Exploring the value of *Mycobacterium tuberculosis* modified lipoprotein as a potential biomarker for TB detection in children

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Abstract

Background: Current TB diagnostic methods available have been developed for adults and development efforts have neglected the differences in disease and sampling that occur between adults and children. Diagnostic challenges are even greater in HIV co-infected children and infants.

Methods and results: We established a sandwich ELISA assay to detect *Mycobacterium tuberculosis* modified lipoprotein (TLP) ex vivo in plasma. The study population contains plasma samples from 21 patients with active TB and 24 control samples with no TB, collected in the International Maternal Pediatric Adolescent AIDS Clinical Trials (IMPAACT) P1041 study. Retrospective analysis was performed and the results demonstrate that the median plasma levels of TLP in control subjects are 2.7 fold higher than the median plasma values in active TB subjects (p < 0.001).

Conclusions: Plasma levels of TLP are elevated with active TB disease in HIV positive subjects and deserves further exploration as an indicator for TB detection in children.

Keywords: Childhood TB, HIV co-infection, Diagnosis, Sandwich ELISA, Lipoprotein, Low-density lipoprotein, apoB, Biomarker

Background

Childhood TB is estimated to contribute 12% of the disease burden, with approximately 10 million cases in 2019 worldwide [1]. Existing gold-standard diagnostic culture tools fail to confirm TB in most children, who typically have low bacterial counts and cannot produce sputum like adults. Diagnostic challenges are great in HIV co-infected children and infants [2], where the clinical presentation of pulmonary TB may be non-specific [3], acute [4, 5], and chest radiographs may be atypical [2]. Although rates of bacteriological confirmation appear similar in HIV-infected and uninfected children [6], diagnostic delay and HIV-related immune pathology contribute to higher rates of TB disease progression with increased disease severity, morbidity, and mortality [7–10]. Infants, young children, and HIV-infected children are at increased risk of developing TB following infection and of disseminated or severe disease, including TB meningitis [11]. Because TB in children is typically paucibacillary, even if a sputum sample or gastric aspirate is obtained, the utility of PCR based diagnostic methods like GeneXpert is limited. More sensitive and child-friendly diagnostic tools are urgently needed to diagnose TB in children [12].

Mycobacterium tuberculosis (*Mtb*) is an intracellular pathogen that is capable of surviving and replicating within macrophages, the frontline of the innate host defense, and has evolved multiple mechanisms to escape these immune cells [13]. One of the immune evasion strategies of *Mtb* is the deregulation of lipid metabolism, leading to the formation of foamy macrophages (FM),

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a hallmark of granulomata in tuberculosis lesions [14]. However, only pathogenic mycobacterial strains including *M. tuberculosis* [15], *M. avium, M. abscessus, or M. bovis*, but not non-pathogenic mycobacterial strains, like *M. smegmatis*, induce foam cell formation upon infection [16, 17]. Foam cells are associated with chronic inflammation in many metabolic diseases and certain cancers besides infectious diseases [18, 19]. For example, foamy macrophages are critical to the initial formation, development, and instability of atherosclerotic plaques and are therefore therapeutic targets in atherosclerosis. Foam cell formation is induced primarily by malondialdehyde-modified low density lipoprotein (MDA-LDL) and not by native or extensively oxidized LDL in atherosclerosis [20]. Elevated plasma levels of MDA-LDL in patients are associated with acute coronary syndromes and are used as diagnostic tools clinically [21]. In contrast, the detailed mechanism of FM formation in *Mtb* is unknown. The current state of knowledge was that *Mtb* infection and *Mtb* trehalose dimycolate can induce FM formation [22]. Inspired by the association of MDA-LDL with FM and therefore atherosclerosis, we asked whether the modification of lipoprotein by *Mtb* may contribute the formation of FM and relate to the disease status of TB.

In this study we identified human lipoprotein modification specific to exposure to *Mtb*. The pathogen modified host lipoprotein, TLP, is detectable ex vivo in plasma using a sandwich ELISA assay. We further evaluated the association between the presence of TLP and TB disease status in children.

