomatic disease or resolving the disease (Figure 5)[36].

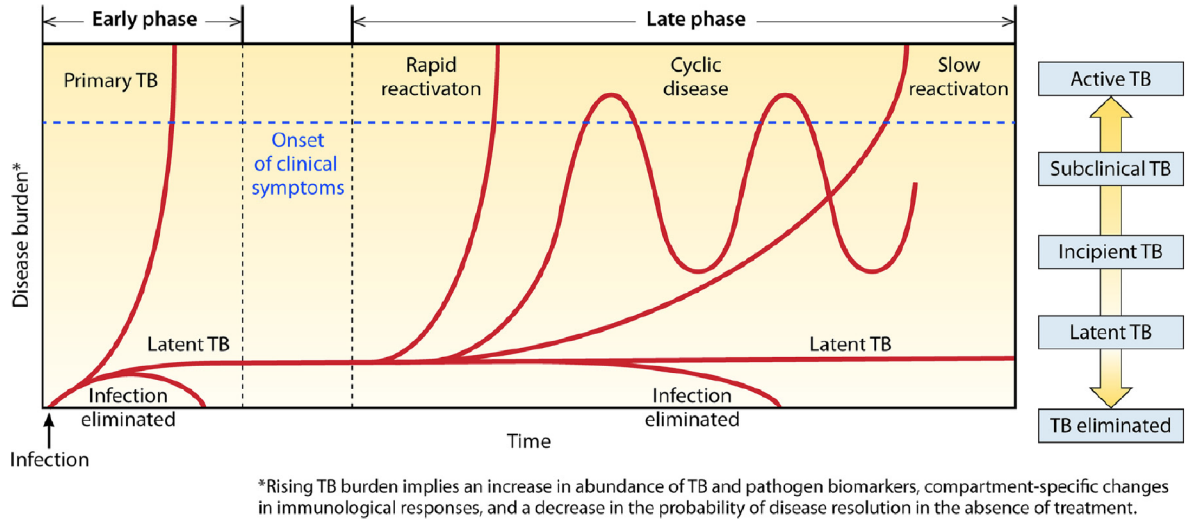

Figure 5. Pathways of tuberculosis disease progression. From Drain, P. K. et al. Incipient and subclinical tuberculosis: A clinical review of early stages and progression of infection. Clin. Microbiol. Rev. 31, 1–24 (2018). Copyright © 2018 American Society for Microbiology. Reprinted with permission from American Society for Microbiology.

The risk of progression from infection to active TB depends on the interaction between host, pathogen, and extrinsic factors. Host immune status, age, presence of co-infections and non-communicable co-morbidities, bacterial pathogenicity, infectious dose, or environmental factors may contribute to the progression of infection to active TB or exacerbate the course of the disease[4,39,40]. HIV co-infection is the most important risk factor associated with TB disease progression[27]. These risk factors are critical for improving the detection and diagnosis of active TB.

3.5. Diagnosis

Early diagnosis and correct treatment of TB are key for reducing the disease burden and TB control. Current diagnostic tools are based on the detection of LTBI and active TB.

3.5.1. Diagnosis of Mycobacterium tuberculosis infection

The tuberculin skin test (TST), classically known as the Mantoux test, measures the delayed hypersensitivity reaction (at 48-72 hours) following intradermal injection of 0.1 ml of 5 tuberculin units (TU) of purified protein derivative (PPD) into the upper layers of the skin of the forearm. Although the TST is the most commonly used method for the diagnosis of *M. tuberculosis* infection[41], it has limited sensitivity in groups with immunosuppression such as people living with HIV or malnourished people. In addition, cross-reactivity with BCG vaccine and non-tuberculous mycobacteria (NTM) can cause false positive results[42–44].

As an alternative to the TST, IFN-γ release assays (IGRAs) measure the detection of IFN-γ secreted by effector T-cells stimulated with *M. tuberculosis*-specific antigens which contain peptides that induce a T-lymphocyte cellular response in individuals previously exposed to *M. tuberculosis*[45]. There are currently two types of IGRAs used for the detection of *M. tuberculosis* infection, one that stimulates a volume of whole blood with *M. tuberculosis*-specific antigens overnight (approximately 16-20 hours) and then quantifies the IFN-γ released by ELISA, and another that stimulates a number of peripheral blood mononuclear cells (PBMCs) with *M. tuberculosis*-specific antigens overnight and then counts the number of cells that have secreted IFN-γ (considering that one spot is equivalent to one cell) by ELISPOT.

As examples of extensively used IGRAs there are the ones from Qiagen (Hilden, Germany; the previous QuantiFERON-TB Gold In-Tube and the current QuantiFERON-TB Gold Plus assay) and Oxford Immunotec (Abingdon, United Kingdom; T-SPOT.TB assay)[46]. QuantiFERON-TB Gold Plus test (QFT-Plus) consists of four tubes, the negative control (nil), positive control (mitogen), and two antigen tubes. The first antigen tube (TB1) contains the ESAT-6 and CFP-10 antigens that stimulate CD4+ T cells and the second antigen tube (TB 2) contains the same CD4+ antigens as TB1, plus other CD8+ antigen peptides of its own[47]. Finally, in the T-SPOT.TB assay a standard number of PBMCs (both CD4+ and CD8+) are added into specially designed plates and stimulated with *M. tuberculosis* specific antigens, ESAT-6 and CFP10[48].

Unlike TST, the region of difference (RD) 1 encoding the ESAT-6 and CFP-10 antigens and the RD11 encoding TB7.7 antigen (this last, present in the previous Qiagen version) of the IGRAs are absent in the *M. bovis* BCG vaccine strain and in most common NTM strains, providing better diagnostic specificity[49]. However, IGRAs have a low sensitivity in children under five years of age[50–52], probably due to the immaturity of their immune responses. Neither the TST nor the IGRAs, nor the combination of both, are capable of

distinguishing between TB infection or disease[53]. However, the difference between TB infection or disease is crucial in determining appropriate treatment, especially in countries where TB is endemic, and in populations, such as children, where the clinical manifestations of TB often overlap with other common conditions such as pneumonia, HIV-associated lung disease, and malnutrition[42].

3.5.2. Diagnosis of tuberculosis disease

The reference diagnostic methods for the diagnosis of TB are based on detecting the presence of *M. tuberculosis* bacilli. These methods are smear microscopy (microscopic examination), culture, and rapid molecular tests[54].

Culture-based methods are the gold standard for TB diagnosis, evaluation of treatment outcome to ensure recovery, and detection of drug resistance. There are two types of media for mycobacterial culture: solid and liquid. While the solid medium allows the morphology of the colonies to be appreciated and reveals indicative data for subsequent identification, the liquid medium has the advantage of shortening the detection of mycobacteria by 2-3 weeks[55]. However, the incubation time to obtain a result (2-4 weeks), the infrastructure, and the level of biosafety required are not accessible in all laboratories[56].

Microscopic examination is the oldest and most widely used method due to its relative simplicity and low implementation cost[57]. The most commonly used techniques for the detection of acid-fast bacilli (AFB) are Ziehl-Neelsen staining (with phenylated fuchsin) and staining with fluorochromes, such as auramine staining. Although direct examination allows rapid presumptive diagnosis, its sensitivity is much lower than that of culture or molecular methods[58,59].

In the last ten years, the introduction of molecular methods based on the amplification and detection of MTBC nucleic acids resulted in a major change for the rapid diagnosis of TB disease and for the detection of resistance to certain anti-TB drugs. Real-time polymerase chain reaction assays (RT-PCR), such as Xpert MTB/RIF (Cepheid, Sunnyvale, US), Xpert MTB/RIF Ultra (Cepheid, Sunnyvale, US), or Truenat (Molbio/bigtec Diagnostics, India); line probe assays (LPA), such as GenoType MTBDRplus v1 and v2 (HAIN Lifescience, Nehren, Germany), Genoscholar NTM+MDR TB II (Nipro, Osaka, Japan), and GenoType MTBDRsl (Hain Lifescience, Nehren, Germany), or loop-mediated isothermal amplification assays (LAMP), such as TB-LAMP (Eiken, Tokyo, Japan), have shown potential as substitutes for smear microscopy[54,60]. However, affordability and ease-of-use requirements limit their integration and use in primary care settings[57].

3.5.3. Diagnosis in high-priority populations

Bacteriological confirmation by culture or sputum-based molecular methods is the

gold standard for the diagnosis of active TB. However, for people with extrapulmonary or miliary TB, people with immunosuppressive conditions, and children, the main impediment to accurate diagnosis of active TB is the paucibacillary nature of the disease (small number of bacilli in sputum), difficulties in sputum production, and/or expectoration[40,61,62]. Consequently, current methods have a low diagnostic sensitivity in these groups[63].

Despite advances in rapid molecular diagnosis of TB, the use of sputum-based techniques means that most children with TB are diagnosed through radiological and clinical scoring systems limited by the clinical presentation of the disease, making diagnosis difficult and delaying therapeutic strategies for TB control. The search for new biomarkers should focus on the development of a rapid, child-friendly, non-sputum-based test able to diagnose the early stages of paediatric TB, improving detection of the source of TB infection in the community, and preventing progression to severe forms of the disease, such as disseminated TB and TB meningitis[3,64,65]. In recent years, novel biomarkers have been identified for the diagnosis of paediatric TB based on plasma, serum, urine, and faeces[42,66]. We commented this in an editorial published in *Revista Enfermedades Emergentes* (See Annex 2)[67]. However, there is still no assay with sufficient sensitivity to develop a diagnostic test for TB in children.

Unlike HIV uninfected people, people living with HIV have poor quality sputum and higher rates of extrapulmonary and disseminated TB[68]. In addition, the non-specificity of symptoms and misleading or atypical radiographic features in HIV-positive people hamper the identification of TB. Because of the high risk of HIV-positive people developing severe forms of TB, early diagnosis of TB is crucial to start treatment as soon as possible to prevent death[69]. Therefore, in HIV-positive people, non-sputum-based point-of-care (POC) tests are key to improving TB detection and ensuring prompt treatment[63].

Over the last few years, the Determine TB LAM Ag lateral flow test (AlereLAM), a POC immunoassay based on antigen-antibody reaction, has been developed and approved for the detection of LAM antigen (constituent of the *M. tuberculosis* cell wall, Figure 3) in urine in HIV-positive patients with presumptive TB and in patients with severe HIV disease[70–72]. AlereLAM is a non-invasive, easy-to-use test that gives immediate time results. Despite the promising characteristics of this test, the diagnostic performance demonstrated so far makes this test unsuitable for other populations[73]. However, a new LAM detection assay, Fujifilm SILVAMP TB LAM (FujiLAM, Fujifilm, Tokyo, Japan), could be promising for future TB diagnosis in HIV-infected and uninfected patients[70,72]. This POC test combines high-affinity monoclonal antibodies against *M. tuberculosis*-specific LAM epitopes and a silver amplification step to increase the visibility of test and control lines[71].

3.6 Treatment of tuberculosis

Due to the slow growth and complex cell envelope of *M. tuberculosis* (see section 2 of the introduction), the treatment regimen for TB patients includes the combination of several drugs over a prolonged period of time to avoid the natural development of *M. tuberculosis* resistance and also to eliminate intracellular and intermittently growing bacteria that occur in the caseus.

In drug-susceptible TB patients, the treatment regimen preferably consists of a 2-month intensive phase of isoniazid, rifampicin, pyrazinamide and ethambutol, and a 4-month (to 7-month) continuation phase of isoniazid and rifampicin. Surveillance during TB treatment is essential to ensure adherence to treatment and to monitor treatment response. This is usually monitored by sputum microscopy, cultures, and X-rays. Improper or incorrect use of antimicrobials, administration of ineffective drug formulations (single drug use, poor quality or poorly stored drugs) and early discontinuation of treatment can cause emergence of *M. tuberculosis* strains with resistance to one or more drugs[74]. Thus, monoresistance is caused by strains of *M. tuberculosis* resistant to a first-line anti-TB drug, Isoniazid-resistant TB (TB-Hr) is caused by isoniazid-resistant and rifampicin-susceptible strains of *M. tuberculosis*, Rifampicin-resistant TB (RR-TB) is caused by strains resistant to rifampicin, Multidrug-resistant TB (MDR-TB) is caused by strains of *M. tuberculosis* resistant to at least isoniazid and rifampicin. Pre-extensively drug-resistant TB (Pre-XDR-TB) is TB resistant to rifampicin and any fluoroquinolone (a class of second-line anti-TB drug), and Extensively drug-resistant TB (XDR-TB) is resistant to rifampicin, plus any fluoroquinolone, plus at least one of the drugs bedaquiline and linezolid[1, 74].

Treatment regimens will vary according to the type of drug resistance, which can be detected by drug susceptibility testing (DST) specific to the drug susceptibility of bacteria. These may include culture tests, nucleic acid amplification molecular tests, and Next Generation Sequencing (NGS) of MTBC[74,75]. Molecular testing and NGS can provide substantially faster results than traditional phenotypic culture. Although NGS can provide data on all clinically relevant resistance mutations for the diagnosis of drug-resistant TB (DR-TB), its application requires the isolation of cultures to generate a sufficient bacterial load for successful sequencing. Currently, a wide range of molecular tests (e.g. Xpert MTB/RIF and Ultra, Truenat, or GenoType) are being developed and evaluated for TB screening that include DST and are targeted at point-of-care and peripheral health care settings[76].

The emergence of DR-TB represents a threat to TB control with an increase in treatment failure. New WHO recommendations aim to accelerate detection of drug resistance and improve access so that patients receive a definitive treatment regimen using a shorter and cheaper regimen[77].

3.7. Challenges and future directions in tuberculosis diagnostics

3.7.1. Closing the tuberculosis gap in low- and middle-income countries

In 2014, the WHO End TB strategy was launched with the goal of ending the TB epidemic by reducing TB incidence by 90% between 2015 and 2035[78]. Since then, great progress has been made and many deaths have been averted. However, in 2020 the gap between new TB cases notified in national TB programmes and those not communicated was 4.1 million people[1]. The substantial reduction in TB case detection and notification between 2019 and 2020 (18%) is likely to reflect both supply- and demand-side disruptions to TB diagnostic and treatment services generated by COVID-19[1].

In 2010, the REACH TB initiative, led by the StopTB strategy, was launched with the aim of increasing the number of people diagnosed and treated for TB in low- and middle-income countries. Although active case-finding is key for diagnosing and treating the missing millions affected by TB, many of the difficulties are due to limited accessibility (either physical, social or cultural) to health services, underdevelopment of health information systems, insufficient of human resources, and poor linkages between private providers and national authorities[79,80].

For decades, TB has been diagnosed with outdated and inefficient tools, resulting in the loss of large numbers of people. Since then, great progress has been made with the emergence of new diagnostics such as Xpert MTB/RIF and Ultra, LPAs, AlereLAM testing, and IGRAs. However, many people with TB (or TB symptoms) have difficulties accessing an adequate initial diagnosis, with only a small proportion of TB tests performed with new technologies (such as molecular testing)[81]. Considering that the highest incidence of TB accounted in low- and middle-income countries[1], in 2015, WHO developed high-priority target product profiles (TPPs) to define the needs of next-generation assays and align them with the requirements and specifications for the development of new diagnostics that are more accurate, affordable, and accessible where patients first seek care[82]. Prioritisation was done on the basis of the impact the test could have on TB transmission, morbidity and mortality, the potential market for the test, and the ease of implementation and scale-up of the test. The three diagnostic priorities identified were: (1) the development of a biomarker-based, non-sputum-based POC to detect all forms of TB, (2) development of a simple, low-cost POC test that could be used as a screening test to identify those in need of further testing, and (3) development of a rapid sputum test that could be used in microscopy centres to detect pulmonary TB to replace smear microscopy and have the ability to diagnose drug susceptibility[63,82]. To end the TB epidemic, we need to increase investment, embrace innovation for the development of rapid and accurate diagnostics, and address implementation gaps to ensure that new technologies reach those who need them[81,83]. With the emergence of COVID-19, massive financial efforts

have been made to accelerate research and development, as well as innovation in delivery systems. The innovation and detection systems provided by this pandemic must be harnessed to improve TB care and control[84].

3.7.2. Omics in the diagnosis of tuberculosis

There is a growing interest in understanding biological mechanisms not only at the molecular level, but also as a whole (biological functionality). In this sense, systems biology aims to explore how the interactions between biological components (genes, proteins, metabolites, etc.) contained in a biological tissue, cell, fluid or organism affect the functionality (biological processes) of an organism as a whole, making it possible to characterise a biological system in a complete and integrated way[85]. Currently, the development of omics, such as genomics, transcriptomics, proteomics, or metabolomics, has had a great impact. These disciplines allow the by allowing the analysis of a large number of biomolecules and the selection of those that really provide information based on algorithms of complex biological systems[86]. Thus, omics allow the generation of knowledge on the underlying mechanisms of the disease[87]. Omics have highlighted a new approach in biochemical and biomedical research, as it is no longer necessary to start from restricted starting hypotheses, but several metabolic pathways and routes can be explored simultaneously. Figure 6 shows the correlation between the omics approaches and their respective biomolecules of study[88].

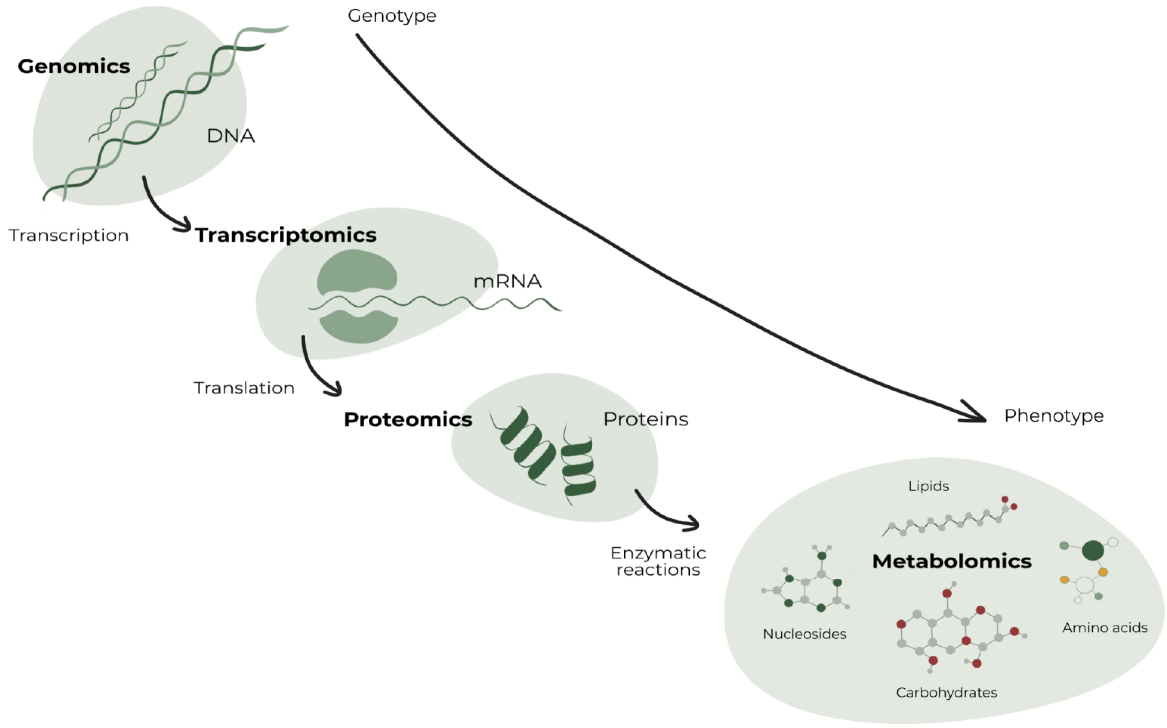

Figure 6. A correlation between the main omics strategies used in systems biology studies. Adapted from Sussulini, A. Metabolomics: From Fundamentals to Clinical Applications. Copyright © 2017, Springer Nature. Figure adapted/reprinted with permission from Springer Nature. Rev. 31, 1–24 (2018).

Advances in the field of systems biology in relation to TB aim to address more precisely clinically relevant issues in the future such as early diagnosis of TB, especially in groups in which diagnosis is more difficult (such as patients with extrapulmonary TB and children), individualizing preventive therapies, regarding the choice of anti-TB drugs and host-directed therapies administered, and predicting the outcome of a treatment regimen[89]. We commented this in a review published in the European Respiratory Review (See Annex 3)[90].

In recent years, different omics strategies have identified several sets of TB-associated biomarkers with the potential to optimise TB prevention, diagnosis, and treatment. However, only a few of these biomarkers have been evaluated in clinical cohorts of patients and implemented in clinical practice, limiting the applicability of these biomarkers in TB diagnosis and clinical management of patients[90,91].

In this context, metabolomics is one of the most powerful bioanalytical strategies to obtain a picture of the metabolites of an organism in the course of a biological process and is considered as a phenotyping tool[92]. Metabolomics allows the identification of a set of metabolites (or unique chemical fingerprint) present in a cellular system or a biofluid in biological samples (tissues, cells, fluids or organisms) under normal conditions compared to altered states promoted by disease, pharmacological treatment, dietary intervention, or environmental modulation[93]. Metabolites are molecules of low and medium molecular weight (< 1,500 Dalton) such as sugars, amino acids, lipids, which are involved in cellular processes and reveal how metabolism is functioning in a given organ or living organism[88]. The absence or presence of some of these metabolites, as well as their relative concentration, can be an indicator of disease states or disease risk factors[94]. These metabolites can be measured using different non-invasive methods comprising nuclear magnetic resonance (NMR) and mass spectrometry (MS) (usually with a chromatographic separation step), providing comprehensive structural and conformational information on multiple chemical groups in a single analytical procedure[95,96].

Metabolomics has provided insight into the pathogenesis of TB, thereby facilitating the understanding of host-pathogen interactions during TB infection and progression[92,97]. Although the use of metabolomics could improve the prediction of disease progression and detection, only a few studies have focused on the discovery of metabolomic biomarkers for TB diagnosis[91].

4

Justification

Justification

Tuberculosis (TB) is the active and contagious form of the disease caused by bacteria of the *M. tuberculosis* complex (MTBC) and the second leading cause of death in 2020 from a single infectious agent (after COVID-19). In this regard, the emergence of COVID-19 only exacerbated the TB situation, committing human, financial, and TB equipment resources to the response to the pandemic. This has negatively influenced TB prevention and care programmes, with a decrease in the diagnosis of new TB cases and treatment follow-up, with a particular focus on low- and middle-income countries, where the highest incidence of TB resides (Annex 1).

Spread by airborne transmission from person to person, it is estimated that a quarter of the world's population is infected with *M. tuberculosis*. However, only one in ten infected people will develop TB.

Although TB can affect anyone, anywhere, people infected with *M. tuberculosis* with a compromised immune system, due to young age, HIV infection or non-communicable conditions, among others, are at increased risk of developing TB and severe forms of the disease. Furthermore, most of the new cases of TB worldwide reside in low- and middle-income countries.

Bacteriological confirmation by culture or sputum-based molecular methods are the gold standard for TB diagnosis. However, the limitations of current diagnostic methods pose difficulties in diagnosing TB, especially in people with extrapulmonary or miliary TB, in people with HIV, and in children. Moreover, the use of invasive techniques is not well accepted among children. Consequently, the lack of a reference test leads to most of the children with TB being diagnosed by radiological and clinical scoring systems which are limited by the wide clinical spectrum of the disease. Difficulties in the early diagnosis and treatment of TB contribute to developing severe forms of developing severe forms of disease and increase disease and increase of spreading. In this regard, this thesis includes in Annex 2 a brief review of the limitations and future needs in the diagnosis of TB in children.

Despite advances in the rapid molecular diagnosis of TB, the affordability, accessibility, and ease-of-use requirements of new technologies limit their integration and use in primary care settings where patients seek care for the first time. In 2015, the WHO developed "High-priority target product profiles" to define the needs for next-generation testing. Among these, sensitive and non-invasive point-of-care tests need to be developed and implemented to diagnose pulmonary and extrapulmonary TB earlier and at all healthcare systems.

In 2018, the WHO published the "Roadmap towards ending TB in children and adolescents" to highlight the priority actions and increased investment urgently needed. Recently, several initiatives have emerged using different specimens (such as serum, plasma, or urine) to diagnose TB in children more accurately and less invasively. Although most of the identified biomarkers are promising, further development and optimisation are needed to improve their diagnostic performance.

In order to address these issues, the studies conducted for this thesis focused on a paediatric cohort from Haiti, and two cohorts of adults with presumptive TB from Nigeria and Europe, to identify and evaluate rapid point-of-care TB diagnostic methods based on non-invasive specimen biomarkers and alternatives to sputum to improve TB detection and access to diagnosis at all levels of the health care system.

Focusing on the **host-response biomarkers to *M. tuberculosis*** infection and disease, a combination of cytokines and individual factors in serum and plasma were identified and evaluated by immunoassays to distinguish between TB infection and disease in children (Article 1 and Patent 1).

Regarding ***M. tuberculosis*-specific biomarkers**, urinary LAM antigen detection using the Fujifilm SILVAMP TB-LAM assay was evaluated in seropositive and seronegative patients with presumptive TB attending three district hospitals in Nigeria (Article 2) and in children attending a paediatric hospital in Haiti (Article 3).

With a focus on **biomarkers from the interaction between the host and *M. tuberculosis***, a metabolic profile of TB in urine was identified and validated using high- and low-field nuclear magnetic resonance (NMR) spectroscopy (Article 4 and Patent 2). In addition, a metabolic fingerprint in urine was detected to characterise the disease in children with presumptive TB (Article 5) and the use of urine NMR-based metabolomics to assess the outcome of TB treatment was described (Article 6). Finally, the available evidence from a metabolomic and proteomic approach with potential for TB management was reviewed (Annex 3).

Although it is a treatable and curable disease, TB remains one of the leading causes of death worldwide. TB diagnosis is critical at every stage of the response to the epidemic to help limit the spread of TB, and provide reliable information for life-saving decisions. Closing the gap between estimated, diagnosed, and notified TB cases relies largely on developing more accurate, rapid, and accessible point-of-care diagnostic tools. This thesis aims to discover biomarkers using different approaches that may contribute to future TB diagnostics.

5

Objectives

Objectives

Host-response biomarkers:

1. Identify and evaluate a combination of cytokine biomarkers and individual factors in serum and plasma by immunoassays to distinguish between *M. tuberculosis* infection and disease in children (Article 1 and Patent 1).

Pathogen-specific biomarkers:

2. Evaluate the use of Fujifilm SILVAMP TB-LAM assay in urine to improve the diagnosis of tuberculosis in HIV-positive and -negative patients with presumptive tuberculosis (Article 2).

3. Evaluate the use of Fujifilm SILVAMP TB-LAM assay in urine to improve the diagnosis of tuberculosis in children with presumptive tuberculosis (Article 3).

Host-pathogen interaction biomarkers:

4. Identify and characterise a metabolomic profile of TB in urine by high-field nuclear magnetic resonance and evaluate its discriminatory potential using low-field benchtop NMR spectrometry (Article 4 and Patent 2).

5. Associate the NMR metabolomic fingerprint of TB in urine with standardised case definitions for the classification of paediatric TB to improve the characterisation of the clinical spectrum of the disease (Article 5 and Patent 2).

6. Describe the use of high-field nuclear magnetic resonance spectroscopy to assess the outcome of TB treatment (Article 6).

6

Results

Article 1

A Model Based on the Combination of IFN-gamma, IP-10, Ferritin and 25-Hydroxyvitamin D for Discriminating Latent from Active Tuberculosis in Children

Comella-del-Barrio, Patricia; Abellana, Rosa; Villar-Hernandez, Raquel; Coute, Mariette Doresca Jean; Sallés Mingels, Beatriz; Canales Aliaga, Lydia; Narcisse, Margareth; Gautier, Jacqueline; Ascaso, Carlos; Latorre, Irene; Dominguez, Jose; Perez-Porcuna, Tomas M.

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A Model Based on the Combination of IFN-γ, IP-10, Ferritin and 25-Hydroxyvitamin D for Discriminating Latent From Active Tuberculosis in Children

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In recent years, pediatric research on tuberculosis (TB) has focused on addressing new biomarkers with the potential to be used as immunological non-sputum-based methods for the diagnosis of TB in children. The aim of this study was to characterize a set of cytokines and a series of individual factors (ferritin, 25-hydroxyvitamin D [25(OH)D], parasite infections, and nutritional status) to assess different patterns for discriminating between active TB and latent TB infection (LTBI) in children. The levels of 13 cytokines in QuantiFERON-TB Gold In-Tube (QFT-GIT) supernatants were analyzed in 166 children: 74 with active TB, 37 with LTBI, and 55 uninfected controls. All cytokines were quantified using Luminex or ELISA. Ferritin and 25(OH)D were also evaluated using CLIA, and *Toxocara canis* Ig-G antibodies were detected with a commercial ELISA kit. The combination of IP-10, IFN-γ, ferritin, and 25(OH)D achieved the best diagnostic performance to discriminate between active TB and LTBI cases in children in relation to the area under receiver operating characteristic (ROC) curve 0.955 (confidence interval 95%: 0.91–1.00), achieving optimal sensitivity and specificity for the development of a new test (93.2 and 90.0%, respectively). Children with TB showed higher ferritin levels and an inverse correlation between 25(OH)D and IFN-γ levels. The model proposed includes a combination of biomarkers for discriminating between active TB and LTBI in children to improve the accuracy of TB diagnosis in children. This combination of biomarkers might have potential for identifying the onset of primary TB in children.

Keywords: pediatrics, biomarkers, immune response, vitamin D, ferritin, enzyme-linked immunoassays, *Mycobacterium tuberculosis*, cytokines

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INTRODUCTION

At least one-quarter of the world's population is infected with *Mycobacterium tuberculosis* (World Health Organization [WHO], 2018a). Childhood tuberculosis (TB) represents at least 10% of the burden of the disease worldwide, being one of the most significant causes of childhood morbidity and mortality (World Health Organization [WHO], 2018b). Estimations show that there are far more children with TB globally than previously thought, with the majority being undiagnosed and untreated (Dodd et al., 2017). Underdiagnosis in children can occur for multiple reasons, such as the lack of a non-specific clinical presentation that delays seeking healthcare and diagnostic suspicion; the lack of a more accurate diagnostic test; and poor geographical and financial access to healthcare in some areas (Kyu et al., 2018; World Health Organization [WHO], 2018a).

Infants and young children have a higher risk of progressing to TB following a primary *M. tuberculosis* infection, usually due to a child being exposed to an infectious adult with active TB (Augustynowicz-Kopeć et al., 2012) and to the development of severe forms of the disease (Marais et al., 2004). Therefore, household detection of index TB cases, together with early detection of the infection and TB disease, followed by a prompt treatment, are fundamental in preventing disease progression (Dodd et al., 2018).

The diagnosis of pulmonary TB is a significant challenge in children due to the paucibacillary nature of the disease and the difficulties in expectorating. Although rapid molecular diagnosis represents a significant advance as an alternative to conventional microscopy and culture methods, its sensitivity remains unacceptably low in children since the technique is based on detecting *M. tuberculosis* from respiratory specimens (World Health Organization [WHO], 2018b). As for immunodiagnostic assays, both the tuberculin-skin-test (TST) and interferon-gamma release assays (IGRAs)—that measure cell-mediated immune responses following *M. tuberculosis* infection—are unable to distinguish between active TB and LTBI (Latorre and Domínguez, 2015) and limit the accuracy of *M. tuberculosis* detection (Dominguez et al., 2009; Velasco-Arnaiz et al., 2018). As a result, most active TB cases are diagnosed through clinical—and radiological when possible—scoring systems which have limitations due to the clinical presentation of the disease (Graham et al., 2015).

The immature immune system of young children added to the dynamic process of the host–pathogen interaction from *M. tuberculosis* infection, hamper defining the transition from latent infection to acute TB disease in children. Recently, a diversity of genetic and individual factors that may contribute to the outcome of the host immune set-points during *M. tuberculosis* infection have been described (Bastos et al., 2018). A good understanding of the diversity of these factors might be crucial to assess the impact of the host-pathogen interactions that occur during *M. tuberculosis* infection. In this sense, the WHO guidelines for the management of malnourished children, as well as guidelines for national TB programs, underline the importance of the association of malnutrition and TB in children (World Health Organization [WHO], 2013). In addition, the impact of alterations in iron homeostasis and vitamin D deficiencies highlight profound effects on immune function and host defenses due to mechanisms that have not yet been resolved. Lastly, comorbidities by helminth infections impair the inflammatory and immune mechanisms involved in the control of *M. tuberculosis* infection (Ibrahim et al., 2017).

In recent years, pediatric research has focused on finding new biomarkers in non-sputum-based samples for the detection of *M. tuberculosis* infection (Nicol et al., 2015). Current studies have highlighted the importance of diverse forms of biomarkers with the potential to be used in immunological methods for the diagnosis of childhood TB (Togun et al., 2018). Furthermore, there is increasing data showing that modified interferon gamma release assays (IGRAs) based on the analysis of a combination of different markers could enhance the diagnostic accuracy (Chegou et al., 2014). However, only a few studies with small cohorts have identified some cytokine responses in QuantiFERON-TB Gold In-Tube (QFT-GIT, QIAGEN, Germany) supernatants which have the potential to monitor specific immunity against *M. tuberculosis* as candidate combinations of markers for the discrimination between LTBI and active TB in children (Togun et al., 2018). Despite these initial approaches, a clear pattern has not yet emerged, and the diagnostic performance of the cytokine biomarkers reported do not meet the minimum targets recommended by the WHO for a new diagnostic or triage test for TB in children (World Health Organization, 2017).

To our knowledge, no cytokine biomarkers based on QFT-GIT supernatants combined with individual factors can distinguish between active TB and LTBI in children. To improve the accuracy of the diagnosis of TB, the aim of this study was to characterize a set of cytokines and a series of individual factors (ferritin, 25-hydroxyvitamin D [25(OH)D], parasite infections, and nutritional status) and to assess different patterns for distinguishing between active TB and LTBI in children.

MATERIALS AND METHODS

Study Population

A prospective case-control study was conducted from August 2015 to December 2016 in the pediatric hospital of Saint Damien in Port-au-Prince (Haiti). Children (0–14 years old) who presented signs and symptoms compatible with active TB and/or documented TB exposure were screened for suspected TB. According to the hospital program, siblings (0–14 years old) of the children diagnosed with TB were also screened for TB or LTBI. Children (0–14 years old) from a school and a kindergarten were screened as uninfected controls. The exclusion criteria were: children with known immunodeficiency, on current immunosuppressive treatment, with a condition that could potentially compromise the immune system (e.g., children from oncology, rheumatology, nephrology and those who had undergone organ transplantation), children who had been under anti-TB treatment or preventive treatment during the previous year, and children not providing informed consent.

The following information was collected: age, sex, weight, height, previous medical history (including TB history,

TB exposure, HIV status, and comorbidities, hemogram), vaccines, and current and previous medication (antibiotics, corticosteroids, antiparasitic drugs).

The following signs and symptoms were evaluated: cough and/or fever ≥2 weeks (with no improvement after at least a 7-day course of amoxicillin), recent unexplained weight loss, and asthenia/fatigue. The TST was performed by a trained laboratory technician. Intradermal injection of 0.1 ml of Tubersol (bioequivalent to 5 tuberculin units; Sanofi Pasteur, Toronto, ON, Canada) was placed into the ventral surface of the lower arm and read after 72 h. A positive TST result was defined as an induration ≥10 mm in BCG-vaccinated children (those with a BCG scar), and ≥5 mm in non-BCG-vaccinated children or with a known adult TB contact (Bass et al., 1990; American Academy of Pediatrics Tuberculosis, 2009). A standardized specific *Z*- score for detection of nutritional status weight-for-age (WAZ) was determined by WHO Anthro Plus 1.0.4 software (World Health Organization [WHO], 2009). Children with WAZ scores below −2 standard deviation (SD) were defined as underweight, and a WAZ score above 2 SD was defined as overweight.

A chest radiograph (anterior–posterior image) was performed in all the children screened for TB or LTBI. Chest radiographs were read by two external experts who were blinded to clinical data using a standardized reporting form and a third reader to resolve discordant opinions (Graham et al., 2015). In addition, nasopharyngeal aspirates and induced sputum were collected on three consecutive days for smear examination (auramine stain) by direct fluorescence microscopy in children suspected of having active TB. Histological examination was performed in children with suspicion of extrathoracic TB (ETB) with lymph node adenopathies.

Of all the children screened for TB or LTBI, only those with a positive TST result and/or microscopic confirmation were invited to participate in the study. Of all the children screened in the schools, only those with a negative TST result were invited to participate in the study as uninfected controls.

Treatment and preventive treatment were prescribed in all the children diagnosed with TB or LTBI, respectively. During TB treatment and preventive treatment, the patients were followed monthly until the end of treatment. Children with a positive sputum smear were retested at the fifth and sixth month of treatment.

Definitions for Classification of the Children Enrolled in the Study

Children were classified according to their clinical history, chest radiographs, smear examination, molecular diagnostic test, TST and QFT-GIT results. Active TB cases were defined as confirmed TB —children with relevant signs and symptoms and microbiologic confirmation of *M. tuberculosis*—, and unconfirmed TB —children without bacteriological confirmation but with relevant signs and symptoms, positive TST and/or QFT-GIT, radiological findings suggestive of TB, known TB contact, and clinical response to anti-TB treatment. Depending on the TB location, active TB cases were classified as ETB or intrathoracic TB. Children with intrathoracic TB were differentiated into those with pulmonary involvement (PTB) and children with isolated mediastinal lymphadenopathy in the absence of lung parenchyma involvement (Mediastinal TB). LTBI cases were defined as children with documented TB exposure, positive TST and/or QFT-GIT, normal chest radiographs, and no clinical signs of TB development in the last 6 months after diagnosis. Finally, uninfected controls were defined as asymptomatic children with no history of TB exposure, and negative TST and QFT-GIT.

Laboratory Tests Performed

Molecular Diagnostic Test

Sputum samples were collected from children with microscopy confirmation and/or radiological findings to perform GeneXpert MTB/RIF (Cepheid, United States) according to the manufacturer's instructions.

QuantiFERON-TB Gold In-Tube

In all the participants, a 3 ml blood sample was drawn for conventional QFT-GIT; the antigen tube (ESAT-6, CFP-10, and TB-7.7), and positive (phytohemagglutinin, mitogen) and negative (no antigen, nil) controls. Tubes were incubated at 37°C for 16–24 h and centrifuged according to the manufacturer's instructions. Plasma was harvested and stored at −20°C until performing the enzyme-linked immunosorbent assay (ELISA). After the screening and recruitment of cases, plasma samples were sent to the laboratories of the Research Institute Germans Trias i Pujol (IGTP, Badalona, Spain) following optimum conservation conditions. Once there, the supernatants were analyzed and the results were interpreted according to the manufacturer's instructions.

Ferritin, 25(OH)D, and *Toxocara* spp. Detection

One milliliter of blood from all the participants was added to the biochemical tube to perform ferritin, 25(OH)D, and *Toxocara canis* determination. Ferritin and 25(OH)D concentrations were analyzed at the biochemistry laboratory of the Germans Trias i Pujol Hospital (Badalona, Spain) with a Chemiluminescence Immunoassay (CLIA) method using a Liaison instrument (DiaSorin Liaison, Stillwater, MN, United States). 25(OH)D is an indirect method to measure vitamin D in the blood. According to the literature, serum 25(OH)D levels equal or above 20 ng/ml were considered normal levels of vitamin D (Michael and Holick, 2007). To avoid variations during blood sampling, the blood was almost always collected on the same day of the week at approximately the same hour (13–15 h). Seasonal fluctuations did not affect sampling because the weather seasons in Haiti are barely defined. *T. canis* Ig-G antibodies were detected at the IGTP laboratory using a commercial ELISA kit (Ridascreen, R-Biopharm AG, Germany). Results with a sample index above 1.1 were considered positive according to the manufacturer's instructions.

Detection of Soil-Transmitted Parasite Infections

Stool samples were collected to detect intestinal parasites in all the participants. Stool samples were processed on the same day of collection at the laboratory of the pediatric

hospital of Saint Damien using the Kato-Katz and formalin–gasoline technique (a modification of the formalin-ether sedimentation technique) as described previously (Katz et al., 1972; Ahmadi and Damraj, 2009) and were examined with optical microscopy.

