Newer and novel sputum versus non-sputum-based tools for the diagnosis of active tuberculosis in different patient sub-populations

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PHD DECLARATIONS

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I confirm that I have been granted permission by the University of Cape Town's Doctoral Degrees Board to include the following publication (s) in my thesis, and where co-authorships are involved, my co-authors have agreed that I may include the following publications:

Publication 1: **Esmail, A.,** Pooran, A., Sabur, N. F., Fadul, M., Brar, M. S., Oelofse, S., Tomasicchio, M., & Dheda, K. (2020). An Optimal Diagnostic Strategy for Tuberculosis in Hospitalized HIV-Infected Patients Using GeneXpert MTB/RIF and Alere Determine TB LAM Ag. J Clin Microbiol, 58(10). https://doi.org/10.1128/JCM.01032-20

Publication 2: **Esmail, A.,** Tomasicchio, M., Meldau, R., Makambwa, E., & Dheda, K. (2020). Comparison of Xpert MTB/RIF (G4) and Xpert Ultra, including trace readouts, for the diagnosis of pulmonary tuberculosis in a TB and HIV endemic setting. Int J Infect Dis, 95, 246-252. https://doi.org/10.1016/j.ijid.2020.03.025

Publication 3: **Esmail, A.,** Randall, P., Oelofse, S., Tomasicchio, M., Pooran, A., Meldau, R., Makambwa, E., Mottay, L., Jaumdally, S., Calligaro, G., Meier, S., de Kock, M., Gumbo, T., Warren, R. M., & Dheda, K. (2023). Comparison of two diagnostic intervention packages for community-based active case finding for tuberculosis: an open-label randomized controlled trial. Nature Medicine, 29(4), 1009-1016. https://doi.org/10.1038/s41591-023-02247-1

Publication 4: Randall P*, **Esmail A*,** Wilson L, Makambwa E, Pooran A, Tomasicchio M, Dheda K*, Ntsekhe M*. Comparison of GeneXpert MTB/RIF Ultra versus unstimulated interferon gamma (IRISA-TB) for the diagnosis of tuberculous pericarditis in TB endemic setting- accepted for publication on Open Forum Infectious Diseases (ID: OFID-D-23-0123; DOI: 10.1093/ofid/ofae021)- see proof of acceptance in the appendix and final 'proof' copy in the appendix.

(*contributed equally as first authors).

Publication 5: **Esmail A*,** Christophera D J*, Scott A J, Wilson L, Randall P, Thangakunama B, Shankara D, Rajasekara S, Swanepoel J, Kühn L, Perumal T, Pooran A, Oelofse S, Dheda K. Diagnostic performance of unstimulated IFN-γ (IRISA-TBTM) for pleural tuberculosis: a prospective study in South Africa and India- submitted for peer review -Open Forum Infectious Diseases

(*contributed equally as first authors). (Manuscript is currently undergoing peer review- see proof of submission to Open Forum Infectious Diseases in the appendix)

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SUMMARY OF PUBLICATIONS INCLUDED IN THIS THESIS

Publication	**Journal Impact Factor (IF)**	**Knowledge gaps addressed by the publication**	**Impact of the publication**
Manuscript 1 corresponding to chapter 1 in this thesis: An optimal diagnostic strategy for tuberculosis in hospitalized HIV-Infected patients using Xpert and LAM.	IF: 9.40 **Journal of Clinical Microbiology**	Diagnosis of TB in HIV-infected and hospitalized patients is challenging. Both Xpert Ultra and Urinary LAM have been approved for use in this sub-population. However, the manner of integrating these tests optimally is unclear. (An optimal diagnostic strategy for TB in hospitalized HIV-infected patients: Xpert vs. LAM study)	***1) Main findings:*** the incremental yield of LAM over Xpert was 29.6% (45/152) and that of Xpert over LAM was 75% (84/11). The costs per TB case diagnosed were similar for the sequential and concurrent testing strategies ($1,617 to $1,626). ***2) Clinical Impact:*** Performing Xpert plus urine LAM *concurrently* in hospitalized patients with suspected TB is the optimal diagnostic strategy, and is only associated with a marginal increase in cost given that the urinary LAM test is under R50. These data have informed South African LAM guidelines.