Methods

1) Establish that TLP is 'real' and can be measured

Preparation of malondialdehyde-conjugated LDL (MDA-LDL) [20] and Mtb-modified lipoprotein (TLP)

HepG2 human liver cells (ATCC HB-8065) were grown to 80% confluence in HepG2 growth media (DMEM, 10% fetal bovine serum, 20 mM ʟ−glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES). Cells were grown for 4–5 days and culture supernatants were then harvested and concentrated. LDL particles were separated by density gradient ultracentrifugation and desalted by ultrafiltration through a 100-kDa molecular weight filter. MDA-LDL was prepared by incubation 2 mg protein/mL of LDL with 2 μM acrolein at 37 °C for 24 h under nitrogen atmosphere. *Mycobacterium tuberculosis* was cultured in Middlebrook 7H9 (broth) supplemented with 0.2% glycerol, 0.5% BSA, 0.08% NaCl, 0.05% (v/v) tyloxapol to OD ∼ 0.7. LDL was added and the culture was incubated at 37 °C for 7 days. TLP was isolated from the culture supernatant, concentrated and washed with PBS.

Agarose gel electrophoresis and western blot analysis

Agarose gel electrophoresis was performed in 0.06 M barbital buffer (pH 8.6). The gel was 0.8% and stained with Sudan Red 7B. Lipoproteins were separated on a 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to a PVDF membrane and immunoblotted with specific antibodies. The primary antibodies used for western blot were monoclonal anti-LDL (MDA oxidized) antibody (Abcam) and polyclonal anti-apoB Ab (H-300, Santa Cruz). Membranes were treated with anti-mouse, or anti-rabbit IgG HRP conjugates as secondary antibodies.

2) Explore if induced TLP it potentially pathogenic by contributing to FM formation

THP-1 macrophage preparation and treatment

THP-1 cells (ATCC TIB-202) were maintained in RPMI-1640 medium containing heat-inactivated 10% fetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol, 100 U/mL penicillin and 100 mg/mL streptomycin (RPMI complete medium). The cells were plated into 6-well plates containing coverslips (1.5×10^6 /well), treated with phorbol 12-myristate 13-acetate (PMA, 150 nM) in RPMI complete media. Media was replaced with fresh RPMI complete media at 48 h after plating. At 72 h after media change, THP-1 macrophages were treated with LDL, MDA-LDL and TLP at 200 μg/mL in PBS for 24 h. All cells were incubated in a humid atmosphere at 37 °C with 95% air and 5% CO_2.

Lipid body staining and immunostaining

Following lipoprotein treatment, macrophages were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. Cells were stained with Oil Red O solution for 20 min at room temperature. The slides were then counterstained with haematoxylin and observed under an inverted microscope (Zeiss Axiovert 200 M). Percent foam cell formation was quantified by counting stained versus total cells in 10 fields.

3) Perform initial pilot evaluation of potential diagnostic value in a HIV + infant cohort

Generation of anti-TLP antibodies [23, 24]

Monoclonal antibodies against TLP were identified utilizing Human Combinatorial Antibody Library (HuCAL, Bio-Rad AbD Serotec GmbH). The HuCAL phage display library was depleted of antibodies that recognize intact LDL. The depleted library was panned for three rounds of binding, elution, and amplification to isolate antibodies specific for TLP. Binding of anti-TLP antibodies to control antigens including BSA, HSA, N1-CD33-His6 and intact LDL, was checked by

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indirect ELISA assays, with anti-TLP antibodies as primary antibodies and an anti-Fab-AP conjugate (Bio-Rad) as a secondary antibody.

Sandwich ELISA assay

Briefly, 96 well plates were coated overnight at 4 ℃ with 5 µg/mL capture antibody (AbD28580, amino acid sequence shown in Additional file 1: Table S2) in phosphate buffered saline (PBS). Plates were washed with PBS containing 0.05% Tween-20 (PBST) 3 times, blocked with 3% BSA in PBST for 1 h at room temperature. Then serial dilutions of standards and test samples in HISPEC assay diluent (Bio-Rad) were loaded and allowed to react for 2 h at room temperature. Plates were then washed 3 times with PBST and treated with 2 µg/mL of HRP conjugated detection antibody (AbD28582) in HISPEC assay diluent for 1 h at room temperature. After washing 6 times with PBST, the plates were developed with Quanta-Blu fluorogenic peroxidase substrate kit (Thermo Scientific) for 30 min at room temperature. Fluorescence was recorded (ex. 320 ± 25 nm, em. 430 ± 35 nm).