Cytokine Measurement

Frozen supernatants remaining from the QFT-GIT tubes were used for the measurement of cytokine concentrations using a bead-based multiplex assay (Luminex 11-plex cytokine kit, R&D Systems, United Kingdom) and measured by Bioplex manager software (version 5.0, Bio-Rad, United States) according to the manufacturer's instructions. After optimization experiments, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-gamma (γ), interleukin (IL)-2, IL-5, IL-10, IL-13, IL-22, IL-17, and tumor necrosis factor (TNF)-alpha (α) were analyzed in a 1:8-fold dilution, while IL-1RA and induced protein (IP)-10 were analyzed in 1:8, 1:80, and 1:160-fold dilutions. In this way, values were within the detection limits marked in the standard curve. IL-32 and vascular endothelial growth factor (VEGF) were measured by DuoSet ELISA (R&D Systems, United Kingdom) because of incompatibilities with the human custom multiplex cytokine kit. ELISA was performed according to the manufacturer's instructions.

Statistical Analysis

Qualitative variables were described using frequencies and percentages. Qualitative variables were described using median and interquartile ranges (IQR), or, using mean and standard deviation (SD) in case of variables were with a normally distribution, using mean and standard deviation. The independent variables analyzed were: sex, age, weight by age, BCG, hemoglobin levels, 25(OH)D levels, ferritin levels, the presence of intestinal helminths, IgG *T. canis* antibodies, and cytokines responses. The concentration of released cytokines released in response to *M. tuberculosis* antigens (Ag-TB) and phytohemagglutinin (PHA) was calculated by subtracting the concentration measured in the nil tube (Ag-TB, antigen minus nil; and PHA, mitogen minus nil). For all the variables with a normal distribution, the comparison between several groups was performed using the analysis of variance or Student's t-student test (only two groups). However, for variables non-normally distributed variables, the comparison between groups was performed using Kruskal–Wallis and Mann–Whitney tests. In the case of qualitative variables, comparisons were performed using the Fisher's exact test or chi-squared test. The Tukey method (normal distribution) or Benjamini and Hochberg method (non-normal distribution) was used to correct *p*-values in multiple comparisons. The cytokines that showed significant differences between study groups were evaluated and considered.

Multivariate logistic regression was performed to detect variables able to classify individuals among the three different study groups (active TB, LTBI, and uninfected). The forward method was used for selecting the best combinations of cytokines. Participants with a positive QFT-GIT result (TB and LTBI) were analyzed separately (Binomial) from those with a negative QFT-GIT result (TB, LTBI, and uninfected; Multinomial). The results were expressed using the odds ratios (OR) and their confidence intervals. Sensitivity and specificity were calculated for assessing the value of performing a diagnostic test. The receiving operating characteristic (ROC) curve was used to evaluate diagnostic accuracy. The optimal cut-off value was identified to maximize the difference between true positive and false positives subjects. The level of significance was set at 0.05.

These analyses were performed using the statistical software IBM SPSS Statistics v. 25 (SPSS, Chicago, IL, United States), R package v.3.0.5 (R Foundation for Statistical Computing, Vienna, Austria), and GraphPad PRISM v. 5 (GraphPad Software, Inc., San Diego, CA, United States).

RESULTS

Study Subjects

A total of 305 children suspected of having LTBI or TB were screened in the hospital, whereas a total of 96 uninfected children were screened in a school and a kindergarten. Among the patients screened at the hospital, 156 were recruited, and 149 were excluded because they did not meet the inclusion criteria (informed consent not obtained, negative TST). Of the 156 participants, 111 (71.2%) were analyzed in the study, and 45 (28,8%) were lost to follow-up and were therefore not included in the analysis due to lack of complementary tests necessary for clinical assessment (chest radiographs, biopsies). Among the children screened at school and kindergarten, 55 were recruited and analyzed, and 41were excluded because they did not meet the inclusion criteria (informed consent not obtained, positive TST). Among the 166 children analyzed (111 screened at the hospital and 55 screened at school and kindergarten), 74 had active TB (44.6%), 37 had LTBI (22.3%), and 55 were uninfected (33.1%). Of the 74 active TB cases, 8 had ETB, and 66 had intrathoracic TB. Among the intrathoracic TB children, 48 had PTB, and 18 had Mediastinal TB (**Figure 1**).

Table 1 shows the description, demographic, and clinical characteristics among study groups according to their QFT-GIT result. Significant differences were observed in age and body WAZ *z*-scores ($p = 0.001$ and $p = 0.001$, respectively) among the study groups with different QFT-GIT results (positive, negative, and indeterminate). Whereas LTBI cases with positive QFT-GIT were older than active TB cases with negative QFT-GIT ($p = 0.002$), active TB cases with positive QFT-GIT had lower WAZ *z*-scores than uninfected controls ($p = 0.001$). However, comparisons among QFT-GIT results within each study group (TB, LTBI, and non-infected) were performed, and no significant differences were observed.

Characterization of Subjects With a Positive QFT-GIT Test

Comparisons between the groups with active TB and positive QFT-GIT were performed (**Table 2**). The Ag-TB levels of IL-17A, GM-CSF, together with the PHA levels of TNF-α, GM-CSF and, IL-13, and median hemoglobin levels showed significant differences between active TB groups ($p = 0.009$, $p = 0.027$, $p = 0.027$, $p = 0.040$ and $p = 0.042$, $p = 0.006$, respectively).

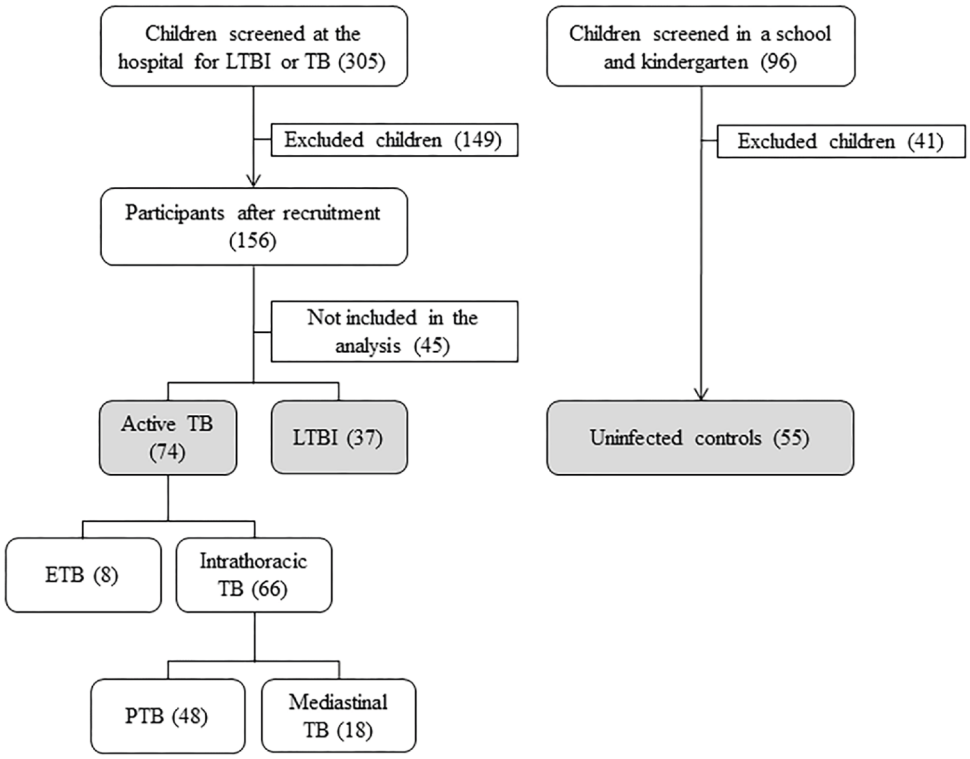

FIGURE 1 | Flow diagram of enrollment. TB, tuberculosis; LTBI, latent tuberculosis infection; ETB, extrathoracic TB; PTB, intrathoracic TB with pulmonary involvement; Mediastinal TB, intrathoracic TB with isolated mediastinal lymphadenopathy in the absence of lung parenchyma involvement.

Furthermore, pairwise comparisons showed that Ag-TB levels of IL-17A and GM-CSF, together with the PHA levels of TNF-α and IL-13, were higher in children with Mediastinal TB than in those with PTB (p = 0.009 and 0.042, p = 0.025 and p = 0.050, respectively). Likewise, hemoglobin levels were significantly higher in children with Mediastinal TB than in children with PTB or ETB (p = 0.014 and p = 0.014, respectively). No biomarker showed significant differences between the PTB and ETB groups. Therefore, the groups of children with PTB and extrapulmonary TB were merged and analyzed as a single group (TB, n = 44), while children with Mediastinal TB (n = 18) were analyzed separately.

Comparisons between the TB group (n = 44) and LTBI cases with a positive QFT-GIT (n = 22) were performed (**Table 3**). The Ag-TB levels of IP-10 along with ferritin levels were significantly higher in children with TB than in children with LTBI (p = 0.005 and p = 0.019, respectively). However, the PHA levels of IFN-γ were significantly lower in children with TB than in those with LTBI (p < 0.001). Although nil levels of IL-5 showed significant differences between groups (p = 0.007), this cytokine was not considered in the analysis because almost all the results of the LTBI cases (20/22) and more than half of those of the children who belonged to the TB group (24/44) were below the standard curve. Regarding the association of *T. canis* with IL-5, no significant differences were found between Ag-TB, PHA and nil IL-5 levels and *T. canis* in the groups with positive QFT-GIT (p-values of 0.202, 0.508, and 0.053, respectively). Moreover, the eosinophil count was determined, but because only 25% of the children had an eosinophil count, this variable was not included in the analysis of the study. Despite the few results, no significant differences were found between Ag-TB, PHA and nil IL-5 levels and eosinophil count in the groups with a positive QFT-GIT (p-values of 0.848, 0.923, and 0.768, respectively).

Characterization of Subjects With a Negative QFT-GIT Test

Comparisons among active TB cases (n = 9), LTBI cases (n = 12) and uninfected controls (n = 55) with a negative QFT-GIT (n = 22) were performed (**Table 4**). The PHA levels of IL-10, IL-13, and IL-32, together with the nil levels of TNF-α and IL-10, the underweight levels, and 25(OH)D levels showed significant differences (p < 0.001, p = 0.007, and p = 0.004, p = 0.001 and p = 0.044, p = 0.001, and p = 0.037, respectively). Furthermore, pairwise comparisons showed that PHA levels of IL-10 were significantly higher in TB and LTBI cases than in uninfected controls (p = 0.020 and p = 0.001, respectively), whereas the PHA levels of IL-32 were significantly higher in LTBI cases than in TB cases, and uninfected controls (p = 0.034 and p = 0.004, respectively), and the PHA levels of IL-13 were significantly higher in LTBI cases compared to uninfected controls (p = 0.006). Otherwise, the nil TNF-α levels were significantly higher in uninfected controls compared to LTBI cases (p < 0.001). Regarding levels of malnourishment, the WAZ z-scores were significantly lower in TB and LTBI

TABLE 1 | Description and comparison of the demographic and clinical characteristics among the study groups according to QFT-GIT results.

Study groups classified by QFT-GIT result (*n* = 166)								
	Active TB (*n* = 74)			**LTBI (*n* = 37)**			**Uninfected (*n* = 55)**	**p. overall**
	Positive *n* = 62	**Negative *n* = 9**	**Indeterminate *n* = 3**	**Positive *n* = 22**	**Negative *n* = 12**	**Indeterminate *n* = 3**	**Negative *n* = 55**	
Gender								0.548
Female	26 (41.9%)	5 (55.6%)	2 (66.7%)	12 (54.5%)	6 (50.0%)	3 (100%)	25 (45.5%)	
Male	36 (58.1%)	4 (44.4%)	1 (33.3%)	10 (45.5%)	6 (50.0%)	0 (0%)	30 (54.5%)	
Age in years	85.3 (44.8)	47.3 (51.6)	59.0 (32.0)[a]	114 (45.6)[a]	80.6 (44.6)	48.7 (19.5)	76.5 (35.2)	**0.001**
Range								**0.008**
≤5 years	25 (40.3%)	7 (77.8%)	2 (66.7%)	5 (22.7%)	6 (50.0%)	3 (100%)	32 (58.2%)	
>5 years	37 (59.7%)	2 (22.2%)	1 (33.3%)	17 (77.3%)	6 (50.0%)	0 (0%)	23 (41.8%)	
Body weight-for-age								
Z-score (SD)	−1.39 (1.70)[a]	−1.70 (1.09)	−1.78 (1.06)	−0.66 (0.98)	−1.57 (1.50)	−0.92 (1.23)	−0.30 (1.00)[a]	**0.001**
BCG scar								0.424
Yes	41 (69.5%)	7 (87.5%)	2 (66.7%)	15 (71.4%)	9 (100%)	1 (50.0%)	39 (72.2%)	
Hemoglobin (g/dl)	10.4 (2.02)	9.89 (0.97)	9.57 (0.84)	11.2 (1.30)	11.0 (1.37)	11.0 (3.33)	11.5 (0.66)	0.457

Categorical variables are expressed as frequencies (n) and percentages (%), and quantitative variables are expressed as median and interquartile ranges (IQR) or standard deviation (SD). Statistical differences between pairwise comparisons (a). QFT-GIT, QuantiFERON-TB Gold In-Tube; TB, tuberculosis; LTBI, latent tuberculosis infection; Z-score, default classification system used to present child nutritional status; BCG, Bacillus Calmette-Guérin. Bold values, statistical significative values with a p-value under 0.05.

TABLE 2 | Description and comparison of demographic, clinical, and cytokine responses among active TB cases with positive QFT-GIT.

Study subjects with active TB and a positive QFT-GIT assay classified by disease location (*n* = 62)				
	PTB *n = 36*	**ETB** *n = 8*	**Mediastinal TB** *n = 18*	**p. overall**
Gender				0.935
Male	20 (55.6%)	5 (62.5%)	11 (61.1%)	
Age in years	76.0 [34.5; 115]	89.5 [55.5; 100]	99.0 [70.2; 130]	0.187
Range				0.406
<5 years	17 (47.2%)	3 (37.5%)	5 (27.8%)	
>5 years	19 (52.8%)	5 (62.5%)	13 (72.2%)	
Body weight-for-age				
Z-score	−1.24 [−2.74; 0.08]	−2.07 [−2.79; −1.13]	−0.98 [−2.37; −0.21]	0.334
BCG scar				0.854
Yes	23 (69.7%)	5 (62.5%)	13 (72.2%)	
Hemoglobin (g/dl)	10.3 [8.90; 11.6][a]	9.00 [8.15; 10.9][b]	12.0 [10.8; 12.1][ab]	**0.006**
Intestinal parasites				
Protozoa (cyst)	0 (0%)	0 (0%)	2 (33.3%)	0.308
Helminths (ova)	0 (0%)	1 (33.3%)	0 (0%)	0.120
Serology *Toxocara* spp.				0.099
Negative	31 (86.1%)	5 (62.5%)	11 (64.7%)	
Positive	5 (13.9%)	3 (37.5%)	6 (35.3%)	
Ferritin (ng/ml)	98.4 [33.0; 201]	138 [78.5; 218]	64.4 [47.4; 117]	0.276
25 (OH) D(ng/ml)	26.6 [20.6; 34.5]	30.2 [26.3; 34.2]	28.6 [21.8; 31.2]	0.735
Range				1.000
Normal levels	27 (75.0%)	6 (75.0%)	14 (77.8%)	
Deficiency	9 (25.0%)	2 (25.0%)	4 (22.2%)	
Cytokine response (pg/ml)				
Ag-TB				
TNF-α	105 [−46.08; 399]	53.1 [36.3; 248]	136 [66.2; 386]	0.400
IP-10	31153 [12825; 47686]	37425 [10304; 57206]	33127 [29369; 48717]	0.778
IL-10	39.5 [23.9; 59.3]	36.4 [16.9; 56.7]	52.4 [47.2; 62.0]	0.060
IFN-γ	3922 [1150; 10449]	3855 [2957; 6969]	7020 [4866; 13847]	0.200
IL-1RA	27796 [12141; 42882]	25015 [13848; 66213]	23673 [15354; 51940]	0.928
IL-17A	7.58 [1.51; 12.6][a]	5.67 [0.00;19.2]	23.1 [9.13; 35.9][a]	**0.009**
GM-CSF	41.4 [8.04; 97.1][a]	28.9 [11.5; 54.8][b]	83.6 [39.6; 201][ab]	**0.027**
IL-13	716 [315; 1350]	1342 [534; 1876]	1326 [984; 1755]	0.071
IL-5	3.32 [0.00; 7.05]	0.00 [0.00; 13.5]	8.59 [3.53; 25.7]	0.204
IL-32	30.1 [−122.86; 64.1]	59.4 [−25.44; 396]	77.1 [−10.03; 360]	0.222
VEGF	−381.77 [−784.77; −120.49]	−903.69 [−1128.04; −499.36]	−468.37 [−931.27; −173.86]	0.150
PHA				
TNF-α	4961 [2833; 8837][a]	4850 [3446; 7646]	9762 [4829; 12489][a]	**0.027**
IP-10	4718 [2862; 7972]	5335 [2623; 16865]	6952 [814; 19877]	0.840
IL-10	−2.79 [−11.05; 0.00]	0.00 [−0.30; 0.91]	0.00 [−1.99; 0.92]	0.071
IFN-γ	1102 [632; 3913]	1029 [876; 1878]	1828 [1131; 8723]	0.272
IL-1RA	46679 [27310; 80379]	39813 [17196; 46881]	82727 [29720; 142991]	0.311
IL-17A	39.8 [13.4; 129]	9.07 [6.54; 13.1]	19.8 [8.22; 81.8]	0.057
GM-CSF	65.2 [34.1; 118]	26.7 [9.59; 46.5][b]	74.7 [54.8; 112][b]	**0.040**
IL-13	1211 [664; 1970][a]	1536 [1132; 2023]	2150 [1423; 2608][a]	**0.042**
IL-5	4.28 [0.00; 7.38]	0.00 [0.00; 0.00]	2.98 [0.52; 4.76]	0.064
IL-32	43.9 [−74.11; 243]	7.99 [−98.12; 686]	243 [69.0; 739]	0.109
VEGF	−62.75 [−368.65; 228]	−434.83 [−559.52; −156.10]	−73.02 [−503.76; 517]	0.297
Nil				
TNF-α	151 [40.3; 250]	33.6 [17.3; 178]	106 [55.3; 209]	0.388
IP-10	673 [412; 1220]	745 [529; 1082]	973 [616; 1201]	0.573

(Continued)

TABLE 2 | Continued

Study subjects with active TB and a positive QFT-GIT assay classified by disease location (*n* = 62)				
	PTB ***n* = 36**	**ETB** ***n* = 8**	**Mediastinal TB** ***n* = 18**	**p. overall**
IL-10	8.52 [1.62; 16.3]	0.88 [0.00; 5.10]	5.45 [3.49; 9.86]	0.168
IFN-γ	197 [114; 509]	310 [106; 454]	200 [171; 451]	0.872
IL-1RA	14867 [9160; 22252]	11778 [6783; 27489]	12406 [8385; 20155]	0.765
IL-17A	0.00 [0.00; 11.3]	0.00 [0.00; 0.00]	3.33 [0.00; 19.7]	0.239
GM-CSF	1.92 [0.00; 16.2]	0.00 [0.00; 2.41]	2.67 [0.85; 5.27]	0.414
IL-13	311 [147; 622]	257 [0.00; 423]	341 [292; 444]	0.604
IL-5	0.00 [0.00; 5.88]	0.00 [0.00; 8.95]	3.26 [0.00; 5.52]	0.937
IL-32	1096 [508; 2241]	1606 [699; 2579]	1558 [1101; 2840]	0.376
VEGF	702 [175; 1801]	935 [726; 1128]	693 [368; 1323]	0.830

Categorical variables are expressed as frequencies (n) and percentages (%), and quantitative variables are expressed as median and interquartile ranges (IQR) or standard deviation (SD). Statistical differences between pairwise comparisons (a, b). QFT-GIT, QuantiFERON-TB Gold In-Tube; TB, tuberculosis; PTB, Intrathoracic TB with pulmonary involvement; ETB, Extrathoracic TB; Mediastinal TB, Intrathoracic TB with isolated mediastinal lymphadenopathy in the absence of lung parenchyma involvement; Z-score, default classification system used to present child nutritional status; BCG, Bacillus Calmette-Guérin; 25(OH)D, 25-hydroxyvitamin D; Ag-TB, antigen-dependent response; PHA, mitogen-induced response. Bold values, statistical significative values with a p-value under 0.05.

TABLE 3 | Description and comparison of demographic, clinical, and cytokine response between groups with a positive QFT-GIT.

Subjects with a positive QFT-GIT assay (*n* = 66)			
	Active TB* ***n* = 44**	**LTBI** ***n* = 22**	**p. overall**
Body weight-for-age			
Z-score	−1.32 [−.74; −0.17]	−0.64 [−1.58; 0.08]	0.061
Intestinal parasites			
Protozoa (cyst)	0 (0%)	1 (50.0%)	0.200
Helminths (ova)	1 (5.88%)	1 (16.7%)	0.462
***Toxocara* spp.**			0.324
Negative	36 (81.8%)	13 (68.4%)	
Positive	8 (18.2%)	6 (31.6%)	
Ferritin(ng/ml)	109 [38.2; 201]	52.2 [32.5; 70.9]	**0.019**
25 (OH) D(ng/ml)	27.6 [20.8; 34.5]	24.7 [21.2; 30.3]	0.563
Range			0.755
Normal levels	33 (75.0%)	18 (81.8%)	
Deficiency	11 (25.0%)	4 (18.2%)	
Cytokine responses (pg/ml)			
Ag-TB			
TNF-α	71.0 [−40.32; 364]	47.5 [−26.19; 151]	0.654
IP-10	31545 [12093; 48379]	7762 [3676; 26629]	**0.005**
IL-10	39.3 [22.1; 59.3]	22.9 [12.1; 44.2]	0.121
IFN-γ	3922 [1201; 10427]	1832 [672; 9569]	0.221
IL-1RA	27796 [12141; 48396]	13918 [4821; 33546]	0.138
IL-17A	7.58 [0.40; 14.9]	10.5 [0.88; 60.2]	0.173
GM-CSF	34.5 [8.04; 97.1]	30.2 [18.4; 60.3]	0.828
IL-13	728 [315; 1468]	427 [171; 1229]	0.118
IL-5	2.90 [0.00; 7.05]	0.00 [0.00; 8.99]	0.579
IL-32	34.7 [−99.73; 72.6]	−63.80 [−176.88; 59.4]	0.206
VEGF	−442.15 [−913.46; −138.11]	−357.76 [−1013.21; −81.31]	0.775
PHA			
TNF-α	4850 [2833; 8837]	6290 [4103; 9904]	0.226
IP-10	4718 [2680; 9102]	4392 [1800; 8974]	0.644

(Continued)

TABLE 3 | Continued

	Subjects with a positive QFT-GIT assay (*n* = 66)		
	Active TB* *n* = 44	**LTBI** *n* = 22	**p. overall**
IL-10	−0.47 [−9.25; 0.00]	−5.14 [−8.32; −1.05]	0.307
IFN-γ	1102 [717;2984]	4420 [2137; 11309]	**<0.001**
IL-1RA	42206 [27310; 69517]	45310 [27013; 112972]	0.812
IL-17A	26.0 [9.06; 112]	99.5 [18.9; 229]	0.053
GM-CSF	55.1 [24.4; 103]	79.4 [47.3; 135]	0.121
IL-13	1232 [683; 1973]	1180 [796; 1812]	0.946
IL-5	2.92 [0.00; 6.98]	0.91 [0.00; 10.1]	0.967
IL-32	32.9 [−74.11; 304]	−1.83 [−57.38; 75.4]	0.391
VEGF	−108.91 [−462.16; 181]	−315.24 [−871.29; −16.37]	0.135
Nil			
TNF-α	139 [35.0; 250]	90.0 [38.0; 185]	0.654
IP-10	680 [423; 1189]	435 [208; 1208]	0.161
IL-10	7.63 [0.19; 14.9]	9.40 [5.28; 13.0]	0.526
IFN-γ	226 [111; 508]	241 [179; 388]	0.523
IL-1RA	14041 [8927; 23001]	11090 [6497; 17128]	0.106
IL-17A	0.00 [0.00; 10.4]	0.00 [0.00; 2.46]	0.764
GM-CSF	0.89 [0.00; 12.3]	0.83 [0.00; 5.30]	0.429
IL-13	302 [114; 595]	256 [146; 499]	0.683
IL-5	0.00 [0.00; 6.96]	0.00 [0.00; 0.00]	**0.007**
IL-32	1112 [556; 2241]	1726 [1033; 2590]	0.334
VEGF	809 [209; 1610]	688 [317; 1381]	0.870

*Categorical variables are expressed as frequencies (n) and percentages (%), and quantitative variables are expressed as median and interquartile ranges (IQR) or standard deviation (SD). QFT-GIT, QuantiFERON-TB Gold In-Tube; TB, tuberculosis; LTBI, latent tuberculosis infection; Z-score, default classification system used to present child nutritional status; BCG, Bacillus Calmette-Guérin; 25(OH)D, 25-hydroxyvitamin D; Ag-TB, antigen-dependent response; PHA, mitogen-induced response. *In this table, children with Mediastinal TB were not included in the active TB cases. Bold values, statistical significative values with a p-value under 0.05.*

cases than in uninfected controls (p = 0.006 and p = 0.006, respectively). On the contrary, the median 25(OH)D levels were significantly higher in LTBI cases compared to uninfected controls (p = 0.022).

Discriminative Biomarker Profiles in Subjects With Positive QFT-GIT

The adjusted logistic model showed a set of 4 biomarkers able to discriminate between active TB and LTBI: ferritin, 25(OH)D, IP-10, and IFN-γ (**Table 5**).

For each increased unit of IFN-γ (in Ag-TB responses), IP-10 (in Ag-TB and PHA responses), ferritin, and 25(OH)D, the odds of being classified as a TB case increased by 1.08, 1.80, 1.13, 1.02, and 1.22, respectively. However, for each increased unit of IFN-γ (in PHA responses), the odds of being classified as a TB case decreased by 0.46. Therefore, the profile to classify a subject with TB but not LTBI is that of low IFN-γ levels (in PHA responses) but high IP-10 (in Ag-TB and PHA responses), 25(OH)D, and ferritin levels.

The model based on the four host-markers mentioned above was able to correctly classify 93.2% of children with TB, and 90.0% of children with LTBI. In addition, the area under the ROC curve of this model was 0.955 (CI 95%: 0.91 to 1.00), and the positive and negative likelihood ratio were 9.32 and 0.08, respectively (**Figure 2**). Furthermore, when Mediastinal TB was included, the model correctly classified 76.2% of these into the TB group. **Supplementary Table 1** summarizes the diagnostic performance of the different combination possibilities of the four host-markers selected in the adjusted logistic model.

Discriminative Biomarker Profiles in Subjects With Negative QFT-GIT

The adjusted logistic model showed a set of 3 biomarkers able to discriminate among active TB, LTBI, and uninfected controls: IL-10, IL-13, and IL-32 (**Table 6**). For each increased unit of IL-13 and IL-32 (in PHA responses), the odds of being classified as LTBI case increased by 1.003 and 1.003, respectively. However, for each increased unit of IL-10 (in PHA responses), the odds of being classified as uninfected control decreased by 0.93 and 0.01, respectively.

The combination of PHA levels of IL-10, IL-13, and IL-32 detected 11.1% of active TB cases, 50.0% of LTBI cases, and 96.4% of uninfected controls.

DISCUSSION

This study aimed to identify promising markers to discriminate between active TB and LTBI in children. We obtained a combination based on IP-10, IFN-γ, ferritin, and 25(OH)D which

TABLE 4 | Description and comparison of demographic, clinical, and cytokine response between groups with a negative QFT-GIT.

	Subjects with a Negative QFT-GIT assay (*n* = 76)			
	Active TB ***n* = 9**	**LTBI** ***n* = 12**	**Uninfected** ***n* = 55**	**p. overall**
Body weight-for-age				
Z-score (SD)	−1.44 [−2.45; −1.00][a]	−1.55 [−2.03; −0.58][b]	−0.59 [−1.08; 0.32][ab]	**0.001**
Intestinal parasites				
Protozoa (cyst)	0 (0%)	3 (50.0%)	0 (0%)	0.464
Helminths (ova)	0 (0%)	0 (0%)	2 (4.55%)	1.000
***Toxocara* spp.**				0.314
Negative	6 (66.7%)	7 (58.3%)	42 (77.8%)	
Positive	3 (33.3%)	5 (41.7%)	12 (22.2%)	
Ferritin (ng/ml)	68.5 [51.0; 84.6]	44.0 [28.4; 77.7]	37.1 [20.3; 55.4]	0.075
25 (OH) D(ng/ml)	29.6 [24.1; 30.1]	30.4 [27.7; 34.4][b]	26.5 [24.6; 30.0][b]	**0.037**
Range				
Deficiency	0 (0%)	0 (0%)	2 (3.84%)	
Cytokine response (pg/ml)				
Ag-TB				
TNF-α	−26.62 [−59.20; 4.27]	−7.89 [−37.82; 0.51]	−57.31 [−321.36; 72.9]	0.385
IP-10	544 [−34.59; 795]	94.6 [49.9; 1332]	219 [85.6; 594]	0.894
IL-10	1.44 [−2.71; 9.21]	3.77 [0.85; 6.40]	0.85 [−1.64; 9.29]	0.918
IFN-γ	88.4 [0.00; 196]	54.4 [−5.32; 239]	23.3 [−2.05; 82.1]	0.525
IL-1RA	4361 [1455; 9833]	3204 [1280; 7608]	3812 [1853; 9435]	0.886
IL-17A	0.00 [0.00; 0.00]	0.00 [−22.20; 6.61]	0.00 [0.00; 1.12]	0.751
GM-CSF	1.45 [−1.10; 5.08]	0.00 [0.00; 1.45]	0.04 [−5.15; 1.52]	0.670
IL-13	361 [67.3; 591]	81.5 [23.5; 565]	56.0 [0.00; 200]	0.240
IL-5	0.00 [−0.46; 0.00]	0.00 [0.00; 0.49]	0.00 [0.00; 1.88]	0.171
IL-32	5.89 [−145.98; 222]	106 [−143.37; 256]	−52.81 [−218.53; −6.17]	0.197
VEGF	−769.46 [−1673.90; 0.00]	−57.79 [−1369.04; 19.9]	−243.45 [−466.44; −22.59]	0.295
PHA				
TNF-α	7244 [2691; 8401]	4609 [2837; 6360]	6932 [5104; 9965]	0.261
IP-10	7177 [4600; 11211]	6415 [5166; 9139]	6524 [3733; 10553]	0.819
IL-10	−8.92 [−13.01; −4.87][a]	−9.43 [−11.82; −7.98][b]	−0.43 [−6.29; 0.00][ab]	**< 0.001**
IFN-γ	3092 [2179; 4970]	4808 [3212; 9358]	3998 [1897; 7064]	0.536
IL-1RA	72506 [42906; 111675]	148877 [70665; 201214]	68339 [30968; 172755]	0.184
IL-17A	105 [8.75; 180]	131 [80.7; 224]	80.0 [30.4; 216]	0.390
GM-CSF	39.3 [33.8; 99.2]	64.1 [46.6; 93.7]	71.5 [42.6; 143]	0.186
IL-13	1948 [1467; 2146]	2400 [2021; 2978][b]	1563 [789; 2236][b]	**0.007**
IL-5	5.24 [0.91; 5.69]	2.99 [0.00; 11.4]	3.42 [1.15; 6.68]	0.789
IL-32	−198.07 [−315.19; −9.88][c]	280 [67.2; 367][bc]	−24.34 [−160.79; 66.4][b]	**0.004**
VEGF	0.00 [−853.06; 782]	0.00 [−780.01; 374]	−27.36 [−358.31; 138]	0.703
Nil				
TNF-α	228 [71.7; 300]	69.5 [40.4; 99.2][b]	312 [155; 809][b]	**0.001**
IP-10	938 [301; 1667]	561 [413; 2085]	487 [317; 770]	0.291
IL-10	12.3 [8.92; 27.5]	14.0 [10.1; 16.5]	7.75 [3.59; 16.0]	**0.044**
IFN-γ	321 [0.00; 862]	255 [67.9; 440]	199 [89.0; 434]	0.911
IL-1RA	11598 [7467; 18694]	10372 [8682; 16165]	13409 [9432; 22543]	0.304
IL-17A	0.00 [0.00; 44.3]	0.00 [0.00; 26.5]	0.00 [0.00; 0.71]	0.220
GM-CSF	4.98 [0.00; 9.43]	0.00 [0.00; 3.76]	1.30 [0.00; 14.6]	0.258
IL-13	1166 [478; 1519]	376 [324; 727]	373 [206; 947]	0.069
IL-5	0.46 [0.00; 4.96]	0.00 [0.00; 2.49]	3.07 [0.00; 5.52]	0.122
IL-32	1242 [1006; 1740]	2020 [1384; 4215]	1532 [657; 3754]	0.332
VEGF	1029 [626; 1674]	1546 [491; 2629]	484 [222; 1189]	0.113

Categorical variables are expressed as frequencies (n) and percentages (%), and quantitative variables are expressed as median and interquartile ranges (IQR) or standard deviation (SD). Statistical differences between pairwise comparisons corrections (a, b, c). QFT-GIT, QuantiFERON-TB Gold In-Tube; TB, tuberculosis; LTBI, latent tuberculosis infection; Z-score, default classification system used to present child nutritional status; BCG, Bacillus Calmette-Guérin; 25(OH)D, 25-hydroxyvitamin D; Ag-TB, antigen-dependent response; PHA, mitogen-induced response. Bold values, statistical significative values with a p-value under 0.05.

TABLE 5 | Classification table of the model for the study groups with positive QFT-GIT results and variables included in the equation.

Classification table of the Active TB* and LTBI cases with positive QFT-GIT results and variables included in the equation

	B	*P*-value	Odds Ratio [CI 95%]
25 (OH) D (ng/ml)	0.20	**0.012**	1.22 [1.05; 1.43]
Ferritin (ng/ml)	0.02	**0.010**	1.02 [1.01; 1.03]
Cytokine response (pg/ml)†			
Ag-TB			
IP-10	0.08	**0.017**	1.08 [1.01; 1.15]
INF-γ	0.12	0.064	1.13 [0.99; 1.29]
PHA			
IP-10	0.59	**0.007**	1.80 [1.18; 2.75]
INF-γ	−0.77	**0.008**	0.46 [0.26; 0.82]
Constant	−8.67	0.004	

*Binomial Logistic Regression. B, regression coefficient; OR, Odds Ratio; CI, Confidence Interval. QFT-GIT, QuantiFERON-TB Gold In-Tube; TB, tuberculosis; LTBI, latent tuberculosis infection; 25(OH)D, 25-hydroxyvitamin D; Ag-TB, antigen-dependent response; PHA, mitogen-induced response. *In this table, children with Mediastinal TB were not included in the active TB cases.†Cytokine levels were multiplied x10. Bold values, statistical significative values with a p-value under 0.05.*

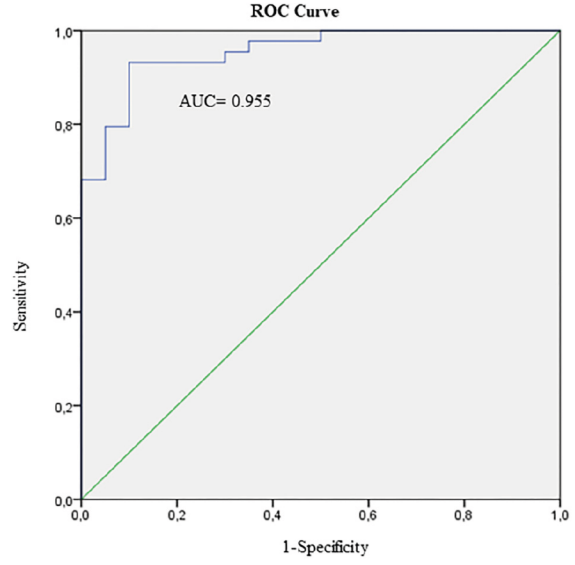

FIGURE 2 | ROC curve of the four markers presented in the model [IFN-γ, IP-10, Ferritin, and 25(OH)D] to discriminate between active TB (N = 44) and latent TB infected cases (N = 37) with positive QuantiFERON-TB Gold In-Tube result. AUC, Area under ROC curve.

showed diagnostic potential and a predictive value higher than the minimum target product profiles (TPPs) recommended.

Cytokines in *M. tuberculosis* Infection and Active Disease

Although IGRAs have become a standard method for the diagnosis of *M. tuberculosis* infection, IFN-γ alone is unable to discriminate between TB and LTBI (Denkinger et al., 2015; Latorre and Domínguez, 2015; Pai and Behr, 2016). Previous studies showed evidence of the low sensitivity of the IGRAs in individuals with a depressed or immature immune system such as immunosuppressed patients or young children (Latorre et al., 2014). In our study, very low Ag-TB cytokine levels were found in subjects with negative QFT-GIT in comparison with those with a positive QFT-GIT. Therefore, no cytokine or combination of cytokines showed potential for the discrimination between uninfected controls and *M. tuberculosis*-infected children with a negative QFT-GIT result. This finding is in line with Lighter-Fisher et al., who reported that LTBI children under 5 years old showed negative QFT-GIT results and very low levels of cytokines (Lighter-Fisher et al., 2010). These results corroborate the weakened immune response of young children in protecting against *M. tuberculosis* and, therefore, their susceptibility to progress to TB disease. Conversely, in the present study, statistically significant differences were found between markers with potential to diagnose TB and to discriminate active TB from LTBI in *M. tuberculosis*-infected children with a positive QFT-GIT result.

Since children become more susceptible to developing intrathoracic and ETB disease following exposure to *M. tuberculosis*, several studies have examined the patterns of expression of a variety of biomarkers to understand the progression of intrathoracic and ETB disease in children (Whittaker et al., 2012). Similar to Kumar et al. (2013) in the present study no marker was able to differentiate between subjects with intrathoracic TB and ETB (Kumar et al., 2013). Although mediastinal TB subjects showed an immune profile similar to that of intrathoracic and ETB, five biomarkers (IL-17, GM-CSF, TNF-α, IL-13, and ferritin) showed potential to identify mediastinal TB subjects in the TB group. Radiographic signs of mediastinal TB have been related to the onset of primary TB

TABLE 6 | Classification table for the study groups with negative QFT-GIT results and variables included in the equation.

Classification table of the study groups with negative QFT-GIT results and variables included in the equation

Model[a]	B	*P*-value	Odds Ratio [CI 95%]
Active TB			
PHA (pg/ml)			
Constant	−4.67		
IL-10	−0.071	**0.036**	0.93 [0.87; 0.99]
IL-13	1.25×10^{-3}	0.073	1.01 [1.00; 1.01]
IL-32	3.42×10^{-4}	0.751	1.00 [0.99; 1.01]
LTBI			
PHA(pg/ml)			
Constant	−8.873		
IL-10	−0.095	**0.009**	0.910 [0.85; 0.98]
IL-13	3.06×10^{-3}	**0.001**	1.003 [1.001; 1.005]
IL-32	2.60×10^{-3}	**0.007**	1.003 [1.001; 1.005]

[a]*The reference category is the uninfected control group. Multivariate Logistic Regression. B, regression coefficient; OR, Odds Ratio; CI, Confidence Interval. QFT-GIT, QuantiFERON-TB Gold In-Tube; TB, tuberculosis; LTBI, latent tuberculosis infection; PHA, mitogen-induced response. Bold values, statistical significative values with a p-value under 0.05.*

in children (Thomas, 2019). Considering that TB represents a dynamic continuum of states in which the dichotomous distinction between infection and disease is often difficult to differentiate (Perez-Velez et al., 2017), the previous cytokines suggest a potential for detecting *M. tuberculosis*-infected children most likely to progress to the disease and/or early stages of TB.

