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CRISPR detection of circulating cell-free *Mycobacterium tuberculosis* DNA in adults and children, including children with HIV: a molecular diagnostics study

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Contributors

ZH, CJL, BN, and TYH conceived and designed the study. ZH and BMY contributed to data collection. ZH, SML, AWK, JS, JNE, WZ, AMM, and CJL contributed to data analysis and interpretation. SML, AWK, JNE, DV, MD, GM, LMC, IN, DCW, EM-O, GJ-S, AD, and AMM contributed to clinical information and sample collection and analysis. ZH and CJL drafted the manuscript. SML, DGC, GJS, AD, AMM, BN, and TYH provided critical revision. ZH, CJL, JS, JNE, and TYH have accessed and verified the data, all authors approved the final manuscript, and TYH was responsible for the decision to submit the manuscript.

Declaration of interests

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Summary

Background—Tuberculosis remains a leading cause of global mortality, especially for adults and children living with HIV (CLHIV) underdiagnosed by sputum-based assays. Non-sputum-based assays are needed to improve tuberculosis diagnosis and tuberculosis treatment monitoring. Our aim in this study was to determine whether ultrasensitive detection of *Mycobacterium tuberculosis* cell-free DNA (*Mtb*-cfDNA) in blood can diagnose tuberculosis and evaluate tuberculosis treatment responses.

Methods—In this molecular diagnostics study we analysed archived serum from two patient populations evaluated for tuberculosis in Eswatini and Kenya to detect *Mtb*-cfDNA, analysing serum from all individuals who had both sufficient serum volumes and clear diagnostic results. An optimised CRISPR-mediated tuberculosis (CRISPR-TB) assay was used to detect *Mtb*-cfDNA in serum at enrolment from adults and children with presumptive tuberculosis and their asymptomatic household contacts, and at enrolment and during tuberculosis treatment from a cohort of symptomatic CLHIV at high risk for tuberculosis, who provided longitudinal serum at enrolment and during tuberculosis treatment.

Findings—CRISPR-TB identified microbiologically and clinically confirmed tuberculosis cases in the predominantly HIV-negative Eswatini adult cohort with 96% sensitivity (27 [96%] of 28, 95% CI 80–100) and 94% specificity (16 [94%] of 17, 71–100), and with 83% sensitivity (5 [83%] of 6, 36–100) and 95% specificity (21 [95%] of 22, 77–100) in the paediatric cohort, including all six cases of extrapulmonary tuberculosis. In the Kenyan CLHIV cohort, CRISPR-TB detected all (13 [100%] of 13, 75–100) confirmed tuberculosis cases and 85% (39 [85%] of 46, 71–94) of unconfirmed tuberculosis cases diagnosed by non-microbiological clinical findings. CLHIV who were CRISPR-TB positive at enrolment had a 2·4-times higher risk of mortality by 6 months after enrolment. *Mtb*-cfDNA signal decreased after tuberculosis treatment initiation, with near or complete *Mtb*-cfDNA clearance by 6 months after tuberculosis treatment initiation.

Interpretation—CRISPR-mediated detection of circulating *Mtb*-cfDNA shows promise to increase the identification of paediatric tuberculosis and HIV-associated tuberculosis, and potential for early diagnosis and rapid monitoring of tuberculosis treatment responses.

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Introduction

There are an estimated 10·0 million people diagnosed with tuberculosis and 1·2 million tuberculosis-related deaths annually.[1] Tuberculosis diagnosis remains challenging and is still widely dependent on sputum-based microbiological methods that have reduced performance in young children and people living with HIV (PLHIV),[2,3] for whom difficult sample collection and immunosuppression can contribute to paucibacillary respiratory samples,[2,4] and individuals with extrapulmonary tuberculosis. Rapid, non-sputum-based tests are thus needed to improve tuberculosis diagnosis and to evaluate tuberculosis treatment responses.[5]

Sensitive detection of pathogen-derived cell-free DNA (cfDNA) in the circulation has potential for diagnosis and treatment evaluation,[6–8] but PCR-based studies that have analysed circulating *Mycobacterium tuberculosis* cfDNA (*Mtb*-cfDNA) have reported poor and highly variable diagnostic sensitivity.[9–11] Clustered regularly-interspaced short palindromic repeats (CRISPR)-Cas12a cleavage activity can be used to enhance detection sensitivity, given that binding of a Cas12a guide RNA (gRNA) complex to its target DNA sequence can be used to cleave a quenched fluorescent probe, causing an enzymatic signal amplification that can markedly enhance the detection of low-concentration target sequences. This approach has been applied to detect *M tuberculosis*-derived DNA targets in sputum,[12,13] but had poor performance when applied to detect circulating *Mtb*-cfDNA,[14] because of the high cfDNA limit of detection (LOD) of the employed diagnostic assay. We adapted an ultrasensitive CRISPR-Cas12a-powered fluorescence assay[15] to detect *Mtb*-cfDNA in blood (CRISPR-TB), and evaluated its diagnostic performance for tuberculosis using cryopreserved samples from adults and children with presumed tuberculosis and their asymptomatic household contacts, and samples from a diagnostically challenging cohort of children living with HIV (CLHIV) who were symptomatic and immunocompromised, which included longitudinal serum obtained during tuberculosis treatment. Our study aim was to determine whether ultrasensitive detection of *Mtb*-cfDNA in blood can diagnose tuberculosis and evaluate tuberculosis treatment responses.

Methods

Study design and study populations

In this molecular diagnostics study, we used cryopreserved serum and clinical data from participants from two cohorts. Adults and children enrolled in the Eswatini cohort (figure 1A; appendix p 21) were considered to have confirmed tuberculosis if diagnosed by *M tuberculosis* liquid culture, Gene Xpert MTB/RIF Ultra (Xpert Ultra, Cepheid, USA), or both. Respiratory samples were collected by gastric aspiration or sputum induction if participants could not produce expectorated-sputum specimens. Patients with clinically diagnosed, presumptive tuberculosis did not have a positive culture or Xpert result, but were determined to have tuberculosis by their treating clinicians on the basis of symptoms and radiographical evidence, and were retrospectively classified as having unconfirmed tuberculosis (children) using National Health Institute paediatric tuberculosis case definitions or were classified as having clinically diagnosed tuberculosis (adults) using WHO guidelines (appendix pp 22–24).[16,17] Controls who did not have tuberculosis were recruited from household contacts of patients who were tuberculosis index cases, and were

asymptomatic or were excluded from having tuberculosis following resolution of mild symptoms or additional tests, and were evaluated at 2 months and 6 months after enrolment to confirm tuberculosis exclusion decisions.

All participants were enrolled from Sept 24, 2020, to June 23, 2021 in Eswatini at Mbabane Government Hospital, Raleigh Fitkin Memorial Hospital in Manzini, Pigg's Peak Government Hospital, Dvokolwako Health Centre, and Baylor Clinic in Mbabane. Serum was collected and cryopreserved at baseline. All individuals provided written informed consent before participation. This work was approved by the Eswatini Health Research Review Board, the Baylor Children's Foundation of Eswatini, and the Baylor College of Medicine ethical review boards. Individuals were eligible for inclusion in the CRISPR-TB diagnostic-performance evaluation part of our study if they had sufficient serum and clear clinical diagnostic results.