Subjects and samples

Retrospective analysis was performed on a total of 45 plasma samples from International Maternal Pediatric Adolescent AIDS Clinical Trails (IMPAACT) P1041 [25, 26]. P1041 was a Phase II/ III, randomized, double-blind, placebo-controlled clinical trial to evaluate the efficacy of isoniazid prophylaxis on TB disease and latent *Mtb* infection free survival in HIV-infected and HIV-exposed, but uninfected infants up to 192 weeks of follow up [26, 27]. Samples include 24 with noTB and 21 with TBDIS, all HIV infected. Patients without *Mtb* infection were categorized as noTB; patients with a positive tuberculin skin test but lacking any clinical, radiographic or laboratory evidence of disease caused by *Mtb* were categorized as latent TB infection (LTBI); and patients presenting clinical, radiographic or laboratory evidence of disease caused by *Mtb* were categorized as TBDIS. Plasma samples were collected, frozen and thawed before use.

Statistical analysis

Categorical variables were compared using t-student test, whenever appropriate. Non-parametric tests (Mann–Whitney) were used for non-normally distributed variables. Assay accuracy, including 95% confidence intervals, was assessed using sensitivity, specificity, predictive values and area under the ROC in the TB and non-TB groups. Statistical calculations were performed with GraphPad Prism® Software.

Results

1) Establish that TLP is distinct from known physiological modifications of LDL

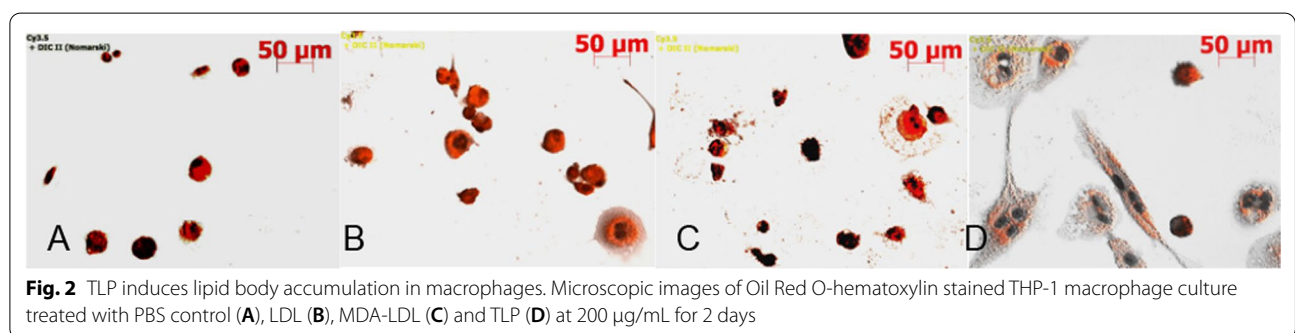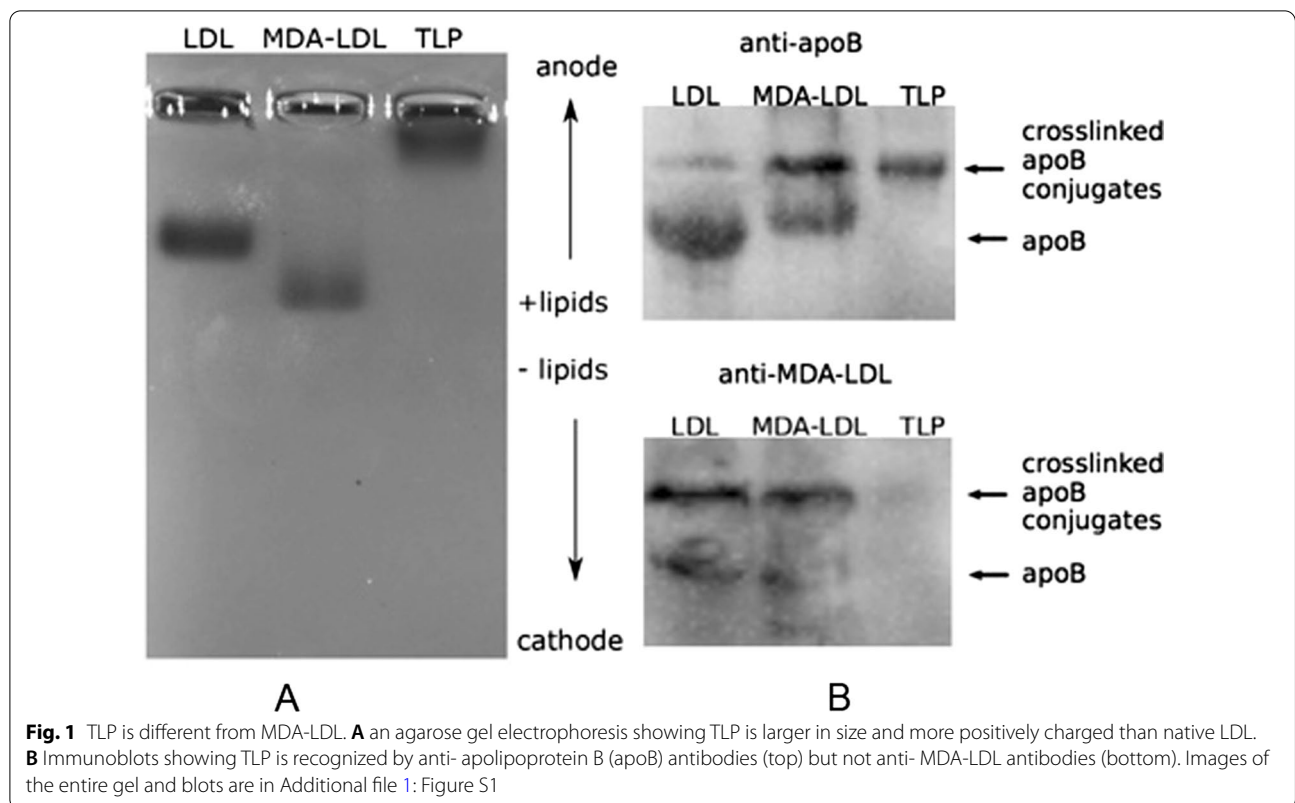#### *Mtb modified lipoprotein (TLP) is different from MDA-LDL*