Manuscript 2 corresponding to chapter 2 in this thesis: Comparison of Xpert MTB/RIF (G4) and Xpert Ultra, including trace readouts, for the diagnosis of pulmonary tuberculosis in a TB and HIV endemic setting.	IF: 8.4 **International Journal of Infectious Diseases**	1) Confirmation about the performance of Xpert Ultra in significant sub-populations (PLHIV and in patients with Previous TB) since only one industry sponsored trial was available at the time. 2) To delineate the epidemiology of trace-readouts which was poorly understood at the time especially regarding how these readouts correspond to active TB.	***1) Main findings:*** The sensitivity of Xpert Ultra was confirmed to be significantly higher than that of Xpert MTB/RIF G4 in limit-of-detection (LOD) experiments (9 vs. 184 cfu/mL). However, trace results contributed marginally (<5%) to the decreased specificity in patients with a smear negative patients with previous history of TB. Overall, trace results 6/9 (66.7%) of trace results were true-positive while 3/9 (33.3%) were likely false positive (culture negative) ***2) Clinical impact:*** Trace readout occur in both TB culture positive and negative patients. Critically ill patients with suspected TB may be initiated on TB treatment based on trace readouts (whilst waiting for a repeat Xpert and TB culture results). However, in selected *stable* patients, waiting for the repeat sputum testing results may be appropriate.

Manuscript 3 corresponding to chapter 3 in this thesis: Comparison of two diagnostic intervention packages for community-based active case finding for tuberculosis: an open-label randomized controlled trial.	IF: 87.24 **Nature Medicine**	The diagnostic performance of the more sensitive Xpert Ultra test in detecting community-based patients (patients who are minimally symptomatic) with active TB has not been established and its impact on interrupting transmission is unknown.	***Main findings:*** Despite the increased sensitivity of Xpert Ultra, it misses ~50% of patients with culture positive TB when used as part of an ACF strategy. However, it detects almost all likely infectious cases. ***Clinical impact:*** Xpert is sub-optimal for community-based active case finding. More sensitive strategies are urgently required. This trial has informed the design of two larger ACF trials (XACT-3 and XACT-19) currently ongoing which are aiming to rapidly screening~40,000 individuals to optimize ACF strategies incorporating CXR/CAD (chest x-rays paired with computer assisted diagnosis) in our communities
Manuscript 4 corresponding to chapter 4 in this thesis: Comparison of GeneXpert	IF: 4.4 Open Forum Infectious Diseases	1) Evaluation of the diagnostic performance of the more sensitive Xpert Ultra (confirmed in publication	***Main finding:*** In patients with definite and probable (response to empiric TB treatment) TB pericardial effusion combined, sensitivity was significantly higher

MTB/RIF Ultra versus unstimulated interferon gamma (IRISA-TB) for the diagnosis of tuberculous pericarditis in a TB endemic setting. (Manuscript DOI: 10.1093/ofid/ofae021; in press Open Forum Infectious Diseases		2 above) in patients with pericardial TB 2) Evaluation of the diagnostic performance of a novel immunodiagnostic test (IRISA-TB) that measures unstimulated interferon gamma level in pericardial effusion compared to conventionally available tests (Xpert Ultra, ADA and TB culture)	with IRISA-TB compared to Xpert Ultra [77.3 versus 37.9; P<0.0001]. Specificity was high for both assays (>95%). ***Clinical impact:*** The performance of Xpert Ultra is sub-optimal in this group of patients with a severe form of EPTB associated with very high mortality. This study supports further development and validation of the IRISA-TB test. This is a major contribution to the field of EPTB diagnosis. Data from this validation study were used to register this novel test with SAHPRA (South African Health Products Regulatory Authority). Data were also used for IRISA-TB to obtain ISO certified and the CE mark. I also leveraged this data to obtain substantive funding for a large multi-country trial evaluating the performance and impact (on treatment decisions) of IRISA-TB in all major forms of TB serositis (pericardial, peritoneal, pleural TB) and TB meningitis

			(Trial currently recruiting NCT06135818; I am the principal investigator for this trial; European Union Funding)
Manuscript 5 corresponding to chapter 5 in this thesis: Diagnostic performance of unstimulated IFN-γ (IRISA-TBTM) for pleural tuberculosis: a prospective study in South Africa and India.	4.4 Open Forum Infectious Diseases	1) Evaluation of the diagnostic performance a novel diagnostic performance of a novel immunodiagnostic test (IRISA-TB) that measures unstimulated interferon gamma level in pleural effusion compared to conventionally available tests (Xpert Ultra, ADA and TB culture)	***Main findings:*** IRISA-TB™ demonstrated markedly better sensitivity and NPV (negative predictive value) than Xpert-Ultra, and excellent specificity for the diagnosis of TPE in TB-endemic settings. ***Clinical impact:*** IRISA-TB™ is a promising novel same-day diagnostic test for the diagnosis of pleural TB. As mentioned above, data was used for registering the test with regulatory authorities and successfully obtain funding for a large-scale multi-country trial of which I am the main principal investigator (NCT06135818)