In subjects with a positive QFT-GIT result, IP-10, IFN-γ, IL-5, and ferritin responses individually showed statistically significant differences between active TB and LTBI. However, the combination of IP-10, IFN-γ, ferritin, and 25(OH)D achieved the best diagnostic performance to discriminate between active TB and LTBI cases. IP-10 and IFN-γ play an important role in the immune response to *M. tuberculosis* infection (Chegou et al., 2014). Several studies have described the good performance of IP-10 when compared or combined with IFN-γ for the diagnosis of TB and the discrimination between TB and LTBI in children, respectively (Ruhwald et al., 2012; Villar-Hernandez et al., 2017). While IP-10 is a chemokine involved in the trafficking and stimulation of monocytes and Th1 cells activated in response to inflammatory foci, IFN-γ is essential for the mediation of the adaptive immune response against *M. tuberculosis* (Ruhwald et al., 2008). In contrast to IFN-γ, IP-10 induces a robust, specific *M. tuberculosis* response not influenced by age (Ruhwald et al., 2012). Indeed, according to previous studies, IP-10 shows the same kinetics as IFN-γ but at levels up to 10-fold higher (Lighter et al., 2009; Ruhwald et al., 2011).

Previous studies in *M. tuberculosis*-infected adults and children observed variations in cytokine responses after PHA-stimulation (Mueller et al., 2008; Ruhwald et al., 2008, 2011; Alsleben et al., 2012; Jeong et al., 2015). In our study, PHA levels of IFN-γ were significantly higher in LTBI cases compared to TB cases. Similar findings have been observed in *M. tuberculosis*-infected adults whose blood was stimulated with QFT-GIT peptides (Jeong et al., 2015). Therefore, PHA-induced responses were considered to evaluate host immune status for the discrimination between TB and LTBI in children. On the other hand, in this study, PHA levels of IP-10 were very similar to those obtained in *M. tuberculosis*-specific stimulation. In this regard, Ruhwald et al. (2008) suggested that PHA was a powerful inducer of IFN-γ. However, the same was not observed with IP-10, with which PHA levels were rather low.

In our study, IL-5 levels in unstimulated samples showed statistically significant differences between TB and LTBI cases. However, this cytokine was not consistently expressed. Studies in adults have shown the potential of IL-5 for discriminating between TB and LTBI cases (Won et al., 2017). However, the diagnostic value of IL-5 in children is relatively unknown (Armand et al., 2014).

In this study, cytokines levels were measured from supernatants remaining from QFT-GIT tubes. Recently, QuantiFERON-TB® Gold Plus (QFT-Plus), has been introduced in the new generation of QFT assay. For this new test there are two TB-specific antigen tubes, called TB1 and TB2. The TB1 tube, contains long peptides derived from ESAT-6 and CFP-10 (for this new test the peptide TB-7.7 has been removed), and it is designed to induce a specific CD4 T cells response. TB2 contains both the same long peptides of TB1 and newly designed shorter peptides to induce interferon (IFN)-g production by both CD4 and CD8 T-cells (Barcellini et al., 2016). The determination of these biomarkers could be performed using the TB1 tube from the QFT-Plus assay, since it contains almost the same relatively long peptides from *M. tuberculosis* antigens (ESAT-6 y CFP-10) to mainly stimulate CD4 + T cells as those used in the QFT-GIT assay antigen tube. Although QFT-Plus does not contain the TB7.7 antigens, it has been shown that the absence of the TB7.7 antigen from the QFT-Plus does not significantly impact assay performance (Theel et al., 2018).

Individual Factors in *M. tuberculosis* Infection and Disease

The mechanism by which host, pathogens and extrinsic factors interact as final determinants of disease outcome and TB transmission is an active area of research (Bastos et al., 2018). In this context, we characterized a series of individual factors, among which ferritin and 25(OH)D were markers that showed potential for the discrimination between active TB and LTBI in children.

Iron is an essential cofactor for mycobacteria propagation during infection (Olakanmi et al., 2000), and successful protective host-immune response (Mainou-Fowler and Brock, 1985). It is known that acute phase proteins stimulate or inhibit their production in response to inflammatory processes such as infections. In our study, ferritin levels in LTBI cases were lower than in active TB cases. Previous findings by our group showed lower ferritin levels in children with a positive QFT-GIT at the onset of *M. tuberculosis* infection (Pérez-Porcuna et al., 2014). Similar studies showed higher median ferritin levels in TB adults than in the other study groups (Jacobs et al., 2016). A possible explanation for this result is that ferritin is a recognized acute phase protein in iron storage processes and is closely linked to host response in *M. tuberculosis* (Thom et al., 2012).

In the present study, no statistically significant differences were found in 25(OH)D alone between study groups. Vitamin D is a steroid hormone with pleiotropic actions in many body tissues and cells, including cells of the immune system (Nair et al., 2018). Several studies suggest that 25(OH)D deficiency (below 20 ng/ml) could compromise antibacterial activity and increase the risk of TB disease by preventing the initiation of immune response mediated by vitamin D (Hewison, 2012).

The analysis of 25(OH)D in combination with IP-10, IFN-γ and ferritin was found to be useful to discriminate between active TB and LTBI cases. We observed an inverse correlation between the 25(OH)D and PHA levels of IFN-γ in active TB cases in comparison to LTBI cases. *In vitro* studies have demonstrated how vitamin D induces innate antimicrobial responses and suppresses proinflammatory cytokine responses (Coussens et al., 2012). Likewise, Ragab et al. (2016) observed a negative association between the addition of vitamin D and PHA-induced IFN- γ levels (Nonnecke et al., 2003) suggesting the multiple mechanisms in which vitamin D is involved in the protection of the host against *M. tuberculosis* infection.

Promising Biomarker-Based Diagnostic Tests in Children

A recent systematic review by Togun et al. (2018) evaluated biomarkers able to diagnose TB in children. All the studies performed in QFT-GIT supernatants presented at least a combination of two markers for the discrimination of active TB and LTBI in children, demonstrating the involvement of several mechanisms mediated by different host markers in *M. tuberculosis* infection and disease (Togun et al., 2018).

Several studies have suggested that IL-2 and TNF-α may be reliable cytokines for the discrimination between active TB and LTBI cases in children (Lighter-Fisher et al., 2010; Gourgouillon et al., 2012). However, we did not find any significant differences in TNF-α, and IL-2 responses in unstimulated and stimulated samples were lower than the minimum concentrations detected in the standard curve of the experiment. Although our findings may not coincide with some mainly previously cited observations, direct comparisons of biomarkers among studies in children are complex due to population variability, the burden of *M. tuberculosis* presented in the different settings, and age variations that could affect the state of maturity of the immune response of the child.

Regarding the possible use of biomarkers in the field, considering the TPPs recommended by FIND/WHO, the sensitivity and specificity achieved by the combination of IFN-γ, IP-10, ferritin and 25(OH)D were optimal (Denkinger et al., 2015). In recent years, two case-control studies presented two models for the diagnosis of TB in children with minimal TPPs higher than those reached in this study for the development of a new diagnostic test (Armand et al., 2014; Zhou et al., 2017). However, they were not able to discriminate between active TB and LTBI cases. In our study, the combination of IP-10, IFN-γ, ferritin, and 25(OH)D achieved the best diagnostic performance with correct classification of active TB cases (93.2%) and LTBI cases (90.0%). Moreover, this combination of biomarkers correctly classified 76.2% of the mediastinal TB subjects. Lastly, this combination could be a good diagnostic test to confirm active TB (LR+ = 9.32) and a very robust test to rule out cases with active TB from cases with LTBI (LR− = 0.08).

Limitations and Strengths of the Study

One limitation of the studies evaluating diagnostic tests for TB in children is the lack of a microbiological gold standard and adequate, validated clinical scoring systems, resulting in low diagnostic sensitivity in children. In this study, TST was defined as a criterion for inclusion of the screened TB and LTBI children to improve patient classification. However, the inclusion criterion mentioned above may have affected the specificity of diagnosis of some risk groups with a vulnerable immune system including malnourished children. Despite attempting to control most of the risk factors, the vulnerability of the study population and context did not allow evaluation of some factors which may affect the immune system (such as cytomegalovirus and allergies). An important strength of this study is the strict criteria for the classification of the study groups (Graham et al., 2015). It should be noted that in this case-control study, the sample size, age ranges according to sex and nutritional status were widely represented. However, according to the results obtained, additional studies are needed to validate the performance of the current model in children with other respiratory infections. The identification of these biomarkers has the same technical difficulty as performance of the QFT-GIT assay. However, the combination of biomarkers presented in this study might also have a great potential to discriminate between TB and LTBI in children. To this end, future studies should be conducted validating the identification of the biomarkers proposed in this study in other geographical areas and different populations.

CONCLUSION

Our findings suggest the diagnostic potential of the combination of IFN-γ, IP-10, ferritin and 25(OH)D detected in supernatants in QFT-GIT tubes for the diagnosis of pediatric TB and discrimination between TB and LTBI. In addition, these markers may be useful for the identification of the onset of primary TB, although future investigations in transversal and prospective longitudinal studies are warranted. This study highlights potential markers with optimal diagnostic accuracy for improving the management of TB diagnosis in children. Finally, developing a rapid diagnostic test based on the immunological biomarkers studied would improve the access of TB services, thereby promoting early diagnosis of pediatric TB and LTBI.

ETHICS STATEMENT

This study was approved by the Health Research Ethics Committee of the University of Barcelona and the Haitian National Ethics Committee (project number IRB00003099). Before participation, written informed consent was obtained from the child's parents or guardian.

AUTHOR CONTRIBUTIONS

PC-d-B, CA, TP-P, RA, and JD conceived and designed the study. PC-d-B, MJC, MN, and JG collected the data. PC-d-B, RV-H, RA, TP-P, IL, LCA, BSM, and JD analyzed and interpreted the data. PC-d-B, TP-P, RA, JD, and RV-H drafted the article. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01855/full#supplementary-material

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Article 2

Fujifilm SILVAMP TB-LAM for the Diagnosis of Tuberculosisin Nigerian Adults

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Article

Fujifilm SILVAMP TB-LAM for the Diagnosis of Tuberculosis in Nigerian Adults

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Abstract: There is a need for diagnostics for tuberculosis (TB) that are easy to use, able to screen non-sputum samples, and able to provide rapid results for the management of both immunocompromised and immunocompetent individuals. The Fujifilm SILVAMP TB LAM (FujiLAM) assay, a new non-sputum based point of need test for the diagnosis of TB, could potentially address most of these needs. We evaluated the performance of FujiLAM in HIV positive and HIV negative patients with presumptive TB attending three district hospitals in Nigeria. Consecutive patients were asked to provide urine samples on the spot, which were tested with FujiLAM. The results were compared against a positive culture and/or Xpert MTB/RIF as the reference standard. Forty-five patients had bacteriologically confirmed TB, and 159 had negative culture and Xpert MTB/RIF (no TB). The FujiLAM test was positive in 23 (sensitivity 65.7%, 95% CI = 48–80) HIV negative and seven (70%, 95% CI = 35–92) HIV positive patients with bacteriological confirmation of TB. FujiLAM was negative in 97 (specificity 99.0%, 95% CI = 94–100) HIV negative and 56 (93.3%, 95% CI = 83–98) HIV positive patients without TB. The FujiLAM test has good diagnostic accuracy for considering its application in both HIV positive and HIV negative patients with TB.

Keywords: tuberculosis; diagnosis; lipoarabinomannan; LAM; HIV; urine; point-of-care

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1. Introduction

Tuberculosis (TB) continues to cause high morbidity and mortality worldwide [1]. Despite increases in TB notifications in recent decades [2] and a major expansion in the use of the World Health Organization (WHO)-recommended molecular diagnostics (WRDs), 2.9 of the ten million estimated people who develop TB are missed by national TB programs each year.

A major drawback of current WRDs is the poor timeliness of test results, which often return to the clinic several hours or days later, when clinical decisions have been taken and the patients have left the premises [3]. It is recognized that, to be impactful, diagnostic test results need to be available at the time of patient management, to guide treatment initiation, and to reduce pre-treatment losses to follow-up [4]. Ideally, assays should be conducted at the point of need, using minimal laboratory skills and examining non- or minimally invasive clinical samples [5,6]. Moreover, large proportions of presumptive individuals cannot expectorate sputum or provide a high-quality sample, and therefore, non-sputum based tests could be helpful for many populations.

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Current non-sputum based, point-of-need prototypes target serological markers, bacterial components, or detritus, including the lipoglycan and virulence factor lipoarabinomannan (LAM). LAM is a heat stable component of the outer cell wall of the bacilli that is released from metabolically active or degenerating bacteria of the genus Mycobacterium. LAM is filtered by the kidney and can be detected in urine, with test prototypes mentioned in the literature since the 1930s [7,8]. Although current LAM assays have low sensitivity, performing better in individuals with HIV and advanced immunosuppression [9], a recent prototype, Fujifilm SILVAMP TB LAM (FujiLAM, Fujifilm, Tokyo, Japan), is reported to have higher sensitivity in both HIV-infected and uninfected individuals [10,11], thanks to the use of high affinity monoclonal antibodies against *Mycobacterium tuberculosis*-specific LAM epitopes and a silver amplification step that increases the visibility of the test lines [11].

We report here a cross-sectional study to assess the diagnostic performance of FujiLAM in consecutive adults with presumptive TB attending ambulatory clinics in Nigeria.

2. Materials and Methods

This was a retrospective study of adults with signs and symptoms suggestive of TB attending TB diagnostic clinics at district hospitals of Abuja, Nigeria. Adults above 18 years old with presumptive TB [12] were enrolled consecutively at the time of submitting samples for diagnosis, regardless of their HIV status. Adults who had formerly been diagnosed as having TB or who had received TB treatment in the previous year were excluded.

After obtaining written informed consent, participants were interviewed to obtain clinical and demographic information. Patients were asked to provide sputum, blood samples, and one midstream urine sample on-site for routine and study assays. All samples except urine were processed locally and were used for patient management. Sputum samples were tested with Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA), and cultured in solid media in duplicate using Löwenstein–Jensen medium. Urine samples were collected in sterile plastic containers and kept in cold boxes until processing for storage the same day. Samples were aliquoted (2 mL) into cryovials and transported frozen to the Institut d'Investigació Germans Trias i Pujol (Badalona, Spain) for LAM testing. One aliquot per participant was thawed at room temperature the day of testing, mixed with a vortex, and tested using FujiLAM following the manufacturers' instructions [11]. Briefly, the reagent tube was filled with urine up to the indicator line, mixed without inverting, and incubated for 40 min at ambient temperature. During this incubation, the gold (Au)-conjugated primary antibody captured the 5-methylthio-D-xylofuranose-lipoarabinomannan antigen present in the patient's urine. The tube was then mixed, and two drops of the contents of the tube were added to the sample well of the test cartridge. Immediately, we pressed the button 2 on the cartridge and waited 10 min until the orange mark appeared on the cartridge readout indicating "go to next step". During this incubation, the sandwich immunocomplex was formed by binding to the immobilized secondary antibody. At the signal to proceed to the next step, the button 3 was pressed, releasing silver particles (10 um in diameter), which cluster around the gold particles and amplify the intensity of the cartridge reader band. The test results could be read approximately 1 min later on the test cartridge reader. Figure 1 shows schematically the test procedure. The test was considered positive if the control and test lines were visible (even if the line was faint) and negative if only the control line appeared. Tests without a control line were considered invalid and were repeated once. A video describing the test procedures is available at https://www.youtube.com/watch?v=aK-QtzkLBug (accessed 26 May 2021). The test lines were read by two investigators blinded to the patients' condition and all other test results. In the case of disagreement with the result, the test was repeated once.

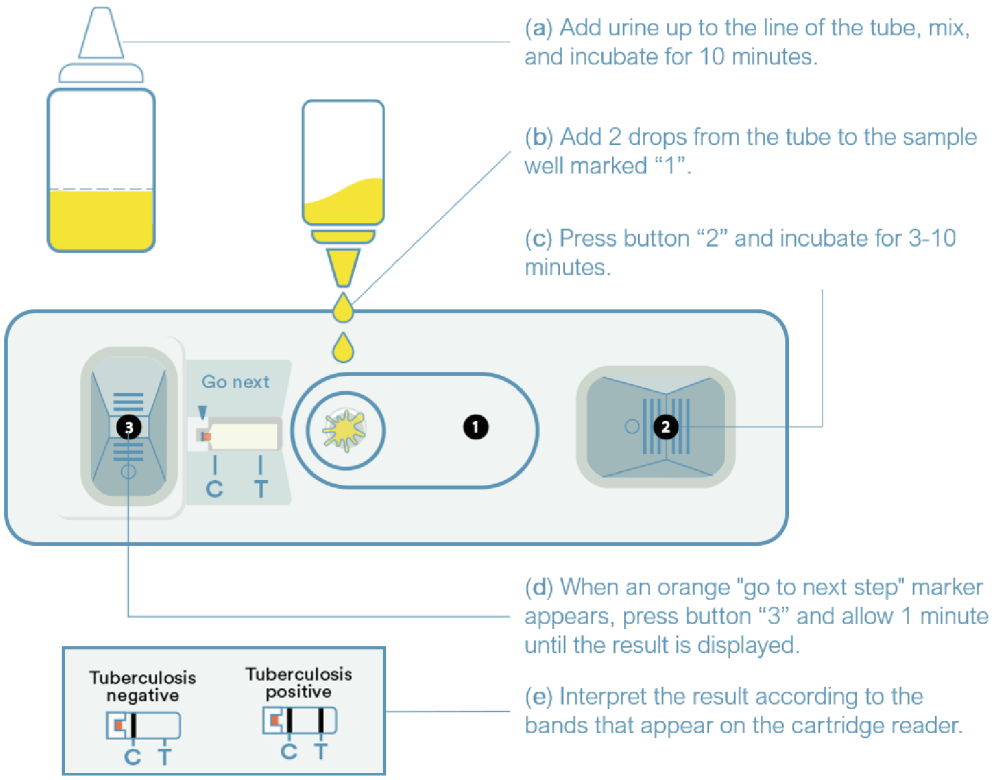

Figure 1. Outline of the procedures to perform the Fujifilm SILVAMP TB-LAM assay.

HIV status was assessed using two rapid antigen tests, and the viral loads of patients with HIV were assessed in plasma using the Xpert HIV-1 viral load (VL) assay (Cepheid, Sunnyvale, CA) according to the manufacturer's instructions. The VL results were interpreted as detected, detected < 40 copies/mL, detected > 10^7 copies/mL, undetected, and undetermined. The range of detection of the Xpert HIV-1 VL test was 40 to 10^7 copies/mL (1.6 to 7.0 $\log_{10}$).

We used the chi-squared and Fisher's exact tests to test parametric data and Student's *t*-tests for continuous variables with normal distributions. Differences were considered statistically significant when the *p*-value was less than 0.05. Analysis was performed using SPSS (SPSS version 26.0, SPSS Inc, Chicago, IL, USA). Xpert and culture results were used to classify participants as bacteriologically confirmed if either the Xpert MTB/RIF or culture results were positive and as non-TB if both tests were negative. The sensitivity and specificity of the FujiLAM test were estimated using the combined results of Xpert MTB/RIF and culture as the reference standard (bacteriologically confirmed). We considered invalid FujiLAM results as negative, but annotated these results in separate rows. Written informed consent was obtained from all participants. The study was approved by the research ethics committees of the Liverpool School of Tropical Medicine, the Nigerian National Ethics Committee, and Ethics Committee of the Hospital Universitari Germans Trias i Pujol.

3. Results

Two hundred and four participants with a mean (SD) age of 37 (12.8) years were enrolled, as shown in Table 1. Thirty-seven (18.1%) had positive *M. tuberculosis* culture, 40 (19.6%) were Xpert MTB/RIF positive, and 45 (22.1%) culture or Xpert MTB/RIF positive (called bacteriologically confirmed). Four (10.8%) culture-positive participants were Xpert MTB/RIF negative, and three (7.5%) and four (10.0%) Xpert MTB/RIF-positive had negative or contaminated culture, respectively. One hundred and fifty-nine (77.9%) participants were culture and Xpert MTB/RIF negative (called not TB) (Table 1). Overall, 70 (34.3%) participants were HIV positive, 133 (65.2%) HIV negative and the HIV status was not known in one. Viral loads among HIV positive participants were undetectable in

11 (15.7%), <40 copies/mL in 16 (22.9%), and between 40 and 10^7 copies/mL in 32 (45.7%), with three (4.3%) and eight (11.4%) participants having indeterminate and missing viral load results, respectively.

Table 1. Characteristics of the study participants.

		All (n = 204)	Not TB (n = 159)	Bact + TB (n = 45)	p-Value
Age	mean (SD)	37.0 (12.8)	37.65 (13.0)	34.60 (12.0)	0.160
Sex	Male	95 (46.6%)	69 (43.4%)	26 (57.8%)	0.088
	Female	109 (53.4%)	90 (56.6%)	19 (42.2%)	
HIV	Negative	133 (65.2%)	98 (61.6%)	35 (77.8%)	0.087
	Positive	70 (34.3%)	60 (37.7%)	10 (22.2%)	
	Unknown	1 (0.5%)	1 (0.6%)	0 (0.0%)	
Culture	Negative	155 (76.0%)	151 (95.0%) [a]	4 (8.9%) [a]	**<0.01**
	Positive	37 (18.1%)	0 (0.0%) [a]	37 (82.2%) [a]	
	Contaminated	12 (5.9%)	8 (5.0%)	4 (8.9%)	
Xpert	Negative	164 (80.4%)	159 (100%) [a]	5 (11.1%) [a]	**<0.01**
	Positive	40 (19.6%)	0 (0.0%) [a]	40 (88.9%) [a]	

SD: standard deviation. The p-value shows the significant differences observed for each variable between the proportions of bacteriologically confirmed TB patients and non-TB patients. [a] Column proportions that differ significantly with a p-value under 0.05 (in bold).

All 204 participants were tested with FujiLAM. Thirty-six (17.6%) were FujiLAM positive, 164 (80.4%) FujiLAM negative, and four (2.0%) had invalid results (Table 2). FujiLAM identified a similar proportion of bacteriologically confirmed individuals by HIV status, and was positive in 30 (66.7%) of 45 patients with bacteriologically confirmed TB, including 23 (65.7%) of the 35 HIV negative and seven (70%) of the ten HIV positive patients ($p = 0.56$). Among the 159 participants with no TB (culture and Xpert MTB/RIF negative), FujiLAM was positive in six (3.8%), invalid in four (2.5%), and negative in 149 (93.7%). Ninety-seven (99%) of the 98 HIV negative and 56 (93.3%) of the 60 HIV positive participants had negative/invalid FujiLAM results. The overall FujiLAM sensitivity and specificity were 66.7% (30/45) (95% CI = 51–80) and 96.2% (153/159) (95% CI = 92–98) among all participants, varying from 65.7% (95% CI = 48–80) and 99.0% (95% CI = 94–100) for HIV negative and 70.0% (95% CI = 35–92) and 93.3% (95% CI = 83–98) for HIV positive patients, respectively (Table 3). Positive and negative predictive values are also shown in Table 3 for patients with bacteriologically confirmed TB (Xpert or culture positive) and patients with no TB (Xpert and culture negative). The positive predictive value was higher among HIV negative patients (96%) than HIV positive (63%), and the negative predictive value was higher among HIV positive (94%) than HIV negative patients (89%). However, the differences were not statistically significant.

FujiLAM results had a similar pattern when analyzed using culture or Xpert MTB/RIF results singly, as shown in Tables 2 and 3, or when disaggregated by gender. FujiLAM results by Xpert MTB/RIF grades and HIV status are shown in Table 2. Although the numbers were too small for statistical analysis, the proportion of participants with positive FujiLAM seemed to be higher among HIV negative participants with high Xpert grades (eight (73%) of 11) than among participants with very low Xpert/RIF grades (four (57%) of seven). However, this pattern was not observed among participants with HIV, as all six HIV positive participants were FujiLAM positive, independently of their Xpert MTB/RIF grade (Table 3). FujiLAM by HIV viral load is shown in Table 4 for bacteriologically confirmed, culture, or Xpert MTB/RIF positive participants and Xpert grade. Most of the patients had high HIV viral loads. The sensitivity seemed higher among patients with a high viral load, but the numbers were too small to conduct a statistical analysis.

Table 2. FujiLAM test results by TB and HIV status.

	All			HIV Negative		HIV Positive		
FujiLAM	**Negative** *n* (%)	**Positive** *n* (%)	**Invalid** *n* (%)	**Negative** *n* (%)	**Positive** *n* (%)	**Negative** *n* (%)	**Positive** *n* (%)	**Invalid** *n* (%)
Bact + TB [a]	15 (33.3%)	30 (66.7%)	0 (0.0%)	12 (34.3%)	23 (65.7%)	3 (30.0%)	7 (70.0%)	0 (0.0%)
(B +) male	9 (34.6%)	17 (65.4%)	0 (0.0%)	7 (35.0%)	13 (65.0%)	2 (33.3%)	4 (66.7%)	0 (0.0%)
(B +) female	6 (31.6%)	13 (68.4%)	0 (0.0%)	5 (33.3%)	10 (66.7%)	1 (25.0%)	3 (75.0%)	0 (0.0%)
Culture pos	11 (29.7%)	26 (70.3%)	0 (0.0%)	9 (30.0%)	21 (70.0%)	2 (28.6%)	5 (71.4%)	0 (0.0%)
Xpert pos	11 (27.5%)	29 (72.5%)	0 (0.0%)	11 (32.4%)	23 (67.6%)	0 (%)	6 (100%)	0 (0.0%)
High	3 (23.1%)	10 (76.9%)	0 (0.0%)	3 (27.3%)	8 (72.7%)	0 (%)	2 (100%)	0 (0.0%)
Medium	5 (29.4%)	12 (70.6%)	0 (0.0%)	5 (35.7%)	9 (64.3%)	0 (%)	3 (100%)	0 (0.0%)
Low	0 (%)	3 (100%)	0 (0.0%)	0 (0.0%)	2 (100%)	0 (%)	1 (100%)	0 (0.0%)
Very Low	3 (42.9%)	4 (57.1%)	0 (0.0%)	3 (42.9%)	4 (57.1%)	0 (%)	0 (0.0%)	0 (0.0%)
Culture neg	143 (92.2%)	8 (5.2%)	4 (2.6%)	92 (97.9%)	2 (2.1%)	51 (85.0%)	5 (8.3%)	4 (6.7%)
Culture cont	10 (83.3%)	2 (16.7%)	0 (0.0%)	8 (88.9%)	1 (11.1%)	2 (66.7%)	1 (33.3%)	0 (0.0%)
Xpert neg	153 (93.3%)	7 (4.3%)	4 (2.4%)	98 (99.0%)	1 (1.0%)	55 (85.9%)	5 (7.8%)	4 (6.3%)
Not TB [b]	149 (93.7%)	6 (3.8%)	4 (2.5%)	97 (99.0%)	1 (1.0%)	52 (86.7%)	4 (6.7%)	4 (6.7%)
Total	164 (80.4%)	36 (17.6%)	4 (2.0%)	109 (82.0%)	24 (18.0%)	55 (78.6%)	11 (15.7%)	4 (5.7%)

[a] Positive culture and/or Xpert. [b] Negative culture and Xpert.

Table 3. Sensitivity and specificity of FujiLAM test by HIV status.

	All		HIV Negative		HIV Positive	
	Sensitivity [*n*/N, %, 95% CI]	**Specificity [*n*/N, %, 95% CI]**	**Sensitivity [*n*/N, %, 95% CI]**	**Specificity [*n*/N, %, 95% CI]**	**Sensitivity [*n*/N, %, 95% CI]**	**Specificity [*n*/N, %, 95% CI]**
Bact + TB [a]	30/45, 66.7%, 51–80	153/159, 96.2%, 92–98	23/35, 65.7%, 48–80	97/98, 99.0%, 94–100	7/10, 70.0%, 35–92	56/60, 93.3%, 83–98
Culture	26/37, 70.3%, 53–84	147/155, 94.8%, 90–98	21/30, 70.0%, 50–85	92/94, 97.9%, 92–100	5/7, 71%, 31–95	55/60, 91.7%, 81–97
Xpert	29/40, 72.5%, 56–85	157/164, 95.7%, 91–98	23/34, 67.6%, 49–82	98/99, 99.0%, 94–100	6/6, 100%, 52–100	59/64, 92.2%, 82–97
	PPV [*n*/N, %, 95% CI]	**NPV [*n*/N, %, 95% CI]**	**PPV [*n*/N, %, 95% CI]**	**NPV [*n*/N, %, 95% CI]**	**PPV [*n*/N, %, 95% CI]**	**NPV [*n*/N, %, 95% CI]**
Bact + TB [a]	30/36, 83%, 67–94	153/173, 88%, 83–93	23/24, 96%, 79–99	97/109, 89%, 82–94	7/11, 63%, 31–89	56/59, 94%, 86–99

[a] Positive culture and/or Xpert. Specificity estimated considering not TB cases as not having TB. CI: confidence interval. PPV: Positive predictive value. NPP: Negative predictive value.

Table 4. Positive FujiLAM test results among HIV positive participants by TB status and HIV RNA viral load.

	HIV Viral Load				
	Undetected	**<40 cps/mL**	**40–10^7 cps/mL**	**Indeterminate**	**Missing**
Bact + TB [a]	1/1 (100%)	1/2 (50%)	3/4 (75%)	0/0 (0%)	2/3 (67%)
Culture pos	0/0 (0%)	1/2 (50%)	3/3 (100%)	0/0 (0%)	1/2 (50%)
Xpert pos	1/1 (100%)	1/1 (100%)	2/2 (100%)	0/0 (0%)	2/2 (100%)
High	0/0 (0%)	0/0 (0%)	1/1 (100%)	0/0 (0%)	1/1 (100%)
Medium	1/1 (100%)	0/0 (0%)	1/1 (100%)	0/0 (0%)	1/1 (100%)
Low	0/0 (0%)	1/1 (100%)	0/0 (0%)	0/0 (0%)	0/0 (0%)
Very Low	-	-	-	-	-
Culture neg	1/10 (10%)	0/14 (0%)	4/28 (14%)	0/3 (0%)	0/5 (0%)
Culture contaminated	0/1 (0%)	0/0 (0%)	0/1 (0%)	0/0 (0%)	1/1 (100%)
Xpert neg	0/10 (0%)	0/15 (0%)	5/30 (17%)	0/3 (0%)	0/6 (0%)
Not TB [b]	0/10 (0%)	0/14 (0%)	4/28 (14%)	0/3 (0%)	0/5 (0%)
Bact + TB					
Sensitivity	100%, 0.05–1	50%, 0.03–1	75%, 0.2–1	0%	67%, 0.1–1
Not TB					
Specificity	100%, 0.7–1	100%, 0.7–1	86%, 0.7–1	100%, 0.3–1	100%, 0.5–1

[a] Positive culture and/or Xpert. [b] Negative culture and Xpert.

4. Discussion

We have evaluated the diagnostic accuracy of FujiLAM in urine samples of adults with signs and symptoms suggestive of TB attending three district hospitals in Abuja, Nigeria. We found a sensitivity and specificity of 65.7% and 99.0% among HIV negative patients and 70.0% and 93.3% among HIV positive patients. These results are higher than reported for the rapid LAM test AlereLAM, which has a reported sensitivity of 42% (95% CI = 31–55) among patients with HIV [13]. AlereLAM is recommended by the WHO for the complementary diagnosis of TB in patients with HIV, but not for HIV negative patients due to its low sensitivity in immune-competent individuals [13].

The overall sensitivity of FujiLAM in HIV positive patients was slightly higher than in HIV negative participants, but this difference was based on a small number of patients, and was not statistically significant. However, the higher sensitivity observed among immunosuppressed individuals with HIV viral loads between 40 and 10^7 copies/mL (75.0%), was similar to studies of HIV positive patients in Ghana and South Africa [11,14]. The higher sensitivity of LAM among HIV positive individuals is said to reflect the increased concentration of LAM in urine due to the hematogenic spread of TB to the kidneys in immunosuppressed individuals [15]. However, LAM detection in urine is not limited to renal involvement [16], and is likely to be associated with the total bacterial burden of *M. tuberculosis* and the severity of disease [17].

The overall sensitivity among HIV negative patients (65.7%) met the minimum target of 65% for the WHO high-priority, non-sputum-based TB diagnostic tests [18]. Our results were higher than reported in a multicenter study of HIV negative patients in Peru and South Africa, which might be explained by the characteristics of the participants. FujiLAM is said to have higher sensitivity in patients with more advanced TB and higher *M. tuberculosis* loads in culture [19], and, as patients in Nigeria were recruited from district hospitals, a high proportion of our participants may have had advanced disease stages.

FujilAM specificity among HIV negative patients was very high (99%) and similar to other studies among HIV negative patients [18,19]. Specificity among HIV positive patients was marginally higher (93.3%) than reported by a study of bio-banked urine samples from HIV positive patients in three Sub-African countries [14], which reported a 90.8% specificity [11]. Patients with HIV experience multiple opportunistic infections, and positive FujiLAM results may well be false positives. However, it is also possible that these results reflect the difficulty of confirming the diagnosis in patients with disseminated TB disease. Culture and WRDs are imperfect tests, which have a lower performance in patients with HIV, extra-pulmonary, and disseminated TB, depending on the quality of the sample and whether the patient is excreting bacilli the day of sampling, and it is possible that patients who do not reach microbiological confirmation may have a missed TB diagnosis [20]. Our six patients with positive urine LAM results but negative sputum tests had the same clinical presentation as patients with bacteriologically confirmed TB. As it is likely that some patients with TB do not have bacilli in sputum in the absence of cavitation or when the disease is disseminated without communications with the airways (e.g., military TB), we cannot rule out that these patients may have been detected by a test based in urine that does not require expectoration of bacilli, and further studies are needed to confirm whether these were true or false positive results. However, it is also true that LAM is a cell wall compound that is present in most Mycobacteria species, and is not exclusive in *M. tuberculosis*. The FujiLAM assays combine high-affinity monoclonal antibodies directed towards the largely *M. tuberculosis*-specific MTX-LAM epitopes, which are expected to increase the sensitivity of the assay by using a silver-based amplification step without affecting the specificity. Despite the potential cross reactivity risk, we feel that the assay would be useful in the field, as it would allow the rapid identification of about two-thirds of patients with TB at the time of the first consultation. The assays would need to be incorporated into diagnostic algorithms, as patients would need to undergo further confirmatory tests to confirm the presence of MTB and then be screened for drug resistance.

Urine is a readily available, non-invasive sample, which would be especially useful in patients unable to expectorate, such as children, the elderly, and adults without a productive cough and individuals with disseminated, extra-pulmonary, and non-cavitary disease. Unfortunately, the information regarding whether the patients included in our study presented cavitary disease or not was not available with the data we collected. FujiLAM is simple to use, does not require sputum samples, which minimizes the risk of aerosols, does not require additional instrumentation, and can be used in decentralized laboratories [21]. Therefore, FujiLAM is a promising test for the early detection and treatment of TB in people with signs and symptoms suggestive of TB, with a particular relevance for low resource health centers in low- and middle-income countries with the highest burden of TB.

Our study has several limitations. We only included patients able to provide sputum, which may underestimate the potential of FujiLAM to identify patients who are difficult to diagnose. In addition, the number of bacteriologically confirmed TB cases is small, especially for patients with HIV, and we were unable to follow-up with participants, which could have re-classified some individuals with negative culture and Xpert MTB/RIF tests as positive, potentially increasing the specificity.

An important issue for the wider use of FujiLAM is that the test costs are currently too high for its wider implementation outside of a research setting. FujiLAM is likely to be most useful in locations with limited resources, and tiered pricing mechanisms will be needed to facilitate access to the tests according to need. Moreover, further cost effectiveness studies are needed. A study in South Africa and Malawi examining patients with HIV reported that FujiLAM combined with Xpert MTB/RIF was more cost effective than using Xpert MTB/RIF alone [22]. However, more studies are needed to assess its cost effectiveness in HIV negative patients, especially at lower levels of the health system in low resource settings.

Despite the development and scale-up of newer, more sensitive TB diagnostic tests over the last decade, these have not lived up to their early promise, because they remain too slow, expensive, and resource-intensive (liquid culture), have been mostly implemented centrally, or have been found to have high diagnostic accuracy only among a subset of patients (e.g., urine LAM for severe HIV-associated illness). However, there is an emerging pipeline of new TB tests and tools that could allow rapid, accurate, point-of-need diagnosis. These tests are probably insufficient when used individually, but their performance could be optimized when used in combination as novel diagnostic algorithms. This is the case of the new generation urine LAM rapid test that we have studied: the FUJILAM test. Although the test has limited sensitivity, it would be able to detect two thirds of patients with bacteriologically confirmed TB at the time of the first consultation. Future research, therefore, should evaluate the potential of FujiLAM in immunocompetent adults and children attending primary healthcare facilities and in patients with extrapulmonary and non-cavitary TB. Further studies are also needed to develop diagnostic algorithms that incorporate drug susceptibility testing for patients identified by FujiLAM at the lower levels of the healthcare system, and whether its combination with other screening tests, such as C Reactive Protein, could be used to develop point-of-care diagnostic algorithms.

There is also a clear need of new technology and stronger efforts in the development, validation, and market shaping initiatives to expand the use of these devices. FujiLAM manufacturing facilities are currently being expanded, and its manufacturer plans to apply for WHO endorsement in 2022. Other LAM prototype developers are also making strides to develop low-cost lateral flow assays with improved monoclonal antibodies, which are expected to become available as research use-only prototypes in 2022, while novel LAM concentration methods are being tested in the field with preliminarily good performance.

In conclusion, testing urine samples with FujiLAM in HIV positive and HIV negative patients with presumptive TB has a higher performance than current urine LAM assays. The use of the FujiLAM test would facilitate the detection and initiation of TB treatment on the same day of consultation in primary health centers.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Research Ethics Committees of the Liverpool School of Tropical Medicine (protocol 17-014; 26 April 2017), the Nigerian National Ethics Committee (protocol NHREC/21/95/2005/00463; 30 March 2017) and Ethics Committee of the Hospital Universitari Germans Trias i Pujol (Protocol PI-16-061; 9 June 2017).

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy reasons.

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Article 3

Diagnostic Performance of the Fujifilm SILVAMP TB-LAM in Children with Presumptive Tuberculosis

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Diagnostic Performance of the Fujifilm SILVAMP TB-LAM in Children with Presumptive Tuberculosis

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Abstract: Current diagnostics for tuberculosis (TB) only manage to confirm a small proportion of children with TB and require respiratory samples, which are difficult to obtain. There is a need for non-invasive biomarker-based tests as an alternative to sputum testing. Fujifilm SILVAMP TB lipoarabinomannan (FujiLAM), a lateral-flow test to detect lipoarabinomannan in urine, is a novel non-sputum-based point-of-care diagnostic reported to have increased sensitivity for the diagnosis of TB among human immunodeficiency virus (HIV)-infected adults. We evaluate the performance of FujiLAM in children with presumptive TB. Fifty-nine children attending a paediatric hospital in Haiti with compatible signs and symptoms of TB were examined using Xpert MTB/RIF, smear microscopy and X-rays, and classified according to the certainty of diagnosis into bacteriologically confirmed TB (n = 5), unconfirmed TB (bacteriologically negative, n = 50) and unlikely TB (n = 4). Healthy children (n = 20) were enrolled as controls. FujiLAM sensitivity and specificity were 60% and 95% among children with confirmed TB. FujiLAM's high specificity and its characteristics as a point-of-care indicate the test has a good potential for the diagnosis of TB in children.

Keywords: tuberculosis; diagnosis; lipoarabinomannan; LAM; children; urine; point-of-care

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1. Introduction

Tuberculosis (TB) is a major cause of morbidity and mortality in children [1]. However, the paucibacillary nature of TB in children and the difficulty of obtaining respiratory samples create significant barriers for diagnosis [2]. Most children with TB, especially in countries with high TB burden [3], are not bacteriologically confirmed, or are missed [3]. The lack of reliable diagnostic methods has highlighted the need for non-sputum-based tests for childhood TB [4,5].