We purified and treated native LDL with *Mycobacterium tuberculosis* in vitro to obtain *Mt*b modified lipoprotein (TLP). Decreased mobility of TLP in agarose electrophoresis indicates that TLP is more positively charged than native LDL (Fig. 1A and Additional file 1: Fig. S1A). In contrast, MDA-LDL, one of the end products of lipid peroxidation, is more negatively charged than native LDL [20]. The increased size of TLP compared to native LDL was also confirmed by dynamic light scattering analysis (Additional file 1: Table S1). Furthermore, TLP is recognized by anti-apolipoprotein B (apoB) antibodies but not anti-MDA-LDL antibodies, indicating TLP does not contain a malondialdehyde-modified apoB derivative (Fig. 1B and Additional file 1: Fig. S1B). We demonstrate that LDL is altered in the presence of *Mtb*, and the resulting TLP is distinct from the typical atherosclerotic species malondialdehyde-modified LDL (MDA-LDL) present in human blood [28].

2) Explore if induced TLP is potentially pathogenic by contributing to FM formation

Mtb modified LDL induces lipid body accumulation in macrophages

The induction of foamy macrophages have been reported as hallmarks in many pathologies associated with chronic proinflammatory stimuli including atherosclerosis [15]. It has also been reported that *Mtb* lipids induce the formation of giant multinuclear macrophages [29]. We therefore used foamy macrophage formation as a tool to assess the bioactive components that trigger a tissue response similar to granuloma formation in TB. Lipid bodies were stained with Oil red O. The buffer control showed no accumulation of lipid bodies and the cells remained rounded (Fig. 2A). LDL treated macrophage showed some lipid accumulation, with a commensurate increase in cell size (Fig. 2B). In contrast, MDA-LDL, commonly associated with atherosclerosis, treated macrophages exhibited more extensive accumulation of lipid bodies than the LDL treated macrophages. THP-1 macrophages treated with PMA do not entirely reproduce the response spectrum of primary monocyte-derived macrophages to activating stimuli [30]. However, foamy macrophage formation is a straightforward biological process and we used this simplified model for comparison to previous studies. These macrophage phenotypes are consistent with previous reports of in vitro foamy macrophage formation