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A huge thank you to my fantastic family who, despite being so far away, provided me with all the love and support I could ever need. To my mother Gulbanu, I thank you for your prayers, for your unconditional love and for sending me tasty treats to keep my spirits up. To my father Sadiq, I thank you for your innumerous sacrifices in educating me, sage advice and for inculcating strong moral values in me. To my sister, for your tremendous love, care and light-hearted humor that always lift my spirits even on the gloomiest of days. To my brother Maza, for being my closest of friends and confidant through bountiful and lean times of life, with whom I have shared the heartiest of my laughter. To my wife Fatema, I thank you for your immense love, friendship, companionship, and unwavering support that you have always displayed towards me. Achieving this degree was only possible because of your many sacrifices. To my children Zahra, Mahdi and Nabeel, looking at your faces and hearing your laughter at the end of my long day would rejuvenate my tired body. This acknowledgement would be incomplete without thanking God for granting me the perseverance to undertake this journey and for ensuring that the journey was shared with highly capable, helpful, and kind individuals.

Finally, I would also like to thank the examiners for taking the time out of their busy schedules to review this thesis.

ABSTRACT

Background

Pulmonary tuberculosis (PTB) has a spectrum of presentation ranging from sub-clinical/minimally symptomatic disease on one end, usually in community-based patients, to overt symptomatic TB with hospitalisation on the severe end of the spectrum. This spectrum of sub-clinical and clinical presentation of TB along with the site of TB (pulmonary vs. Extra-pulmonary TB) generate a number of sub-populations. Current diagnostic tools are not a 'one size fits all' and have important differential limitations in these patient sub-populations including those with pauci-bacillary disease (e.g., in HIV-infected patients, minimally symptomatic patients with low burden disease in the community, and in patients with extra-pulmonary TB). Thus, the overarching objective of this PhD was to evaluate the diagnostic performance of frontline tests, including the newer and more sensitive Xpert Ultra and urine LAM in key active TB patient sub-populations (wherein data are limited). The main themes and sub-populations included in my thesis are as follows:

1) Hospitalised HIV-Infected patients (patients in the severe end of PTB spectrum): To determine the optimal and most cost-efficient diagnostic strategy incorporating sputum and LAM (lipoarabinomannan) for the detection of TB **(chapter 1).**

2) Smear-negative TB: Independent confirmation on the performance of the more-sensitive Xpert Ultra (Cepheid MTB/RIF Ultra) in sputum archived samples **(chapter 2).**

3) Community-based minimally symptomatic patients (patient in the non-severe end of the PTB spectrum): Evaluation of point-of-care (POC) sputum Xpert Ultra for detection of TB in minimally symptomatic/at-risk population for TB and its impact in detecting potentially infectious patients **(chapter 3).**

4) Extra-pulmonary TB (TB serositis): Evaluation on the performance of conventional diagnostic tools, including Xpert Ultra in tuberculous pericarditis **(chapter 4)** and pleuritis **(chapter 5)** compared to a novel immunodiagnostic tool (IRISA-TB).

Methods (sub-populations are underlined)

In **Chapter 1**, I describe an evaluation of an algorithm that describes the optimal & cost-efficient strategy to combine sputum GeneXpert and urine LAM (tests that are recommended by the WHO) in a *hospitalized HIV-infected sub-population*: This comprised a *post-hoc* analysis of 561 HIV-infected sputum-expectorating patients that was part of a larger parent trial. 5 different diagnostic strategies using sputum culture as a reference standard were explored (Xpert alone, LAM alone, sequential Xpert followed by LAM and vice versa [LAM in Xpert-negative patients and Xpert in LAM-negative patients], and both tests concurrently [LAM + Xpert]). A cost-consequence analysis was also performed.