In recent years, attempts have been made to develop diagnostic tests based on the detection of lipoarabinomannan (LAM) antigen, a lipoglycan and virulence factor in the bacteria genus *Mycobacterium* present in the outer cell wall, which is released from

metabolically active or degenerating bacterial cells. As LAM is heat stable, filtered by the kidney and detectable in the urine of people with overt TB, test prototypes have attempted to identify LAM in urine since the 1930s [6,7]. Until recently, urine-based LAM prototypes have had low sensitivity and performed better in individuals co-infected with human immunodeficiency virus (HIV) and advanced immunosuppression [8]. Although the biological mechanisms for the better performance in HIV-positive individuals are not fully understood, the higher concentration of LAM in the urine could be due to a greater difficulty in containing *Mycobacterium tuberculosis* with subsequent hematogenous spread to the kidneys or to a higher body burden of *M. tuberculosis* [9–12]. Compared to the Alere Determine TB-LAM assay, with a reported sensitivity of about 40% for detecting TB in HIV-infected patients [13], a new assay is reported to have higher sensitivity to detect LAM in both HIV-infected and uninfected patients [14,15]. The assay, the Fujifilm SILVAMP TB LAM (FujiLAM, Fujifilm, Tokyo, Japan), combines high-affinity monoclonal antibodies against *M. tuberculosis*-specific LAM epitopes and a silver amplification step to increase the visibility of the test and control lines [15]. However, data on its performance in children is limited, and we report here its performance in a case series of children with signs and symptoms of presumptive TB. TB in children has more disseminated clinical presentations than adults because of their poor containment of TB. In addition, children have difficulty in expectorating sputum, and assays that use non-sputum samples could be useful, particularly in young children [2,11,16–18].

2. Materials and Methods

This was a retrospective case series of the bio-banked urine samples of 59 children with signs and symptoms compatible with TB, and of 20 healthy control children. Children were enrolled prospectively between August 2015 and December 2016 [19]. The children with presumptive TB were between 0 and 14 years old, and were enrolled when attending the Saint Damien paediatric reference hospital in Port-au-Prince, Haiti. Children were initially identified by the diagnostic clinics and enrolled opportunistically. This is a case series, as we did not estimate the number of children required and instead enrolled as many children being investigated for TB as possible over a period of sixteen months. The healthy children, enrolled as controls, were attending a primary school in the same neighbourhood of the hospital and were between 5 and 8 years old. The objective of including a control group was to explore whether any of the children without symptoms of TB had a positive FujiLAM test to better characterize the specificity of the test.

After obtaining informed parental consent, we collected clinical and demographic data, vaccination history (including Bacillus Calmette-Guérin (BCG)) and information about current and previous medications. Children were excluded if they had known immunodeficiencies, were receiving immunosuppressive treatment, or if they had received anti-TB treatment for two or more weeks before enrolment. Tuberculin skin tests (TSTs) were performed by a trained technician using 0.1 mL of Tubersol (bioequivalent to 5 Tuberculin Units; Sanofi Pasteur, Canada) and were considered TST positive if they had $\geq$10 mm induration in the presence of a BCG scar or $\geq$5 mm in non-BCG-vaccinated children with no known contact with adults with TB [20]. Three millilitres of blood was obtained for the QuantiFERON-TB Gold In-Tube test (QFT-GIT, Qiagen, Germany [21]), following the manufacturer's instructions.

Children with presumptive TB underwent anterior–posterior chest X-rays, which were read by two radiologists blinded to the child's condition, and a third reader resolved reading disagreements. Three consecutive sputum samples obtained by induced or nasopharyngeal/nasogastric aspiration were examined by fluorescence smear microscopy stained with auramine, and children with positive smear microscopy or abnormal X-rays underwent Xpert MTB/RIF (Cepheid, Sunnydale, CA, USA) following local diagnostic algorithms. No culture facilities were available. Children with lymph node adenopathy underwent biopsies for histological examination.

Children with presumptive TB were classified using the clinical case definitions into confirmed, unconfirmed, and unlikely TB [22]. Children were classified as having confirmed TB if bacteriologically confirmed by Xpert MTB/RIF; as unconfirmed TB if there was no bacteriological confirmation but a positive TST or QFT-GIT and at least one of the clinical criteria of the Clinical Case Definitions (i.e., X-rays consistent with TB, signs and symptoms of TB, close TB exposure, or positive response to TB treatment), or at least two clinical criteria if the TST and QFT-GIT results were negative; and unlikely TB if the child had only evidence of *M. tuberculosis* infection or presented only one clinical criterion compatible with TB. Enrolled school children had a negative TST and QFT-GIT, and no signs or symptoms of TB. Those with positive TST or symptoms compatible with TB were referred to the hospital for TB screening and were not included as controls.

Participants were asked to provide a midstream urine sample on-site, collected in sterile plastic containers, or in urine bags for children <1 year, and stored at room temperature if they were processed at the time of collection, or stored in a refrigerator if processed in batches at the end of the day. Urine samples were aliquoted (2 mL) into cryovials and kept at −20 °C until processing in Badalona, Spain (IGTP). To test with FujiLAM, urine samples were thawed at room temperature, mixed with a vortex, and processed following the manufacturer's instructions steps: first, the reagent tube was filled with urine up to the indicator line, mixed and left to incubate for 40 min at room temperature. The tube was then mixed, two drops of urine were added to the test device, and the first button was pressed. After 3–10 min of incubation, the second button was pressed to release the silver ions around the gold-conjugated antibody. The results were then read after one minute by two investigators blinded to the participants' TB status, following the manufacturer's instructions. If readers disagreed on the presence of test lines, the test was repeated once.

Categorical variables were described by frequencies and percentages, and quantitative variables described using means and 95% confidence intervals (CIs) if normally distributed, and medians and interquartile ranges (IQRs) if not normally distributed. We used Fisher's exact test, chi-square and Kruskal–Wallis tests to compare between groups, and p-values < 0.05 were considered statistically significant. The sensitivity, specificity, and predictive values of FujiLAM were estimated using Xpert MTB/RIF as the microbiological reference standard, and against a composite of the combination of clinical diagnosis and Xpert MTB/RIF. Diagnostic accuracy was calculated for microbiologically confirmed cases vs. cases with unconfirmed TB, unlikely TB or controls, and for the composite reference standard vs. unlikely TB and controls. Statistical analysis was performed using SPSS (SPSS version 26.0, SPSS Inc, Chicago, IL, USA). The World Health Organization (WHO) Anthro 3.2.2 (<5 years) and Anthro Plus 1.0.4 (≥5 years) were used to estimate z-scores [23,24]. Undernutrition was classified as a weight-for-age z-score < −2 (or as body mass index-for-age z-score < −2 for children above 10 years) and stunting as a height-for-age z-score < −2.

Informed consent was obtained from the parents or legal guardians. The study was approved by the Ethics Committee of the University of Barcelona and the Haiti National Ethics Committee (reference number IRB00003099).

3. Results

Eighty-two children were enrolled, of which three were excluded due to insufficient urine volumes. Of the 59 with presumptive TB, 5 were bacteriologically confirmed, 50 had unconfirmed but clinically diagnosed TB, and 4 were deemed not to have TB. The additional 20 were healthy controls. Demographic characteristics of the study groups are shown in Table 1. Fifty-one (64.6%) children were male, with a median age of 76 (IQR 58–121) months. Their median body mass index was 14.80 (SD ± 2.4); eighteen (30.5%) reported recent and severe weight loss and twelve (20.7%) reported stunted growth. Fifty-six (75.7%) had received the BCG vaccine and had a BCG scar. FujiLAM results are shown in Table 2. Eight (10.1%) samples were positive and seventy-one (89.9%) negative (Table 2). Three of the positive samples belonged to children with bacteriologically confirmed TB, three to children with unconfirmed TB, one to a child with unlikely TB and one to a

control. Among the six FujiLAM-positive children with bacteriologically confirmed and unconfirmed TB, four had intrathoracic and one both intrathoracic and TB spondylitis. If Xpert MTB/RIF was considered the reference standard, the sensitivity of FujiLAM was 60% (95% CI 17–93) and the specificity, considering the control group, 95% (95% CI 73–100). If confirmed and unconfirmed TB were compared against unlikely TB and controls, the sensitivity was 11% (95%CI 5–23) and the specificity 92% (95% CI 72–99). Five (71.4%) of the seven FujiLAM-positive children with presumptive TB were underweight and four (66.7%) were stunted ($p = 0.023$ and $p = 0.014$, respectively). The one case of disagreement of FujiLAM assays between the two readers was resolved after repeating the test.

Table 1. Demographic and clinical characteristics of participants.

	Overall (n = 79)	Confirmed TB (n = 5)	Unconfirmed TB (n = 50)	Unlikely TB (n = 4)	Controls (n = 20)	p-Value
Female	28 (35%)	1 (20%)	21 (42%)	1 (25%)	5 (25%)	0.530
Male	51 (65%)	4 (80%)	29 (58%)	3 (75%)	15 (75%)	
Median age (IQR) months	76 (58–121)	95 (51–128)	76 (51–122)	152 (98–165)	70 (58–94)	0.109
<5 yrs.	24 (30%)	1 (20%)	16 (32%)	0 (0%)	7 (35%)	0.654
≥5 yrs.	55 (70%)	4 (80%)	34 (68%)	4 (100%)	13 (65%)	
BCG scar (n = 74)	56 (76%)	3 (60%)	35 (78%)	2 (50%)	16 (80%)	0.390
TST or QFT-GIT positive	55 (93%)	5 (100%)	46 (92%)	4 (100%)	0 (0%)	1.000
TST positive	52 (88%)	4 (80%)	44 (88%)	4 (100%)	0 (0%)	0.707
QFT-GIT positive (n = 53)	37 (70%)	5 (100%)	28 (64%)	4 (100%)	0 (0%)	0.101
TB contact	50 (85%)	1 (20%)	45 (90%)	4 (100%)	0 (0%)	0.002
Cough	48 (81%)	5 (100%)	39 (78%)	4 (100%)	0 (0%)	0.491
Fever	40 (68%)	4 (80%)	34 (68%)	2 (50%)	0 (0%)	0.718
Lethargy	4 (7%)	3 (60%)	1 (2%)	0 (0%)	0 (0%)	0.002
Weight loss (n = 56)	19 (34%)	3 (60%)	15 (32%)	1 (25%)	0 (0%)	0.517
Adenopathy	22 (37%)	4 (80%)	18 (36%)	0 (0%)	0 (0%)	0.046
Underweight (n = 74)	18 (31%)	3 (60%)	15 (30%)	0 (0%)	0 (0%)	0.201
Stunted (n = 58) [a]	12 (21%)	2 (40%)	9 (18.4%)	1 (25%)		0.418
X-ray consistent with TB	28 (46%)	5 (100%)	23 (46%)	0 (0%)	NA	0.161
Positive smear-microscopy (n = 52)	10 (19%)	3 (60%)	7 (16%)	0 (0%)	0 (0%)	0.062
Treatment completed	50 (85%)	5 (100%)	45 (90%)	0 (0%)	NA	<0.001
Lost to follow-up	8 (14%)	0 (0%)	4 (8%)	4 (100%)	NA	
Died	1 (2%)	0 (0%)	1 (2%)	0 (0%)	NA	
Intrathoracic	18 (31%)	2 (40%)	16 (32%)	0 (0%)	NA	<0.001
Extra-thoracic	7 (12%)	0 (0%)	7 (14%)	0 (0%)	NA	
Both	4 (7%)	3 (60%)	1 (2%)	0 (0%)	NA	
Not defined	30 (51%)	0 (0%)	26 (52%)	4 (100%)	NA	

IQR: interquartile range; BCG: Bacillus Calmette-Guérin; TST: tuberculin skin test; QFT: QuantiFERON-TB Gold. Malnutrition defined as weight-for-age z-score < −2 for children under 10 years old and body mass index -for-age z-score < −2 for children above 10 years. Stunting was defined as height-for-age < −2. [a] Controls have no results on stunting because height measurements were not taken.

Table 2. FujiLAM results by study group.

	FujiLAM		
	Positive n (%)	Negative n (%)	Total
Confirmed TB	3 (60%)	2 (40%)	5
Unconfirmed TB	3 (6%)	47 (94%)	50
Unlikely TB	1 (25%)	3 (75%)	4
Controls	1 (5%)	19 (95%)	20
All	8 (10.1%)	71 (89.9%)	79

4. Discussion

We evaluated the FujiLAM test in a case series of children attending the hospital with signs and symptoms of presumptive TB. Among children with bacteriologically

confirmed TB, the test had a sensitivity of 60%, with a high specificity of 95%. However, the case series is small, which resulted in wide and overlapping 95% CI, and therefore larger studies are needed to confirm our findings. The current LAM assay (AlereLAM) recommended by the WHO in adults [8] and children [25,26] is only recommended for severely immunosuppressed adults with HIV and CD4 counts <100 cells/μL [8]. Therefore, a test that performs well in HIV-uninfected patients would be a significant improvement. Unlike its predecessor, FujiLAM combines a pair of high-affinity monoclonal antibodies targeting largely *M. tuberculosis*-specific LAM epitopes and a silver amplification step to improve the visibility of the test and control lines, allowing the detection of much lower LAM concentrations in urine [6,15]. Furthermore, the FujiLAM test identified three more children who had a clinical diagnosis of TB but could not be bacteriologically confirmed, which would have lent more support to the decision to treat.

Children have a high risk of developing disseminated forms of TB, and the hematogenous and lymphatic spread of *M. tuberculosis* is likely to result in high amounts of LAM in urine [2,9]. However, few studies have reported the performance of FujiLAM in children [27,28]. A multicentre study in four African countries reported a relatively high sensitivity of 67.5% in HIV-negative children [28], but a cohort study in South Africa reported a lower sensitivity of 38.8% [27]. The authors concluded that the differences in their results may have been due to the severity of the TB episodes, and that some of the studies included a high proportion of malnourished children [28]. It has been reported that LAM detection in urine is associated to the overall amount of *M. tuberculosis* in the body, as well as the severity of the disease [10,29]. In our study, sensitivity was higher among bacterially confirmed cases, who are likely to have more advanced disease stages, while sensitivity was lower when children with a clinical diagnosis were included. It is unlikely that all children with a clinical diagnosis of TB had TB (and thus some were misclassified), and it is thus difficult to classify a negative FujiLAM test as false negative. Furthermore, the number of positive cases was proportionally higher among underweight and stunted children [30–32]. Malnutrition has an impact in the several faces of the immune response against infections, including TB; this limits the contention capacity of the bacilli, increasing the amount of LAM in urine samples and, therefore, the sensitivity of the LAM tests [28].

FujiLAM specificity was high, which is consistent with previous findings in adults and children using microbiological (95%) and composite reference standards (91.7%) [10,27,28,33,34]. Although the test did not reach the 98% specificity recommended by the WHO, our sample size is not sufficiently powered to make this distinction and the 95% CI overlap with this value [35].

The FujiLAM's main advantage is its urine-based format, making an easy to do and non-invasive test without the need for additional instrumentation [10], generating opportunities to diagnose TB in patients who do not excrete bacilli, such as people with extrapulmonary or military TB, HIV and children [4]. Especially among children, the test may be able to add value to clinical decision-making as a rule in test for TB, in our study effectively doubling the number of children treated who had confirmation of TB disease. To our knowledge, there are no studies evaluating the economic impact of LAM testing within the diagnostic algorithms for paediatric TB [36]. The inclusion of a test with the performance of the FujiLAM assay could simplify these algorithms and potentially diagnose a greater number of children with TB and increase the number of children initiating specific therapy [37]. Our study has significant limitations, including its small sample size—especially the small number of children with bacteriologically confirmed TB, which resulted in underpowered estimates. In addition, we did not culture the samples, and Xpert MTB/RIF and clinical diagnosis were used as reference standards. Xpert MTB/RIF is known to have lower sensitivity than culture [22], and thus it is likely it underestimated the number of children with bacteriologically confirmed TB. Other limitations concern retrospective testing of stored samples; however, studies using the FujiLAM test have shown good agreement between fresh and frozen urine samples [31,38].

In conclusion, FujiLAM had moderate sensitivity and high specificity in children compared to Xpert MTB/RIF, can process samples that are easier to collect than sputum, and can add a level of certainty to clinical diagnosis. These performance characteristics and the test's operational characteristics would facilitate a rapid diagnosis in non-sputum samples in children—especially in low- and middle-income countries, where facilities available are limited. More extensive evaluations in children are warranted.

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Informed Consent Statement: Informed consent was obtained from the parents or legal guardians of all children included in the study.

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Article 4

Discovery and validation of an NMR-based metabolomic profile in urine as TB biomarker

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Discovery and validation of an NMR-based metabolomic profile in urine as TB biomarker

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Despite efforts to improve tuberculosis (TB) detection, limitations in access, quality and timeliness of diagnostic services in low- and middle-income countries are challenging for current TB diagnostics. This study aimed to identify and characterise a metabolic profile of TB in urine by high-field nuclear magnetic resonance (NMR) spectrometry and assess whether the TB metabolic profile is also detected by a low-field benchtop NMR spectrometer. We included 189 patients with tuberculosis, 42 patients with pneumococcal pneumonia, 61 individuals infected with latent tuberculosis and 40 uninfected individuals. We acquired the urine spectra from high and low-field NMR. We characterised a TB metabolic fingerprint from the Principal Component Analysis. We developed a classification model from the Partial Least Squares-Discriminant Analysis and evaluated its performance. We identified a metabolic fingerprint of 31 chemical shift regions assigned to eight metabolites (aminoadipic acid, citrate, creatine, creatinine, glucose, mannitol, phenylalanine, and hippurate). The model developed using low-field NMR urine spectra correctly classified 87.32%, 85.21% and 100% of the TB patients compared to pneumococcal pneumonia patients, LTBI and uninfected individuals, respectively. The model validation correctly classified 84.10% of the TB patients. We have identified and characterised a metabolic profile of TB in urine from a high-field NMR spectrometer and have also detected it using a low-field benchtop NMR spectrometer. The models developed from the metabolic profile of TB identified by both NMR technologies were able to discriminate TB patients from the rest of the study groups and the results were not influenced by anti-TB treatment or TB location. This provides a new approach in the search for possible biomarkers for the diagnosis of TB.

Tuberculosis (TB) is the leading cause of death by infectious disease worldwide. Despite efforts to improve TB detection through advances in diagnosis and accessibility to treatment, there are still more than 2–3 million

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Variable	All (n = 332)	TB untreated (n = 49)	TB under treatment (n = 140)	PnP (n = 42)	LTBI (n = 61)	Uninfected (n = 40)
Gender						
Feminine	117 (35.2%)	10 (20.4%)	41 (29.3%)	8 (19.0%)	28 (45.9%)	30 (75.0%)
Masculine	215 (64.8%)	39 (79.6%)	99 (70.7%)	34 (81.0%)	33 (54.1%)	10 (25.0%)
Age in years						
Media (SD)	45.9 (17.3)	43.0 (20.3)	43.5 (16.3)	64.4 (13.5)	42.6 (10.3)	43.1 (17.1)

Table 1. Demographic information of the study population. Categorical variables expressed as a number of subjects (n) and percentage (%), and quantitative variables expressed as median and standard deviation (SD). TB, tuberculosis; PnP, pneumococcal pneumonia; LTBI, latent TB infection; Uninfected, individuals without infection.

unidentified TB cases at the moment. Misdiagnosis and late detection of the disease increase the risk of *Mycobacterium tuberculosis* transmission and infection. Progress in controlling TB and mitigating its consequences can be expedited through early diagnosis and treatment[1].

Among current diagnostics, culture is the gold standard for diagnosing TB, but it takes approximately 4–6 weeks to obtain results[2]. Therefore, in low-income countries where the burden of disease is high, smear microscopy and X-ray are the main tests used. However, these have shown limited diagnostic sensitivity and specificity, respectively[3,4]. The introduction of Xpert MTB/RIF (Cepheid, CA, USA) as a molecular method for TB diagnosis has considerably improved the time of diagnosis and detection of resistance to treatment, its use has increased as an alternative to culture and smear microscopy[5]. However, cost and infrastructure requirements prohibit its implantation and use in most microscopy centres[6]. In addition, its poor performance when testing individuals with low bacilli numbers (children, HIV-co-infected patients, extrapulmonary TB cases, early stages of disease), require consumables that are expensive or locally unavailable due to stock-outs[7]. Limitations of accessibility, quality and timing of diagnostic services in low and middle-income countries (LMICs) represent a challenge for current TB diagnostics. Future research should be focused on developing an accurate and rapid biomarker-based test that can diagnose all forms of TB using non-sputum samples, ideally one suitable for use in both primary healthcare centres and regional centres[8].

Metabolomics has emerged from the 'omics' technologies as a tool to obtain a fingerprint of all the metabolites present in a cellular system, allowing discrimination between samples with a different biological status[9]. In this approach, metabolomics has been applied to study the metabolites affected by host–pathogen interactions and identify diagnostic markers to improve diagnosis of different respiratory infectious diseases[10]. In recent years, metabolomic studies have been conducted to gain novel biological insights into TB pathogenesis[11]. Thus, metabolomics has been used to study TB progression and detect metabolic profiles, as well as to assess TB treatment response from different biological specimens[12]. Urine is an abundant sterile, biological sample that is obtained non-invasively and requires little preparation[13]. However, few metabolomic studies focus on the discovery of new urine-based biomarkers for TB detection. Metabolic changes in any type of sample can be measured through different analytical techniques summarised in mass spectrometry (MS) and Nuclear Magnetic Resonance (NMR)[14]. Recently, a benchtop NMR spectrometer has been developed as a potential tool for point-of-care diagnostics in urine samples due to its high performance in a compact size[15,16].

This study aimed to identify and characterise a metabolic profile of TB in urine by high-field NMR spectrometry and assess whether the TB metabolic profile is also detected by a low-field benchtop NMR spectrometer. The identification of a metabolic pattern for urine from an NMR technology would provide a new approach and advance in the search of potential biomarkers for TB diagnosis.

Results

Study population. Three hundred and thirty-two participants were included in this study and classified into the following study groups: 189 active TB patients, 42 pneumococcal pneumonia patients, 61 LTBI individuals, and 40 uninfected individuals. Demographics of the study population are shown in Table 1. Of the 332 patients, 64.8% were men of an average of 46 years old (± 17.3). Regarding study groups, most TB (untreated and under treatment) and pneumococcal pneumonia patients and individuals with LTBI were men (79.6%, 70.7%, 54.1%, and 81.0%, respectively), while 75.0% of the uninfected individuals were women. Patients with pneumococcal pneumonia were older than the rest of the study groups (49.6% older, $p < 0.001$). From the patients with active TB, 49 were enrolled before starting anti-TB treatment and 140 during TB treatment. Patients undergoing TB treatment averaged 39.1 (SD ± 68.9) days of treatment. Most patients (99.3%) had strains sensitive to TB treatment except one patient with rifampicin-resistant strains (Table 2). Among patients with TB, 78.8% had pulmonary TB, 13.8% had extrapulmonary TB (lymph nodal, pleural, peritoneal, osteoarticular, meningeal and miliary TB), and 7.4% had disseminated TB (Table 2).

Of the 332 urine samples obtained from the individual participants, 169 samples were analysed with HF-NMR to identify a metabolic profile that discriminated TB patients from study controls. Then, the remaining 163 samples plus 85 samples previously analysed by HF-NMR were analysed using an LF benchtop NMR spectrometer to detect the TB metabolic profile in a more compact device that can be installed in conventional laboratories and used as a diagnostic tool. The procedure followed to analyse urine samples by NMR is shown in Fig. 1.

Variable	All (n = 189)	TB untreated (n = 49)	TB under treatment (n = 140)
TB type			
Pulmonary TB	149 (78.8%)	37 (75.5%)	112 (80.0%)
Extrapulmonary TB	26 (13.8%)	10 (20.4%)	16 (11.4%)
Disseminated TB	14 (7.4%)	2 (4.1%)	12 (8.6%)
TB treatment			
Drug-susceptible TB	186 (98.4%)	47 (95.9)	139 (99.3%)
RR-TB	1 (0.5%)	0 (0.0%)	1 (0.7%)
Hr-TB	1 (0.5%)	1 (2.0%)	0 (0.0%)
XDR-TB	1 (0.5%)	1 (2.0%)	0 (0.0%)

Table 2. Table describing the location and treatment of TB patients. Variable expressed as a number of subjects (n) and percentage (%). TB: tuberculosis; RR-TB: rifampicin-resistant TB; Hr-TB: isoniazid-resistant TB; XDR-TB: extensively drug-resistant TB.

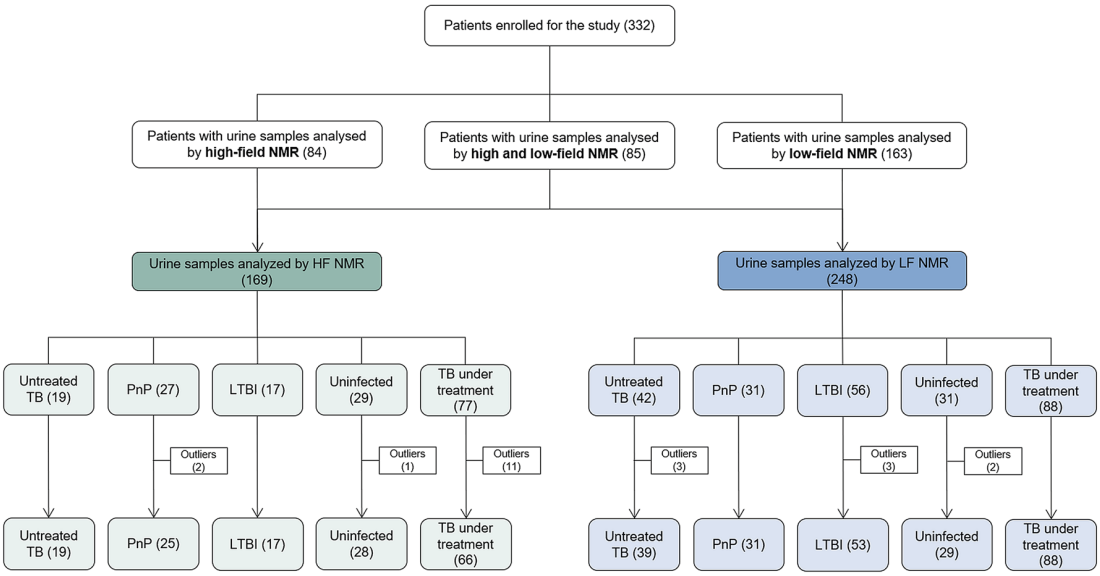

Figure 1. Description of the procedure followed to analyse the urine samples of the 332 participants by high and/or low NMR. NMR, Nuclear Magnetic Resonance; TB, tuberculosis; PnP, pneumococcal pneumonia; LTBI, latent TB infection; uninfected, individuals without infection.

Characterisation of the TB metabolic fingerprint using HF-NMR. We applied an unsupervised Principal Component Analysis (PCA) to the urine spectra acquired by HF-NMR (19 patients with untreated TB, 27 patients with pneumococcal pneumonia, 17 individuals with LTBI and 29 uninfected individuals) and detected 3 statistical outliers (2 patients with pneumococcal pneumonia and 1 uninfected individual) that were excluded from the analysis[17]. PCA score plots identified differential metabolic clusters between patients with untreated TB (n = 19) and pneumococcal pneumonia patients (n = 25), LTBI individuals (n = 17), and uninfected individuals (n = 28) (Fig. 2). The variability observed was not explained by the different participant recruitment centres (Supplementary Figure S1). The spectral regions responsible for the metabolic differences in each PCA scores plots between TB patients and control groups (pneumococcal pneumonia, LTBI, and uninfected) were identified in PCA loading plots by Hotteling's T2 tests[18] and correlated with a total of 31 chemical shift regions in the first two Principal Components (PCs) (Fig. 3). This urinary spectral fingerprint (corresponding to 31 spectral regions) was assigned to the following eight metabolites: aminoadipic acid, citrate, creatine, creatinine, glucose, mannitol, phenylalanine, and hippurate (Supplementary Figure S2). Metabolites were quantified to show the statistically substantial differences between study groups (Table 3).

HF-NMR-based profile to discriminate TB. We applied a supervised Partial Least Squares-Discriminant Analysis (PLS-DA) to establish predictive models from the identified spectral fingerprint that differentiated untreated TB cases (n = 19) from control groups (25 pneumococcal pneumonia, 17 LTBI, and 28 uninfected). Thus, when comparing untreated TB and pneumococcal pneumonia patients, 100% of TB patients were correctly classified, with 100.0% of sensitivity and specificity. Similarly, when comparing TB patients and LTBI individuals, 94.0% (Standard Deviation, SD = 5.6%) of TB patients were correctly classified, with 89.4% (SD = 6.5%) and

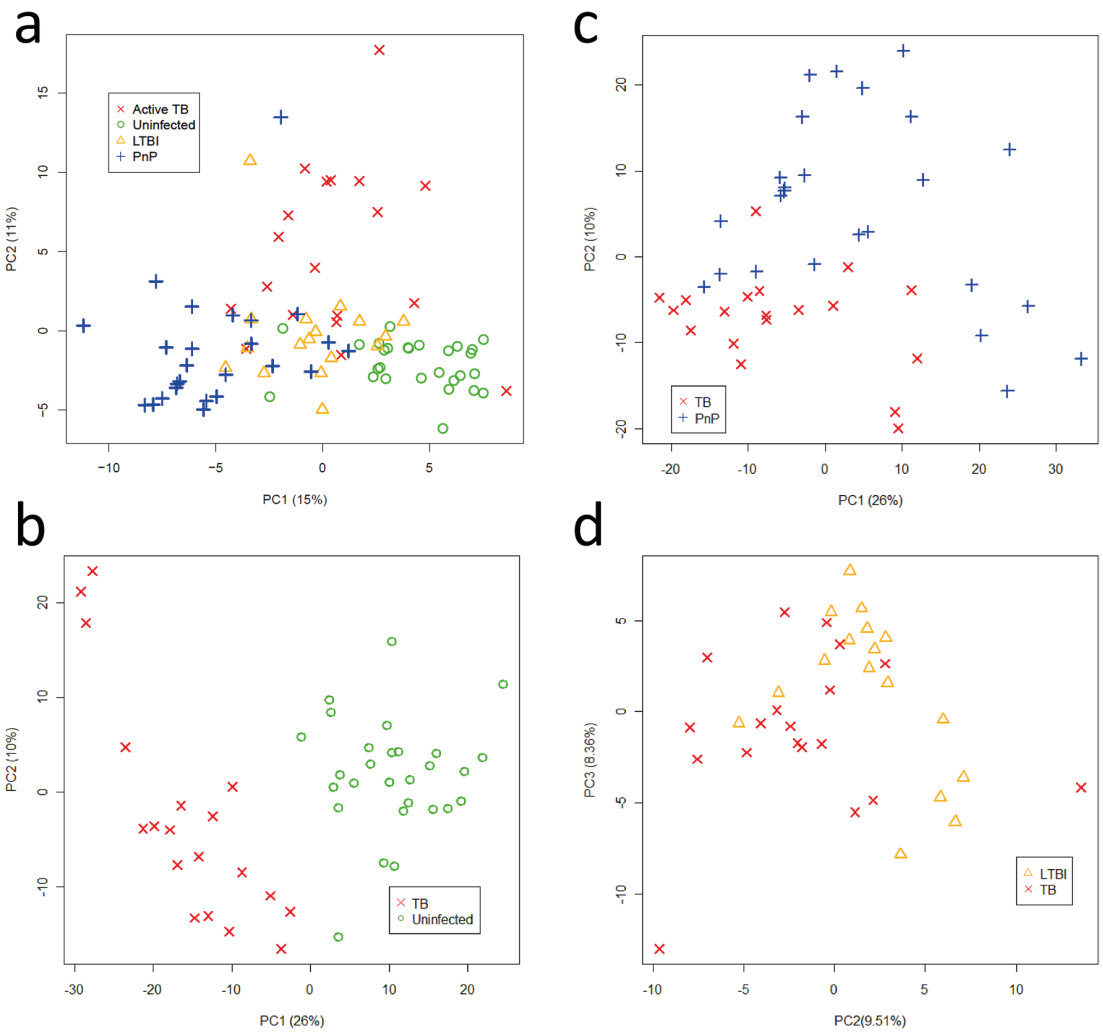

Figure 2. Principal Component Analysis (PCA) score plots of urine spectra analyzed by high-field Nuclear Magnetic Resonance of (**a**) untreated TB patients (n = 19), uninfected individuals (n = 28), pneumococcal pneumonia patients (n = 25) and LTBI individuals (n = 17); (**b**) untreated TB and uninfected individuals; (**c**) untreated TB and pneumococcal pneumonia patients; (**d**) untreated TB and LTBI individuals. TB, tuberculosis; PnP, pneumococcal pneumonia; LTBI: latent TB infection; PC, Principal Component.

93.9% (SD = 5.7%) sensitivity and specificity, respectively. Finally, when comparing TB patients and uninfected individuals, 100% of TB patients were correctly classified, with 100% of sensitivity and specificity, respectively.

We used the model established between untreated TB patients and uninfected individuals to classify the HF-NMR urine spectra of the remaining 66 TB patients (all of them under treatment). The model correctly classified 90.9% of these TB patients in the TB group. Among these TB patients, 51 had pulmonary TB, 11 had extrapulmonary TB, and 4 had disseminated TB. This model also correctly classified 90.9% of the extrapulmonary TB patients in the TB group.

Characterisation of the TB metabolic profile using LF-NMR. To detect the metabolic profile of TB in urine previously characterised using HF-NMR, we applied the unsupervised PCA to the urine spectra acquired by LF-NMR (42 patients with untreated TB, 31 patients with pneumococcal pneumonia, 56 individuals with LTBI and 31 uninfected individuals). We detected 8 statistical outliers (3 untreated TB, 3 LTBIs, and 2 uninfected), which were excluded from the analysis[17]. Thus, the unsupervised PCA was applied to a total of 39 untreated TB patients, 31 pneumococcal pneumonia patients, 53 LTBI individuals, and 29 uninfected individuals (Fig. 1). PCA score plots of the LF-NMR urine spectra did not show as clear a discrimination as the HF-data did (Fig. 4). However, although LF-NMR spectroscopy provides a lower resolution than HF-NMR spectroscopy, we identified the spectral fingerprint assigned to the eight metabolites (aminoadipic acid, citrate, creatine, creatinine, glucose, mannitol, phenylalanine, and hippurate) in the metabolic profile, which enabled the differentiation of patients with TB from the controls (Fig. 5).

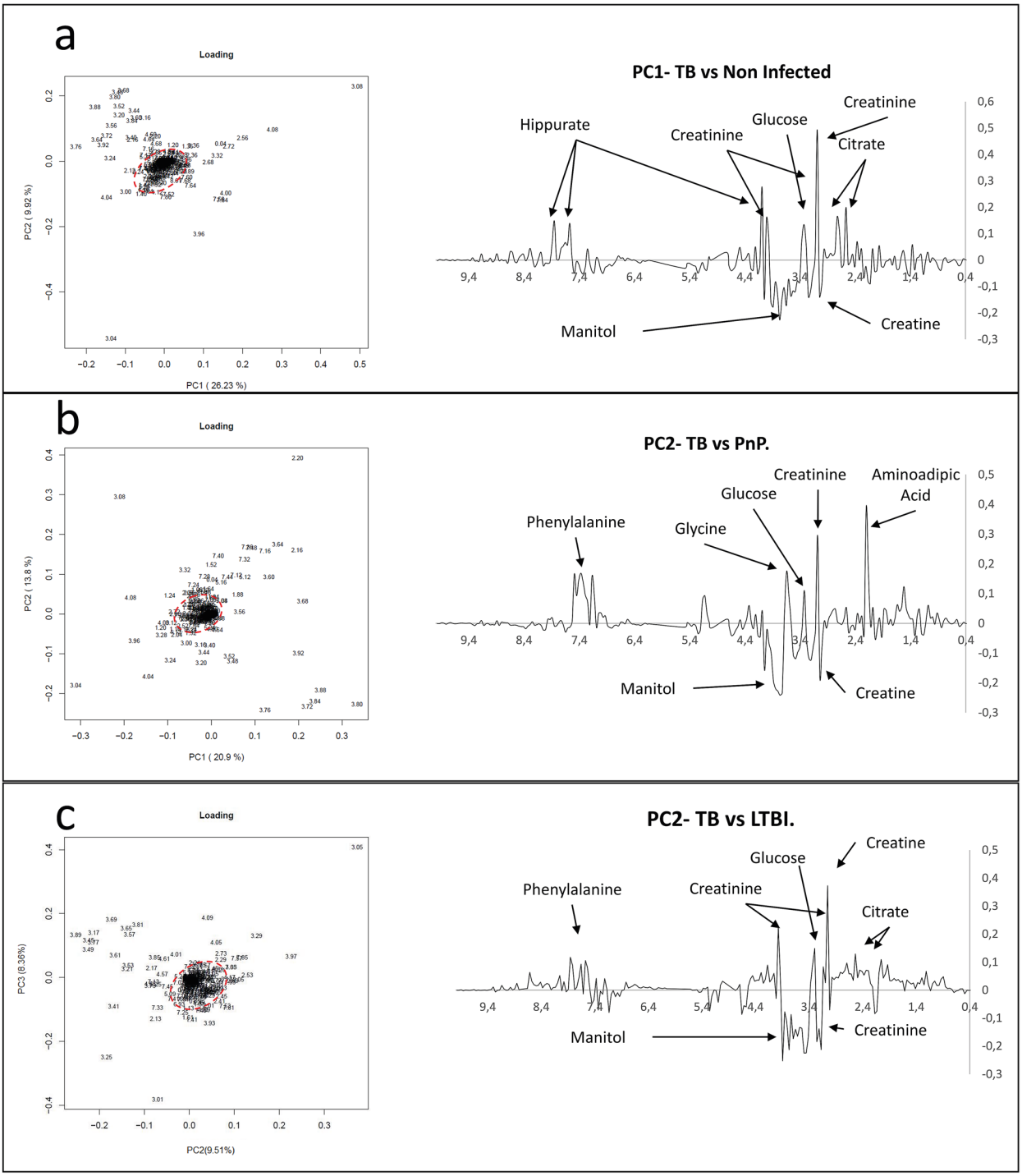

Figure 3. Principal Component Analysis (PCA) loading plots of 89 urine spectra analyzed by high-field Nuclear Magnetic Resonance reveals the metabolomic fingerprint of TB corresponding to 31 chemical shift regions assigned to eight metabolites. (**a**) PCA loading PC1-PC2 biplot and PC1 loading plot between TB patients and uninfected individuals; (**b**) PCA loading PC1-PC2 biplot and PC2 plot between TB patients and patients with pneumococcal pneumonia (PnP); (**c**) PCA loading PC2-PC3 biplot and PC2 loading plot between TB patients and individuals with LTBI. Multiple regions for the discrimination between groups were pointed outside the boundaries of a Hotelling's T2 statistics ellipse (pointed red line) in PCA loading biplots. TB, tuberculosis; PnP, pneumococcal pneumonia; LTBI: latent TB infection; PC, Principal Component.

Metabolite	Percentage change in concentration (%)			Bonferroni corrected T-test		
	TB vs. PnP	TB vs. LTBI	TB vs. Uninfected	TB vs. PnP	TB vs. LTBI	TB vs. uninfected
Aminoadipic	− 67.0	17.3	82.9	**0.0013**	0.6450	0.0975
Citrate	66.2	− 36.7	− 59.6	0.0881	**0.0162**	**0.0001**
Creatine	− 30.3	102.7	21.6	0.2690	**0.0407**	0.4470
Creatinine	91.6	− 19.4	− 32.3	**0.0012**	0.1790	**0.0013**
Glucose	37.8	− 24.6	− 7.9	0.3720	**0.0073**	0.7080
Mannitol	− 47.7	18.1	78.8	0.1070	0.2620	**0.0436**
Phenylalanine	− 33.4	60.4	33.5	**0.0262**	0.0321	0.0626
Hippurate	34.8	− 23.3	− 49.6	0.2700	0.4720	**0.0132**

Table 3. Relative change in the concentration of the identified metabolites. TB, tuberculosis; PnP, pneumococcal pneumonia; LTBI, latent TB infection; uninfected, individuals without infection; PC: Principal Component. Bold values, statistical significance was determined using a Bonferroni corrected Student's t-test assuming significant unequal corrected variance with $p < 0.05$.