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Fig. 1 TLP is different from MDA-LDL. **A** an agarose gel electrophoresis showing TLP is larger in size and more positively charged than native LDL. **B** Immunoblots showing TLP is recognized by anti- apolipoprotein B (apoB) antibodies (top) but not anti- MDA-LDL antibodies (bottom). Images of the entire gel and blots are in Additional file 1: Figure S1

Fig. 2 TLP induces lipid body accumulation in macrophages. Microscopic images of Oil Red O-hematoxylin stained THP-1 macrophage culture treated with PBS control (**A**), LDL (**B**), MDA-LDL (**C**) and TLP (**D**) at 200 µg/mL for 2 days

[20, 31]. Most importantly, TLP treated macrophages had extensive lipid body accumulation per cell compared to macrophages treated with other LDLs (Fig. 2 and Additional file 1: Fig. S2B). Moreover, the TLP-treated macrophage morphology was distinct from other samples. The majority of the TLP-treated macrophages became multinucleated (Fig. 2D and Additional file 1: Fig. S2A) and developed long filopodia consistent with cell migration or exocytosis. Thus, we hypothesize that the modified LDL is detectable in vivo to serve as a biomarker for TB disease, and an antibody specific for recognition of TLP is an *Mtb*-specific indicator of infection and progression to (active) TB disease.

3) Perform initial pilot evaluation of potential diagnostic value of TLP in a HIV + infant cohort

Anti-TLP monoclonal antibodies were generated and a TLP Sandwich ELISA assay was established

We generated 13 anti-TLP monoclonal antibodies (Fad-A-FH, bivalent Fab-bacterial alkaline phosphatase fusion antibody followed by FLAG® and His6-tag) from the Human Combinatorial Antibody Library (HuCAL, Bio-Rad AbD Serotec GmbH) using in vitro selection and counter selection [23, 24]. HuCAL is a phage display

library containing highly specific, fully human monoclonal antibodies, with DNA sequence encoded for each antibody fragment. The HuCAL phage display library was depleted of antibodies that recognize LDL (Additional file 1: Figure S3). The depleted library was panned for three rounds of binding, elution, and amplification to isolate antibodies specific for TLP. Among the antibody hits selected, 13 antibodies were identified as unique by DNA sequencing. These antibodies were produced heterologously for further development. The cross-reactivity with native LDL, human serum albumin, bovine serum albumin, and His$_6$ affinity tag was determined in an indirect ELISA format. Eight antibodies showed a specific signal for TLP at least fourfold above native LDL background (Additional file 1: Figure S3). We tested pairwise combinations of antibodies to identify antibody pairs that have non-overlapping epitopes suitable for use in a sandwich ELISA (Additional file 1: Figure S4A). 5 sandwich pairs were identified with good signal over background and the assay conditions were optimized for the best pair, AbD28582 and AbD28580 (amino acid sequence shown in Additional file 1: Table S2). We established the linear response of the sandwich ELISA (Additional file 1: Figure S4B) to antigen spiked into pediatric plasma. TLP is specifically detected at $> =4$ ppm in the presence of plasma LDL, which is around 10^6 ng/mL [32].

Levels of TLP are increased in patients with active TB compared to no TB controls

We assayed stored plasma samples from the HIV IMPAACT P1041 trial for the presence of TLP. The median plasma levels of TLP was 494 ng/mL in control subjects, and 2.7 fold higher in active TB subjects (Fig. 3A). The difference between control subjects and active TB subjects is significant ($p < 0.001$). The receiver operating characteristic (ROC) analysis describes the relationship between the sensitivity and specificity at any cut-off values. An ROC curve of TLP concentration for active TB versus control subjects is presented in Fig. 3B. The area under the ROC curve (AUC) is 0.86 (95% CI 0.75–0.97, $p < 0.0001$). An optimal cutoff of 1064 ng/mL rendered 71% sensitivity and 88% specificity. Alternatively, a cutoff of 1230 ng/mL provided 57% sensitivity and 96% specificity.