Cryopreserved serum and clinical data were also used from participants in the Paediatric Urgent versus Post-Stabilisation Highly-Active Anti-Retroviral Therapy Initiation Trial (PUSH). The PUSH study was a randomised controlled trial[18] performed in Kenya, which enrolled children younger than 12 years who were hospitalised with HIV and who had not yet initiated antiretroviral therapy (ART). The trial evaluated whether urgent ART (<48 h) versus ART after stabilisation (7–14 days) improved survival (figure 1B).[18] Children with clear or possible central nervous infection were excluded. Clinical management of comorbidities was done according to hospital protocols and Kenyan National guidelines. All CLHIV initiated cotrimoxazole prophylaxis as per clinical guidelines. Participants were categorised by HIV-associated immunosuppression using WHO-defined age-specified CD4 count and CD4 percentage cutoffs.[19] At enrolment, all participants were systematically screened for tuberculosis symptoms and exposure and underwent intensive tuberculosis evaluation, including chest x-ray, sputum or gastric aspirates for Xpert MTB/RIF (Xpert) and culture, urine for lipoarabinomannan testing (urine LAM), and stool for Xpert, irrespective of tuberculosis symptoms.[20] Details regarding the parent trial implementation and primary results, and results of the enrolment tuberculosis diagnostics done in real-time, have been previously reported.[18,20] Serum was collected and cryopreserved at enrolment and at 2 weeks, 4 weeks, 12 weeks, and 24 weeks after enrolment. Diagnostic results were available to the study clinicians and tuberculosis treatment was initiated at their discretion, as per Kenyan guidelines.[21] Enrolment began April 24, 2013, and follow-up was completed on Nov 17, 2015. In the parent study, children were categorised post hoc as having confirmed tuberculosis (microbiological conirmation by either Xpert or culture), unconfirmed tuberculosis (absence of microbiological confirmation), or unlikely tuberculosis (not meeting the criteria for confirmed or unconfirmed tuberculosis) by modified NIH paediatric tuberculosis case definitions, which consider both baseline evaluation and follow-up data (appendix pp 23–24).[16]

After the study was completed, an expert panel reviewed the deaths that had occurred during the study period and judged them to be unlikely, possibly, or likely related to tuberculosis (appendix p 24), with possible or likely tuberculosis-related deaths considered to be additional factors for unconfirmed tuberculosis (appendix p 23). For this study, children with unconfirmed tuberculosis were further stratified by whether they were clinically

diagnosed with tuberculosis during the study (eg, whether they initiated tuberculosis treatment) and children with unlikely tuberculosis were further stratified by presence of any NIH tuberculosis criteria (figure 1B). Children with negative baseline work-ups, but who developed symptoms consistent with tuberculosis during follow-up and received a clinical diagnosis at 14 days or more after enrolment, as indicated by tuberculosis treatment initiation, were considered to have incident tuberculosis.

Children were eligible for inclusion in the CRISPR-TB diagnostic performance evaluation if they had a serum sample available at baseline, when all children underwent intensive tuberculosis evaluation, or within 2 weeks of baseline. Children who initiated tuberculosis treatment but did not have serum collected before, or within 2 weeks of, tuberculosis treatment initiation were excluded from this evaluation. Children evaluated for CRISPR-TB diagnostic performance were also included in the tuberculosis treatment-response analyses if they had at least one subsequent sample after tuberculosis treatment initiation. Baseline characteristics of children without available serum samples for this analysis were similar to the entire PUSH cohort (data not shown).

All participants provided written informed consent to participate in the study. The original trial and post-hoc use of cryopreserved samples was approved by the Kenyatta National Hospital and University of Nairobi Ethics Research Committee, and the University of Washington Institutional Review Board.

Sample collection and transportation

For both cohorts, blood samples were collected in red-top tubes without additives at facility or household visits, processed for serum within 4 h, and stored or transported at −80°C until processed for cfDNA analysis, at which time samples were thawed on ice.

CRISPR-TB assay-based serum test

CRISPR-TB was established and optimised as described (appendix pp 2–10, 25–28). Typically, 50 μL cfDNA was isolated from 200 μL serum, and 5 μL of extracted cfDNA was analysed in a CRISPR-TB assay that detected the multicopy *M tuberculosis*-specific IS6110 DNA sequence (assay reagents and protocol in the appendix p 2). Samples were analysed in triplicate to determine mean values, and results with coefficients of variation of 20% or higher among these replicates were considered invalid. Samples with invalid results were reanalysed if there was sufficient volume; otherwise, all invalid results were excluded from study analyses.

Statistical analysis

We summarised participant characteristics using medians, IQRs, and proportions. We used Wilcoxon rank-sum tests to compare *Mtb*-cfDNA concentrations between tuberculosis classifications, and between survival states. We used Wilcoxon signed-rank tests to compare the concentrations of the three *M tuberculosis* target genes in patient samples, and to compare *Mtb*-cfDNA concentrations before and after tuberculosis treatment and ART initiation. We estimated CRISPR-TB sensitivity and specificity for tuberculosis diagnosis using exact 95% CIs, assuming a binomial distribution. We used Kaplan-Meier survival

curves and univariate and multivariate (adjusting for dichotomised age and CD4 percentage via a median split at baseline) Cox proportional hazard regression to compare mortality for baseline CRISPR-TB positivity in the PUSH cohort during the 6 month follow-up period. All tests were two sided with an α of 0·05. All analyses were done with Stata 14 (version 17.0). GraphPad Prism (version 9.1.2), was used for data visualisation.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

PCR primers and gRNAs for gyrB, esxB, and the *M tuberculosis*-complex-specific IS6110 element (figure 2A; appendix p 25) produced similar CRISPR-TB results with a high concentration of *M tuberculosis* DNA (appendix p 5), although IS6110, which exhibits copy number variation among *M tuberculosis* strains, was detected at lower concentration and was not detected in genomic DNA of non-tuberculous mycobacteria (figure 2B–D; appendix p 26). We thus optimised a CRISPR-TB IS6110 assay for all further analyses (appendix pp 3–4, 6–9, 27) and found that it had a linear quantification range (0·5–1000 copies per µL; figure 2E) fully spanning the reported IS6110 cfDNA range in blood (mean 7·20 copies per µL, SD 29·15).[22] At analysis, serum cfDNA was concentrated four times from its serum concentrations, resulting in a final 0·06 copy per µL LOD, which was markedly lower than the LOD obtained with the same samples by PCR reactions done with the same primers (appendix p 10).