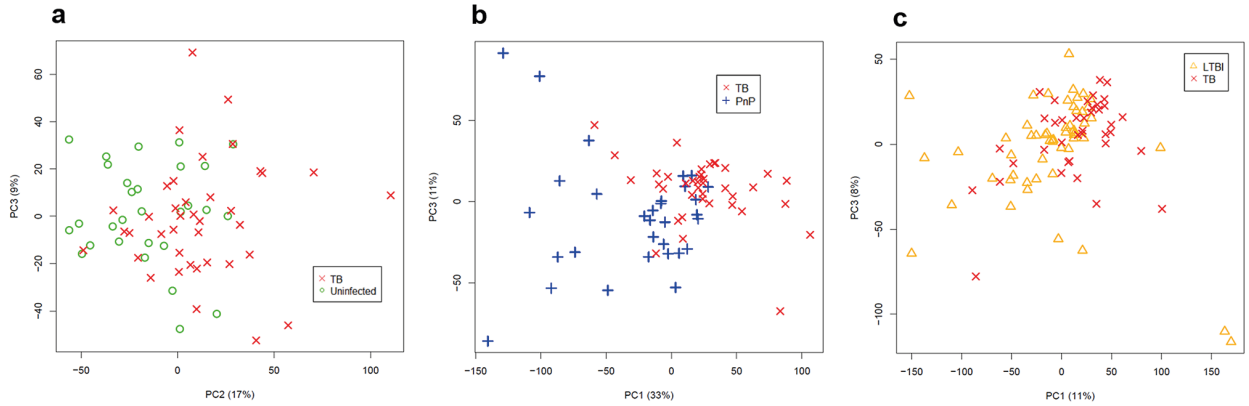

Figure 4. Principal Component Analysis (PCA) score plots of urine spectra analyzed by low-field Nuclear Magnetic Resonance between 39 untreated TB (x) and: (**a**) 29 uninfected individuals (circle), (**b**) 31 pneumococcal pneumonia patients (cross), and (**c**) 53 LTBI individuals (triangle). TB, tuberculosis; PnP, pneumococcal pneumonia; LTBI: latent TB infection; PC, Principal Component.

TB discrimination from LF-NMR-based metabolic profile. PLS-DA was applied to establish predictive models to discriminate untreated TB cases (n = 39) from control groups (31 pneumococcal pneumonia, 53 LTBI, and 29 uninfected) from the LF-NMR urinary spectral fingerprint identified. When comparing TB and pneumococcal pneumonia patients, 87.3% (SD = 7.8%) of TB patients were correctly classified, with 94.4% (SD = 3.6%) and 85.62% (SD = 5.5%) sensitivity and specificity, respectively. Similarly, when comparing TB patients and LTBI individuals, 85.2% (SD = 5.8%) of TB patients were correctly classified, with 91.9% (SD = 4.8%) and 90.2% (SD = 3.5%) sensitivity and specificity, respectively. Finally, when comparing TB and uninfected individuals, 100% of TB patients were correctly classified, with 100% of sensitivity and specificity.

The predictive model established between untreated TB patients and uninfected individuals was applied to classify the LF-NMR urine spectra of the 88 TB patients under treatment. The model correctly classified 84.1% of TB patients that had not been used to create the predictive model (all of them under treatment) in the TB group. Among these TB patients, 68 had pulmonary TB, 12 had extrapulmonary TB, and 8 had disseminated TB. This model also correctly classified 100% of extrapulmonary TB patients in the TB group.

Discussion

In recent years a lot of effort has been made to identify the highest priority needs in order to improve the diagnostic procedures for TB[1,19]. The need to develop accurate and more accessible diagnostic methods in primary healthcare centres has meant an intensification of the search for biomarkers from non-sputum-based biological samples[20].

In this study, we have identified and characterised a metabolic profile of TB in urine from a high-field NMR spectrometer and detected the same profile with a low-field NMR spectrometer. The models developed from the metabolic profile of TB identified by both NMR technologies showed the potential to discriminate between TB patients, pneumococcal pneumonia patients, individuals with LTBI and uninfected individuals. In addition, the results of the models developed from this metabolic fingerprinting were not influenced by anti-TB treatment or TB location.

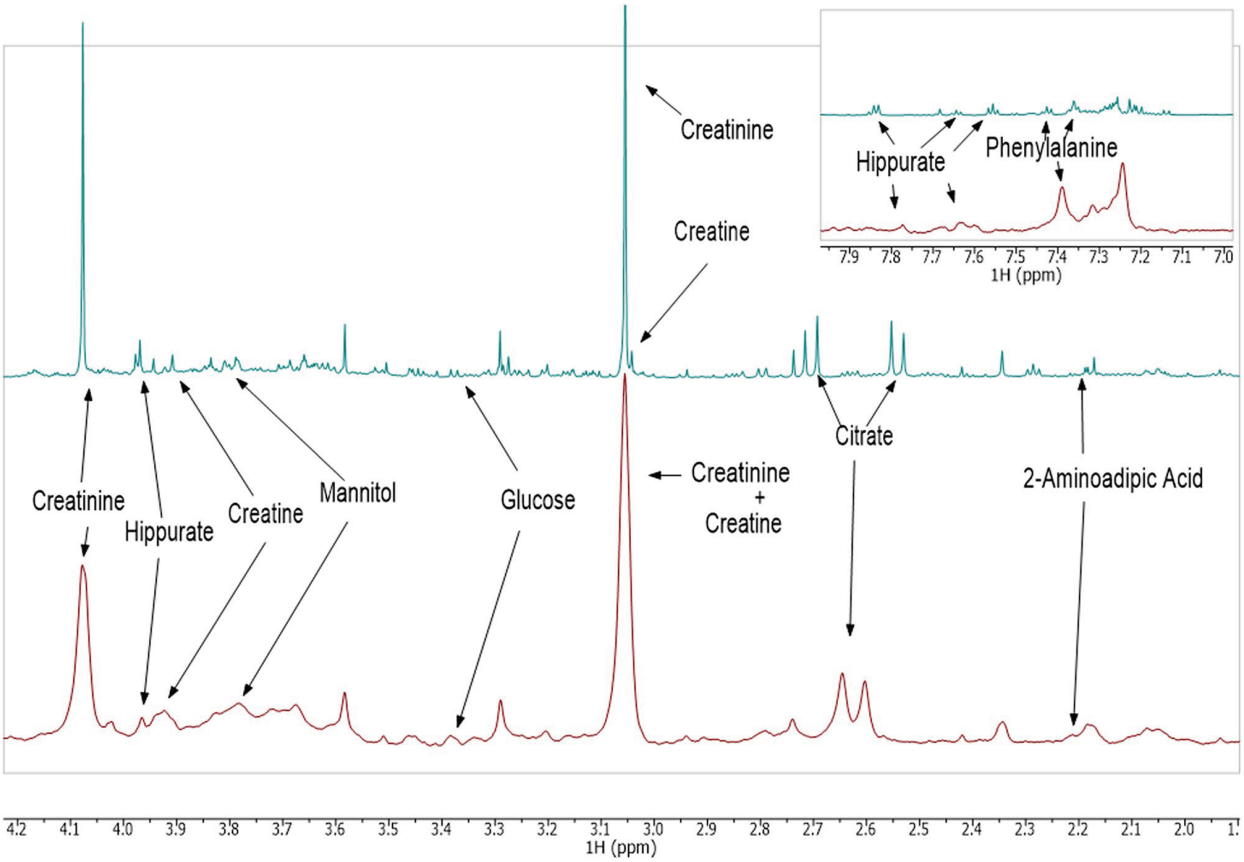

Figure 5. Comparison of the metabolomic fingerprints of tuberculosis (TB) identified in urine spectra analyzed by high-field Nuclear Magnetic Resonance (green) and low-field Nuclear Magnetic Resonance (red) showing the identification of the TB metabolite biomarkers. PC: Principal Component.

When applying the predictive model to the HF and LF-NMR spectra of the TB patients under treatment, 90.9% and 84.1% of the patients in the TB group were correctly classified. Furthermore, the models developed from the HF and LF-NMR-based spectral fingerprints correctly classified TB patients and pneumococcal pneumonia patients with a success rate of 100% and 87.3%, respectively. Therefore, the use of the model developed here could facilitate the identification of patients with TB and rule out those with other respiratory infections, such as pneumococcal pneumonia.

Using the HF-NMR-based spectral fingerprint, our model correctly classified 94.0% of TB patients compared to LTBI individuals, and 85.2% of them when using the LF-NMR-based urine metabolic fingerprint. This may be useful in situations where it is necessary to distinguish between TB and LTBI, such as in children, where early detection of TB is crucial to avoid severe forms of the disease developing. It is well known that neither TST nor IGRAs can distinguish between LTBI and active cases[21,22]. In our experience[23], a model based on the combination of IFN-γ, IP-10, ferritin and 25-hydroxyvitamin D could improve the detection of patients with subclinical TB. The metabolomic approach we described may be useful in detecting the early stages of the disease. Regarding metabolomics, a multi-site study across Sub-Saharan Africa provided a trans-African metabolic biosignature in serum and plasma to predict the onset of TB before active TB manifestation[24].

The metabolomic approach we present showed a classification performance within the minimum standards required by the World Health Organization to develop a non-sputum based biomarker test[19]. Although Xpert for detecting TB is still not as sensitive as culture, its rapidity in diagnosing TB has made shortening the delay between diagnosis and early treatment possible[25]. Also considering speed, the ability of the LF benchtop NMR spectrometer to measure samples quickly and easily would enable the integration of the final decision on the diagnosis into the same visit.

In addition, the use of an easily accessible sample would allow for successful implementation in microscopy centres, health posts and primary-care clinics[26]. A urine-based approach would allow the diagnosis of TB in patients who had difficulty in obtaining a representative sample from the site of infection (patients with extrapulmonary TB or not able to produce sputum), a relevant issue especially in LMICs since these patients often present non-specific symptoms of the disease[1]. The Alere Determine TB LAM Ag assay (AlereLAM, Abbott, Chicago, US) was introduced for TB diagnosis in HIV-positive patients by detecting the presence of lipoarabinomannan (LAM) in urine samples[27]. Subsequently, a new generation of urine LAM assays, Fujifilm SILVAMP TB LAM (FujiLAM) assay (Fujifilm, Tokyo, Japan) has been developed[28], representing improved diagnostics for HIV-positive TB patients. In this regard, the metabolic profile identified in this study might provide a promising tool

for diagnosing TB from urine samples. In addition, the quantification of the statistically significant metabolites identified in this TB metabolic profile would allow this technology to be adapted to a point-of-care test. Although more studies should be conducted in larger TB cohorts, vulnerable populations (children, HIV, comorbidities), and in other geographic regions to validate the performance of the predictive model based on the metabolic profile identified by LF benchtop NMR technology, these first results are promising.

If we consider the target product profiles (TPPs) published by the WHO and partners[26], although the diagnostic sensitivity of this metabolic model did not reach that achieved with the Xpert (diagnostic sensitivity of > 95% in comparison to culture), the sensitivity it achieved was within the minimum requirements established by the TPPs to develop a rapid non-sputum-based biomarker test for pulmonary TB in adults. Furthermore, with 100% of extrapulmonary TB patients correctly classified into the TB group, this model would fall within the optimal requirements recommended by the published TPPs for developing a rapid non-sputum-based biomarker test for extrapulmonary TB in adults[19]. Therefore, this metabolic approach based on LF-NMR could be a promising candidate for detecting all TB types. In addition, its small size and ease of maintenance would allow the implementation of the technology in primary healthcare centres as an alternative, or complementary to the diagnostic tests already available, without the risk of a shortage of cartridges. This robust benchtop NMR technology, straightforward sample preparation and minimal operational requirements would lead to a better diagnostic performance in LMICs; thus, increasing the number of cases diagnosed and allowing prompt treatment, which would reduce the transmission and mortality burden of TB.

TB is known to be a wasting disease involving weight loss, malnutrition and metabolic disorders[29]. The nutritional source of the bacteria is an essential aspect of host–pathogen interaction[24]. *M. tuberculosis* has adapted its metabolism to use different nutrient sources and compounds such as carbon or nitrogen sources to promote bacterial growth[30]. The metabolic profile presented in this study is based on the combination of eight metabolites, which is able to distinguish TB patients from those with pneumococcal pneumonia, LTBI, and non-infection. Previous studies have been conducted on serum samples to identify potential biomarkers for TB diagnosis using MS[31–33] and NMR[34]. Others have identified TB diagnostic markers from plasma samples in adults[35,36] and children[37]. In this study, TB patients showed reduced concentrations of creatine and phenylalanine compared to LTBI patients. Both metabolites are involved in the metabolism of necessary amino acids and derivatives. During infection in macrophages, *M. tuberculosis* shows a preference for amino acids as a source of nitrogen[38]. In line with this, TB patients showed high concentrations of creatinine compared to pneumococcal pneumonia patients. Creatinine is a breakdown product of creatine, so it might have a role in the synthesis of nitrogen-containing molecules. *M. tuberculosis* can co-metabolise multiple amino acids simultaneously[30]. In this metabolic process urea and other amino groups are synthesised from the breakdown of the amino acids. Thus, the low concentration of urine hippurate observed in TB patients might be due to/connected to the synthesis of aromatic amino acids such as tryptophan, tyrosine and phenylalanine[39,40]. Alternatively, Weiner et al. suggested that low serum hippurate concentrations might be related to uremic cytotoxic activity related to vitamin D metabolism[24]. In this study, TB patients showed low glucose and citrate concentrations compared with LTBI patients and uninfected individuals. The involvement of these two metabolites in the oxidative metabolism of carbohydrates, proteins and fats suggests that there is an increase in energy consumption by TB patients[41]. In accordance with this, Ehrt et al. reported the adaptation of *M. tuberculosis* to co-catabolise multiple carbon substrates, so that it grows faster and more extensively on carbon source mixtures than it does on any single source[30]. This might also explain the high concentrations of mannitol found in TB patients compared with non-infected individuals. However, in this study, we found that TB patients showed higher concentrations of citrate and lower concentrations of intermediary compounds of amino acid metabolism compared to pneumococcal pneumonia patients. This could be explained because the Krebs cycle is most likely not used in the *S. pneumoniae* metabolism due to the lack of genes encoding the enzymes involved in this pathway. In contrast, the lack of a complete Krebs cycle for *S. pneumoniae* has a great impact on amino acid synthesis[42]. Ultimately, the metabolites identified in the TB metabolic profile of our study are involved in pathways such as the oxidative metabolism of carbohydrates, proteins and fats, or the metabolism of the necessary amino acids and derivatives[41].

When considering urine metabolomics, a study conducted in Uganda identified a urine-based metabolite biosignature with potential to monitor the response of individuals to anti-TB therapy[43]. In contrast, another study conducted in South Africa characterised the biological basis of a poor treatment outcome using urine samples collected at diagnosis, during treatment, and after treatment completion[44]. In a study of children and adults with TB, LTBI or uninfected, urine neopterin levels were measured using an enzyme-linked immunosorbent assay predicting that the neopterin/creatinine ratio may be a considerable predictor of disease progression[45]. According to our results, our models can detect TB even in patients who have initiated anti-TB treatment. In recent years, new markers have been identified in urine in response to TB treatment[43]. Future metabolomic studies should be conducted to monitor treatment and evaluate treatment or cure success, and also identify possible re-infections. In addition, host metabolites derived from anti-TB treatment identified in urine could be useful for monitoring treatment and improving patient adherence to TB treatment.

Limitations of this study include the relatively small sample size of TB patients before starting anti-TB treatment. Additionally, not all urine samples could be tested by HF and LF-NMR due to the time lapse between the analysis of the samples by the two NMR spectrometers. Therefore, some patients with samples tested for HF were not tested for LF-NMR and vice versa. Although this did not affect the results of the study, future research should test the discriminatory potential of the identified TB spectral fingerprint in a consecutive series of patients in a country with a high incidence of TB and co-infections. This proof of concept represents a first step towards the development of an affordable metabolomic test for the diagnosis of TB.

In this study, we have identified and characterised a metabolic profile for TB in urine with potential to discriminate TB patients from the rest of the study groups; this metabolic profile for TB has also been detected using an LF benchtop NMR spectrometer. The use of benchtop technology would facilitate its implementation

in microscopy centres, health posts and primary care clinics, improving access to TB diagnosis. In addition, the ability of the model developed through this urine-based NMR technology to detect extrapulmonary TB and TB in patients under treatment is a step forward for the search for new diagnostics for TB that are not sputum-based and that can detect all forms of TB.

In summary, the identification of a metabolic profile for TB in urine from NMR with the potential to discriminate TB patients from pneumococcal pneumonia patients, individuals with LTBI, and uninfected individuals highlights the application of metabolomics as a new approach in the search of non-sputum-based new potential biomarkers for TB diagnosis.

Methods

Ethical statement. The study was approved by the ethical review board of the Ethics Committee of the HUGTiP and subsequently for all the Ethics Committee of all the health care centres participating (reference number CEI-PI-15-073). All patients gave written informed consent before being included. Sample collection and all experiments were performed in accordance with relevant guidelines and regulations.

Study population. We have conducted a case–control study, in which we included patients with active TB, patients diagnosed with pneumococcal pneumonia, and healthy controls with latent TB infection (LTBI) and no infection. Participants were recruited through four different health care centres in Spain (Hospital Universitari Germans Trias i Pujol, Unitat de Tuberculosis de Drassanes de l'Hospital Universitari Vall d'Hebron, Serveis Clínics Unitat Clínica de Tractament Directament Observat de la Tuberculosi, and Hospital Sant Joan Despí Moisès Broggi), and one in Germany (Medical Clinic of the Research Center Borstel).

Participants were classified into four study groups: patients diagnosed with active TB, patients with pneumococcal pneumonia, individuals with LTBI, and uninfected individuals.

All active TB patients had clinical and radiological signs compatible with TB and were microbiologically confirmed by culture and/or Xpert MTB/RIF.

Patients with pneumococcal pneumonia were diagnosed by isolating the bacteria in blood culture and/or detecting the pneumococcal urinary antigen. Patients with pneumococcal pneumonia were collected before starting the antibiotic treatment.

LTBI individuals were recruited from contact tracing studies with a positive TST and/or IGRA but without clinical and radiological signs consistent with active TB. To perform the TST, we used a 2-TU dosage of PPD-RT (Statens Serum Institut, Copenhagen, Denmark). The performance and interpretation of the results of the Mantoux test were carried out following the Spanish guidelines[46]. IGRAs testing was performed using the commercially available enzyme-linked immunosorbent assay (ELISA) QuantiFERON-TB Gold In-Tube test (QFT, Qiagen, Hilden, Germany) and/or the Enzyme-Linked Immunospot (ELISPOT) assay T-SPOT.TB blood test (T-SPOT.TB; Oxford Immunotec Ltd, Oxford, UK) following the manufacturer's protocol. The LTBI patients were included before starting chemoprophylaxis.

The uninfected individuals were volunteers with no evidence of *M. tuberculosis* infection and with negative TST and IGRA tests.

Collection and preparation of urine samples for NMR analysis. We collected midstream urine samples from all participants of the study in sterile, universal plastic containers following standardised procedures[13]. Urine samples were aliquoted into 2 ml cryovials with screw caps and frozen at − 20 °C until the NMR experiments were performed. Before analysis, urine samples were thawed at room temperature and vortexed 30 s before use. We then aliquoted 400 µl of urine samples into Eppendorf tubes and added 250 µl of 0.2 M phosphate buffer solution containing 0.09% NaN_3 to adjust the internal pH to 7.4. We adjusted the axis of chemical shifts to a signal reference at 0 ppm adding 0.3 mM trimethylsilyl propanoic acid (TSP) in deuterated water dissolution in the preparation of sample. Azide was added during the preparation of the urine samples to avoid bacterial contamination[14]. Buffered urines were vortexed for 30 s and centrifuged at 12,000*g* for 5 min. Then, we transferred 600 µl aliquot of the supernatant into 5 mm diameter NMR tubes (CortecNet, Les Ulis, France) for proton (^{1}H) NMR acquisition. Figure 1 shows how many of these samples were analysed by HF and LF NMR.

NMR spectral acquisition and processing. HF-NMR urine spectra were acquired using a Bruker 700 MHz NMR spectrometer (CNIO, Madrid, Spain) operating at a frequency of 697.87 MHz. Shimming and NMR preparation time was reduced to a minimum, while the sample for NMR analysis was chilled to 4 °C to minimise metabolic changes. The acquisition of the spectra was performed in accordance with the standardised protocols previously described[47]. A number of bidimensional homonuclear and heteronuclear experiments such as standard gradient-enhanced correlation spectroscopy (COSY), ^{1}H–^{1}H total correlated spectroscopy (TOCSY), and gradient-selected heteronuclear single quantum correlation (HSQC) protocols were performed to carry out component assignments. Between consecutive two-dimensional (2D) spectra, a control ^{1}H NMR spectrum was always measured. No gross degradation was noted in the signals of multiple spectra acquired under the same conditions. Standard solvent-suppressed spectra were grouped into 32,000 data points, averaged over 256 acquisitions. The data acquisition lasted a total of 13 min using a sequence based on the first increment of the nuclear Overhauser effect spectroscopy (NOESY) pulse sequence to effect suppression of the water signal ($\delta = \sim 4.80$ ppm). Sample acquisitions were performed using a spectral width of 8333.33 Hz prior to Fourier transformation, and the free induction decay (FID) signals were multiplied by an exponential weight function corresponding to a line broadening of 0.3 Hz.

LF-NMR urine spectra were acquired using a Magritek Spinsolve 60 Ultra Benchtop spectrometer (Magritek GmbH, Aachen, Germany) at a frequency of 60 MHz using a one-dimensional presaturation (1D PRESAT)

sequence to allow for efficient saturation of the water signal (δ = ~4.95 ppm) following the previously described procedures[15].

NMR data were processed and editing using MestReNova software (v.14; Mestrelab Research, Santiago de Compostela, Spain) according to the established protocols described in a previous study[47]. Metabolite signals of the spectra were shift-aligned using trimethylsilyl propanoic acid (TSP) as a reference signal standard (δ = 0.00 ppm). From the raw NMR spectra, the chemical shift region from 5.00 to 5.20 ppm was excluded from the analysis to remove the random effects of variation in the urine and water resonance suppression (δ = 6.50 to 4.22 ppm). Similarly, the chemical shift region from 0 to 0.04 ppm containing the internal reference (TSP) was excluded from the statistical analyses. Baseline correction was performed automatically using the 'Withaker Smoother' algorithm. Binning (also known as bucketing) was applied to NMR spectra and data-reduced to equal length integral segments (bins) of δ = 0.04 ppm to compensate variations in resonance positions. All bins were normalized by the total sum of the spectral regions (each bin was divided by the sum of all the NMR signals). Thus, the concentration of each metabolite was normalized by the urine concentration to compare these concentrations (in arbitrary units) between samples. Relative intensity was calculated as the original intensity normalized by total sum of the spectral regions to compensate urine concentration and to ensure that all observations were directly comparable.

Data analysis. *Statistical analysis of the study population.* A descriptive analysis of the subjects who participated in the study was performed according to the study groups. Frequencies and percentages described the qualitative variables, while the mean and standard deviation described the quantitative variables. For comparisons between study groups, we used the chi-squared test in the case of qualitative variables, and the analysis of variance (ANOVA) in the case of quantitative variables. The level of significance was fixed at 0.05. Analyses were performed using the statistical software IBM SPSS Statistics v.25 (SPSS, Chicago, US).

Statistical analysis of metabolomic data. Data from ^{1}H NMR spectra were analysed in a multivariate manner using the Metabonomic package of R software (rel.3.3.1)[48]. NMR spectra were data-reduced to equal length integral segments of δ = 0.04 ppm to compensate variations in resonance positions, and they were normalized by total sum of the spectral regions. Prior to multivariate statistical analysis, spectral data were Pareto scaled[49]. Unsupervised (blinded) data were analysed by PCA by the "prcomp" function from the statistical library and supervised (unblinded) analysis was by PLS-DA by the "gpls" function from the "gpls" package allowing separation between no more than two classes of samples.

PCA was applied to represent the variance of all metabolomic variables present in the data-reduced NMR spectra in a low-dimensional space (bins of δ = 0.04 ppm)[50] to identify a differential metabolic pattern of TB to be used as potential biomarkers for TB diagnosis. Thus, all the spectral regions grouped in bins of δ = 0.04 ppm were transformed into a new set of orthogonal variables known as PCs. The first PC was defined by the spectral profile (load) in the data describing most of the variation; the second PC, was the second-best profile describing the variation, and so on, so that the retention of the variation present in the original variables decreased as we went down the order.

The PCs are composed of the scores and loadings. On one hand, the scores hold information about the samples (concentrations). Thus, PCA score plots of the first two or three PCs were used to visually observe the differences between the samples and immediately display sample clustering patterns according to their elemental composition[51,52]. In addition, PCA score plots were used to highlight statistical outliers. We use the Mahalanobis distance to confirm statistical outliers; this consists of calculating the distance from a data point to the centroid of all samples. Mahalanobis distance was calculated for PC1, PC2, and PC3. A single case was considered a statistical outlier if it was placed out of the tolerance ellipse of 97.5%[17]. On the other hand, the loadings hold information about the variables of the data set (chemical changes), indicating the importance of each region in explaining the variance between samples. Therefore, PCA loading plots were used to identify the multiple regions (δ = 0.04 ppm bins) of the ^{1}H NMR spectra responsible for the separation between groups (the so-called metabolic fingerprint). The spectral regions (potential biomarkers for TB diagnosis) selected from PCA loading plots were confirmed by Hotteling's T2 tests[18]. Those regions outside the 95% tolerance ellipse were identified as the spectral regions responsible for the metabolic differences in the PCA plots. Hotteling's T2 test was applied for each PCA: (1) TB and uninfected groups, (2) TB and pneumococcal pneumonia groups, and (3) TB and LTBI groups.

The identification of the metabolites corresponding to the metabolic fingerprint was performed using the Human Metabolome Database[53] and the characteristic cross-peaks from 2D HSQC spectra. The identified metabolites were individually integrated for metabolic quantification applying the Global Spectral Deconvolution analysis algorithm provided by the MestReNova software and corrected by the multiplicity of the NMR signal. Statistical significance was determined using a Bonferroni corrected Student's t-test assuming significant unequal corrected variance with $p < 0.05$[54].

PLS-DA was applied by classifying patients into two groups[51]. We used the algorithm proposed by Ding and Gentleman et al.[55] (tolerance for convergence: 1×10^{-3}, the maximum number of iterations allowed: 100). The number of PLS components used was chosen by the percentage of variance explained, the R2, and the mean squared error of cross-validation graphics. Thus, PLS-DA was applied to the δ = 0.04 ppm bucketed NMR spectra of the following groups: TB vs. pneumococcal pneumonia, TB vs. LTBI, and TB vs. uninfected. PLS-DA predictive models were performed to assess and validate the diagnostic accuracy of the fingerprints of the metabolites present used to discriminate TB cases from the control groups. Before the comparison between groups (TB vs. pneumococcal pneumonia, TB vs. LTBI, and TB vs. uninfected), samples from each group were divided into two sets: the training set (50%) and the test set (50%). For training purposes, the classification functions derived from the probability of belonging to each group were computed with a number of random testing subjects. These

classification functions were used afterwards to classify the rest of the subjects for internal validation. This process was repeated 100 times with random permutations of the training and test sets to reduce type I errors[55]. The percentages of correct classification were calculated as a measure of model performance.

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Author contributions

Study design and supervision: J.L.I.G., J.R.C., and J.D. Recruitment of study subjects: M.L.S.G., M.A.J., J.R.M., Z.S., A.G., J.P.M., I.M.P., X.C., J.S., F.S., C.M., C.P.A., and C.H. Acquisition of data: J.L.I.G., P.C.B., M.S.V., E.G.G., B.M.M. Analysis and interpretation of data: J.L.I.G., P.C.B., M.S.V., J.R.C., and J.D. Drafting the article: J.L.I.G., P.C.B., J.R.C., and J.D. Revising the manuscript critically for important intellectual content and final approval of the version to be published: all authors.

Competing interests

José Luis Izquierdo-Garcia, Patricia Comella-del-Barrio, Cristina Prat-Aymerich, and José Domínguez are registered as inventors on a patent filed by the Institut d'Investigació Germans Trias i Pujol and CIBERES, disclosing the use of NMR-based urine metabolomic profile for TB diagnosis. Ramón Campos-Olivas, Raquel Villar-Hernández, Maria Luiza De Souza-Galvão, María A Jiménez, Juan Ruiz-Manzano, Zoran Stojanovic, Adela González, Mar Serra-Vidal, Esther García-García, Beatriz Muriel-Moreno, Joan Pau Millet, Israel Molina-Pinargote, Xavier Casas, Javier Santiago, Fina Sabriá, Carmen Martos, Christian Herzmann, and Jesús Ruiz-Cabello declare no competing interests.

Additional information

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Article 5

Urine NMR-based TB metabolic fingerprinting for the diagnosis of TB in children

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Urine NMR-based TB metabolic fingerprinting for the diagnosis of TB in children

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Tuberculosis (TB) is a major cause of morbidity and mortality in children, and early diagnosis and treatment are crucial to reduce long-term morbidity and mortality. In this study, we explore whether urine nuclear magnetic resonance (NMR)-based metabolomics could be used to identify differences in the metabolic response of children with different diagnostic certainty of TB. We included 62 children with signs and symptoms of TB and 55 apparently healthy children. Six of the children with presumptive TB had bacteriologically confirmed TB, 52 children with unconfirmed TB, and 4 children with unlikely TB. Urine metabolic fingerprints were identified using high- and low-field proton NMR platforms and assessed with pattern recognition techniques such as principal components analysis and partial least squares discriminant analysis. We observed differences in the metabolic fingerprint of children with bacteriologically confirmed and unconfirmed TB compared to children with unlikely TB ($p = 0.041$ and $p = 0.013$, respectively). Moreover, children with unconfirmed TB with X-rays compatible with TB showed differences in the metabolic fingerprint compared to children with non-pathological X-rays ($p = 0.009$). Differences in the metabolic fingerprint in children with different diagnostic certainty of TB could contribute to a more accurate characterisation of TB in the paediatric population. The use of metabolomics could be useful to improve the prediction of TB progression and diagnosis in children.

One-quarter of the world's population is infected with *Mycobacterium tuberculosis*, and 10 million people fell ill with tuberculosis (TB) in 2019[1]. TB is also a major cause of morbidity and mortality in children, with an estimated one million dying from TB each year[1]. Under-detection of childhood TB is common in low- and middle-income countries[2] as its clinical presentation overlaps with other respiratory infections, children have low sputum bacillary loads and are often unable to produce sputum, making its diagnosis difficult[3,4].

Metabolomics, or the systematic study of a unique chemical fingerprint present in a cellular system or bio-fluid, increasingly allows discrimination between samples with different physiological or pathological states[5]. These fingerprints can be measured in biological samples, such as urine, serum or plasma, using non-invasive methods such as nuclear magnetic resonance (NMR) spectroscopy[6], and have been used to monitor metabolic

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Variable	All (n = 117)	Presumptive TB (n = 62)	Controls (n = 55)	p-value
Gender				0.717
Girls	49 (41.9%)	25 (40.3%)	24 (43.6%)	
Boys	68 (58.1%)	37 (59.7%)	31 (56.4%)	
Age in years				
Mean (SD)	6.89 (3.56)	7.28 (4.05)	6.45 (2.90)	0.203
Range				0.640
≤ 5	40 (34.2%)	20 (32.3%)	20 (36.4%)	
> 5	77 (65.8%)	42 (67.7%)	35 (63.6%)	
BCG scar				0.259
Yes	81 (69.2%)	43 (69.4%)	38 (69.1%)	
No	30 (25.6%)	14 (22.6%)	16 (29.1%)	
Unknown	6 (5.1%)	5 (8.1%)	1 (1.8%)	

Table 1. Demographic and clinical characteristics of the study participants. Categorical variables expressed as number of subjects (n) and percentage (%), and quantitative variables expressed as median and standard deviation (SD). *TB* tuberculosis, *BCG* Bacillus Calmette-Guérin.

changes over time induced by pathogens[7]. Furthermore, the application of metabolomics to low-field (LF) NMR spectrometry has facilitated the development of smaller platforms suitable for primary and secondary medical centres laboratories[8,9]. In recent years, metabolomics has facilitated gaining insights into TB pathogenesis[10], disease progression, and evaluation of treatment responses[11].

A few studies have focused on the discovery of urine-based biomarkers for TB diagnosis. Urine is a non-invasive sample that requires minimal preparation[12] and would be an easily obtained clinical sample for diagnosis, especially for individuals unable to produce sputum, such as children. In this study, we aimed to describe a urine proton (1H) NMR-based metabolic fingerprint for the diagnosis of TB in children.

Results

One hundred and seventeen children were included, of which 62 had presumptive TB and 55 were apparently healthy (controls). Sixty-eight (58.1%) were male, and their mean (SD) age was 7 (3.6) years (Table 1). There were no sex or age differences between children with presumptive TB and controls. Eighty-one (69.2%) participants had received the Bacillus Calmette et Guérin (BCG) vaccine and had a BCG scar (Table 1). Among the 62 children with presumptive TB, 6 had bacteriologically confirmed TB, 52 unconfirmed TB (bacteriologically negative) and four were considered to be unlikely to have TB (unlikely TB), as described in Table 2. Eighteen (29%) of the 62 children with presumptive TB had X-rays compatible with intrathoracic TB, nine (14.5%) had X-rays and clinical findings of extra-thoracic TB, and four (6.5%) had both intra- and extra-thoracic TB. Thirty-one (50%) children's X-rays were considered inconclusive. Fifty-seven (91.9%) children had positive tuberculin skin test (TST, 54, 88.5%) and/or QuantiFERON-TB Gold In-Tube test (QFT-GIT, 39, 70.9%), with 63.2% (36/57) agreement between the tests. Fifty-three (85.5%) had documented exposure to an index case of TB. Seven (11.3%) of the 62 children had five clinical criteria of TB, while 21 (33.9%) had four, 28 (45.2%) had three, and six (9.7%) had two clinical criteria.

Thirty-one (43.6%) of the 55 controls were male, and their mean (SD) age was 6.5 (2.9) years, as shown in Table 1.

Performance of the TB metabolic fingerprinting. The metabolic fingerprint of the urine samples (n = 117) were measured using both high-field (HF) and LF 1H NMR spectroscopy, as detailed in Fig. 1. Representative spectra obtained with the HF and LF 1H NMRs are shown in Supplementary Fig. 1.

An unsupervised principal component analysis (PCA)[13] was applied to the HF 1H NMR urine spectra of the six bacteriologically confirmed, 52 unconfirmed and four unlikely TB, and the 55 controls not showing clustering patterns between samples. Two children's samples (one bacteriologically confirmed TB and one control) were considered outliers in the PCA score plot and were excluded[14] (Supplementary Fig. 2). A supervised partial least squares discriminant analysis (PLS-DA) was applied to identify a discriminatory metabolic pattern between presumptive TB and control groups to the remaining 115 urine samples. We observed groupings along the scores of the first component of the PLS-DA (PLS-DA component 1) (Fig. 2). The robustness parameters of the HF PLS-DA model were tested by Leave-One-Out Cross-Validation (LOOCV) using the PLS-DA component 1 showing a performance accuracy to discriminate between presumptive TB and controls of 0.68, with R2 and Q2 values of 0.61 and 0.13, respectively; and an Area Under the Curve of Receiver Operating Characteristic (AUC-ROC) of 0.65. The Variable Importance in Projection (VIP) scores for the PLS-DA component 1 identified the main spectral regions of the metabolic fingerprint to differentiate between children with presumptive TB and controls (Supplementary Table 1). There was a trend in the PLS-DA component 1 scores with the certainty of TB diagnosis (Fig. 2). Thus, children with bacteriologically confirmed (n = 5) and unconfirmed TB (n = 52) had higher median PLS-DA component 1 scores (883.3 ± 751.1 and 913.3 ± 716.6) than children with unlikely TB (n = 4; − 385.2 ± 417.3) (p = 0.026 and p = 0.005, respectively; Fig. 3a). The PLS-DA component 1 scores also varied with the number of clinical criteria for TB. Children with five (n = 7), four (n = 21), and three (n = 27)

Variable	All (n = 62)	Confirmed TB (n = 6)	Unconfirmed TB (n = 52)	Unlikely TB (n = 4)	p-value
Gender					0.742
Girls	25 (40.3%)	2 (33.3%)	22 (42.3%)	1 (25.0%)	
Boys	37 (59.7%)	4 (66.7%)	30 (57.7%)	3 (75.0%)	
Age in years					
Mean (SD)	7.3 (4.1)	6.7 (3.9)	7.0 (4.0)	11.6 (3.3)	0.090
Range					0.250
≤ 5	20 (32.3%)	3 (50.0%)	17 (32.7%)	0 (0.0%)	
> 5	42 (67.7%)	3 (50.0%)	35 (67.3%)	4 (100.0%)	
BCG scar					0.540
Yes	43 (69.4%)	4 (66.7%)	37 (71.2%)	2 (50.0%)	
No	14 (22.6%)	2 (33.3%)	10 (19.2%)	2 (50.0%)	
Unknown	5 (8.1%)	0 (0.0%)	5 (9.6%)	0 (0.0%)	
TB type					**< 0.001**
Intrathoracic	18 (29.0%)	2 (33.3%)	16 (30.8%)	0 (0.0%)	
Extrathoracic	9 (14.5%)	1 (16.7%)	8 (15.4%)	0 (0.0%)	
Both	4 (6.5%)	3 (50.0%)[a, b]	1 (1.9%)[a]	0 (0.0%)[b]	
Not defined	31 (50.0%)	0 (0.0%)[a, b]	27 (51.9%)[a]	4 (100.0%)[b]	
Immunologic evidence of *M. tuberculosis* infection					0.619
Yes	57 (91.9%)	5 (83.3%)	48 (92.3%)	4 (100.0%)	
No	5 (8.1%)	1 (16.7%)	4 (7.7%)	0 (0.0%)	
TB exposure					**0.001**
Yes	53 (85.5%)	2 (33.3%)[a, b]	47 (90.4%)[a]	4 (100.0%)[b]	
No	9 (14.5%)	4 (66.7%)[a, b]	5 (9.6%)[a]	0 (0.0%)[b]	
Symptoms/signs suggestive of TB					**0.006**
≤ 2	32 (51.6%)	0 (0.0%)[a, b]	28 (53.8%)[a]	4 (100.0%)[b]	
≥ 3	30 (48.4%)	6 (100.0%)[a, b]	24 (56.2%)[a]	0 (0.0%)[b]	
Lymphadenopathy					0.092
Yes	22 (35.5%)	4 (66.7%)	18 (34.6%)	0 (0.0%)	
No	40 (64.5%)	2 (33.3%)	34 (65.4%)	4 (100.0%)	
Chest radiograph					**0.006**
Abnormal	30 (48.4%)	6 (100.0%)[a, b]	24 (46.2%)[a]	0 (0.0%)[b]	
Normal	32 (51.6%)	0 (0.0%)[a, b]	28 (53.8%)[a]	4 (100.0%)[b]	
Response to TB treatment					**< 0.001**
Treatment completed	52 (83.9%)	5 (83.3%)[a]	47 (90.4%)[b]	0 (0.0%)[a, b]	
Lost to follow-up	8 (12.9%)	0 (0.0%)[a]	4 (7.7%)[b]	4 (100.0%)[a, b]	
Died	2 (3.2%)	1 (16.7%)	1 (1.9%)	0 (0.0%)	
Anti-TB treatment or preventive treatment					0.302
Under treatment	24 (38.7%)	4 (66.7%)	19 (36.5%)	1 (25.0%)	
Untreated	38 (61.3%)	2 (33.3%)	33 (63.5%)	3 (75.0%)	

Table 2. Demographic information and clinical criteria of children with presumptive TB. [a,b]Significant differences in variables when comparing proportions between study groups. If a pair of values is significantly different, the values have the same superscript letters assigned to them. Bold values, significative statistical values with a p-value under 0.05. Categorical variables expressed as number of subjects (n) and percentage (%), and quantitative variables expressed as median and standard deviation (SD). *TB* tuberculosis, *BCG* Bacillus Calmette-Guérin.

clinical criteria had significantly higher median PLS-DA component 1 scores (1262.4 ± 649.8, 1062.7 ± 839.3, and 736.3 ± 644.6, respectively) than children with two (n = 6) criteria (− 100.5 ± 582.7) (p = 0.014, p = 0.007, and p = 0.021, respectively; Fig. 3b). Children with unconfirmed TB with X-rays compatible with TB (n = 24) had higher PLS-DA component 1 scores than unconfirmed cases with normal X-rays (n = 28) (1080.8 ± 736.3 and 796.2 ± 634.4) (p = 0.043).