We also monitored apoB levels, i.e., LDL levels, of all the samples using Human apoB ELISA kits (ab190806, Abcam) and found there was no linear correlation between TLP and apoB levels (Additional file 1: Figure S6A), suggesting that plasma LDL fluctuation does not interfere with TLP detection. There is a weak correlation between TLP levels and time before or after diagnosis (Additional file 1: Figure S6B). The samples collected before diagnosis may represent disease progression from no infection or infection but without clinical signs of disease to outward manifestation of disease. Samples collected after diagnosis may represent a mixed outcome of disease progression and anti-tubercular treatment. We do not have additional sample sets to demonstrate if anti-tubercular treatment affects TLP levels. However, the discrimination of TLP levels between control subjects and active TB subjects (post diagnosis with treatment less than 40 weeks) was improved with the
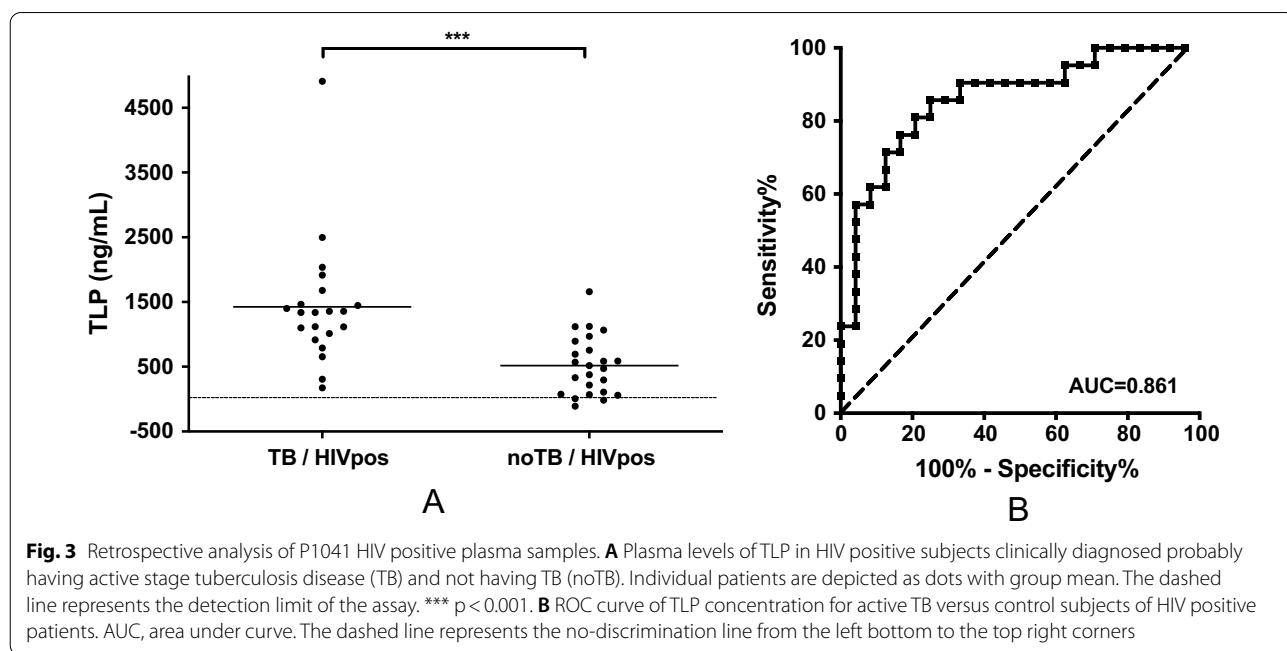