In **Chapter 2**, I evaluated the performance of GeneXpert Ultra in 272 selected and well characterized archived sputum samples including 104 patients with smear negative TB and 102 non-TB with a history of previous TB to accentuate and evaluate the clinical significance of trace readouts (i.e., a readout that usually corresponds to the detection of a very small amount of TB DNA). Assay-specific limit-of-detection (LOD) experiments were conducted using serial dilutions of *Mycobacterium tuberculosis H37Rv* to confirm the assay's detection threshold. In

In **Chapter 3,** I evaluated the diagnostic performance of point-of-care (POC) Xpert Ultra for the detection of TB in minimally *symptomatic community-based sub-population*: 5,274 participants were rapidly screened to enrol 584 patients with suspected pulmonary TB from peri-urban high burden communities of Cape Town, South Africa. The utility of POC-Xpert Ultra in detecting likely infectious patients was also specifically evaluated.

In **Chapter 4**, I described the diagnostic performance of pericardial fluid unstimulated interferon gamma (measured using the novel IRISA-TB™ test) and compared it to other same-day tests including Xpert Ultra and adenosine deaminase (ADA) in 99 South African patients with suspected pericardial TB using a composite reference standard including pericardial fluid, pericardial tissue TB culture, pericardial tissue histopathology and response to TB treatment.

Similarly, In **Chapter 5**, I described the diagnostic performance of pleural fluid unstimulated interferon gamma (measured using the novel IRISA-TB™ test) and compared to other same-day tests including Xpert Ultra and adenosine deaminase (ADA) in 207 individuals from Cape Town, South Africa and Vellore, India. A composite reference standard including pleural fluid, pleural tissue TB culture, pericardial tissue histopathology and response to TB treatment was used to define the composite reference standard for TB.

***Results**:*

Chapter 1: In the HIV-infected hospitalised patient sub-population, the incremental yield of LAM over Xpert was 29.6% (45/152) and that of Xpert over LAM was 75% (84/11). The incremental yield of LAM increased with decreasing CD4 count. The costs per TB case diagnosed were similar for the sequential and concurrent strategies ($1,617 to $1,626).

Chapter 2: Xpert Ultra had a lower sputum-specific LOD compared to that of the G4 version of Xpert MTB/RIF (9 vs. 184 cfu/mL). <5% of culture-negative patients, but with a history of previous TB, had a likely false-positive trace readout.

Chapter 3: The incorporation of POC-Xpert Ultra within a scalable mobile community-based active case finding strategy was feasible but only detected 52% of individuals with culture-

positive TB . However, Xpert Ultra detected almost all of the probably infectious patients compared with smear microscopy (94.1% versus 23.5%, P = <0.001).

Chapter 4: In patients with microbiologically confirmed TB pericarditis, IRISA-TB™ was more sensitive than Xpert Ultra [88.6% (74.1;95.5) versus 71.5% (55.0;83.7); n=53], and significantly more sensitive in HIV-uninfected participants (100% [72.3;100.0] versus 60% [31.3;83.2]; P=0.03). However, in patients with definite and probable TB pericarditis (clinical response with empiric TB treatment) combined (n=84), the over-all sensitivity was significantly higher with IRISA-TB™ [77.3 (65.9;85.8) versus 37.9 (27.2;50.0); P<0.0001].

Chapter 5: For pleural TB, the sensitivity of IRISA-TB™ was significantly better than Xpert-Ultra (81.8% [70.4-90.2] vs 32.9% [22.1-45.1], p<0.001).

The specificity of IRISA-TB was high >94% in both forms of EPTB in chapters 4 & 5.

***Conclusions**:*

Overall, the performance of currently available diagnostic tools varied in different subpopulations due to differential mycobacterial load, the compartment interrogated, patient phenotype (e.g. HIV-infected), testing strategy, and clinical context (e.g. hospitalized versus community-based). Main findings from each chapter as summarized as follows:

Main conclusion in chapter 1: Xpert-Ultra had a considerably lower limit-of-detection compared to older Xpert-MTB/RIF assay, however, trace-readouts in the context of previous TB, reduced the specificity of the assay slight (<5%). A third of the trace results (3/9) were likely false positive.

Main conclusion in chapter 2: In sputum-expectorating hospitalized patients with advanced HIV and access to both Xpert and LAM, concurrent testing with sputum Xpert and urine LAM may be the best and most cost-effective strategy for diagnosing TB in this sub-population.

Main conclusion in chapter 3: POC-Xpert Ultra, when used as part of a community-based ACF, missed ~50% of patients with culture-positive TB. However, Xpert detected almost all likely infectious cases.