If children who had already started treatment (for less than 15 days) are excluded from the analysis (4 confirmed TB, 19 unconfirmed TB and 1 unlikely TB), children with bacteriologically confirmed TB or unconfirmed TB still had significantly higher median PLS-DA component 1 scores (1333.4 ± 636.5 and 480.3 ± 508.5, respectively) than children with unlikely TB (− 437.9 ± 125.1) (p = 0.005 and p = 0.010, respectively; Fig. 4).

The PCA applied to the 117 LF 1H NMR acquired urine fingerprints detected eight outliers (two bacteriologically confirmed TB, 4 unconfirmed TB and two controls), which were excluded from the PLS-DA (Supplementary

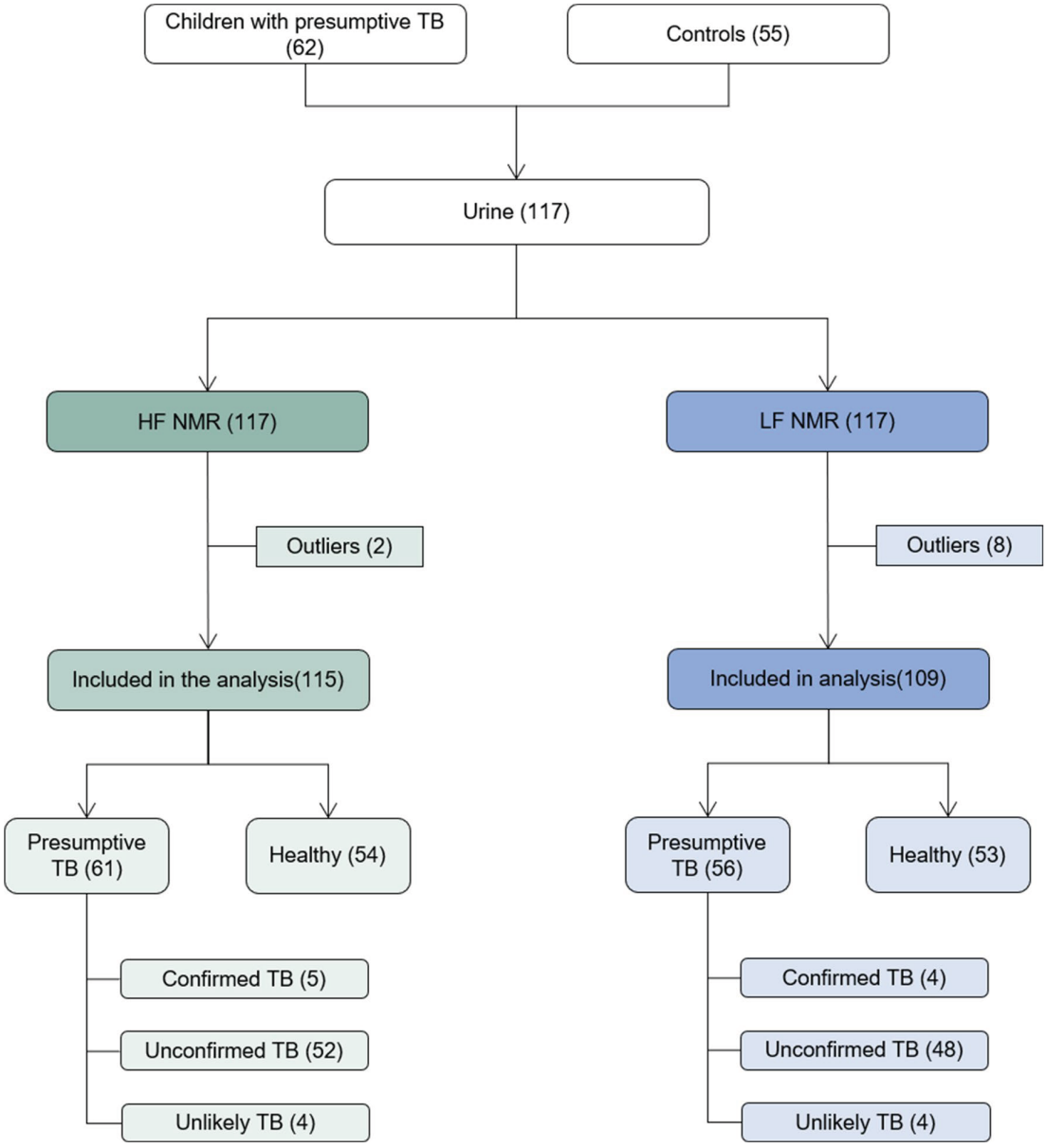

Figure 1. Description of the children who participated in the study according to the nuclear magnetic resonance equipment used to acquire the urine samples spectra and the classification of the patients in the study group. *NMR* nuclear magnetic resonance, *HF* high-field, *LF* low-field, *TB* tuberculosis.

Fig. 3). A PLS-DA was applied to the remaining 109 urine fingerprints to identify a discriminatory metabolomic pattern between presumptive TB and control groups. We observed groupings between presumptive TB and control groups along the scores of the first component of the PLS-DA (PLS-DA component 1) (Fig. 5). PLS-DA component 1 scores were higher in children with presumptive TB than controls. The robustness parameters of the LF PLS-DA model were tested by LOOCV using the PLS-DA component 1 (performance accuracy to discriminate between presumptive TB and controls = 0.70, R2 = 0.76, Q2 = 0.08, and AUC-ROC = 0.65). Supplementary Table 1 shows the VIP for PLS-DA component 1 responsible for differentiating between children with presumptive TB and controls. Children with bacteriologically confirmed TB (n = 5) had higher median PLS-DA component 1 scores (825.2 ± 1236.52) than children with unlikely TB (n = 3; − 316.5 ± 1464.3) (p = 0.040 Fig. 6a). The PLS-DA component 1 scores also varied with the number of clinical criteria for TB. Children with five (n = 6) clinical criteria had significantly higher median PLS-DA component 1 scores (1426.1 ± 1088.1) than children with two (n = 5) criteria (− 46.3 ± 1229.8) (p = 0.009; Fig. 6b). The median PLS-DA component 1 scores among children with unconfirmed TB with compatible TB X-rays (n = 23) and normal X-rays (n = 28) were similar (1518.7 ± 1136.3 and 1920.1 ± 1419.3) (p = 0.643).

Figure 2. Partial least squares discriminant analysis (PLS-DA) score plot of urine spectra measured using high-field proton (1H) nuclear magnetic resonance spectroscopy of children with presumptive TB (n = 61) and healthy children (n = 54). Two-dimensional view showing the distribution of the groups according to the first two components of the PLS-DA model. TB, tuberculosis. Metaboanalyst 5.0. (https://www.metaboanalyst.ca).

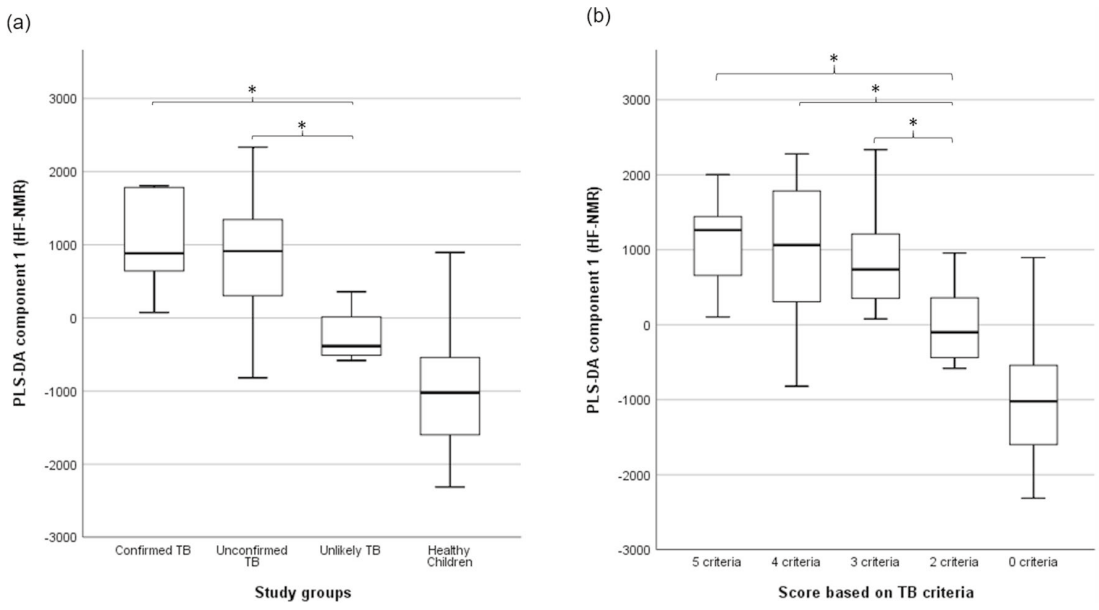

Figure 3. Association between partial least squares discriminant analysis (PLS-DA) scores and (**a**) study groups, and (**b**) the number of clinical criteria compatible with TB in 115 urine spectra acquired by high-field proton (1H) nuclear magnetic resonance. The central horizontal line within the boxes represents the median. The boxes comprise the first and third quartiles, the tiles indicate the maximum and minimum values, and the asterisk indicates statistically significant differences (p-value < 0.05) between groups. TB: tuberculosis; PLS-DA 1, the first component of the PLS-DA model. IBM SPSS Statistics 26 (https://www.ibm.com).

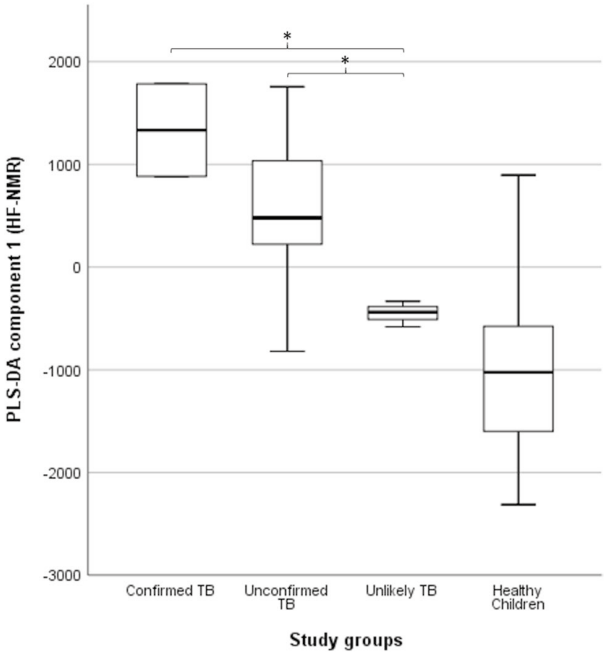

Figure 4. Association between partial least squares discriminant analysis (PLS-DA) scores and study groups in 92 urine spectra of children without TB-treatment acquired by high-field proton (1H) nuclear magnetic resonance. The central horizontal line within the boxes represents the median. The boxes comprise the first and third quartiles, the tiles indicate the maximum and minimum values, and the asterisk indicates statistically significant differences (p-value < 0.05) between groups. *TB* tuberculosis; PLS-DA 1, the first component of the PLS-DA model. IBM SPSS Statistics 26 (https://www.ibm.com).

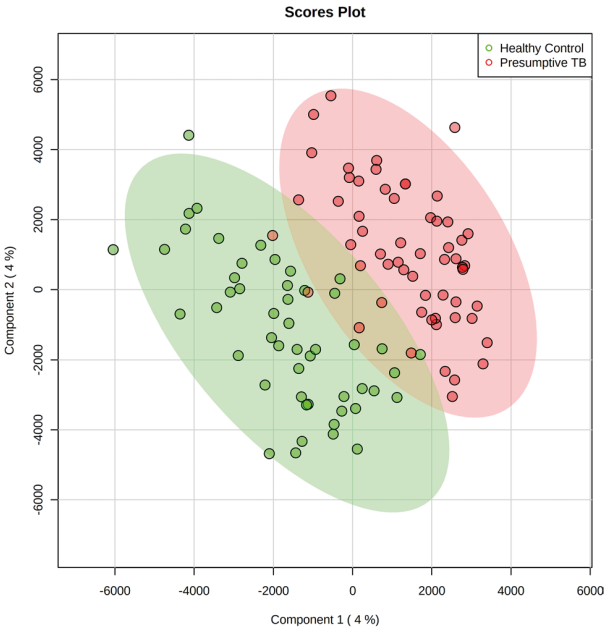

Figure 5. Partial least squares discriminant analysis (PLS-DA) score plot of urine spectra measured using low-field proton (1H) nuclear magnetic resonance spectroscopy of children with presumptive TB (n = 56) and healthy children (n = 53). Two-dimensional view showing the distribution of the groups according to the first two components of the PLS-DA model. TB, tuberculosis. Metaboanalyst 5.0. (https://www.metaboanalyst.ca).

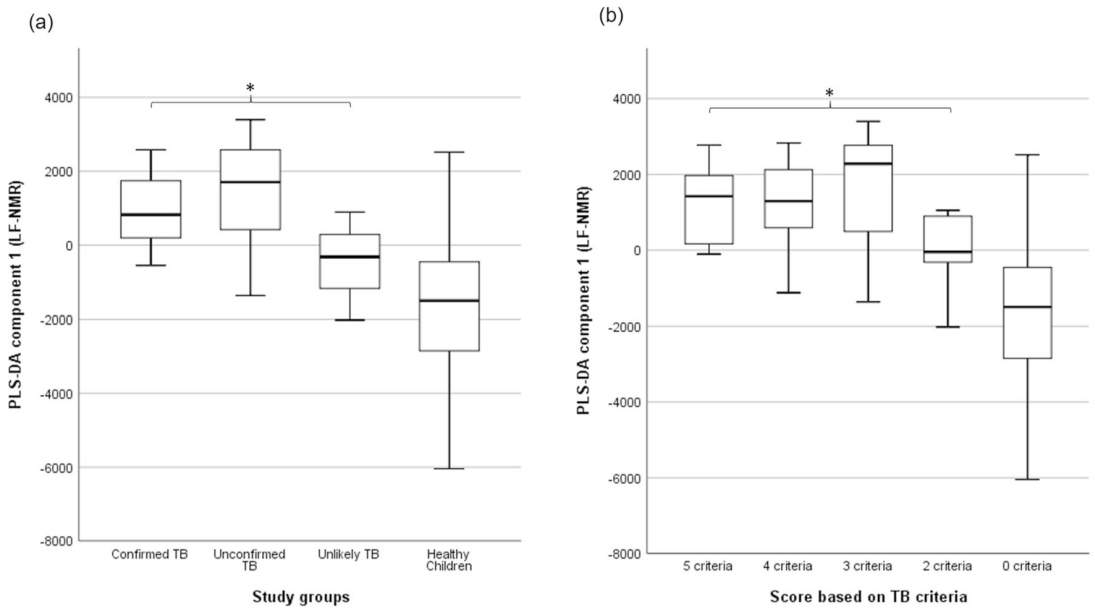

Figure 6. Association between partial least squares discriminant analysis (PLS-DA) scores and (**a**) study groups, and (**b**) the number of clinical criteria compatible with TB in 109 urine spectra acquired by low-field proton (1H) nuclear magnetic resonance. The central horizontal line within the boxes represents the median. The boxes comprise the first and third quartiles, the tiles indicate the maximum and minimum values, and the asterisk indicates statistically significant differences (p-value < 0.05) between groups. TB: tuberculosis; PLS-DA 2, the second components of the PLS-DA model. IBM SPSS Statistics 26 (https://www.ibm.com).

Discussion

Early diagnosis and treatment are crucial to stop the epidemic of childhood TB[2]. The search for biomarkers in non-invasive biological samples as alternatives to sputum is needed to improve the diagnostic sensitivity of TB in this population[1,15]. We report here a urine NMR-based metabolic fingerprint associated with bacteriologically and clinically diagnosed TB in children[16].

Recently, the detection of TB from Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) in urine has been evaluated in children[17]; however, the results have not achieved the accuracy desired to improve TB diagnosis in this population. Other emerging alternative diagnostic include Fujifilm SILVAMP TB (FujiLAM, Fujifilm, Tokyo, Japan), a new assay generation for detecting lipoarabinomannan (LAM) in urine[18,19]. In studies evaluating FujiLAM in children, the sensitivity and specificity reported were 50% and 92%, respectively, in a South African cohort, and 64.9% and 83.8%, respectively, in a multicentre evaluation in Africa [20,21]. Despite its low sensitivity, its high specificity could help confirm the disease in children with a high probability of TB (e.g. children living in high-TB burden areas and those with HIV or malnutrition).

Biofluid metabolomics provides a snapshot of all the mechanisms that act during the disease, thus facilitating understanding the interaction between host and pathogen during infection and TB disease progression[22,23]. Previously, metabolomic profiles have been described in serum[24–29] and plasma[24,25,30,31] by NMR spectroscopy and mass spectrometry for the prediction and detection of TB. In children, two studies have reported different metabolomic profiles for TB detection in plasma[32] and serum[33] analysed by 1H NMR spectroscopy. However, neither of these studies reported whether the metabolic profile could discriminate between children with different diagnostic certainty of TB[32]. We have previously identified an NMR-based metabolomic profile in urine to diagnose TB in adults[34] and here we extend their potential application to the diagnosis of TB in a paediatric population and we have demonstrated differences in the urinary metabolic fingerprint of children with different certainty in the TB diagnosis.

TB in children is characterized by a continuum of conditions correlated with bacterial load, host immune responses, clinical manifestation, and the detection of *M. tuberculosis*[3]. Inflammatory host biomarkers in plasma have potential to discriminate latent TB infection from overt TB in children, and to identify the onset of TB disease [35–37]. During latent infection, the host is able to contain the infection, the bacteria has restricted metabolic activity and there are no clinical manifestations. However, with progression to active TB, the infection overcomes the host immune system, the bacilli replicate, and the increased metabolomic activity of the mycobacteria modifies the tissues physiopathology, with changes in the host metabolome. The metabolic fingerprinting analysed by HF 1H NMR spectroscopy showed metabolic differences between children with presumptive TB with two or fewer clinical criteria and three or more clinical criteria. Moreover, children with high diagnostic certainty of TB showed metabolic fingerprints similar to that of children with bacteriologically confirmed TB. This metabolic response could be attributed to the physiological stimuli that occurs during disease progression[23,38].

The paucibacillary nature of TB in children, combined with the limitation of current microbiological methods, results on a high dependence on chest X-rays for diagnosis[39]. In this study, children with unconfirmed TB and

abnormal X-rays had differences in their metabolic fingerprint compared with those with normal X-rays. The differences in the metabolic fingerprint are consistent with studies interpreting the occurrence of radiological features from the pathway of incipient TB infection to subclinical and symptomatic TB[40,41].

One limitation of this study is the low confirmation rate of TB (8% and 7% in HF and LF NMR metabolic fingerprint approach, respectively). This low rate, together with the inherent resonance overlap phenomenon in LF spectrometers (60 Hz)[8,9] might have hindered the pattern recognition process in the LF metabolic fingerprinting approach, losing its discriminatory power between the study sub-groups with presumptive TB. The compact and portable size and the successful performance of this approach, demonstrated in previous studies[8,9,34], makes LF benchtop NMR-based metabolic fingerprinting a promising diagnostic tool. However, further analysis with a larger group of children with confirmed TB is needed to evaluate the full potential of this approach in children as the small final number of bacteriologically confirmed TB cases (9.7%) in our study, prevented the development of a TB-specific discriminatory model.

In summary, this study identified an association between the urine NMR-based metabolic fingerprint and the clinical case definitions used for the classification of TB in children, and observed differences in the metabolic response of children with different diagnostic certainty of TB. This finding could contribute to the identification and classification of childhood TB, which would improve the characterization of the clinical spectrum of the disease and the search for new diagnostic and prognostic biomarkers of TB in children.

Methods

This was a prospective case series of children aged 0 to 14 years old with presumptive TB attending the St. Damien Paediatric Hospital, Port-au-Prince, Haiti, in 2015 and 2016, and healthy children attending a local primary school in the same neighbourhood.

Clinical and demographic information obtained at the time of enrolment included age, sex, weight, medical history and clinical presentation (history and exposure of TB, presence of cough, fever for ≥ 2 weeks, unexplained weight loss, and asthenia/fatigue, TB treatment, HIV status, and comorbidities), vaccines received (including BCG), and current and previous medications. Children with known immunodeficiencies, those receiving immunosuppressive treatment, or those starting TB treatment more than 15 days ago were excluded. The Mantoux TST (Sanofi Pasteur, Canada) and the QFN-GIT (Qiagen, Germany) assays were performed and interpreted according to the manufacturer's instructions.

All children with presumptive TB had a chest X-ray and induced or aspirated nasopharyngeal/nasogastric sputum collected on three consecutive days. Sputum was examined using fluorescent smear microscopy (auramine stain). Children with positive smear microscopy or abnormal X-rays were tested with Xpert MTB/RIF. Children with lymph node adenopathy underwent biopsies, and specimens underwent histological examination from a pathologist.

Children with presumptive TB were classified, following the updated clinical case definitions for classification of intrathoracic TB in children into confirmed, unconfirmed, and unlikely TB[16]. Children were classified as: confirmed TB, if bacteriological confirmation was attained by Xpert MTB/RIF; unconfirmed TB, if there was not bacteriological confirmation, but evidence of *M. tuberculosis* infection (i.e., TST or QFT-GIT positive) and at least one clinical criteria of the clinical case definition (i.e., X-ray consistent with TB, symptoms and signs suggestive of TB, close TB exposure, or positive response on TB treatment), or two clinical criteria, if TST and QFT-GIT results were negative. Children were considered unlikely TB if the child had only evidence of *M. tuberculosis* infection or presented only one clinical criterion compatible with TB. School children were enrolled as controls if they had negative TST and QFT-GIT and no signs or symptoms of TB.

Urine collection. Midstream urine samples were collected from all participants in sterile plastic containers following standardized procedures[12]. In children who attended the hospital, urine samples were collected within the first week of the TB diagnosis. Two millilitres of urine were aliquoted in cryovials with screw caps that were frozen at − 20 °C until the 1H NMR analysis. According to a protocol established in a previous study[34], 400 µl of urine were mixed with either 200 µl of the standard deuterated buffer for HF 1H NMR measurements or 250 µl for LF 1H NMR measurements. The standard deuterated buffer was a 0.2 M phosphate buffer solution dissolved in 99.9% deuterated water to adjust the internal pH to 7.4, containing 0.09% sodium azide and 0.3 mM trimethylsilyl propanoic acid (TMSP). Six hundred µl of buffered urine was transferred into 5 mm diameter NMR tubes (CortecNet, Les Ulis, France) for 1H NMR spectra acquisition.

Acquisition of NMR spectra. All 1H NMR urine spectra were measured following the procedures previously described[9,42] using two different instruments operating at HF and LF, respectively: (1) a Bruker Avance 700 MHz spectrometer at a 1H frequency of 700 MHz (CNIO, Madrid, Spain) and (2) a Magritek Spinsolve 60 Ultra benchtop NMR spectrometer at a 1H frequency of 60 MHz (Magritek GmbH, Aachen, Germany). Briefly, HF 1H NMR spectra were measured using a pulse sequence based on the first increment of the nuclear Overhauser effect spectroscopy (NOESY) with pre-saturation to effect suppression of the water signal (δ = ~ 4.80 ppm). The spectra were acquired using the following parameters: 32,000 data points over a spectral width of 8,333.33 Hz and 256 scans resulting in acquisition times of 13 min per sample. LF 1H NMR spectra were measured using a one-dimensional presaturation (1D PRESAT) sequence to allow for efficient saturation of the water signal (δ = ~ 4.95 ppm). The spectra were acquired using the following parameters: 64 scans, an acquisition time of 6.4 s, and shimming of the sample after each new one to maintain a line width below 0.55 Hz. Data were zero-filled before Fourier transformation, and free induction decays (FIDs) were multiplied by exponential line broadening of 0.3 Hz.

Processing spectral data. Spectral data were processed using the MestReNova program (v.14; Mestrelab Research, Santiago de Compostela, Spain). According to the established protocols described in previous studies[34,42], metabolite signals of the spectra were shift-aligned using TMSP as a reference signal standard ($\delta = 0.00$ ppm), and the chemical shift regions of the raw HF 1H NMR spectra from 6.50 to 4.22 ppm were excluded from the analysis to remove the random effects of variation in urea and water resonance suppression[34,42]. Then, the chemical shift region around 0.00 ppm containing the internal reference (TMSP) was excluded, and baseline correction was performed using the 'Withakker Smoother' algorithm[34,42]. Binning (also known as bucketing) was applied to 1H NMR spectra and data-reduced to equal length integral segments (bins) of 0.04 ppm to compensate variations in resonance positions. All bins were normalized by the total sum of the spectral regions (each bin was divided by the sum of all the 1H NMR signals) [34,42]. Thus, the concentration of each metabolite was normalized by the urine total metabolite content to compare these concentrations (in arbitrary units) between samples[34,42]. Before multivariate statistical analysis, spectral data were Pareto scaled[43], where the square root of the standard deviation is used as the scaling factor.

Multivariate analysis of spectral data. Processed 1H NMR data were analysed in a multivariate manner using the Metabonomic package of R software (rel.3.3.1)[44,45] and MetaboAnalyst v.5.0[46]. The analysis included all urine spectra acquired using the HF and LF 1H NMR equipment. Graphs were plotted using SPSS statistical software for windows (SPSS version 26; SPSS Inc, Chicago, IL, USA).

Unsupervised data were analysed by applying the PCA to reduce the dimensionality of NMR-processed data and to observe clustering patterns according to their elemental composition[13,14]. In addition, PCA score plots were used to highlight statistical outliers based on Mahalanobis distance. Mahalanobis distance was calculated from the data point to the centroid of all samples in PC1, PC2, and PC3 three-dimensional space. A single case was considered a statistical outlier if it was placed out of the tolerance ellipse of 97.5%[47].

Supervised PLS-DA[13] was applied to the metabolic fingerprint of children with presumptive TB and controls to detect a discriminatory metabolic pattern between groups. Thus, all spectral regions grouped in bins of 0.04 ppm were transformed into a new set of orthogonal components obtained by maximising the covariance between spectral data and the class membership (presumptive TB and controls).

The robustness of the HF and LF PLS-DA models using the PLS-DA component 1 was validated using the LOOCV procedure (performance accuracy, R2, Q2, AUC-ROC). The VIP scores for PLS-DA were calculated to identify the spectral regions of the metabolic fingerprint most important for differentiating between children with presumptive TB and controls. Statistical significance was determined using Student's t-test.

Since PLS-DA scores were trained to maximise the covariance between spectral data and class membership (presumptive TB vs controls), we hypothesised that the same PLS-DA scores should be sensitive also to differences within the group of children with presumptive TB (sub-categorised into bacteriologically confirmed TB, unconfirmed TB, and unlikely TB). Thus, the resulting PLS-DA component 1 (first latent variable) scores were used to evaluate metabolic differences between children with presumptive TB classified according to the standardized case definitions for TB and with the number of clinical criteria of TB.

Statistical analysis. Clinical and demographic characteristics were described using descriptive statistics. Categorical variables were described using frequencies and percentages, while continuous data were described using means and standard deviations (SD). Variables normally distributed were compared using parametric tests, including analysis of variance, and Student' T-tests, and with non-parametric tests for comparisons of proportions. Comparison of PLS-DA scores among the children with presumptive TB groups and clinical criteria score groups were performed using the Kruskal–Wallis test with Dunn's post hoc comparisons. Differences were considered statistically significant when a p-value was < 0.05. Analyses were performed using the SPSS 26 software package (SPSS, Chicago, USA).

Ethical statement. The study protocol was approved by the ethical review board of the Ethics Committee of the University of Barcelona and the Haiti National Ethics Committee (reference number IRB00003099). Written informed consent was obtained from the children's parents or legal guardians before enrolment. Sample collection and all experiments were performed in accordance with relevant guidelines and regulations.

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Author contributions

Study design and supervision: P.C.B, T.M.P.P, J.G., J.L.I.G., L.E.C., J.R.C., and J.D. Recruitment of study subjects: T.M.P.P., J.D., M.J.C.D., J.G., and P.C.B. Acquisition of data: P.C.B., J.L.I.G., R.C.O., C.M.S., and B.M.M. Analysis and interpretation of data: P.C.B., J.L.I.G., R.A., C.P.A., L.E.C., J.R.C., and J.D. Drafting the article: P.C.B., J.L.I.G., J.R.C., L.E.C., and J.D. Revising the manuscript critically for important intellectual content and final approval of the version to be published: all authors.

Competing interests

José Luis Izquierdo-Garcia, Patricia Comella-del-Barrio, Cristina Prat-Aymerich, and José Domínguez are registered as inventors on a patent filed by the Institut d'Investigació Germans Trias i Pujol and CIBERES, disclosing the use of NMR-based urine metabolomic profile for TB diagnosis. Jacqueline Gautier, Mariette Jean Coute Doresca, Ramón Campos-Olivas, Clara M. Santiveri, Beatriz Muriel-Moreno, Rosa Abellana, Tomas M. Pérez-Porcuna, Luis E. Cuevas, and Jesús Ruiz-Cabello declare no competing interests.

Additional information

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Article 6

Use of NMR-based urine metabolomics to evaluate TB treatment response and outcome. A proof of concept study

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TECHNICAL REPORT

Use of NMR-based urine metabolomics to evaluate TB treatment response and outcome. A proof of concept study

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Abstract

Surveillance during tuberculosis (TB) treatment is essential to monitor treatment adherence and response. We have characterised a urine metabolic fingerprint of patients successfully responding to anti-TB treatment to provide a baseline for further studies evaluating treatment outcome.

This was a case series of adults with drug-sensitive pulmonary TB (DS-TB) and healthy controls. Individuals with DS-TB received the standard 2-month intensive phase (isoniazid, rifampicin, pyrazinamide, and ethambutol) and the 4-month continuation phase (isoniazid and rifampicin). Urine samples were collected at start of treatment and at two, four- and six-months treatment. All patients were considered clinically and microbiologically cured at the end of treatment. We characterized the urinary metabolomic profile using Nuclear Magnetic Resonance (NMR) spectroscopy and estimated their principal component analysis (PCA) scores to identify differences between samples. We compared the PCA scores of patients with TB and healthy controls.

The scores of patients with DS-TB were different from controls at the start of treatment and then changed over time. At the end of treatment, the scores of patients and controls were similar.

The urine metabolic fingerprint can differentiate patients with and without TB at the start of treatment. The scores changed over the course of treatment. This baseline can be used to compare how the metabolic scores differs from patients with poor treatment outcome.

Keywords: Tuberculosis, Metabolomics, Biomarker, Nuclear Magnetic Resonance, Urine, Anti-tuberculosis therapy, follow-up, *Mycobacterium tuberculosis*, Metabolites

Introduction

The emergence of drug-resistant tuberculosis (DR-TB) presents a challenge to TB control with an increase in treatment failure [1]. Surveillance during TB treatment is essential to ensure treatment adherence and to monitor treatment response [1,2]. Detecting viable *Mycobacterium tuberculosis* bacilli in sputum is cumbersome. Sputum may contain contagious dormant and viable mycobacteria along with dead mycobacteria [3]. As smear microscopy with auramine or Ziehl-Neelsen staining is unable to distinguish them [4], additional laboratory protocols are required to detect the bacilli that are live [5] or metabolically active [3]. New approaches are thus needed to monitor treatment responses of patients with TB.

Metabolomics can be used to obtain a fingerprint of the metabolites present in a biological sample and this fingerprint varies in response to host-pathogen interactions [6–8]. We have previously characterised a nuclear magnetic resonance (NMR)-based metabolomic profile in urine as biomarkers able to differentiate patients with TB from patients with pneumococcal pneumonia and between individuals with latent TB infection and uninfected individuals [9]. Recently, compact NMR instruments have become available, facilitating the application of metabolomics outside reference and research laboratories [10,11]. Metabolomics therefore could be useful for the rapid identification of markers of TB treatment, which could facilitate treatment monitoring. In this proof of concept, we aimed to describe the urinary metabolic fingerprint of TB patients over the course of treatment of patients receiving TB treatment over the intensive and continuation phase for DS-TB. This information would be useful to later compare changes in the fingerprint during the course of treatment among patients with successful treatment and patients with poor treatment outcome.

Materials and Methods

This proof of concept study was based on NMR urine spectra obtained through a study of patients with TB and uninfected individuals (controls) [12]. Patients with pulmonary TB (PTB) were inpatients of the Clinical Unit of the Serveis Clínics in Barcelona, Spain. Controls were individuals attending the University Hospital of Germans Trias i Pujol (Hospital Universitari Germans Trias i Pujol [IGTP]) and the TB Unit of the University Hospital Vall d'Hebron (Unitat de Tuberculosis de Drassanes de l'Hospital Universitari Vall d'Hebron) [12].

Patients with PTB were confirmed by culture and/or Xpert MTB/RIF and underwent phenotypic drug susceptibility testing (DST). Controls had negative tuberculin skin tests (TST) and interferon-gamma release assays (IGRAs). Patients with PTB received a 2-month intensive phase of isoniazid, rifampicin, pyrazinamide, and ethambutol and 4- or 7-month continuation phase of isoniazid and rifampicin. The duration of the continuation phase treatment varied according to the negative culture test results of the patients. Patients were considered to have a successful outcome if they completed treatment, had a negative culture and resolved symptoms [13]. Adults with known immunodeficiencies, immunosuppressive treatment, extrapulmonary forms of TB or drug resistance were excluded.

Demographic and clinical data included age, sex, comorbidities, history of TB, immunological evidence of TB infection, anatomical site of the disease, anti-TB treatment received, and treatment outcome.

All participants were asked to collect urine samples in sterile plastic vials. Urine samples from patients with PTB were collected serially from the start of treatment and at 0-2, 3-5, and 6-9) months follow up. Fresh samples were kept cold and transferred to the IGTP Research Institute, where they were aliquoted and frozen at -20ºC until analysis [14].

Before NMR processing, 400 µl of urine were mixed with 250 µl of 0.2 M phosphate buffer solution dissolved in 99.9% deuterated water to adjust the internal pH to 7.4, containing 0.09% sodium azide and 0.3mM trimethylsilyl propanoic acid (TMSP). Spectrometry was conducted with a Magritek Spinsolve 60 Ultra benchtop NMR spectrometer operating at 60MHz (Magritek GmbH, Aachen, Germany) as previously described [11,12,15] and the spectra generated were processed as previously described [9,15]. NMR spectra were data-reduced using bins of equal length integral segments (0.04 ppm) to compensate variations in resonance positions, and were normalized by the sum of the spectral regions. Bins were divided by the sum of all the NMR signals [9,15]. Before multivariate analysis, spectral data were Pareto scaled [16] to normalize different bins/signals to unit variance.

Categorical data were described using frequencies and percentages and continuous variables were described as means and standard deviations (SD). Chi-Square and Fisher's exact tests were used to test differences among categorical variables and Student's T-tests for continuous variables with normal distributions. Differences with P-values < 0.05 were considered statistically significant. Analysis were performed using SPSS (SPSS version 26.0, SPSS Inc, Chicago, IL, USA).

The multivariate analysis of the spectral data was analysed by the Metabonomic package in R (rel.3.3.1) to develop a fingerprint of the metabolic composition in urine [8]. Graphs were plotted using SPSS. Unsupervised principal component analysis (PCA) were applied to all spectra to observe clustering patterns according to their elemental composition [17,18]. Reduced spectra were transformed into a new set of orthogonal variables known as principal components (PCs) defined by the scores (holding information on sample concentrations) and loadings (holding information on chemical shifts in the dataset). The first PC (PC1) represented the axis describing most of the variation in the samples. Assuming that the PCA scores should be sensitive to metabolomic changes as treatment progresses and the disease resolves, we used PC1 scores to observe these differences during treatment. The PC1 scores of patients and controls were measured using the Kruskal-Wallis test. Significance values were adjusted by the Bonferroni correction [19].

The study protocol was approved by the Ethics Committees of the University Hospital of IGTP and the participating health care centres (reference number CEI-PI-15-073). Written informed consent was obtained from all participants.

Results

We included nine patients with PTB with DS-TB and nine controls. Nine of the cases and two of the controls were men (Table 1). Patients with PTB had a mean (SD) age of 44 (16) years, and three (23%) had received treatment for TB in previous occasions.

Table 1. Characteristics of participants

Variable	Pulmonary TB (n=9)	Controls (n=9)	p-value
Men	9 (100%)	2 (22.2%)	<0.01
Mean age ± SD	44.3 (16.4)	46.8 (20.5)	0.784
History of previous TB treatment	3 (23.1%)	0 (0.0%)	0.103

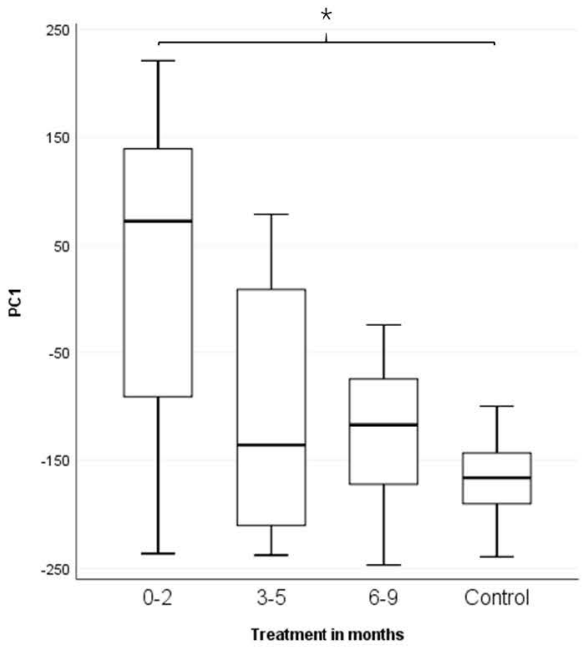
PCA was applied to 37 urine spectra, of which 28 were from nine patients with PTB and the nine were from controls, as shown in table 2.

Table 2. Time of urine sample collection

	Duration of TB treatment (in months)									
	0 mo.	1 mo.	2 mo.	3 mo.	4 mo.	5 mo.	6 mo.	7 mo.	8 mo.	9 mo.
Patient 1	1		2				1			
Patient 2	1		1							
Patient 3		1		1			1	1		1
Patient 4			1	1						
Patient 5			1	1		1				
Patient 6		1		1		1				
Patient 7	1		1	1						
Patient 8			1		1				1	
Patient 9		1		1			1			
Controls	9									

The PCA showed a statistically significant polarisation in the PC1 scores between cases and controls (p=0.014). Patients receiving TB treatment in the intensive phase (months 0-2) had significantly higher median metabolomic scores (30.1±151.2) than controls (-168.2±41.8) (p=0.011). The metabolomic profile changed over time. The median scores at months 3-5 were lower than during the intensive phase (-101.0±118.0), and these in turn were higher than the scores after 6-9 of treatment (-125.3±78.2), although these differences were not statistically significant (p = 0.263 and 0.373, respectively). At the end of treatment, patients with PTB had similar scores than controls (Figure 1).

Figure 1. Box plot of the principal component scores of patients with PTB and controls.



The central horizontal line represents the median, boxes represent first and third quartiles and tiles indicate the range. PC1 is the first principal component of the PCA model.

4. Discussion

The low-field NMR fingerprint in urine of patients with TB changed over time after initiation of TB treatment. In the early stages of treatment, all patients had urine metabolic profiles consistent with disease. However, as treatment progressed, the differences in the metabolomic scores of patients and controls decreased, and at the end of treatment patients and controls had similar profiles.

The development of omics technologies (genomics, transcriptomics, proteomics, metabolomics) in recent years has driven the application of systems biology in TB biomarker research for disease prediction and TB treatment response and outcomes [20]. However, few metabolomics studies have focused on monitoring treatment response [21–24]. In two African cohorts of adults with recently diagnosed TB monitored during treatment, a set of urinary molecular signatures were identified by liquid chromatography-mass spectrometry (LC-MS) that changed in abundance as TB treatment progressed [22]. The change in the metabolomic profile with treatment may have indicated an effective response to treatment.

In this proof of concept, all patients completed treatment and were cured. However, the South African cohort identified baseline differences in the metabolic profile of patients who responded to treatment and one patient with treatment failure [23], and thus metabolomics may be able to predict treatment failure at the time of diagnosis [23]. Similarly, *Fitzgerald et al.* reported a decrease in urine Seryl-Leucine Core 1*O*-glycosylated peptide (SLC1G) levels one week after treatment initiation in patients with successful treatment compared to patients who failed treatment [21].