Fig. 3 Retrospective analysis of P1041 HIV positive plasma samples. **A** Plasma levels of TLP in HIV positive subjects clinically diagnosed probably having active stage tuberculosis disease (TB) and not having TB (noTB). Individual patients are depicted as dots with group mean. The dashed line represents the detection limit of the assay. *** p < 0.001. **B** ROC curve of TLP concentration for active TB versus control subjects of HIV positive patients. AUC, area under curve. The dashed line represents the no-discrimination line from the left bottom to the top right corners

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exclusion of samples collected after 40 weeks of treatment (p < 0.0001), which may represent patients clear of TB (Additional file 1: Figure S6). ROC (Receiver operating characteristic) analysis provided an AUC (area under the ROC curve) of 0.88 (95% CI 0.76–0.99, p < 0.0001). An optimal cutoff of 632 ng/mL gave 67% sensitivity and 93% specificity. The results provide further evidence that TLP levels are associated with active TB disease. Overall, the results clearly highlight the potential of TLP as a biomarker in the diagnosis of active TB in HIV positive children.

Discussion and conclusions

The wide spectrum of disease observed in children, and the non-specific signs and symptoms especially in young and HIV + children, contribute to diagnostic delay and missed opportunities to detect TB, which in turn, favors TB disease progression and poor treatment outcomes. There is urgent need to search for a "Rapid Non-sputum-based Biomarker Test for Tuberculosis Detection" [12]. Most of the biomarkers currently studied are host-derived biomarkers including metabolites and cytokines [33, 34], the levels of which can be affected by many confounding factors. On the other hand, *Mtb* products can be detected directly in blood, sputum or urine, and are increasingly being used for diagnosis [35]. We have characterized a unique biomolecule, *Mtb* modified lipoprotein, which results from a combination of host and *Mtb* pathogen activity. Functionally, TLP increases the flux of lipid into activated macrophages, thereby stimulating their conversion into foamy multi-nucleated macrophages. The TLP-stimulated macrophages accumulate lipid bodies but have a distinct phenotype from MDA-LDL-stimulated macrophages (Fig. 2). We reason that detection of this biomolecule in patient plasma may withstand the heterogeneity associated with variations derived from host and be pathogen or disease specific. Thus, TLP detection may be used as TB diagnosis in young children.

The lipoprotein modification can take place in macrophages in contact with *M. tuberculosis*. There are also reports demonstrating that extracellular vesicles carrying bacterial components are released from infected macrophages and circulated beyond the site of infection [36], where host lipoproteins could be modified via contact with the extracellular vesicles. In addition, the intracellular bacilli are able to avoid killing by escaping phagosome-lysosome fusion. In certain individuals, especially children below 5 years of age and immunosuppressed subjects, the extracellular bacilli may disperse to distant metastatic sites via lymphatics and the bloodstream [37], where host lipoproteins may be modified by direct contact with *M. tuberculosis*. Our data demonstrate that the modified lipoprotein in the present study is distinct from other commonly recognized disease associated LDLs, which are typically negatively charged and smaller in particle size compared to native LDL [38].

The present study established a rapid, non-invasive, and robust sandwich ELISA assay for the detection of a novel biomolecule TLP in plasma. It would be beneficial to further refine the sensitivity and selectivity of the ELISA assay and determine the minimum required dilution to optimize the matrix effect, in order to standardize the test and use it as a diagnostic kit. We demonstrate that plasma levels of TLP are distinguishable in HIV positive subjects with active TB disease compared to no TB controls (Fig. 3). The elevation of plasma levels of TLP in HIV positive subjects with active TB disease suggests the detection of TLP is associated with TB active disease in HIV positive subjects. The AUC value of 0.86 evidenced the potential diagnostic value of TLP for TB detection in child. The target product profiles (TPP) adopted by the World Health Organization (WHO) in 2015 for a "Rapid Non-sputum-based Biomarker Test for Tuberculosis Detection" is a diagnostic sensitivity ≥ 66% for microbiologically confirmed pediatric TB and 98% specificity for a pediatric test [12]. The TPP proposed sensitivity is similar to the sensitivity of the Xpert MTB/RIF assay in microbiologically confirmed samples. Our assay renders 57% sensitivity and 96% specificity (21 TB and 24 control samples), which has the potential to meet the WHO TPP for detection of active TB in children, given the highly heterogenous population of P1041 samples, with respect to the bacteriological and clinical status.