We first evaluated CRISPR-TB in an Eswatini cohort of adults and children with microbiologically confirmed (n=30) or clinically diagnosed tuberculosis (n=4), and their household non-tuberculosis controls (n=39). CRISPR-TB signal was higher in adults and children with microbiologically confirmed tuberculosis, and adults with clinically diagnosed tuberculosis, than their asymptomatic controls (figure 2F). Using a tuberculosis definition that included cases identified by both microbiological results and clinical findings, a negative response threshold defined by healthy serum (appendix p 28) identified adult tuberculosis with 96% sensitivity (27 of 28 adults, 95% CI 80–100) and 94% specificity (16 of 17 adults, 71–100), and paediatric tuberculosis with 83% sensitivity (five of six children, 36–100%) and 95% specificity (21 of 22 children, 77–100; figure 2F–G; appendix p 29). Notably, CRISPR-TB identified all six extrapulmonary tuberculosis cases (lymph node, bone, joint, and pleura involvement) in this cohort (figure 2F, 2G), although this subgroup and the paediatric tuberculosis group was too small to estimate assay performance.

Next, we did an analysis on 153 participants in the PUSH trial (figure 1B; table 1) to evaluate assay performance in a diagnostically challenging cohort of CLHIV who were ART naive, at high risk of tuberculosis, and admitted to hospital. This group had a median age of 2·0 years (IQR 0·8–5·1), and 73 (48%) were female and 80 (52%) were male. Most children had severe immunosuppression (104 [68%] of 152) and presented with at least one symptom suggestive of tuberculosis (139 [91%] of 193; figure 3A), including children who

were categorised as unlikely tuberculosis cases, who presented with symptoms (50 [76%] of 75) and chest x-ray findings (18 [26%] of 69) suggestive of tuberculosis.

CRISPR-TB detected all children with confirmed tuberculosis (13 [100%] of 13; 95% CI 75–100) and most with unconfirmed tuberculosis (52 [80%] of 65), including most with unconfirmed tuberculosis (13 [68%] of 19) who did not receive a tuberculosis diagnosis or tuberculosis treatment during the study and four of five children with extrapulmonary tuberculosis (figure 3A–B; table 2; appendix p 30). Urine LAM assay results (which have been proposed for non-sputum paediatric tuberculosis diagnosis) had poor sensitivity for confirmed (four [44%] of 9) and unconfirmed tuberculosis (one [6%] of 50) cases with valid test results (figure 3A), and moderate specificity for unlikely tuberculosis cases that did not meet tuberculosis criteria (11 [85%] of 13).

CRISPR-TB negative rates markedly differed among unlikely tuberculosis cases with and without NIH tuberculosis criteria (27 [44%] of 61 *vs* 11 [79%] of 14; figure 3A; table 2), suggesting the potential for nascent tuberculosis in the former group. CRISPR-TB specificity estimated for children with no NIH criteria was 79% (11 of 14, 95% CI 49–95%), which was at the lower end of the Eswatini cohort specificity estimate (appendix p 29).

CRISPR-TB detected *Mtb*-cfDNA (median 0·13 copies per µL, IQR 0·07–1·57; mean 5·58 copies per µL, SD 18·25) in the earliest available serum of 102 children (figure 3A; appendix p 11), and about half had *Mtb*-cfDNA concentrations lower than the LOD of PCR (0·25 copies per µL; appendix p 10) in agreement with previously reported PCR analytical[22] and diagnostic sensitivity estimates.[9–11] Median CRISPR-TB signal of children with confirmed and unconfirmed tuberculosis was significantly higher than among children with unlikely tuberculosis who met no NIH tuberculosis criteria. Median CRISPR-TB among children with unlikely tuberculosis with any NIH tuberculosis criterion was higher than those with no NIH criteria, but not significantly so (figure 3C; table 2; appendix p 12).

Children in the PUSH cohort who died of any cause (28 [18%] of 153) tended to have higher baseline *Mtb*-cfDNA concentrations than children who survived to 6 months (appendix p 13). 6-month overall mortality (figure 3D) also appeared to be higher among children with positive baseline *Mtb*-cfDNA signal versus those with negative *Mtb*-cfDNA signal, but not significantly so (hazard ratio [HR] 2·4, 95% CI 0·9–6·4; p=0·073), even after adjusting for age and baseline CD4 percentage (adjusted HR [aHR] 2·4, 0·9–6·4; p=0·076). Among the 95 children who did not receive tuberculosis treatment, the risk of mortality was higher among children who were *Mtb*-cfDNA positive at baseline (HR 3·9, 1·3–11·7; p=0·015; appendix p 14). However, among the 58 children who received tuberculosis treatment, baseline positive *Mtb*-cfDNA signal was not associated with increased mortality (HR 0·9, 0·1–7·5; p=0·94; appendix p 14).

Most children in the PUSH cohort who were clinically diagnosed with incident tuberculosis after their baseline evaluation visit had *Mtb*-cfDNA-positive serum samples at baseline (ten [83%] of 12; appendix pp 15, 31). These children initiated tuberculosis treatment more than 14 days after enrolment and initial evaluation, with a median time of 43·5 days (IQR

24·5–83·0) from the initial CRISPR-TB positive result to clinical tuberculosis diagnosis, indicating the potential for early diagnosis.

CRISPR-TB analysis of serum from 51 children (appendix p 16) with samples available before and after tuberculosis treatment initiation (median 5·6 months after tuberculosis treatment initiation, IQR 3·2–5·8) also detected a 2·8-times decrease in *Mtb*-cfDNA after tuberculosis treatment initiation (figure 3E), with similar decreases detected in confirmed and unconfirmed tuberculosis cases, and one unlikely tuberculosis case that received tuberculosis treatment (table 2). Most *Mtb*-cfDNA-positive tuberculosis treatment recipients revealed symptom improvement or weight gain (42 [89%] of 47), and *Mtb*-cfDNA decreases (35 [83%] of 42) with or without *Mtb*-cfDNA clearance (14 [33%] of 42) by study completion (appendix p 17). A majority of individuals treated for tuberculosis who had four or more serum samples revealed *Mtb*-cfDNA decreases by 1 month after tuberculosis treatment initiation (14 [52%] of 27; median 15·5 days, IQR 13–23) and most exhibited complete or near-complete clearance by their last available sample (19 [70%] of 27; median 172 days after enrolment, IQR 167–175; appendix pp 18, 32), with all but two recipients demonstrating decreases (25 [93%] of 27). *Mtb*-cfDNA concentrations tended to immediately decrease or spike and then decrease after tuberculosis treatment initiation (appendix p 18).

Several children in the PUSH cohort who were serum *Mtb*-cfDNA positive at baseline were deemed post hoc to represent unlikely tuberculosis cases because of symptom resolution or weight gain after initiation of ART in the absence of tuberculosis treatment, and revealed decreases in *Mtb*-cfDNA after ART, suggestive of *M tuberculosis* containment by the reconstituting immune system (figure 3A; appendix pp 19, 33).