The emerging acquisition of resistance to anti-TB drugs, which is largely due to the suboptimal management of TB treatment, has led to an increase in treatment failure [2,25]. The development of a urine-based NMR approaches, may be able to detect differences in the metabolic profile over time, which may correlate with unfavourable treatment outcomes. A non-invasive and easy to obtain urine-based method would facilitate monitoring treatment among people unable to provide sputum, and people with extrapulmonary TB and children [26]. The robustness and high reproducibility of NMR coupled with the small size and easy maintenance of a NMR benchtop device would enable its implementation at microscopy centres [11].

We have established that the urine profile of patients with PTB changes over time. This change is progressive and is similar to the profile of controls by the time of treatment completion. The limitations of this case series is the small sample size and the scarcity of samples for some of the time periods. The strength of this study is the standardisation of sample collection and processing [14].

The application of metabolomics from low-frequency NMR spectroscopy of urine has been shown to be a potential tool for TB detection [9] and could be a promising technique for monitoring TB treatment response and disease outcome. Future studies should describe the urinary metabolic profile of patients with drug-resistant TB and patients fail treatment.

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Author Contributions

Study design and supervision: JRC, JLI, LEC, JD. Recruitment of study subjects: CPA, JPM, IMP, JS, LDS, MAJ. Acquisition of data: PC, BMM, EGG. Analysis and interpretation of data: PC, JLIG, JRC, JD. Writing – original draft: PC, JLIG, CPA, JRC, LEC, JD. Writing – review & editing, All authors; Supervision, JRC, JLI, JD; Project administration, JD; Funding acquisition, JD.

Conflicts of Interest

José Luis Izquierdo-Garcia, Patricia Comella-del-Barrio, Cristina Prat-Aymerich, and José Domínguez are registered as inventors on a patent filed by the Institut d'Investigació Germans Trias i Pujol and CIBERES, disclosing the use of NMR-based urine metabolomic profile for TB diagnosis.

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7

General Discussion

Discussion

TB remains one of the world's leading causes of death and its diagnosis is essential to prevent and control the disease[1]. Nevertheless, current diagnostic methods present limitations in patients with difficulties in obtaining a representative sample from the site of infection (patients with extrapulmonary TB, children, HIV co-infected patients or individuals in early stages of the disease). For instance, over half of the children with TB are not diagnosed or are not reported[98]. In addition, the affordability, accessibility, and ease of use of molecular methods challenge their integration into primary health care centres, where the majority of new TB cases first seek care[82].

To improve TB diagnosis, efforts should be made to develop more accurate and accessible non-sputum-based diagnostic methods especially in low- and middle-income countries since these patients often present non-specific symptoms of the disease[54,99,100]. The studies included in this thesis focus on the identification of host-response biomarkers in plasma for TB diagnosis in children (article 1 and patent 1), the evaluation of a *M. tuberculosis*-specific biomarker in urine for TB diagnosis at the point of TB care (articles 2 and 3), and the application of metabolomics for the discovery of urine-based biomarkers resulting from the interaction between host and *M. tuberculosis* for the diagnosis of TB (articles 4, 5, and patent 2) and assessment of TB treatment response (article 6).

TB is a major cause of morbidity and mortality in children[36,101]. Therefore, early detection of paediatric TB is essential to prevent progression and development of severe forms of the disease after exposure to *M. tuberculosis*[40,102]. However, the non-specific clinical presentation of the disease in children and difficulties in obtaining a quality sputum sample pose a major diagnostic challenge[103,104]. In this regard, a combination of four biomarkers was detected in QFT-GIT tube supernatants with potential for the detection and discrimination of TB in children (article 1). The model based on IFN-γ, IP-10, ferritin, and 25(OH)D correctly classified active TB cases (93.2%) and LTBI cases (90.0%), as well as 76.2% of children with mediastinal TB into the TB group. Although IGRAs are unable to discriminate between TB and LTBI[53,105], previous studies performed in QFT-GIT demonstrated the involvement of several mechanisms mediated by different host markers in *M. tuberculosis* infection and disease[42,66]. The biomarkers identified above have previously been described separately for their involvement in the immune response against *M. tuberculosis* infection. While IP-10 has been described for its good performance in the diagnosis of TB in children when compared or combined with IFN-γ[106–109], ferritin has been proposed in other diagnostic algorithms as an adjuvant biomarker for TB diagnosis[110–114], and vitamin D has been reported in multiple mechanisms of host protection against *M. tuberculosis* infection[115–117]. Future studies should validate this combination of four blood-based biomarkers in other geographical areas and using the current QFT-Plus test tubes, as

its development could be of great interest for the detection of paediatric TB in early stages of the disease[98].

Another priority for improving TB case detection is the need to develop accessible diagnostic methods in low- and middle-income countries, where the greatest burden of new TB cases resides[1,80,118]. So far, the urine lateral-flow LAM assay AlereLAM is the only commercially available non-sputum-based TB diagnostic recommended by the WHO for the complementary diagnosis of TB in patients with HIV[69, 73]. In recent years, the LAM-based prototype, FujiLAM, has reported higher sensitivity in HIV-infected individuals than its predecessor[71,119]. Evaluation of this assay in other populations is of great interest to improve access to TB diagnosis in high-burden settings. In article 2, the FujiLAM assay was evaluated using urine samples from a cohort of HIV-positive and HIV-negative adults with signs and symptoms suggestive of TB attending three district hospitals in Nigeria. The sensitivity among HIV-negative and HIV-positive patients (65.7% and 70.0%, respectively) was higher than that reported for the rapid LAM test AlereLAM among patients with HIV [42% (95% CI = 31–55)][69]. Although it has been argued that the higher concentration of LAM in the urine of HIV-positive individuals reflects an increased haematogenous spread of TB to the kidneys[120], this does not appear to be limited to renal involvement, but the detection of LAM in urine may also be associated with the total bacterial load of *M. tuberculosis* and the severity of the disease[72,121]. This would explain the high sensitivity found among HIV-negative patients (65.7%) in contrast to other studies[72,73,122], as the patients were recruited from district hospitals in Nigeria and a high proportion of our participants might be in advanced stages of the TB disease. Accordingly, the FujiLAM assay was evaluated using urine samples from a cohort of children with presumptive TB attending a paediatric hospital in Haiti (article 3). Sensitivity was higher in children with more advanced stages of the disease, and the number of positive cases was proportionally higher among underweight and stunted children. This has also been previously described in other studies[123–125] suggesting that the immunomodulatory effect of undernutrition against TB may have limited the containment capacity of the bacilli, increasing the amount of LAM in urine samples and thus the sensitivity of LAM tests[126]. However, the small sample size of children with confirmed TB has underpowered estimates in this study. Although LAM is a cell wall component present in most *Mycobacteria* species with potential cross reactivity risk, we found a high specificity in both HIV-positive (93.3%) and HIV-negative (99%) adults and children (95%). This has also been previously reported[69,72,82,126,127], suggesting the role of FujiLAM assay to clinical decision-making as a rule in test for TB.

Another approach to step forward in the improvement of TB control is the use of omics technologies as a tool for TB biomarker discovery[128,129]. Metabolomics provides a snapshot of all the mechanisms involved in the interaction between the host and *M. tuberculosis* during disease[94,130]. In article 4, we identified a urine NMR-based

metabolic fingerprint of TB with potential to diagnose TB patients (regardless of TB location or early treatment initiation) and discriminate them from patients with pneumococcal pneumonia, individuals with LTBI, and uninfected individuals. This metabolic fingerprinting is based on eight metabolites involved in the oxidative metabolism of carbohydrates, proteins, and fats, as well as the metabolic pathways of the necessary amino acids and derivatives needed to promote bacterial growth and macrophage infection[131]. This metabolic fingerprint was also detected with a low-field benchtop NMR spectrometer. Despite its lower resolution compared to the high-field spectrometer[132], it was sufficient to detect the metabolic profile of TB. The compact and portable size and the successful performance of this approach, demonstrated in previous studies[132–134], makes low-field benchtop NMR-based metabolic fingerprinting a promising diagnostic tool. Miniaturisation to a portable NMR device would allow this technology to be integrated into clinical reference laboratories. With regard to the paediatric population, we identified an association between the urine NMR-based metabolic fingerprint and the clinical case definitions used for the classification of TB in children (article 5). The urinary metabolic fingerprinting showed metabolic differences between children with presumptive TB with two or less clinical criteria and three or more clinical criteria suggestive of TB. Moreover, children with high diagnostic certainty of TB showed metabolic fingerprints similar to that of children with bacteriologically confirmed TB. This metabolic response could be attributed to the physiological stimuli that occurs during disease progression, in which the infection overcomes the host immune system, the bacilli replicate, and the increased metabolic activity of the mycobacteria modifies the tissues physiopathology, with changes in the host metabolome[93,135]. Previous studies in children have reported different metabolomic profiles analysed by NMR spectroscopy for TB detection in plasma and serum[136,137]. However, neither of these studies reported whether the metabolic profile could discriminate between children with different diagnostic certainty of TB[136]. Despite the small final number of bacteriologically confirmed TB cases in our study that prevented the development of a TB-specific discriminatory model in this population, we observed differences in the urinary metabolic fingerprint of children with different certainty in the TB diagnosis. However, further analysis with a larger group of children with confirmed TB is needed to evaluate the full potential of this approach in children. The articles 4 and 5 represent a first step towards the development of an affordable metabolomic approach for the diagnosis of all types of TB. However, its performance should be evaluated in other geographical areas. In addition, quantification of the statistically significant metabolites identified in this TB metabolic fingerprint would allow this technology to be adapted to a point-of-care test[138].

In recent years, new markers have been identified for disease prediction and TB treatment outcomes[90]. However, few metabolomics studies have focused on monitoring treatment response[139–142]. In article 6, we detected a urinary metabolic

fingerprint by low-field NMR spectrometry in patients with drug-susceptible TB monitored during the intensive and continuation phase of treatment. The differences observed in the course of treatment suggest that metabolomics may be able to predict treatment response. In this study, all patients completed treatment and were cured. However, previous studies have identified differences in the metabolic profile of patients who have responded and those who have failed treatment[141,142]. The emerging acquisition of resistance to anti-TB drugs, which is largely due to the suboptimal management of TB treatment, has led to an increase in treatment failure[1]. This proof of concept may be used as a benchmark for comparing urinary metabolomic differences observed in future treatment response monitoring studies of patients with drug-resistant TB, which may correlate with unfavourable treatment outcomes.

The identification of new blood biomarkers of host response to *M. tuberculosis*, the evaluation of *M. tuberculosis*-specific urinary antigens, and the discovery of urinary metabolites resulting from the interaction between the host and *M. tuberculosis* have contributed to the development of new approaches to improve TB diagnosis in patients unable to expectorate, such as children, the elderly and adults without productive cough, and individuals with disseminated, extrapulmonary, non-cavitary disease. Also, the application of metabolomics could be a promising tool to monitor progress and assess response to treatment.

Diagnostics are an essential part of a well-functioning and high-quality health system[143]. However, constraints in accessibility, quality, and time of diagnostic services in low- and middle-income countries represent a challenge for current TB diagnosis, where a high burden of TB resides[54,80,83,144]. Consequently, currently, about 41.1% of patients with TB are not diagnosed or reported, contributing to the estimated 1.5 million annual deaths globally[1]. The studies in this thesis have presented different biomarkers using alternative non-sputum-based and non-invasive approaches for the future development of TB diagnostics, with a special focus on vulnerable populations and low- and middle-income countries[80]. Aware of the prolonged treatment of TB[145], the poor adherence to treatment[146–148], and the emergence of drug-resistant strains[1,74], this thesis also presented a preliminary approach to assess response to TB treatment based on the metabolomic profile of TB in urine.

8

Conclusions

Conclusions

Host-response biomarkers (Article 1 and Patent 1)

The combination of IFN-γ, IP-10, ferritin, and 25(OH)D increase sensitivity to discriminate between children with active TB and LTBI classifying correctly 93.2% and 90.0% of children, respectively.

The combination of IFN-γ, IP-10, ferritin, and 25(OH)D detected in the supernatants of the QFT-GIT tubes, would improve access to TB services, promoting early diagnosis of TB and LTBI in children.

Pathogen-specific biomarkers (Article 2 and Article 3)

Detecting LAM with Fujifilm SILVAMP TB-LAM assay in adults either HIV positive or HIV negative with presumptive TB has a higher performance than current urine LAM assays.

LAM detection with Fujifilm SILVAMP TB-LAM had 60% sensitivity and 95% specificity in children microbiologically confirmed with TB, but a number of positive results in children were not diagnosed by conventional methods. Larger studies are needed to assess the real performance of the test in children population.

The Fujifilm SILVAMP TB-LAM assay enables rapid, non-invasive TB diagnosis from urine samples tested easily and without additional instrumentation, generating opportunities to diagnose TB in children or HIV patients, who cannot expectorate and are paucibacillary.

The diagnostic and operational characteristics of the Fujifilm SILVAMP TB-LAM assay may help to add value to clinical decision-making as a supportive test for TB in children, especially in low- and middle-income countries, where available facilities are limited. This assay would facilitate the detection and initiation of TB treatment on the same day of consultation in primary health centres.

Host-pathogen interaction biomarkers (Article 4, 5, 6, and Patent 2)

The urine-based NMR metabolic profile identified in adults (assigned to eight metabolites: aminoadipic acid, citrate, creatine, creatinine, glucose, mannitol, phenylalanine, and hippurate) allows discrimination of TB patients from those with pneumococcal pneumonia, LTBI, and non-infected individuals; and could provide an

alternative method to sputum-based tools for the diagnosis of all forms of TB in adults.

This technology transferred to a low-field benchtop NMR device could improve its implementation in microscopy centres.

The urine NMR-based metabolic fingerprinting may help to observe differences in the metabolic profile of children with different diagnostic certainty of TB and improve the characterization of the clinical spectrum of the disease as well as the search for new diagnostic and prognostic biomarkers of TB in children.

The NMR-based urine metabolic profile of patients with drug-sensitive pulmonary TB changes during anti-TB treatment. This change is progressive and similar to the profile of uninfected controls by the time of treatment completion. This may enable the detection of differences in the metabolic profile over time, which may correlate with unfavourable treatment outcomes.

The application of metabolomics from high-field and low-frequency NMR spectroscopy may help to monitor TB treatment response and disease outcome.

9

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Annexes

Annex 1

Impact of COVID-19 on Tuberculosis Control

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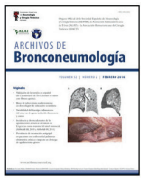

Editorial

Impact of COVID-19 on Tuberculosis Control

Impacto de la COVID-19 en el control de la tuberculosis

Dear Editor,

The onset of the COVID-19 pandemic came through a World Health Organization (WHO) statement declaring an outbreak of pneumonia, similar to severe acute respiratory syndrome (SARS), in a population in central China.[1] What appeared to be a common outbreak of a zoonotic virus in Southeast Asia, where most outbreaks of respiratory disease originate, turned into an epidemic that triggered chaos in hospitals and primary care services until they collapsed in a matter of weeks. On March 11, the WHO declared COVID-19 as a global pandemic.[2] Since then, the irruption of COVID-19 has led to the suspension of routine health care services in almost every country.[3] Low- and middle-income countries (LMICs) experienced substantial difficulties with interruptions in services for the three of the main health priorities, specifically HIV/AIDS, tuberculosis (TB), and malaria.[4]

TB is the most deadly infectious disease worldwide (above HIV/AIDS), with an estimated 10 million new cases and 1.5 million deaths per year.[5] TB and COVID-19 are airborne infectious diseases that primarily attack the lungs. Both diseases have similar symptoms such as cough, fever and shortness of breath. However, testing for COVID-19 or TB should be conducted according to the clinical characteristics, history, and local burden of TB to ensure that TB patients' diagnostic needs are not neglected while testing for COVID-19.[6] In LMICs where the burden of TB is highest, the differential diagnosis of COVID-19 and TB is key to detect the co-infection and prevent a bad evolution and even death.[7,8] COVID-19 pandemic response, particularly containment measures, reassignment of health care personnel and equipment are affecting TB prevention and care programs.[9–12] After the first three months of the pandemic, a survey was sent to 165 countries, of which 42% reported partial disruptions in TB case detection and treatment.[3] A study conducted in 33 centres in 16 countries on five continents reported that during confinement 82% of centres showed reductions in TB-associated hospital discharges, 84% of centres reported a decrease in newly diagnosed active TB cases, 95% showed a decrease newly LTBI outpatient visits, and 75% and 81% of centres showed reductions in TB and LTBI outpatient visits, respectively.[13] Estimates of the impact of the COVID-19 pandemic on the TB response suggest that a 3-month lockdown and a 10-month protracted recovery could result in 6.3 million additional TB cases between 2020 and 2025, and 1.4 million additional TB deaths during this time. These numbers would imply a regression of at least 5–8 years in the fight against TB.[6] Over the last few months, an impressive number of molecular assays ($n = 378$) and immunoassays ($n = 444$) have emerged for the diagnosis of COVID-19.[14] Most of them have taken advantage of existing technologies to develop COVID-19 diagnostics. Among which is the Xpert® Xpress SARS-CoV-2 test for use in GeneXpert® devices.[6] Due to the health emergency, countries with fewer resources have experienced the shared use of GeneXpert for COVID-19 and TB.[15] The diversion of attention and resources away from TB could have a devastating effect on the diagnosis and treatment of the disease, especially in LMICs.[5] A 25% reduction in global TB detection over three months is predicted to lead to a 13% increase in TB deaths, bringing us back to the TB mortality figures reported in 2015.

Pulmonology, infectious medicine, and microbiology services have been overwhelmed by the pandemic. All resources have been devoted to the fight against COVID-19, leading to the paralysis of most of the TB screening programs in public dining rooms, shelters, and drug cessation centres. This fact, associated with the difficulty of patients to access primary care, has reduced the number of TB cases diagnosed since the beginning of the pandemic. Economic and social problems arising from the pandemic suggest an increase in the delay of diagnosis and the severity of cases being diagnosed. However, containment and the use of personal protective equipment could have had a positive impact on TB control by helping to mitigate community transmission of the disease. Thus, the reduction of mass gatherings in spaces such as buses, trains, cinemas, or sports events, as well as the intensive use of masks, may have contributed to reducing the risk of TB transmission during the pandemic and after confinement. It would be interesting to include these variables in predictive analyses in order to know the long-term effect these measures could have on TB control.

COVID-19 could have a severe impact on case detection and treatment success.[11] However, TB may contribute to the control of COVID-19 with lessons learned and possible direction for future COVID-19 preventive treatment strategies. Thus, public health strategies employed in TB programs could serve as examples to identify and mitigate potential risks of SARS-CoV-2 infection.[15] Taking TB as precedent, contact studies developed by TB prevention and control services have been applied to identify contacts of patients infected with SARS-CoV-2.[16] *Bacille de Calmette et Guérin* (BCG) has been the vaccine against TB for almost one hundred years. However, BCG has also demonstrated a protective effect on the immune system that reduces overall mortality during the first few

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years of life by improving responses to other respiratory infections such as respiratory viruses.[17] This evidence has led to clinical trials to assess whether BCG would be able to provide non-specific protection to mitigate the outbreak before a specific COVID-19 vaccine is developed.[18] However, until these trials are completed, continued routine use of the vaccine exclusively for TB is strongly recommended so as not to jeopardise the necessary supply of BCG to protect children against TB in high-incidence areas.[19]

The pandemic has altered sustainable development goals into a challenge for humanity in the coming years. Global support should be provided to enable the countries and communities most affected by TB to respond to the pandemic while ensuring the maintenance of TB services. The pandemic response should be integrated into existing disease workflows to create synergies and strengthen health systems in a transversal approach. Over the next few years, we must promote bi-directional screening, multi-pathogen tests, and work using connectivity solutions for TB/COVID-19 surveillance.

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Annex 2

Future approaches to the diagnosis of TB in children

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validez del test *Tuberculin Skin Test* (TST) y Quantiferon-TB *Gold In-Tube Assay* (QFT) (Perez-Porcuna *et al.*, 2016). El estudio incluye una muestra de 29 niños menores de 6 años sin ningún contacto reciente conocido con TB y otro grupo de 92 niños con contacto a un caso índice en los 12 últimos meses. Fueron excluidos del análisis 16 niños porque presentaron un resultado indeterminado con QFT. El reclutamiento de los datos se realizó en la Policlínica Cardoso Fontes y en la Fundação de Medicina Tropical Dr. Heitor Vieira Dorado, Manaus (Brasil) entre marzo del 2009 a febrero del 2010. Todos los casos índices adultos tenían esputo y cultivo positivo. Los individuos que recibieron tratamiento o profilaxis de TB fueron excluidos.

Para estimar la prevalencia y la sensibilidad y especificidad de ambos test se asumió para la prevalencia una distribución priori no informativa y basándonos en la literatura de LTBI, se consideró que la sensibilidad y la especificidad de los dos test se encuentran dentro del rango 50%–100%.

Treinta y cuatro niños (32,7%) mostraron resultado positivo con el QFT y treinta y tres (31,4%) con el TST, pero sólo 19 niños (23,9%) dieron positivos para ambos test. Los dos test mostraron una baja concordancia de 0,364 (p <0,001). La prevalencia de la infección en el grupo de niños sin contacto con un caso índice fue de 0,04 (95% ICr [0,00; 0,20], siendo de 0,50 en niños con contacto con un caso índice (95% ICr [0,28; 0,81]). La sensibilidad del QFT fue de 0,58 (95% ICr [0,41; 0,78]) y 0,75 (95% ICr [0,49; 0,94]) para el TST. La especificidad del QFT fue de 0,79 (95% ICr [0,67;0,91]) y 0,92 (95% ICr [0,78; 0,98]) para el TST. En el grupo de niños con el contacto con un caso índice, la probabilidad de que un niño diagnosticado como positivo realmente este infectado fue de 0,74 (95% ICr [0,47; 0,95]) para QFT y 0,91 (95% Crl [0,61; 0,99] para TST. Por otra parte, la probabilidad de que un niño diagnosticado como negativo realmente no este infectado fue de 0,65 (95% Crl [0,27; 0,88]) para QFT y 0,79 (95% Crl [0,31; 0,96]) para TST.

Conclusión

La estimación de la prevalencia de una enfermedad y los parámetros (sensibilidad, especificidad y valores predictivos) de los test utilizados para estudiar la presencia de una enfermedad en ausencia de *gold standard* es un importante avance para la evaluación de test de diagnóstico. Esto es de especial relevancia en el actual contexto epidemiológico y diagnóstico del estudio de la infección por *Mycrobacterium Tuberculosis* en la que es imposible determinar si un individuo está realmente infectado o si esta persona presenta respuesta inmune a una infección previa.

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Future approaches to the diagnosis of TB in children

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It is estimated that there are more children with tuberculosis (TB) than those diagnosed. This is mostly because currently available methods are based on sputum samples. However, young children have a low number of bacilli in their sputum or are unable to expectorate. Therefore, despite advances in rapid molecular TB diagnostics, methods based on respiratory specimens have a low diagnostic sensitivity in the paediatric population. As for the immunodiagnostic assays, both tuberculin-skin-test (TST) and interferon-gamma release assays (IGRAs)—that measure cell-mediated immune responses following *Mycobacterium tubercu-*

losis infection—provide information about likely M. tuberculosis infection but are unable to distinguish between latent infection and active TB disease. As a result, there is currently no *gold standard* for the diagnosis of childhood TB. Therefore, most children are diagnosed through radiological and clinical scoring systems limited by the clinical presentation of the disease hampering their diagnosis and delaying therapeutic strategies for TB control. Moreover, its diversity of clinical manifestations often overlaps with other common childhood conditions as pneumonia, HIV-associated lung disease, and malnutrition.

In order to close the gap in detecting cases of childhood TB, the search for new biomarkers should be focused on developing a rapid, child-friendly and non-sputum-based test able for diagnosing the early stages of paediatric TB. This would improve the detection of the source of TB infection in the community and prevent progression to severe forms of the disease, such as disseminated TB and TB meningitis.

Recently promising biomarkers for paediatric TB diagnosis in non-sputum-based specimens have been reported. According to the WHO-endorsed minimal targets product profiles (TPPs) for new TB diagnostic test in children, the minimal targets of diagnostic performance recommended should meet sensitivity and specificity of ≥66% and ≥98%, respectively[1].

Blood-based tests for TB diagnosis. Two case-control studies by Armand *et al.* and Zhou *et al.* reported a combination of cytokines in plasma (IP-10, IL-2 and IL-13) and a combination of circulating miRNAs in PBMCs (miR-1, miR-155, miR 31, miR 146a, miR 10a, miR 125b, miR 150, and miR 29) respectively, with sufficient diagnostic performance to develop a new TB diagnostic test in children. However, neither could discriminate between active TB and LTBI cases. In this approach, IL-2 and TNF-α cytokines showed in the potential for discriminating between active and latent TB with diagnostic but did not reach the minimum target product profiles for new TB diagnostics[2]. Recently, we showed that the combination of the cytokines IP-10, IFN-γ, ferritin, and 25-hydroxyvitamin-D in the QuantiFERON Gold in Tube (QFT) Nil, Ag-TB, and PHA tubes, and biochemical tubes, with Luminex and chemiluminescence immunoassays, has potential to discriminate between active and latent TB and to identify the onset of primary TB in children[3]. This combination achieved the best diagnostic performance with a correct classification of active TB (93.2%) and LTBI (90.0%) cases. Also, this combination of biomarkers classified correctly 76.2% of the mediastinal TB cases showing the potential of these biomarkers for early diagnosis of paediatric TB.

Stool-based tests for TB diagnosis. Nucleic acid amplification testing of stool samples could be a promising non-invasive and easy to collect alternative to the sputum-based methods for TB diagnosis in children. In recent years, studies have been conducted to evaluate the diagnostic accuracy of stool testing with the Xpert MTB/RIF assay and in-house test in the paediatric population[4]. However, these studies reported high heterogeneity of stool test sensitivities from 12% to 100% with Xpert stool test, and 19%-67% with another in-house test. Although molecular stool tests have demonstrated potential as diagnostic screening tests, the variation between studies in test methodology and procedures presents a challenge in optimizing test sensitivity. Furthermore, data of PCR stool tests in children under five years old for diagnostic accuracy evaluation remains insufficient.

Urine-based tests for TB diagnosis. Urine is a sterile, non-invasive, and easily collectable sample that requires little sample preparation, which makes it a promising sample for TB diagnosis. Alere Determine TB LAM Ag immunoassay (AlereLAM, Abbott, Chicago, US) has been introduced as a new alternative for TB diagnosis from urine samples with higher sensitivity in HIV patients. However, the diagnostic accuracy showed insufficient sensitivity and specificity (48.3% and 60.8%, respectively) for diagnosing TB in children attributed to the protocol used for urine collection. Recently, a new generation of urine LAM assays, Fujifilm SILVAMP TB LAM (FujiLAM) has been developed[5], demonstrating an improved sensitivity for diagnosing TB in HIV patients compared to AlereLAM (in patients with pulmonary TB, extrapulmonary TB, or both only FujiLAM detected 60%, 67%, and 91% respectively, compared to, 19%, 41%, and 61%respectively for AlereLAM). Children may also have a higher amount of LAM antigen in urine (as people with HIV) as they have an immature immune system leading to a bad contention of the TB infection. Therefore, FujiLAM assay should be evaluated in children as a promising assay for TB diagnosis.

Metabolomics has emerged from the 'omics' as a tool for obtaining a fingerprint of all the metabolites presents in a cellular system, allowing the discrimination between samples from different biological status. In this approach, urine-based metabolomics can be applied to the study of metabolites affected by host-pathogen interactions and the identification of diagnostic markers for TB disease. A recent study of metabolic profiling in children's urine by NMR has been conducted to correlate the broad spectrum of disease manifestations and TB severity to clinical case definitions for the classification of TB in children. Further exploration of the utility of this technology in children should be assessed to improve research into the diagnosis of TB in children (manuscript pending to submit).

We aimed to address the challenges of diagnosing TB in children and highlight the latest advances focused on the development of rapid, accurate and child-friendly tools for TB diagnosis in the paediatric population.

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Infección tuberculosa en niños *"Visiting Friends and Relatives"* que viajan a países con una elevada incidencia de TB

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Introducción

Al juliol de 2015 es va presentar la nova guia de consens "Recomanacions per a la prevenció i el control de la tuberculosi pediàtrica a Catalunya" en la qual s'indicava la realització d´una prova de tuberculina (PT) a tots els nens i nenes, sobretot als menors de 5 anys d'edat, que tornessin de països amb una incidència de tuberculosi (TB) de com a mínim 3 vegades (>50 casos per cada 100.000 habitants) la incidència de TB a Catalunya[1].

Encara que la majoria dels casos de TB pediàtrica registrats a Catalunya a l'any 2017 corresponen a nens autòctons (87,7%), els fills de famílies d'immigrants representen >50% del total de casos declarats[2]. Molts d'ells són nens nascuts al nostre país o a l'estranger que visiten les seves famílies d'origen, que viuen en països de major incidència de TB que la nostra, i es defineixen com a *"Visiting Friends and Relatives"* (VFRs)[3]. Els objectius d'aquest estudi van ser estimar la taxa d´infecció TB latent (ILTB) recent, adquirida durant el viatge, en nens i nenes que són viatgers VFRs. També volíem determinar quins factors podien estar associats als resultats positius de PT i tests d´alliberació d´interferon gamma (IGRAs) en aquesta població i veure si hi havia factors de discordança entre els resultats de PT i IGRAs.

Annex 3

Perspectives for systems biologyin the management of tuberculosis

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REVIEW
I. KONTSEVAYA ET AL.

Perspectives for systems biology in the management of tuberculosis

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Shareable abstract (@ERSpublications)
We are at the doorstep of a new era in which systems biology approaches will contribute to the management of patients with tuberculosis including prediction of risk for disease progression and severity, response to therapy and treatment outcome. https://bit.ly/36DQegb

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Abstract

Standardised management of tuberculosis may soon be replaced by individualised, precision medicine-guided therapies informed with knowledge provided by the field of systems biology. Systems biology is a rapidly expanding field of computational and mathematical analysis and modelling of complex biological systems that can provide insights into mechanisms underlying tuberculosis, identify novel biomarkers, and help to optimise prevention, diagnosis and treatment of disease. These advances are critically important in the context of the evolving epidemic of drug-resistant tuberculosis. Here, we review the available evidence on the role of systems biology approaches – human and mycobacterial genomics and transcriptomics, proteomics, lipidomics/metabolomics, immunophenotyping, systems pharmacology and gut microbiomes – in the management of tuberculosis including prediction of risk for disease progression, severity of mycobacterial virulence and drug resistance, adverse events, comorbidities, response to therapy and treatment outcomes. Application of the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) approach demonstrated that at present most of the studies provide "very low" certainty of evidence for answering clinically relevant questions. Further studies in large prospective cohorts of patients, including randomised clinical trials, are necessary to assess the applicability of the findings in tuberculosis prevention and more efficient clinical management of patients.

Introduction

Tuberculosis, the infectious disease caused by *Mycobacterium tuberculosis*, remains a leading cause of morbidity and mortality worldwide. The World Health Organization (WHO) estimates that 10 million people developed tuberculosis and approximately 1.4 million people died from this disease in 2019 [1]. Currently, the management of patients with tuberculosis is based on clinical and laboratory assessments that have a number of limitations; importantly, the standardised approach does not consider the individual variability in host immunity or the pathogenicity of *M. tuberculosis*.

Systems biology is a rapidly growing field of computational and mathematical analysis that integrates various technologies, including genomics, transcriptomics, proteomics, metabolomics, lipidomics and microbiome profiling. These technologies leverage very large datasets from entire genomes or global sets of proteins/metabolites. Analysis of these data involves artificial intelligence, a branch of engineering that implements novel concepts and novel solutions to resolve complex challenges. In medicine, artificial intelligence is applied in two branches: physical (robots, medical devices) and virtual, *i.e.* represented by various machine learning algorithms [2, 3]. These approaches are extensively used in cancer research providing insight into tumorigenesis and accelerating integration of precision cancer medicine into clinical routine [4]. Recent examples of successful application of machine learning techniques include identification of differentially expressed microRNAs that may be considered as diagnostic biomarkers for hepatocellular carcinoma *via* a random forest algorithm [5] or applying various machine learning models for intrinsic subtyping of breast cancer which may help in estimating the risk of relapse after surgery or survival [6].

In recent years, systems biology approaches have been increasingly applied in tuberculosis research to identify biomarkers that can indicate changes in pathogen viability or its susceptibility to antituberculosis drugs, elucidate host signalling in response to the pathogen, and predict treatment response. Such biomarkers could be used as treatment monitoring tools, for example, and could inform the development of individualised rather than standardised approaches to future tuberculosis management that would be based on the individual characteristics of host and pathogen. It is especially important for optimising the treatment of patients infected with resistant forms of tuberculosis, including rifampicin-resistant and multidrug-resistant (MDR) tuberculosis, that require longer therapy with more toxic drugs and have generally higher risks of unfavourable treatment outcomes. Although the novel biomarkers are promising, in many cases, their clinical relevance and certainty of evidence supporting their use in clinical practice have still to be fully evaluated.

The aim of this review is to summarise the currently available key evidence and to illustrate how different fields of systems biology may contribute to the improvement of the future management of patients with tuberculosis. We have assessed the current state of use of novel biomarkers in clinical practice as well as the quality of evidence for answering clinically relevant questions and forming recommendations for implementation of these biomarkers in clinical practice.

Bacterial biomarkers derived from systems biology approaches

Mycobacterial genomics

Next generation sequencing (NGS) technology provides rapid and inexpensive sequencing of DNA that can be used to identify drug-resistance-conferring mutations in the *M. tuberculosis* genome. It is currently implemented in routine tuberculosis diagnostics, which renders added benefit to tuberculosis patient care [7]. In recent years, the key challenges of NGS-based drug-resistance predictions have been intensively addressed: the ability to predict susceptibility, to provide results more rapidly than phenotypic drug-susceptibility tests (pDST), and eventually standardisation with automated results interpretation [8].

Validation of resistance prediction to all first-line drugs using NGS was an important step to bring this technology into clinical practice [7, 9]. Now, patients and clinicians can potentially receive faster and cost-effective drug-resistance reports based on mutation profiles compared with routine reference laboratory reports [8]. Results from pilot studies even propose sequencing and analysis within 1 day from direct patient material; however, routine practice is currently limited by low bacterial loads and potential contaminations in sputum specimens [10, 11]. Targeted NGS methods, as opposed to whole genome sequencing (WGS), amplify defined DNA sequences implicated in drug resistance to overcome limitations of specimens exhibiting minimal amounts of mycobacterial DNA and are able to detect heteroresistance, *i.e.* presence of a resistant bacterial sub-population [12].

In order to provide comparable results across different platforms and between different laboratories, it is crucial to standardise computational algorithms and to develop a consensus on genetic markers that indicate the exclusion of a respective drug from the antituberculosis regimen [13, 14]. Yet, even with the latest automated interpretation tools, considering over 1000 molecular markers, we would not meet the WHO target product profile (TPP) for sensitivity (90%) and specificity (95%) for all drugs used in MDR tuberculosis therapy [15]. Thus, partially characterised mutations will still require focused pDST and ideally determination of the minimum inhibitory concentrations (MIC) of drugs to provide patients an optimal personalised therapy. International consortia such as the CRyPTIC project (www.crypticproject.org) further aim to improve the accuracy of resistance predictions to MDR tuberculosis drugs by aligning thousands of genotypic and phenotypic datasets and by determining MICs to multiple antituberculosis medicines.

On a small scale, predicted MDR tuberculosis regimens based on NGS data, and jointly interpreted with MIC data, already demonstrate a 93% overlap with guideline-driven regimens based on routine diagnostics in a tuberculosis reference centre [16]. When it comes to "big data" analysis and making new inferences about the phenotypic impact of noncanonical mutations, machine learning algorithm or genome-wide association studies are often employed [17, 18]. However, especially for new and repurposed drugs for which resistance is not yet widespread, *e.g.* bedaquiline, clofazimine or linezolid, mutant libraries and phenotypic characterisation are the main instruments to complement the knowledge of relevant mutations [19].

In coming years, NGS technologies will likely become the first choice to generate rapid and comprehensive tuberculosis drug-resistance profiles that initiate early treatment decisions. Moreover, mycobacterial genomics might also be employed to predict the risk of relapse and treatment failure linked to particular mutations in the bacterial genome, which enhances the likelihood of developing unresponsiveness or resistance during therapy, and thus to expand the spectrum of diagnostics in personalised tuberculosis medicine [20, 21]. Its full-scale implementation in clinical and public health applications is still limited by a number of factors: lack of standardised end-to-end solutions and the technical requirements of laboratories. The high costs associated with implementation and maintaining of NGS technologies, internet infrastructure and cloud computing may limit its application in low- and middle-income settings [14].

Mycobacterial transcriptional profile

To date, transcriptomics research has been largely focused on the human transcriptome response to tuberculosis while the corresponding literature describing pathogen adaptation is relatively sparse.

In response to antibiotic treatment *M. tuberculosis* shows diverse transcriptomic responses. A study of 75 different antituberculosis agents that used a DNA array and patterns of responses clustered together by mechanism of drug action identified this as a useful approach that since then has been used along with RNA sequencing for the identification of responses to individual agents [22, 23]. For example, *M. tuberculosis* responds to bedaquiline by induction of the dormancy survival regulon *DosR* and activation of ATP-generating pathways, thereby maintaining bacterial viability during initial drug exposure. This may contribute to the recognised slow response to this antibiotic and affect the evaluation of its efficacy in early bactericidal activity studies [24].

Quantifying 16S ribosomal RNA has been employed to determine the number of viable *M. tuberculosis* cells in a highly sensitive molecular bacterial load assay (MBLA) that detects as few as 10 $CFU \cdot mL^{-1}$ over many weeks of treatment [25]. As 16S rRNA is present in higher copy numbers than mRNA and is more stable, it makes an excellent target to monitor treatment response [26]. It correlates closely with culture and, as it is species specific, bacterial contamination does not result in loss of data from the sample [26–28].

A promising new tool for dissection of the host–pathogen interplay during infection is dual RNA sequencing allowing unbiased and simultaneous profiling of host and pathogen transcription that captures the transcriptome in its entirety. It has been used for studying the metabolic regulation of host and pathogen and the molecular dynamics during mycobacterial infection both *in vitro* and *in vivo* [29, 30].

Mycobacterium tuberculosis-*specific proteomic signatures*

Similar to transcriptomics, pathogen proteomic biomarkers have been less studied compared with host proteomic signatures. However, different sets of *M. tuberculosis* peptides have been identified in sera from patients with active tuberculosis [31] and latent *M. tuberculosis* infection (LTBI) [32] using multiplexed multiple reaction monitoring mass spectrometry (MS); their applicability as biomarkers is yet to be investigated.

Proteomic profiling of *M. tuberculosis* isolates has contributed to the understanding of drug-resistance mechanisms and the virulence factors of the bacilli that can be used for classification of disease and as promising targets for drug and vaccine development [33, 34].

Finally, through modulation of significant host proteins, MS-based proteomics has promoted the advancement of host-directed therapies (HDTs). For example, induction of endogenous antimicrobial peptides may be advantageous for effective targeting of drug-resistant tuberculosis due to lower bacterial resistance to antimicrobial peptides compared with antibiotics. An important example here is vitamin D supplementation in antituberculosis treatment that induces production of an antimicrobial peptide cathelicidin [35] that has been shown, although inconclusively, to improve sputum and culture conversion

rates in cohorts of patients with tuberculosis [35–37]. It is important to note that observational studies on HDTs are associated with a high risk of bias and the clinical trials published to date often provide evidence with only low certainty and a serious risk of bias [37].

Different gut microbiomes as tuberculosis signatures

The microbiome is defined as all microbes that colonise human epithelial surfaces [38]. In the context of tuberculosis, some data suggest that changes in gut microbiota may be linked with lung immunity [39] and may even influence the outcome of *M. tuberculosis* infection [40]; however, convincing evidence is lacking. Comparative microbiome studies in patients with tuberculosis *versus* healthy controls have not revealed specific changes characterising the tuberculosis microbiome. Small differences such as a nonsignificant decrease in microbial alpha-diversity in patients with tuberculosis have been reported [41]. One study described increased numbers of short-chain fatty acid-producing bacteria associated with regulatory immune functions in tuberculosis [42] while the opposite was found in another study [43]. First attempts also used microbiota profiling as a marker for disease severity in rhesus macaques [44]. However, further research of factors that might affect gut microbiota are needed before microbiome data can be employed as a disease biomarker; for example, to complement conventional diagnostic methods in cases where they are less feasible (*e.g.* in children).