Besides limited numbers of samples used in the current study, the heterogeneity of the clinical status of the samples may affect the accuracy of the test. Samples used in our study were obtained from IMPAACT P1041 clinical trial. The accuracy of TB diagnosis for the P1041 trial samples is not known due to the absence of good pediatric TB diagnostics. In addition, microbiological confirmation of TB disease was not obtained for any of these patients in the P1041 trial. TB disease subjects used in the present study are patients infected with TB presenting clinical symptoms of disease. The ELISA assays were performed retrospectively and the variance in storage conditions might affect the antigen integrity and therefore the accuracy of the assay. We have insufficient samples to include other clinical characteristics of the patients into data analysis. The clinical characteristics include but are not limited to BCG administration, immunosuppression status like CD4 counts, or clinical manifestation of tuberculosis (pulmonary and extra-pulmonary TB). The consideration of those characteristics may help define the limitation of our TLP assay or render improved accuracy with the combination of other clinical factors.

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We focused on HIV positive subjects in the current study. TB diagnosis is particularly difficult among HIV co-infected individuals who may have atypical, nonspecific clinical presentation, high rates of smear negative disease and high rates of extrapulmonary TB [39]. P1041 samples were obtained from TB endemic settings, therefore patients were typically being diagnosed at more advanced stages than those living in the US. Therefore, the heterogenous stages of TB disease may contribute to the wide dispersity of TLP levels in TB disease subjects (Fig. 3A), which is common among TB diagnostic assays. In addition, the duration of anti-tubercular treatment was not consistent among patients in the present study. Change of LDL modification is a dynamic process and may have the potential to monitor treatment outcome. Although not statistically significant, the slight decrease in the TLP level post diagnosis/ anti-tubercular treatment of 40 weeks may be related to a reduction in microbial load (Additional file 1: Figure S6A and B). Further studies are required to elucidate the effect of different disease status and/or treatment status on TLP levels in plasma, and to extend our preliminary results to other populations such as HIV negative subjects and adults. Although the accessibility and better-defined TB status of adults might be beneficial for feasibility testing, the matured immune system of adults might add complexity to interpretation of the assay.

It is a growing notion in the field that a single biomarker will not be sufficient for distinguishing TB status in different patient groups, and multiple biomarkers may be used to increase sensitivity and specificity. Further gains in clinical sensitivity and specificity may be obtained by combining the current assay with other biomarkers or diagnostic tests.

Abbreviations
TB: Tuberculosis; *Mtb*: *Mycobacterium tuberculosis*; ELISA: Enzyme-linked immunosorbent assay; FM: Foamy macrophages; MDA-LDL: Malondialdehyde-modified low density lipoprotein; TLP: *Mycobacterium tuberculosis* Modified lipoprotein; ROC: Receiver operating characteristic; AUC: Area under the ROC curve.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12879-022-07140-9.

Additional file 1. Supplemental Information.

Authors' contributions
SN, MFW, XY, NSS conceived the experiments. SN, MFW, obtained sample resource. XY performed the experiments. XY, NSS wrote the main manuscript text. XY prepared all figures. All authors read and approved the final manuscript.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All methods were carried out in accordance with relevant guidelines and regulations. The NIH-IMPAACT Scientific Leadership Group (SLG) reviewed and approved the use of P1041 trial samples under NWCS127. The Wits Health Consortium, University of Witwatersrand, South Africa provided deidentified samples to Stony Brook University for which informed consent had been obtained from a parent and/or legal guardian of all participants. The Stony Brook University CORIHS determined the experiments (IRB# 687246-1) in this manuscript were not human subjects research (exemption 4) under either United States Department of Health and Human Services 45 CFR part 46, subpart A, "the Common Rule" or United States Food and Drug Administration (FDA) Regulations 21 CFR parts 50, 56, 312 and 812.

Consent for publication
Not applicable.

Competing interests
NSS is President and co-owner of Chronus Pharmaceuticals, Inc. XY was an employee of both Chronus Pharmaceuticals, Inc and Stony Brook University. All intellectual property is currently owned by Stony Brook University. SN and MFW have no conflict of interest.

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