Discussion

We observed that detection of serum *Mtb*-cfDNA in adults and children, including CLHIV, provided a sensitive approach to identify individuals with tuberculosis, including paucibacillary tuberculosis cases not detected by microbiological methods. Serum *Mtb*-cfDNA concentrations rapidly decreased following tuberculosis treatment initiation, suggesting its potential for tuberculosis treatment monitoring. *Mtb*-cfDNA detection also identified CLHIV most likely to die, who were predominantly missed by conventional tuberculosis diagnostics. Intriguingly, *Mtb*-cfDNA was detected in serum collected from CLHIV weeks before their diagnosis with tuberculosis by other methods and might thus have potential utility for early diagnosis.

Currently, available sputum-based diagnostics have suboptimal diagnostic performance especially in children, PLHIV, and individuals with extrapulmonary tuberculosis. Sensitive detection of circulating *Mtb*-cfDNA could potentially identify both pulmonary tuberculosis and extrapulmonary tuberculosis given that *Mtb*-cfDNA released during apoptosis and *M tuberculosis* lysis[9,23] should accumulate in the circulation during tissue perfusion irrespective of its sites of origin. However, previous studies have determined that *Mtb*-cfDNA circulates at very low concentrations,[22] resulting in poor and highly variable diagnostic sensitivity,[9–11] which was not improved by use of a CRISPR assay with a high

LOD.[14] Here we provide evidence that the combination of an assay optimised for serum cfDNA extraction and sensitive and specific target amplification and cleavage can sensitively detect serum *Mtb*-cfDNA in most patients with tuberculosis, including those missed by sputum-based diagnostics. This assay analyses cfDNA isolated from about 200 μL of serum, has a 2 h sample-to-answer time, detects its *Mtb*-cfDNA target across its full reported concentration range, and can be adapted to a portable format suitable for resource-limited settings (appendix p 20).

New approaches are needed for rapid analysis of tuberculosis disease severity in populations in which respiratory samples are challenging to collect and often have low yield. Currently used sputum-based diagnostic assays yield results that typically reflect pulmonary, but not systemic, *M tuberculosis* burden; perform poorly in PLHIV and children; and exhibit poor correlation with tuberculosis disease severity.[24] Several groups are investigating the ability of host biomarkers to evaluate *M tuberculosis* burden,[5] but any identified host biomarkers will require careful independent validation in large, well designed cohort studies to confirm their specificity. Evidence indicates that circulating microbial cfDNA concentrations, including *Mtb*-cfDNA, may reflect disease severity.[6–8,25] In this study, baseline serum *Mtb*-cfDNA concentrations in CLHIV admitted to hospital tended to correspond with increased mortality, suggesting that *Mtb*-cfDNA positivity could predict short-term mortality in this population, particularly in the absence of tuberculosis treatment. However, similar to other biomarkers, the utility of serum *Mtb*-cfDNA concentration to predict early mortality and estimate *M tuberculosis* burden will require validation in prospective cohorts powered to address these crucially important questions.

Evaluation of tuberculosis treatment effectiveness currently relies on positive-to-negative *M tuberculosis* culture conversion and clinical improvement, which do not permit rapid or quantitative measures. Xpert and Xpert Ultra results are rapid but cannot distinguish DNA contributed by viable and non-viable *M tuberculosis* to evaluate rapid changes in *M tuberculosis* burden following tuberculosis treatment initiation.[26,27] In patients with paucibacillary disease, including children, baseline culture and Xpert results are often negative, and cannot be used to evaluate tuberculosis treatment response. Collection of longitudinal samples from patients with extrapulmonary tuberculosis for microbiological evaluations is also not typically feasible because of their invasive nature. In an attempt to circumvent the limitations of respiratory or tissue-biopsy samples, several studies have analysed host-derived factors or biomarker patterns in liquid biopsies as indicators of tuberculosis treatment response.[24] Nevertheless, these approaches provide only indirect evidence of *M tuberculosis* burden as reflected by non-specific gene changes that might exhibit latency and might not detect residual concentrations of viable pathogen that are insufficient to provoke robust gene expression. However, circulating *Mtb*-cfDNA is measured from readily-accessible blood specimens and reflects material released from dying *M tuberculosis* and apoptotic cells in real time, rather than accumulated material reflecting earlier *M tuberculosis* burden. Hence, serial concentrations of *Mtb*-cfDNA during tuberculosis treatment could provide an estimate of *M tuberculosis* burden and correlate with tuberculosis treatment response. Although this exploratory study observed that serum *Mtb*-cfDNA concentrations decreased during tuberculosis treatment, we interpret this novel finding with caution because semiquantitative microbiological data were not available

for comparison and the mechanisms responsible for the patterns of *Mtb*-cfDNA decrease following tuberculosis treatment initiation are not clear and require further research studies.

This study has limitations that could affect its interpretation. First, in this study we analysed a multicopy DNA target (IS6110), which is absent in some *M tuberculosis* strains, to enhance sensitivity.[28] However, CRISPR-TB assays for *M tuberculosis* gyrB cfDNA, which circulates at slightly lower abundance, could avoid false negatives for IS6110-deletion strains. Second, we analysed cryopreserved serum that might have influenced cfDNA concentration.[29] Third, paediatric tuberculosis diagnoses were primarily made without microbiological confirmation; however, our study used the NIH consensus case definition that supports standardised case reporting in paediatric tuberculosis diagnostic studies.[16] Fourth, *Mtb*-cfDNA detected in the serum of children in PUSH cohort unlikely tuberculosis cases might represent a false-positive signal. This cohort was composed of CLHIV who were immunocompromised and at high risk for tuberculosis; however, 80% of children classified as unlikely tuberculosis cases had symptoms, signs, or chest x-ray findings consistent with tuberculosis. Specificity estimates for the PUSH cohort thus used only CRISPR-TB data from children in the unlikely tuberculosis group who did not meet any criteria of the NIH consensus definition to reduce the risk of including children with potential tuberculosis in the specificity estimates. This strategy appears to represent the best available choice for this population, but might have underestimated or overestimated test specificity. We note that the assay had higher specificity (94% for adults and 95% for children) when assessed in asymptomatic household contacts available within the Eswatini cohort, although this estimate was limited by sample size. Further, although tuberculin skin tests were done in the PUSH cohort, other evaluations for latent tuberculosis infection were not available.

The strengths of our study include optimisation of a CRISPR-based assay to detect *Mtb*-specific cfDNA in a small blood volume, analysis of two well characterised cohorts, inclusion of adults and children with microbiological tuberculosis confirmation and matched asymptomatic healthy controls, and inclusion of a cohort of CLHIV at highest risk of tuberculosis-related morbidity and mortality, who had longitudinal samples to permit assessment of early tuberculosis diagnosis and *Mtb*-cfDNA response to tuberculosis treatment. Future analyses in larger prospective paediatric cohorts are required to better understand its performance along the diverse spectrum of disease presentations, with appropriate negative controls, including individuals with latent tuberculosis infection.