Standard treatment with rifampicin, isoniazid, pyrazinamide and ethambutol in drug-susceptible tuberculosis has been demonstrated to provoke a small decline in microbial alpha-diversity and a change in the community structure [41]. Changes in gut microbiota persisted for more than 1 year after the treatment has been stopped [45]. MDR tuberculosis treatment consisting of different broad-spectrum antibiotics given over a long period of time has the potential to provoke serious and long-lasting dysbiosis in patients with tuberculosis. A 16% decrease in microbial richness linked with altered lipid metabolism has been seen in patients 3–8 years after recovery from MDR tuberculosis ($p=0.018$) [46]. Future studies need to differentiate the effects of individual second-line drugs on gut microbiota composition to evaluate the relevance of microbiome data for the choice of second-line antituberculosis drugs and the putative influences of changes in microbiota on tuberculosis disease severity, risk of developing adverse events, treatment outcome as well as the general effects of tuberculosis disease and treatment on a patient's health.

Human biomarkers derived from the systems biology approaches

Human genetics and epigenomics in the management of patients with tuberculosis

The contribution of human genetic factors to the risk of tuberculosis, clinical presentation and management of the disease has long been the subject of intense investigation. A summary of human genetic susceptibility factors for tuberculosis has recently been published [47]. Many of these factors were identified using genome-wide linkage studies and genome-wide association studies – two systematic, powerful and unbiased approaches for identifying disease-associated variants in population samples.

Although genetic studies of human susceptibility to tuberculosis have contributed to a better understanding of the pathogenesis of tuberculosis, to date only three genetic factors are known that in the future may directly impact the management of patients with tuberculosis. First, very rare mutations leading to impairment of either response or production of IFN-γ, such as in case of the Mendelian susceptibility to mycobacterial disease, can underlie severe childhood tuberculosis cases that can be treated with IFN-γ [48, 49]. Second, a common amino acid variant of *TYK2*, P1104, is a strong risk factor for tuberculosis (relative risk 8–16) that selectively impairs IL23-dependent IFN-γ production [50]. Approximately 1 in 550 Europeans carry the high-risk homozygous genotype; it was estimated that throughout history, approximately 10 million deaths attributed to tuberculosis in Europe occurred due to this mutation [51]. Inclusion of *TYK2* genotyping in contact investigations of tuberculosis can therefore be clinically relevant in European populations.

Finally, the *LTA4H* C/T promoter polymorphism can have an impact on the management of tuberculous meningitis (TBM) and is potentially the most clinically important out of the three. *LTA4H* encodes for the leukotriene A4 hydrolase responsible for synthesis of the pro-inflammatory and pro-resolving lipid mediators, LTB_4 and LXA_4, which both, despite their counterregulatory functions, promote mycobacterial growth. Lipidome-based detection of LTB_4 and LXA_4 has been indicated as a severity marker for TBM [52]. The genotype of the *LTA4H* promoter polymorphism correlated with pre-treatment cerebrospinal fluid leukocyte counts and survival from TBM, and the beneficial effects of adjunctive dexamethasone therapy were most pronounced for carriers of the hyperinflammatory TT genotype [53]. Two large follow-up studies provided inconsistent results about the role of *LTA4H* polymorphism in TBM [54]; its impact on clinical management of TBM is currently being further investigated in a randomised trial [55].

A major focus of epigenetics is the description of chromatin structure, DNA modifications by methylation and modifications of histones, and the link of these changes with gene expression [56–58]. In the context of human tuberculosis patients, most studies have been concerned with gene expression changes in blood cells due to *M. tuberculosis* without additional characterisation of underlying DNA or chromatin changes. However, the potential importance of epigenetics for tuberculosis management is highlighted by the persistent dampening of key antimycobacterial immune response pathways even after successful therapy [57]. Also, recent studies revealed epigenetic alterations upon Bacillus Calmette–Guérin (BCG) vaccination, which can educate myeloid cell-mediated innate immune responses thereby contributing to nonspecific vaccine efficacy [59, 60]. Future studies are needed to differentiate between trained immunity as induced by attenuated *versus* virulent *M. tuberculosis* as predictors for better immune control.

Human transcriptional signatures

Studies dealing with host transcriptomic data have yielded different RNA signatures that may be able to predict future onset of disease and have the potential to be developed into diagnostic and therapy monitoring tools for active tuberculosis [61–63]. Many of these signatures include genes involved in IFN-γ driven signalling that are important for the host response to *M. tuberculosis* [61, 64]. A recent systematic review demonstrated eight RNA signatures that predicted the onset of active tuberculosis 0–3 months to disease with sensitivities ranging between 47.1% and 81.0% and specificities over 90% meeting or approximating the WHO TPP criteria; however, the accuracy of the signatures decreased with increasing time-periods before the onset of disease [62]. A 11-gene version of a previously validated 16-gene correlate of risk (COR) mRNA signature [65] has been shown to have a specificity of 84% and sensitivity of 71% for predicting tuberculosis up to 1 year before the onset of disease in a cohort of adolescents [66]; its prognostic performance in unselected populations in a high-burden setting is being currently evaluated in the CORTIS study (ClinicalTrials.gov NCT02735590) [67]. In a prospective observational study, four transcriptional signatures were able to discriminate individuals with tuberculosis from those without with sensitivities of 83.3–90.7%. These signatures either met or approximated to the minimum WHO TPP for a tuberculosis triage test (90% sensitivity, 70% specificity) but not the optimum criteria or the minimum criteria for a confirmatory test [68]. A three-gene signature is proposed to not only predict the conversion from latent to active disease, but also has been proven useful to monitor progress of treatment and correlate with lung pathology indicating patients at high risk of treatment failure [69, 70]. In a systematic review with meta-analysis, it was the only signature that met the minimum TPP criteria for both positive predictive value ⩾5.8% and 75% sensitivity and 75% specificity for a 6-month period prior to TB diagnosis [71]. An alternative five-gene signature strongly correlated with positron emission tomography-computed tomography (PET-CT) defined lung inflammation and with high accuracy (area under curve (AUC) 0.72, p=0.04) only 1 week after treatment initiation identified patients who eventually failed treatment [72]. Another transcriptomic signature, RISK6, is suggested to be used for identifying individuals at risk of incipient disease (AUC 0.87 within 0–12 months and AUC 0.74 within 12–24 months before the diagnosis of tuberculosis), as a screening test for subclinical *versus* clinical tuberculosis and for monitoring tuberculosis treatment [63]. Transcriptomic models allowing the individualisation of the duration of antituberculosis therapy necessary for achieving cure are yet to be published.

Recently published RNA signatures from whole blood are short nucleotide sequences and therefore easily translatable to point-of-care tests, which may dramatically change tuberculosis disease management in the future. Despite the promising results, none of the transcriptomics signatures have been implemented into clinical practice so far. Future studies assessing applicability of these signatures in management of patients with tuberculosis are required, including those on prospective cohorts of patients.

Human proteomic signatures for tuberculosis and antituberculosis treatment responses

Entire proteomes in patient samples are usually analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Plasma CC and CXC chemokines differentiated between active tuberculosis and LTBI allowed treatment responses to be monitored and were indicative for pulmonary cavitation [73] (table 1). Other studies revealed a serum six-protein signature (sensitivity 90%, specificity 80%) [80], an eight-protein signature in stimulated whole blood culture able to distinguish between tuberculosis and nontuberculosis, including healthy controls, LTBI and nontuberculosis pneumonia (sensitivity 75%, specificity 84%) [81] as well as sputum signatures discriminating tuberculosis from other respiratory diseases using a set of inflammatory cytokines (sensitivity 85%, specificity 96%) [82]. In a cohort of children with symptoms compatible with tuberculosis, a three-protein signature distinguished between tuberculosis, irrespective of microbiological confirmation, and other respiratory diseases with a sensitivity of 72.2% and a specificity of 75% [83]. Sputum proteomics identified a five-protein signature differentiating between tuberculosis and nontuberculosis patients (sensitivity 70%, specificity 79%) [84]. Differential regulation of pro-inflammatory, lipid and iron transport proteins were revealed between patients with active tuberculosis and LTBI patients [74]. A serum three-protein signature distinguished MDR from

TABLE 1 Proteins and metabolites defining progression from LTBI to active TB disease, response to antituberculosis treatment and treatment outcome

Clinical question	Proteins	Metabolites	Material
LTBI to active TB transition	Chemokines CCL1, CCL3, CXCL1, CXCL2, CXCL9, CXCL10 [73], kallikrein 1B, C-reactive protein, haptoglobin, alpha-1-acid glycoprotein 1 haptoglobin, complement component C9, apolipoprotein A1, serotransferrin [74]	Cortisol, mannose, gamma-glutamylglutamine, cysteine, glycocholenate sulfate, phenylalanine, histidine, citrulline, glutamine, tryptophan [75]	Blood
Treatment response	Chemokines CCL1, CCL3, CXCL1, CXCL9 [73], nectin-life protein 2, Ephrin type-A receptor 1, gp130, beta-Ala-His dipeptidase, transforming growth factor-beta receptor III, mannose receptor C type 2, disintegrin and metalloproteinase domain-containing protein 9, cell adhesion molecule-related/downregulated by oncogenes [76]		Blood
	Matrix metalloproteinases MMP-2, MMP-3, MMP-8, MMP-9, tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 [77]		Sputum
		Seryl-leucine core 1 O-glycosylated peptide [78]	Urine
Treatment outcome	Chemokines CCL1, CCL2, CXCL2, CXCL9, CXCL10, CXCL11 [73]		Blood
	Matrix metalloproteinases MMP-1, MMP-3, MMP-8, tissue inhibitor of metalloproteinases TIMP-2 [77]		Sputum
		Tryptophan [79]	CSF (TB meningitis)

LTBI: latent tuberculosis infection; TB: tuberculosis; CSF: cerebrospinal fluid.

drug-susceptible tuberculosis (sensitivity 81.2%, specificity 90%) and healthy controls (sensitivity 94.7%, specificity 80%) [85]. Proteomics also defined two prognostic signatures comprising five (TRM5) or three proteins (3PR) that predict disease progression to active tuberculosis; however, their performance did not achieve the WHO TPP for an incipient tuberculosis test [86] as well as a serum signature predicting 8-week culture status with 95% sensitivity and 90% specificity [76]. Increased levels of matrix metalloproteinases in serum were also shown to be associated with disease severity ($p<0.05$) [77]. Proteome analysis of bronchoalveolar lavage fluid identified distinct proteomic signatures stratifying active and clinically cured patients with TB into distinct groups; moreover, this study indicates the potential of identifying patients prone to relapse using proteomic analysis at the site of disease [87].

Lipidomic profiling to identify tuberculosis biomarkers

The role of host lipid metabolism as a biomarker of disease severity and progression has recently been emphasised [88]. Entire lipidomes are commonly analysed by LC-MS/MS. Yet, few patient studies are available to date. Small lipid mediators such as leukotrienes (LT), prostaglandins (PG), lipoxins (LX), resolvins, and protectins have been found to be associated with clinical features of tuberculosis by lipidomics [52, 88–90]. The pro-inflammatory LTB_4 is abundantly found in sputum and pleural effusions of patients with tuberculosis where it is likely to be involved in neutrophil recruitment and immunopathology [90]. Recently, MS analyses of plasma from tuberculosis patients with and without diabetes revealed significantly increased concentrations of the pro-resolving LXA_4 and the pro-inflammatory PGE_2; reduced amounts of LTB_4 were associated with enhanced lung pathology and bacterial burden [88]. In contrast, MS-based analysis of cerebral fluid from patients with TBM showed a clear correlation of disease severity with decreased levels of LXA_4 and PGE_2 [52]. These potent immune regulators and their downstream signalling pathways represent interesting targets for HDT and, as biomarkers thereof, can be employed to stratify patients for HDT [91]. Further studies on clinical samples are needed to validate these and identify other host lipids that can be used as single biomarkers or included into global biosignatures of tuberculosis and treatment response.

Human metabolic biomarkers of tuberculosis

In recent years, metabolic studies employing chromatography-MS or nuclear magnetic resonance have revealed novel host and pathogen biomarkers for monitoring the course of diseases, their stages and mechanisms [92]. However, only a few studies so far have presented metabolites as biomarkers for

tuberculosis progression, treatment response and outcome when detected in blood, urine, or cerebrospinal fluid (CSF) of patients [78, 79, 93, 94] (table 1).

Metabolite fingerprinting in blood can predict active tuberculosis months before the onset of clinical manifestations: a metabolic biosignature of tuberculosis comprising 10 metabolites demonstrated a performance of 69% sensitivity and 75% specificity within 5 months of diagnosis in cohorts of household contacts who either progressed to tuberculosis or remained healthy [94].

In urine, the level of seryl-leucine core 1 O-glycosylated peptide (SLC1G) was higher in patients with active tuberculosis compared with healthy controls ($p<0.0001$). During treatment, SLC1G level decreased in patients with tuberculosis who were eventually cured but did not change significantly in patients with treatment failure. A significant difference in SLC1G was observed at Week 24 in patients who were cured compared with patients for whom treatment had failed ($p=0.038$) making SLC1G a potential point-of-care biomarker of treatment response [78].

Low tryptophan concentration in CSF has been demonstrated to be a predictive biomarker for positive outcome (survival) in TBM, the most severe manifestation of tuberculosis resulting in 30% deaths or neurological disability. In cohorts of patients with definite and probable TBM, CSF tryptophan level in survivors was 9-times lower than in nonsurvivors and 31-times lower than in healthy controls ($p<0.001$) [79].

Further studies on clinical samples are needed to identify applicability of metabolomic biomarkers in clinical practice.

Immunophenotyping for the management of patients with tuberculosis

Immune phenotype is the outward interaction between immune cells and their environment. Systems biology is contributing to immune phenotyping in a number of ways: identifying simple signals within the complexity of multidimensional data, elucidating immune cell interactions, and integrating immunophenotyping with epidemiology and other datasets derived from the systems biology studies.

Instead of measuring a single analyte at a time, flow cytometry easily allows simultaneous measurement of 30 and more parameters. Using advanced tools, these multiplex data can be analysed for characterisation of multiple functions from a single cell or multiple cell types. Important examples of application of these tools in studies of tuberculosis include using SPICE (Simplified Presentation of Incredibly Complex Evaluations) to demonstrate the tuberculosis-induced immunosuppression when $CD4^+$ T-cells from patients with tuberculosis produce less IL-2 and IFN-γ compared with individuals with LTBI; an assay based on single tumour necrosis factor (TNF)-α discriminated active tuberculosis from individuals with LTBI with sensitivity 67% and specificity 92% [95]. CITRUS (Clustering Identification, Characterisation and Regression) tool was used to identify that healthy controls who did not progress to tuberculosis had more robust cytotoxic functioning (production of granzyme, perforin and IFN-γ) from $CD8^+$ T-cells, NK cells and γδ T-cells [75]. Excess granzyme production by these cells of nonprogressors, *i.e. M. tuberculosis*-exposed individuals who remained asymptomatic, was demonstrated using t-distributed stochastic neighbour embedding (t-SNE). While cytotoxic and cell-mediated immunity is a critical component of the host immune response against *M. tuberculosis*, integrating SPICE with quantitative PET-CT and pulmonary function testing demonstrated that exuberant immunity was associated with decreased lung function 12 months after completion of antituberculosis therapy [96]. These findings have a potential to become the basis for more precise predictions of progression to disease, its severity and response to treatment in the future. Also, applying systems biology approaches to immune phenotyping should identify the immune function of individuals suitable for short-course therapies or those at increased risk of relapse [97].

Immune phenotyping has contributed to the development of the concept of tuberculosis endotypes, *i.e.* distinct molecular pathways through which an individual can develop tuberculosis. The best described tuberculosis endotypes are deficiencies in IL-12-IFN-γ or TNF signalling pathways [98, 99]. Understanding of endotypes may help in developing endotype-specific HDTs targeting specific host immune pathways, which may eventually support antibiotic efficacy, improve disease outcomes and avoid long-term sequelae. However, before implementing HDTs in clinical practice, tuberculosis endotypes should first be defined at the cellular level and here immune phenotyping with transcriptomic, proteomic, metabolic, lipidomic, epigenomic studies and clinical data will play an important role.

Systems pharmacology for tuberculosis management

In pharmacology, quantitative approaches are commonly used to predict treatment efficacy. There are two main branches: pharmacokinetics is the study of drug distribution within the body, while

pharmacodynamics is the study of the effects and modes of drug action. Progress in these fields is difficult due to the lack of universal determination of the levels of drugs in routine practice in many settings and the gaps in our understanding of both drug levels and activity in the target tissue [100]. The latter, in particular, is a major problem for understanding and predicting the efficacy of tuberculosis treatments.

Systems biology methods coupled to pharmacokinetic and pharmacodynamics have the potential to address these issues [101, 102]. The recently emerged multiscale approaches of systems pharmacology have been gaining traction to better understand how and why single drugs or drug regimens work. Physiologically based pharmacokinetic (PBPK) approaches combine mechanistic and small-scale understanding of tissue properties to capture the movement of drugs in the body. In tuberculosis, the most prominent PBPK model used data from patients undergoing lung resection surgery to estimate distribution patterns to different lesion types in the lungs for many of the first- and second-line drugs [100].

Systems pharmacology-based pharmacodynamic models use our knowledge of the mode of action of antibiotics to predict the effects of antibiotics on bacteria; for instance, by modelling drug-target interactions [103]. Those models can capture why some antibiotics work better when given infrequently at high doses while others work better at low but constant dosing. Currently, our understanding of antibiotic action is expanding rapidly [104], therefore novel pharmacodynamic models may allow us to better predict how bacteria are affected by antibiotics within the human body at different sites of infection and at different dosing regimens.

Drug–drug interactions (DDIs) affect both pharmacokinetics and pharmacodynamics. Predicting pharmacokinetic DDIs has been successful in other diseases and can be targeted for tuberculosis as well [105, 106]. Predicting and modelling DDIs in pharmacodynamics can be complex even for pairs of two drugs [107, 108]. However, machine learning-based and other statistical methods can potentially predict higher order interactions [109–111].

Being a relatively novel, although a promising, field for improving the clinical management of patients with tuberculosis, systems pharmacology requires extensive further research and development of the models allowing the analysis of complex multidimensional data derived from pharmacokinetic/pharmacodynamic and DDI studies.

Outstanding questions and future directions

Systems biology approaches have the potential to contribute to various aspects of the management of patients with tuberculosis (figure 1). The diagnostic and analytic developments in the fields of systems biology will allow physicians and scientists to address clinically relevant questions more accurately in the future (table 2). This progress will include a wide range of clinically relevant areas with the prospect of personalising decisions for preventive therapies and the choices of antituberculosis medicines and HDTs administered to individual patients, and enabling outcome prognosis for treatment episodes. These developments will also lead to an earlier diagnosis of tuberculosis, especially in those groups where the diagnosis is more challenging, *e.g.* patients with extrapulmonary tuberculosis and children. The translation of systems biology findings into clinical practice promises to eventually implement precision medicine and drive cost-savings as patients will receive the correct treatment earlier, benefit from shorter treatment regimens associated with fewer adverse events, and experience lower rates of disease relapse and emergence of bacillary drug resistance.

Although every -omics approach described in this review has identified biomarkers associated with tuberculosis, only few of these biomarkers have been assessed in clinical cohorts of patients and implemented in clinical practice (table 2). Some of these biomarkers will be implemented eventually but, before that, their applicability in clinical practice needs careful evaluation. We applied the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) approach [112] for rating the certainty of evidence that the studies included in the review provide for answering clinically relevant questions and forming recommendations of implementation of the biomarkers in clinical practice (table 2). Bacterial and human genomics, as well as bacterial transcriptomic studies, present either “high” or “moderate” evidence in addressing questions on vulnerability to tuberculosis, presence of bacteria, and its resistance to first-line antituberculosis drugs. In all other cases, the grading resulted in a “very low” certainty due to the observational nature of the studies, indirectness in addressing patient-important outcome questions, and other factors downgrading the quality of evidence. As evident from the GRADE criteria, to demonstrate the applicability of the findings in tuberculosis prevention and more efficient clinical management of patients, further studies in large prospective cohorts of patients with matching control groups, including randomised clinical trials, are urgently needed.

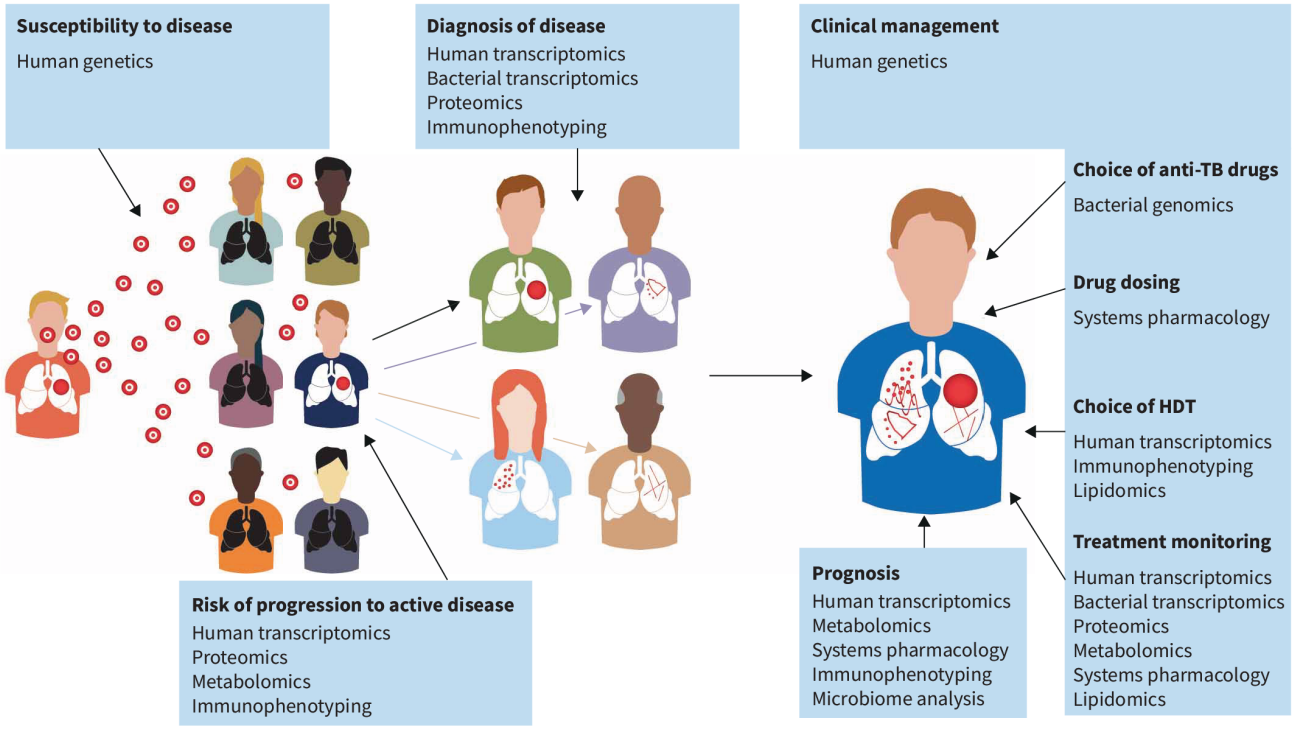

FIGURE 1 Role of systems biology fields in the management of patients with tuberculosis (TB). A patient with active TB disease disseminates droplets with *Mycobacterium tuberculosis* that can be inhaled by healthy contacts who, depending on their genetic susceptibility to TB, may further develop an active disease. However, only approximately one out of 10 latently infected individuals will develop an active disease; risk of progression to active disease can be predicted by human transcriptomics, proteomics, metabolomics and immunophenotyping. Active TB manifests in various clinical forms and can be detected using methods of human and bacterial transcriptomics, proteomics and immunophenotyping. Systems biology methods have the potential to contribute to clinical management of patients with TB. Choice of antituberculosis drugs and host-directed treatment (HDT) can be determined and adjusted according to data obtained using bacterial genomics, human transcriptomics, immunophenotyping and lipidomics. Drug dosing can be adjusted and monitored by systems pharmacology. For monitoring of patient responses to treatment, human and bacterial transcriptomics, proteomics, metabolomics, systems pharmacology and lipidomics may be employed. Human transcriptomics, metabolomics, systems pharmacology, immunophenotyping, and microbiome analysis may play a role in prognosis of adverse drug events, treatment outcomes and TB relapse after treatment end.

Integrating data obtained in different areas of systems biology by artificial intelligence and machine learning techniques will help to refine and focus molecular signatures. By implementing network analysis, this multiomics approach will help in identifying previously unappreciated pathways to determine modules of metabolites, genes, lipids, proteins and the microbiome in the treatment of responsive *versus* nonresponsive patients. The transformative potential of such an approach will open up novel therapeutic vulnerabilities with combination therapies against these interacting multi-omic molecular pathways.

Conclusions

We are at the doorstep of a new era of an individualised, precision medicine-guided approach to treatment where innovations of systems biology approaches will have an impact on the diagnosis, management and treatment outcomes of patients with tuberculosis, especially rifampicin-resistant and MDR tuberculosis, and provide valuable information that will aid clinicians in making medical decisions. Before some of these innovations can be implemented in clinical practice, further studies on large cohorts of patients with tuberculosis, household contacts, and control groups are needed to assess the applicability of these biomarkers and biosignatures in tuberculosis prevention and clinical management of patients. There will also be a significant challenge in translating the exciting results into large-scale clinical interventions. Only those innovations making a difference on the burden of tuberculosis in high-incidence countries will make significant contributions on the way to eliminating tuberculosis in the future.

TABLE 2 Impact of advances in systems biology fields on the management of patients with tuberculosis (TB)

Clinical question	Field of systems biology	Clinical relevance of the method	Method in clinical practice Yes/no	Quality of evidence (GRADE) [112]	
				Study	Body of evidence
Who is susceptible to TB?	Human genetics	The clinical utility of *LTA4H* polymorphism for TB management is in phase 3 randomised clinical trial [55]	No	Moderate	Moderate
		Patients with certain genetic aetiologies of MSMD and paediatric TB are treated with adjuvant IFN-γ therapy [49]	Yes	Moderate	
Who will develop TB when latently infected?	Human transcriptional profile	RISK6 signature predictive of progression to active TB within 1 (AUC 87.6%, 95% CI 82.8–92.4) and 2 years (AUC 74.0%, 95% CI 66.0–82.0) [63]	No	Very low	Very low
		11-gene version of 16-gene COR signature predicts TB 1 year before the onset of disease (sensitivity 71%, specificity 84%) [66]	No	Very low	
	Proteomics	TRM5 and 3PR plasma protein signatures predict disease progression to active TB within 1 year (sensitivity 46–49%, specificity 75%) [86]	No	Very low	
	Metabolomics	Metabolic TB biosignature predictive of progression to active TB (sensitivity 69%, specificity 75% within 5 months of diagnosis) [94]	No	Very low	
	Immunophenotyping	Different immune profiles between healthy controls, individuals with LTBI and with active TB [75]	No	Very low	
		Identification of TB-specific endotype for application of host-directed therapy [98, 99]	No	Very low	
Who has active TB?	Human transcriptional profile	RISK6 signature detected active TB (sensitivity 90%, specificity 93.4% in HIV-uninfected and 72.5% in HIV-infected persons) [63]	No	Very low	Very low
		3-gene signature discriminates active TB from healthy controls (AUC 0.90, 95% CI 0.85–0.95), latent TB (AUC 0.88, 95% CI 0.84–0.92), and other diseases (0.84, 95% CI 0.80–0.95) [69]	No	Very low	
	Bacterial transcriptomics	Highly sensitive 16S rRNA MBLA accurately quantifies *M. tuberculosis* viable bacillary load to as low as 10 CFU per mL [25]	No	Moderate	
	Proteomics	Plasma CC and CXC chemokines as markers of disease severity, predicting increased bacterial burden and delayed culture conversion [73]	No	Very low	
		Serum 6-protein signature discriminates TB from other respiratory diseases (sensitivity 90%, specificity 80%) [80]	No	Very low	
		A set of three inflammatory cytokines discriminates TB from other respiratory diseases (sensitivity 85%, specificity 96%) [82]	No	Very low	
		8-protein signature in stimulated whole blood culture discriminates between TB and nonTB, including healthy controls, LTBI and nonTB pneumonia (sensitivity 75%, specificity 84%) [81]	No	Very low	
		3-protein signature distinguishes between TB and other respiratory diseases (sensitivity 72.2%, specificity 75%) in cohort of children [83]	No	Very low	
	Immunophenotyping	Single-positive TNF-α *M. tuberculosis*-specific $CD4^+$ T-cell response assay discriminates between LTBI and active disease (sensitivity 67%, specificity 92%) [95]	No	Very low	
Is the specific strain of *M. tuberculosis* susceptible to anti-TB drugs?	Bacterial genomics	Genotypic prediction of phenotypic drug susceptibility by NGS technologies using validated mutation catalogues [8]	Yes	High	High

Continued

TABLE 2 Continued

Clinical question	Field of systems biology	Clinical relevance of the method	Method in clinical practice Yes/no	Quality of evidence (GRADE) [112]	
				Study	Body of evidence
How severe is TB disease?	Human transcriptional profile	RESPONSE5 signature correlates with pulmonary inflammation predicting Week 24 PET-CT status at baseline, Week 1 and Week 4 (AUC 0.72–0.74, p<0.02) [72]	No	Very low	Very low
	Lipidomics	Elevated plasma eicosanoid LXA4 and 15-epiLXA4 levels are associated with disease severity defined by extended lung pathology and bacterial burden [88]	No	Very low	
How is the patient responding to anti-TB treatment?	Bacterial transcriptomics	16S rRNA MBLA detects *M. tuberculosis* viable count in real time that correlates with culture result and can be used for treatment monitoring [25, 26, 28]	No	Moderate	Very low
	Proteomics	Serum proteomic signature predicts 8-week culture status (sensitivity 95%, specificity 90%) [76]	No	Very low	
	Metabolomics	Changes in SLC1G level in urine correlate with different treatment response outcomes [78]	No	Very low	
	Systems pharmacology	PK/PD models assess penetration of anti-TB drugs in cavitary lesions [100]	No	Very low	
		Drug–drug interaction models can help set dosing for drugs by predicting the change in the efficacy of a given dose when used together with other drugs [109–111]	No	Very low	
What will be the outcome of anti-TB treatment?	Human transcriptional profile	RISK6 signature predicts treatment failure before the treatment initiation (AUC 77.1, 95% CI 52.9–100) and at the end of treatment (AUC 95.2, 95% CI 87.5–100) [63]	No	Very low	Very low
		9-gene DISEASE signature predicts treatment failure 1 week (AUC 0.70, p=0.04) and 4 weeks (AUC 0.72, p=0.03) after treatment initiation [72]	No	Very low	
	Metabolomics	Cerebral tryptophan metabolism can predict the outcome of TB meningitis being 9-times lower in survivors than nonsurvivors (p<0.001) [79]	No	Very low	
	Systems pharmacology	PK/PD models identify patients at risk of treatment failure due to suboptimal drug concentrations in TB lesions [100]	No	Very low	
Why does a patient develop unwanted drug reactions?	Microbiome profiling	The microbiota accounts for elevated levels of low-density lipoprotein cholesterol and total cholesterol that might be associated with metabolic disorders [46]	No	Very low	Very low
Will the patient experience TB relapse?	Immunophenotyping	Serum biosignature combining 4 immunological with 2 clinical parameters measured at diagnosis predicts TB relapse (sensitivity 75% and specificity 85% in the discovery cohort; sensitivity 83% and specificity 61% in the validation cohort) [97]	No	Very low	Very low

GRADE: Grading of Recommendations, Assessment, Development and Evaluation; MSMD: Mendelian susceptibility to mycobacterial disease; IFN-γ: interferon-γ; AUC: area under curve; LTBI: latent tuberculosis infection; MBLA: molecular bacterial load assay; CFU: colony forming unit; TNF: tumour necrosis factor; NGS: next generation sequencing; PET-CT: positron emission tomography-computed tomography; PK/PD: pharmacokinetic/pharmacodynamic.

Search strategy and selection criteria

Data for this review were identified by searches in MEDLINE, Web of Science, PubMed, and references from relevant articles using the search terms “system biology”, “omics”, “genomics”, “transcriptomics”, “proteomics”, “lipidomics”, “metabolomics”, “immunophenotyping”, “systems pharmacology”, “microbiota” and “tuberculosis”. Only articles published in English between 2000 and 2020 were included.

Provenance: Submitted article, peer reviewed

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Annex 4

Patent. Identification of Metabolomic Signatures in Urine Samples for Tuberculosis Diagnosis (WO2019243347A1)

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(54) Title: IDENTIFICATION OF METABOLOMIC SIGNATURES IN URINE SAMPLES FOR TUBERCULOSIS DIAGNOSIS

(57) Abstract: The authors of the present invention have identified a series of metabolic markers present in the urine samples collected from patients diagnosed of tuberculosis (TB, n=19), respiratory infections caused by *Streptococcus pneumoniae* (RI, n=25) and healthy controls (HC, n=29). These metabolic markers selected are significantly differentiated between Healthy Controls (HC) and patients diagnosed of tuberculosis, between tuberculosis patients versus patients affected by respiratory infections caused by *S. pneumoniae*, and between patients affected by respiratory infections caused by *S. pneumoniae* and HC. These metabolic markers can thus be used in a non-invasive diagnostic method identifying and classifying patients.

Annex 5

Patent. In Vitro Method for Discriminating Latent from Active Tuberculosis (WO2021013933A1)

Perez-Porcuna, Tomas M; **Comella-del-Barrio, Patricia**; Dominguez, Jose; Abellana, Rosa

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(54) Title: IN VITRO METHOD FOR DISCRIMINATING LATENT FROM ACTIVE TUBERCULOSIS

(57) Abstract: In vitro method for discriminating latent from active TB. The present invention refers to an in vitro method for the differential diagnosis between active TB, preferably early stages of TB, and LTBI.

11

About the author

Patricia Comella del Barrio

Patricia Comella-del-Barrio

Born in Barcelona (Spain), I attended primary, secondary and scientific baccalaureate at La Salle Bonanova school, and then I studied Biology at the *Universitat Autònoma de Barcelona* (UAB, Barcelona, Spain).

During my undergraduate studies (Sept. 2010 - Mar. 2011), I received an Erasmus scholarship at the *Università degli Studi di Sassari* (UNISS, Sardinia, Italy), where I did an internship in parasitology of aquatic organisms with Dr Paolo Merella at the Department of Animal Biology, and in marine invertebrate genetics with Dr Fabio Scarpa at the Department of Zoology and Evolutionary Genetics. I also did an internship at the Department of Animal Biology, Plant Biology and Ecology of the Faculty at the UAB with Dr Maite Carrassón, which led to my final degree project on the analysis of the parasitofauna of *Helicolenus dactylopterus* (Delaroche, 1809) in the north-western Mediterranean and the study of its possible seasonal variation. I also participated in the ANTROMARE I (CTM2009-12214-C02-01-MAR) campaign, where fish samples collected in two contrasting oceanographic environments were analysed onboard the oceanographic vessel García del Cid of the *Consejo Superior de Investigaciones Científicas* (CSIC, Barcelona, Spain). In September 2011, I graduated in Biology, specialising in Animal Biology by the UAB.

During the last years of my career and onwards, I volunteered (2009-2014) and co-founded (2010) the non-profit association *Sonríe y Crece* (sonrieycrece.es), which has been cooperating for more than 10 years in education, health, and housing projects in isolated communities in the southwest of the Dominican Republic, near the border with Haiti. Aware of the international health challenges, after finishing my scientific career, I received an Official Master's Degree in International Health from the *Universitat de Barcelona* (UB, Barcelona, Spain).

During the master's degree, I collaborated in the Department of Public Health at the UB, developing an epidemiological study of intestinal parasitosis in the Amazon (Brazil) between an urban and a rural area under the mentorship of Dr Alessandra Queiroga Gonçalves and Dr Carlos Ascaso. In June 2012, I obtained my master's degree after defending my dissertation entitled "Prevalence of intestinal Parasites and Risk Factors in an area of the Rio Negro, State of Amazonas, Brazil", which contributed to a subsequent publication in 2016 (doi.org/10.1590/0037-8682-0128-2015).

In 2013 I started my PhD in the medicine programme of the UB studying risk factors (helminthiasis and malnutrition) for the control and management of tuberculosis (TB) and worked in the coordination of the Official Master in International Health of the UB

and the Master in Research in Tropical Medicine and International Health of the Bolivian Bioregions of the UB/Universidad Mayor de San Andrés under the mentorship of Dr Carlos Ascaso.

From May 2015 to May 2017, I did a research stay at the *Hôpital pédiatrique Saint Damien* (Port-au-Prince, Haiti), coordinating the implementation and execution of the research project on the development of new diagnostic methods to improve the detection of TB infection and disease in children.

At the end of 2017, I visited the *Institut d'Investigació Germans Trias i Pujol* (IGTP, Barcelona, Spain) to analyse samples collected in Haiti. Soon after, I joined the IGTP's "Innovation in respiratory infections and tuberculosis diagnosis" group (@Oneandahalf_Lab) to work on respiratory infections and TB, improving the understanding of host-pathogen interaction and exploring new approaches in the diagnosis and treatment under the supervision of Dr Jose Dominguez and Dr Cristina Prat-Aymerich (May 2018 to February 2021).

In June 2018, I changed my PhD programme to Microbiology at the UAB with the mentorship of Dr Jose Dominguez, Dr Tomas M. Pérez-Porcuna and Dr Rosa Abellana. During this time, I have been mainly involved in projects related to evaluating different techniques to improve the diagnosis of TB by exploring new biomarkers based on blood, urine, or exhaled air VOC samples. But I have also participated in the study of mechanisms of adaptation of *Staphylococcus* aureus to the respiratory tract through the development of new diagnostic and therapeutic approaches that allow more rational and effective use of antimicrobials, in the evaluation of diagnostic techniques for COVID-19, and in data management for clinical research by designing and managing electronic databases (using REDCap) of different on-going projects. I also trained in the acquisition and processing of Nuclear Magnetic Resonance spectra during short stays at the *Centro Nacional de Investigaciones Oncológicas* (CNIO, Madrid, Spain) and CICBiomaGUNE (San Sebastian, Spain) under the supervision of Dr Jose Luis Izquierdo-García and Dr Jesús Ruiz-Cabello, at the *Centro Nacional de Investigaciones Cardiovasculares* (CNIC, Madrid, Spain) under the supervision of Dr Ramón Campos and Dr Clara Santiveri, and at Magritek GmbH (Aachen, Germany) under the supervision of Dr Federico Casanova. From February to May 2019, I did a research stay within the framework of the INNOVA4TB project (innova4tb.com) at the primary immunodeficiency laboratory of the Jeffrey Modell Centre at the *Universidad de la Frontera* (UFRO, Temuco, Chile) under the supervision of Dr Anessi Giacaman, for the study of host-pathogen interaction through phenotypic characterisation of primary immunodeficiencies. As a result of my research in TB diagnosis, I am registered as inventor of a patent filed by the IGTP and *Consorcio Centro de Investigación Biomédica en Red* (CIBER, Madrid, Spain), which discloses the use of eight metabolites in urine for TB diagnosis, and a second patent filed by the *Fundació Mútua Terrassa*, UB, and IGTP

which discloses the use of IFN-γ, IP-10, ferritin and 25(OH)D in the blood to discriminate between latent TB infection and active TB in children. The results of the work conducted during the PhD programme in microbiology are presented in this thesis.

From March to October 2021, I worked at the Barcelona Institute for Global Health (ISGlobal, Barcelona, Spain) with Dr Alberto García-Basteiro. During these months, I have coordinated the implementation and recruitment of the research project "PreFIT - Predicting the Future: Incipient Tuberculosis" (predictingtb.org) at the *Centro de Investigação em Saúde de Manhiça* (CISM, Manhiça, Mozambique) to validate assays to improve the prediction of progression from latent TB infection to active TB.

In the near future, I would like to link science to development cooperation by working on TB research projects that promote the identification, validation, and implementation of new technologies that could be brought closer to the populations that need them most.

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