In conclusion, these findings provide strong preliminary evidence for the utility of serum *Mtb*-cfDNA analysis to provide non-sputum-based tuberculosis diagnosis and a potential secondary application for tuberculosis treatment monitoring. Further, among CLHIV, these findings highlight potentially high-impact applications, including early tuberculosis diagnosis and identification of children at increased risk of tuberculosis-related mortality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data sharing

The main data supporting the results in this study are available within the paper and its appendix. The raw datasets generated and analysed during the current study are available from the corresponding author on reasonable request following publication.

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Research in context

Evidence before this study

Tuberculosis is a leading cause of death from infectious disease, particularly in adults and children living with HIV. However, tuberculosis diagnosis is challenging in young children and individuals living with HIV because of the difficulty in obtaining respiratory samples, and their frequent paucibacillary nature. This difficulty has led to a recognised need for more effective non-sputum-based tuberculosis diagnostics in these populations. Xpert MTB/RIF and Xpert MTB/RIF ultra nucleic acid amplification assays, recommended as initial diagnostic tests for tuberculosis diagnosis, also have reduced performance when used to analyse respiratory samples from children and individuals living with HIV or non-respiratory specimens. We searched Google Scholar to identify English-language articles published between Jan 1, 2001, and March 1, 2022, that investigated the diagnostic performance of circulating *Mycobacterium tuberculosis*-derived cell-free DNA (*Mtb*-cfDNA) for tuberculosis diagnosis. In this literature search we used the search terms "tuberculosis", "cell-free DNA", and "blood" to identify articles that included all three search terms. This search identified two systematic reviews that evaluated a total of 18 studies that used nucleic acid amplification to investigate *Mtb*-cfDNA in patient samples for tuberculosis diagnosis, including five studies that analysed peripheral blood samples, and ten additional blood-based studies not included in these two reviews. These studies primarily analysed small casecontrol cohorts of adults diagnosed with tuberculosis with microbiological evidence or a composite reference standard, and yielded a median diagnostic sensitivity of 41% (IQR 27·1–54·2) and a median specificity estimate of 97% (93·2–100). However, the two studies that analysed paediatric cohorts reported much lower sensitivities (26·2% and 5·4%).

Added value of this study

CRISPR collateral cleavage activity has been used to improve the detection sensitivity for pathogen-derived material in several assays, although no reported studies have successfully used this approach to detect circulating *M tuberculosis* genomic DNA for tuberculosis diagnosis with good diagnostic performance. This study evaluated the diagnostic sensitivity and specificity of an ultrasensitive CRISPR-based fluorescence assay to detect *Mtb*-cfDNA in blood samples from adult and children, including children with HIV. This assay demonstrated high diagnostic sensitivity and specificity (>90%) in a pooled adult and paediatric group, and diagnostic sensitivity remained high (85%) in children diagnosed with tuberculosis by non-microbiological clinical findings.

Implications of all the available evidence

This platform has strong potential to improve the diagnosis of tuberculosis in cases in which diagnostic sputum samples are difficult to obtain (eg, extrapulmonary, paediatric, or HIV-associated tuberculosis cases) and in which low bacillary yield might compromise the accuracy of the nucleic amplification tests recommended for initial diagnosis. Notably, this platform also has potential to support early tuberculosis diagnosis and predict tuberculosis mortality while rapidly evaluating tuberculosis treatment response in

children living with HIV. Hence, this platform addresses important unmet clinical needs required to improve tuberculosis diagnosis and management.

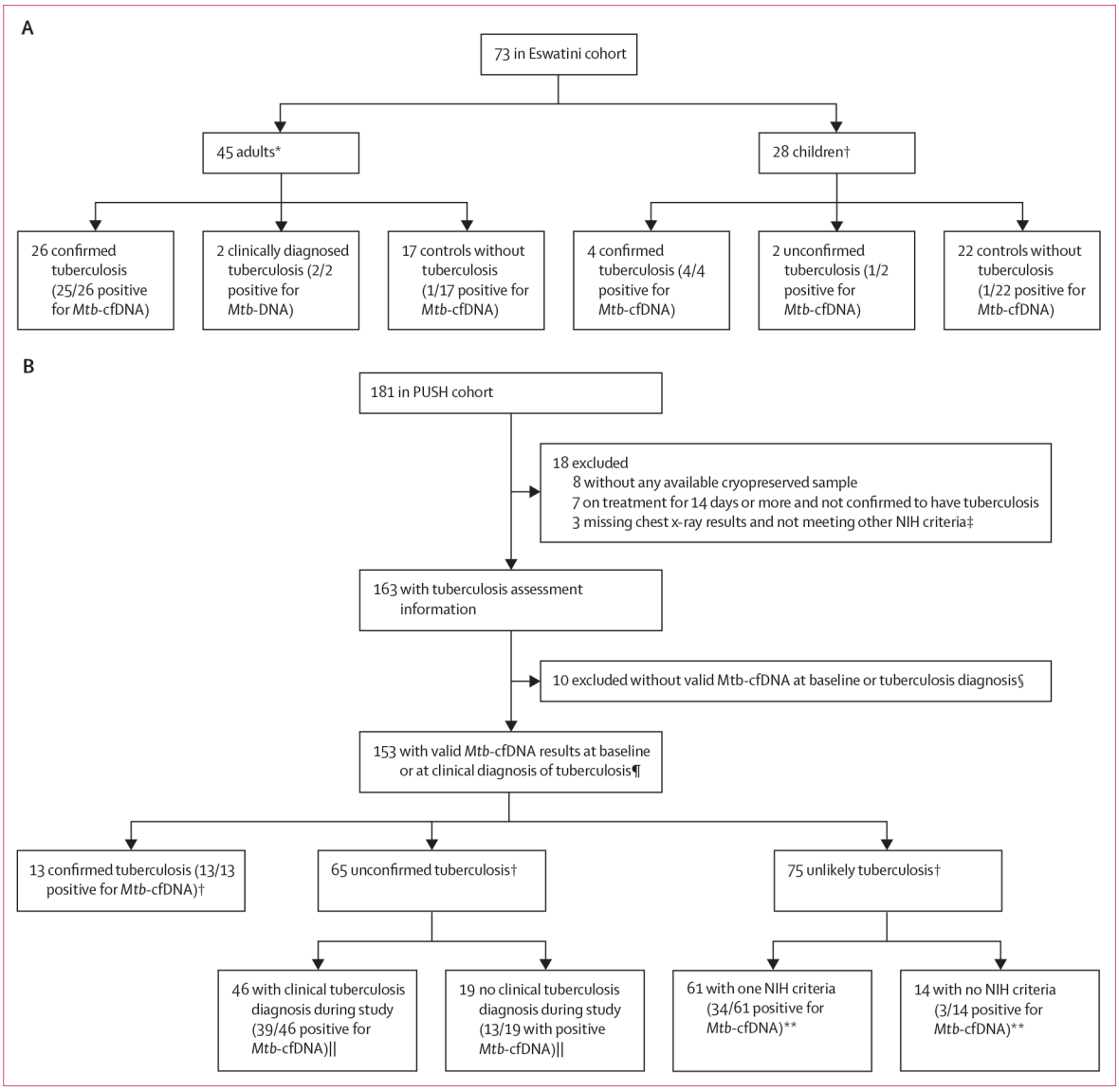

Figure 1: Study participants

CRISPR-TB diagnostic performance was evaluated by blind analysis of serum before tuberculosis treatment from a cohort of adults and children with presumptive tuberculosis and their asymptomatic household contacts (Eswatini cohort), and longitudinal serum collected at enrolment and during tuberculosis treatment in a cohort of children living with HIV at high risk for tuberculosis (PUSH cohort). *Mtb*-cfDNA=*Mycobacterium tuberculosis* cell-free DNA. *Adults were classified using WHO guidelines (appendix p 22). †Children were classified using modified NIH classification criteria (appendix p 23). ‡Children with missing chest x-ray results were excluded because they could not be classified as having unconfirmed or unlikely tuberculosis. §Triplicate CRISPR-TB results that had coefficients of variation of 20% or higher were considered invalid. ¶142 children had serum available at baseline; 11 children had their first serum sample available at a later visit. ||People without a confirmed tuberculosis diagnosis who initiated tuberculosis treatment were considered to have a clinical tuberculosis diagnosis. People without a confirmed tuberculosis who did not

initiate tuberculosis treatment were considered to have no clinical tuberculosis diagnosis.
**NIH tuberculosis symptoms suggestive of tuberculosis (appendix p 23).

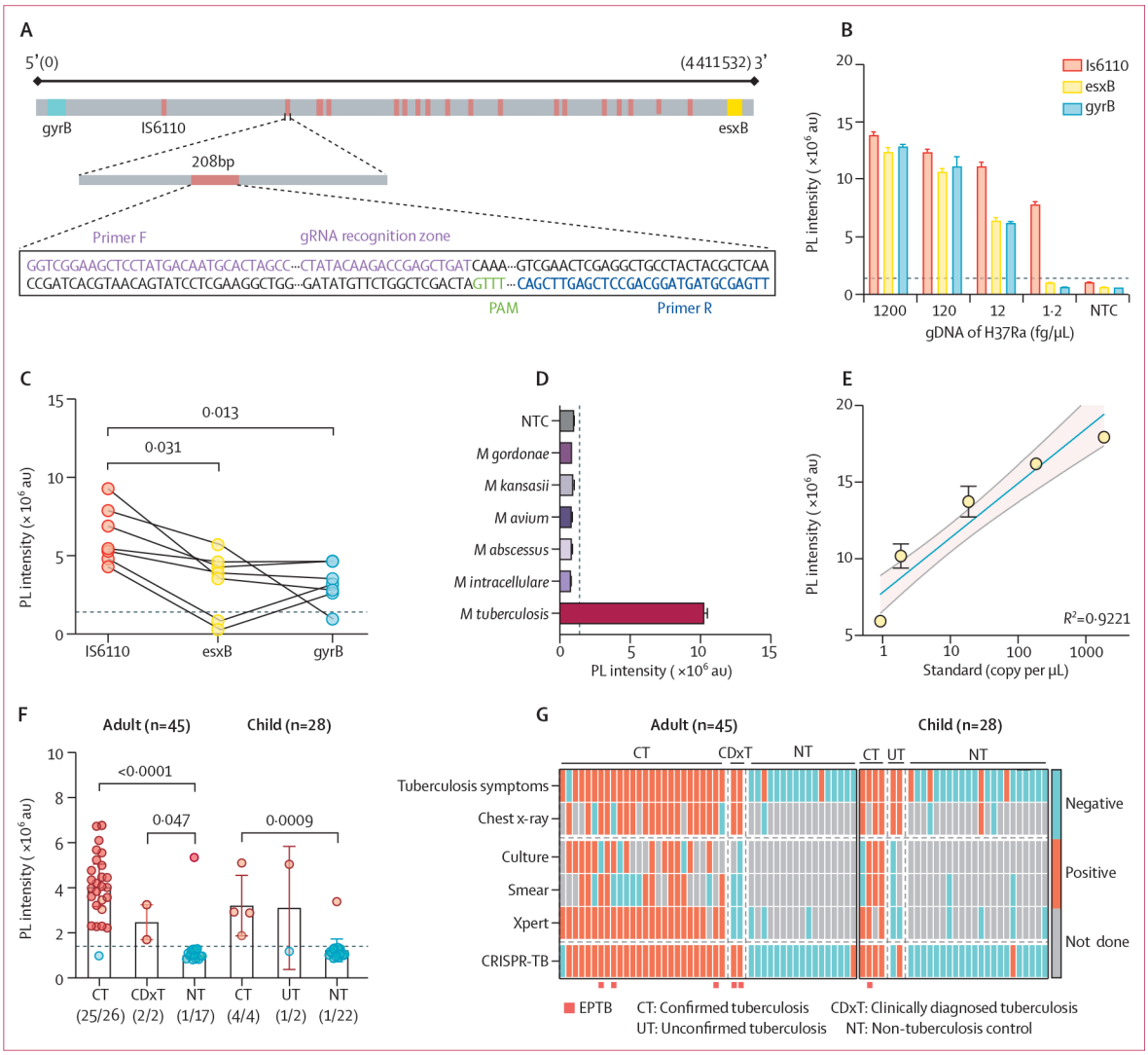

Figure 2: Analytical validation of the CRISPR-TB assay

Three CRISPR-TB target sequences mapped in the genome of *Mycobacterium tuberculosis* H37Ra (A), and their signal detected in serial dilutions of extracted *M tuberculosis* (H37Ra) gDNA (B) and in serum samples of patients with culture-confirmed tuberculosis (C; n=7), indicating two-sided p values in the Wilcoxon signed-rank tests. (D) CRISPR-TB signal detected with healthy human plasma spiked with or without gDNA from the indicated *M tuberculosis* or NTM species. (E) CRISPR-TB standard curve, in which shading denotes the 95% CI of the fitted line. CRISPR-TB serum values (F) and clinical and assay data for 45 adults and 28 children (E; Eswatini cohort), categorised as confirmed tuberculosis, clinically diagnosed tuberculosis, unconfirmed tuberculosis, and non-tuberculosis control by study physicians with expertise in pulmonary medicine using results of standardised tuberculosis tests, indicating two-sided p values for Wilcoxon rank-sum tests. Red squares indicate EPTB cases. The mean (SD) of three technical replicates for each sample (B–E) or the median (IQR; F) are presented. The blue dashed line indicates the lowest limit of positive signal cutoff threshold. au=arbitrary units. CT=confirmed

tuberculosis. CDxT=clinically diagnosed tuberculosis. EPTB= extrapulmonary tuberculosis. gDNA=genomic DNA. NT=non-tuberculosis control. NTC=no template control. NTM=non-tuberculous mycobacteria. PL intensity= photoluminescence intensity. UT=unconfirmed tuberculosis. Xpert=Gene Xpert MTB/RIF Ultra.

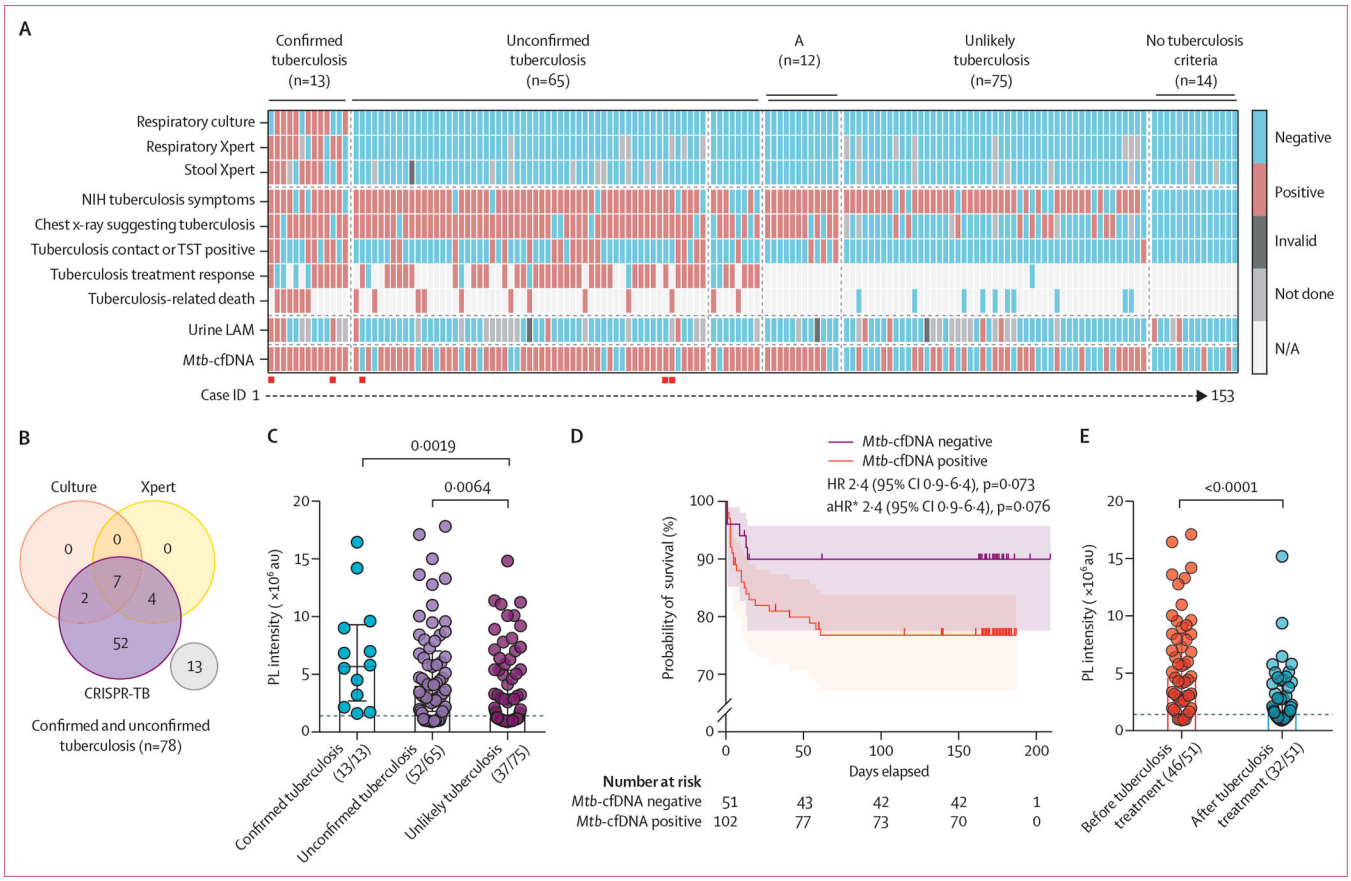

Figure 3: CRISPR-TB evaluation in children with HIV at high risk of tuberculosis (PUSH cohort)

(A) Clinical and assay data for children with HIV who are immunocompromised and symptomatic classified with confirmed, unconfirmed, and unlikely tuberculosis by NIH consensus diagnostic criteria with the addition of death possibly or likely to be related to tuberculosis, as judged by an expert review panel (appendix p 23). Urine LAM and serum *Mtb*-cfDNA results were not used for classification. Subgroup A (cases 79–90) included children who had symptom improvement after antiretroviral therapy initiation without tuberculosis treatment initiation (appendix p 33). Red squares indicate EPTB cases. (B) CRISPR-TB agreement with microbiological and clinical data of confirmed and unconfirmed tuberculosis cases. Grey circle indicates 13 children classified as having unconfirmed tuberculosis with negative CRISPR-TB, Xpert, and culture. (C) CRISPR-TB signal in children with confirmed, unconfirmed, and unlikely tuberculosis, with the number of signal positive and total cases in each group, and the two-sided p values from the Wilcoxon rank-sum test. (D) Survival curve for children segregated by high and low CRISPR-TB signal, HRs from a Cox proportional hazard model before and after adjustment for age and CD4 percentage differences. (E) CRISPR-TB signal initiation in 51 children who received tuberculosis treatment and had two or more longitudinal serum samples available for analysis (appendix p 16), with two-sided p values from the Wilcoxon signed rank test. (C, E) Median (IQR) presented for each group. The blue dashed line indicates the lowest limit of positive signal cutoff threshold. au=arbitrary

units. aHR=adjusted hazard ratio. EPTB=extrapulmonary tuberculosis. HR=hazard ratio. LAM=mycobacterial lipoarabinomannan. *Mtb*-cfDNA=*Mycobacterium tuberculosis* cell-free DNA. NIH=National Institutes of Health. TST=tuberculin skin test. Xpert=Gene Xpert MTB/RIF.

Table 1:

Baseline characteristics of CLHIV at high risk for tuberculosis who were ART naive and hospitalised (PUSH cohort)

	Number of participants with results	Overall (n=153)	Confirmed tuberculosis (n=13)*	Unconfirmed tuberculosis (n=65)*	Unlikely tuberculosis (n=75)*
Age, years	153	2·0 (0·8–5·1)	4·0 (1·3–6·6)	2·0 (1·1–5·1)	1·7 (0·7–4·4)
Sex					
Female		73 (48%)	6 (46%)	30 (46%)	37 (49%)
Male		80 (52%)	7 (54%)	35 (54%)	38 (51%)
Clinical presentation					
CD4 cell count, cells/μL	152	699·0 (308·0–1220·5)	438·0 (104·0–799·0)	621·0 (194·5–1169·0)	767·0 (491·0à1328·0)
CD4 cell percentage	152	15·2 (9·0–22·1)	11·0 (6·0–17·6)	12·9 (6·5–19·5)	17·0 (11·4–24·0)
Severe immunosuppression†	152	104 (68%)	10 (77%)	48 (75%)	46 (61%)
Wasting (WHZ lower than −2 or MUAC <12·5 cm)‡	114	70 (61%)	7 (88%)	31 (65%)	32 (55%)
Underweight (WAZ lower than −2)	153	98 (64%)	9 (69%)	46 (71%)	43 (57%)
Tuberculosis features					
NIH criteria signs and symptoms of tuberculosis§	153	115 (75%)	10 (77%)	55 (85%)	50 (67%)
TST ≥5 mm	140	7 (5%)	1 (8%)	5 (9%)	1 (1%)
Tuberculosis contact	153	26 (17%)	7 (54%)	15 (23%)	4 (5%)
Chest x-ray suggestive of tuberculosis	147	86 (59%)	11 (85%)	57 (88%)	18 (26%)
Positive respiratory culture or positive Xpert¶	153	12 (8%)	12 (92%)	0	0
Positive stool on Xpert//	135	8 (6%)	8 (67%)	0	0
Positive urine lipoarabinomannan**	122	13 (11%)	4 (44%)	3 (6%)	6 (10%)
Tuberculosis treatment initiated	153	58 (38%)	11 (85%)††	46 (71%)	1 (1%)
Positive tuberculosis treatment response	58	47 (81%)	7 (64%)	40 (87%)	0
Deaths‡‡	153	28 (18%)	6 (46%)	12 (18)	10 (13%)

Data are median (IQR) or n (%). MUAC=mid-upper-arm circumference. NIH=National Institutes of Health. TST=tuberculin skin test. WAZ=weight-for-age Z score. WHZ=weight-for-height Z score. Xpert=Xpert MTB/RIF.

*On the basis of international consensus clinical case definitions for paediatric tuberculosis (appendix p 23) via post-hoc classification.

† WHO age-specified CD4 percentage cutoffs for severe immunosuppression (<12 months, <25%; 12–35 months, <20%; ≥36 months, <15%) or, in absence of CD4 percentage data, in terms of CD4 count (age <12 months, <1500 cells/μL; 12–35 months, <750 cells/ μL; ≥36 months, <350 cells/μL).

‡ Among children aged 5 years and younger, WHZ lower than −2 or MUAC lower than 12·5 cm.

§ Persistent cough (>14 days), fever (>7 days), failure to thrive, or lethargy (>7 days). Failure to thrive means wasted (WHZ lower than −2 or MUAC <12·5 cm) or underweight (WAZ lower than −2) at enrolment (growth trajectories unavailable before enrolment).

¶ Sputum or gastric aspirate.

‖ Includes one child with unconfirmed tuberculosis with indeterminate stool Xpert results.

** Includes one child with unconfirmed tuberculosis and two children with unlikely tuberculosis who had invalid urine LAM results. Colour change corresponding to manufacturer reference card grade 1 or higher was considered positive at the time of the study.

†† Two children with confirmed tuberculosis died before initiating tuberculosis treatment.

‡‡ Within 6 months of enrolment.

Table 2:

CRISPR-TB *Mtb*-cfDNA signal in CLHIV at high risk for tuberculosis who were hospitalised (PUSH cohort)

	Microbiologically confirmed tuberculosis (n=13)*	Unconfirmed tuberculosis* (n=65)		Unlikely tuberculosis* (n=75)	
		Clinical tuberculosis diagnosis (n=46)†	No clinical tuberculosis diagnosis (n=19)†	Any NIH tuberculosis criterion (n=61)‡	No NIH tuberculosis criteria (n=14)‡
***Mtb*-cfDNA detection of tuberculosis diagnostic performance (n=153)** §					
Number of children positive for *Mtb*-cfDNA	13	39	13	34	3
Sensitivity, % (95% CI)	100 (75–100)	85 (71–94)	68 (43–87)	..	..
Specificity, % (95% CI)	..	..	..	44 (32–58)	79 (49–95)
Deaths of people with untreated tuberculosis	2/2 (100%)	..	8/19 (42%)	10/60 (17%)	0/14 (0%)
Deaths of people with treated tuberculosis	4/11 (36%)	4/46 (8%)	..	0/1 (0%)	..
Mtb-cfDNA signal ($\times 10^6$ au), median (IQR)¶	5·8 (3·2–9·0)	4·3 (2·0–7·3)	3·0 (1·1–6·5)	2·0 (1·1–5·4)	1·1 (0·99–1·2)
p value‖	0·0003	0·0017	0·049	0–059	Ref
***Mtb*-cfDNA signal ($\times 10^6$ au) at earliest available visit and after tuberculosis treatment initiation (n=51), median (IQR)** **					
Before tuberculosis treatment (n=51)††	7·0 (5·8–9·6)	4·3 (2·5–7·9)	..	9·2	..
After tuberculosis treatment initiation (n=51)‡‡	2·8 (1·4–5·1)	1·5 (1·1–3·0)	..	3·8	..
p value‡‡	0·0077	<0·0001	..	NA	..

Mtb-cfDNA=*Mycobacterium tuberculosis* cell-free DNA. au=arbitrary units. MUAC=mid-upper-arm circumference. NIH=National Institutes of Health. TST=tuberculin skin test. WAZ=weight-for-age Z score. WHZ=weight-for-height Z score.

*Assessed post hoc on the basis of international consensus clinical case definitions for paediatric tuberculosis (appendix p 23).

†People without a confirmed tuberculosis diagnosis who initiated tuberculosis treatment were considered to have a clinical tuberculosis diagnosis. People without a confirmed tuberculosis who did not initiate tuberculosis treatment were considered to have no clinical tuberculosis diagnosis.

‡Positive chest x-ray, tuberculosis exposure within the past 2 years, TST ≥5 mm, or NIH tuberculosis symptoms (persistent cough >14 days, fever >7 days, failure to thrive, or lethargy >7 days). Failure to thrive indicated wasting (WHZ lower than −2 or MUAC <12·5 cm) or underweight (WAZ lower than −2) at enrolment.

[§] Includes 142 people with samples analysed at baseline and 11 people with samples analysed at a later visit (confirmed tuberculosis includes 12 people with samples analysed at baseline and one with a sample analysed at week 2; unconfirmed tuberculosis includes 59 people with samples analysed at baseline and six people with samples analysed at week 2; and unlikely tuberculosis includes 71 people with samples analysed at baseline and four people with samples analysed at week 2).

[¶] The *Mtb*-cfDNA signal as detected by CRISPR-TB, with a cutoff threshold of $1{\cdot}4 \times 10^6$ au.

[//] As compared with unlikely tuberculosis with no NIH criteria as established by Wilcoxon rank-sum test.

[**] Includes participants who initiated tuberculosis treatment with at least two sample times available (appendix p 16).

[††] Median 5·6 months (IQR 3·2–5·8) between tuberculosis treatment initiation and after tuberculosis treatment initiation.

[##] As compared with before tuberculosis treatment and after tuberculosis treatment initiation as established by Wilcoxon signed rank test.