Main conclusions in chapters 4 & 5: Conventional TB diagnostic tests performed poorly in pericardial and pleural TB. IRISA-TB™, a novel same-day immunodiagnostic test, outperformed Xpert Ultra for the diagnosis of pericardial and pleural TB. These data have implications for the clinical utility of newer diagnostic tools in patient sub-populations from TB and HIV endemic settings.

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An optimal diagnostic strategy for tuberculosis in hospitalized HIV-Infected patients using Xpert and LAM. Journal of Clinical Microbiology. J Clin Microbiol 2020; **58**(10); IF 9.4

Publication 2: Chapter 2

Comparison of Xpert MTB/RIF (G4) and Xpert Ultra, including trace readouts, for the diagnosis of pulmonary tuberculosis in a TB and HIV endemic setting. **Int J Infect Dis.** 95: 246-252; IF 12

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Publication 4: Chapter 4

Comparison of GeneXpert MTB/RIF Ultra versus unstimulated interferon gamma (IRISA-TB) for the diagnosis of tuberculous pericarditis in a TB endemic setting.

(Manuscript DOI: 10.1093/ofid/ofae021; currently in-press accepted for publication in Open Forum Infectious Diseases- see proof of acceptance in the appendix)

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Publication 1: Chapter 1

An optimal diagnostic strategy for tuberculosis in hospitalized HIV-Infected patients using Xpert and LAM. Journal of Clinical Microbiology. 2020 Jul 29.

Publication 2: Chapter 2

Comparison of Xpert MTB/RIF (G4) and Xpert Ultra, including trace readouts, for the diagnosis of pulmonary tuberculosis in a TB and HIV endemic setting. International Journal of Infectious Diseases. 2020 Apr 2.

Publication 3: Chapter 3

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Figure 1 | Consort schematic summarizing the recruitment strategy and overall findings.

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Publication 4: Chapter 4

Comparison of GeneXpert MTB/RIF Ultra versus unstimulated interferon gamma (IRISA-TB) for the diagnosis of tuberculous pericarditis in a TB endemic setting. (Manuscript DOI: 10.1093/ofid/ofae021; currently in-press accepted for publication in Open Forum Infectious Diseases- see proof of acceptance in the appendix)

Figure 1 | Study overview of patient groups, investigations performed, and tests undertaken.

Figure 2 | Performance of IRISA-TB in differentiating Definite TB and/or Probable TB from non-TB, using pericardial fluid from patients with suspected pericardial TB.

Publication 5: Chapter 5

Diagnostic performance of unstimulated IFN-γ (IRISA-TBTM) for pleural tuberculosis: a prospective study in South Africa and India. (Submitted for publication in Open Forum Infectious Diseases)

Figure 1 | Study overview of patient groups. Participant classification: Definite TPE, (i) at least one positive *M. tuberculosis* Xpert Ultra and/or culture on pleural fluid and/or biopsy specimen; and/or (ii) granulomatous inflammation with caseation and/or necrosis on histological examination of pleural biopsy tissue; and/or (iii) acid fast bacilli on histological examination of pleural biopsy tissue. Non-TPE, patients with no microbiological or histological evidence of *M. tuberculosis* and/or an available alternative diagnosis (not started on TB treatment). Probable TPE, not meeting the criteria for definite or non-TPE but initiated on TB treatment.

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Figure 2 | Area under the receiver operator characteristic curves (AUROC) for IRISA-TB™ and ADA.

ABBREVIATIONS

ACF	Active case finding
ART	Antiretroviral therapy
AUROC	Area under the ROC curve
BDQ	Bedaquiline
CAD	Compute assisted diagnosis
CI	Confidence interval
COVID-19	Coronavirus-19
CRP	C-reactive protein
CXR	Chest X-ray
eCRF	Electronic case report form
DNA	Deoxyribonucleic acid
DR-TB	Drug-resistant TB
EPTB	Extrapulmonary TB
EDCTP	European and Developing Countries Clinical Trials Partnership
FN	False negative
FP	False positive
FujiLAM	Fujifilm SILVAMP
Hb	Haemoglobin
HIV	Human Immunodeficiency Virus
IQR	Interquartile range
LAM	Lipoarabinomannan
MGIT	Mycobacteria growth indicator tube

NNT	Number needed to test
NPV	Negative predictive value
PLHIV	People living with HIV
POC	Point-of-care
PPV	Positive predictive value
PTB	Pulmonary TB
PTM	Pretomanid
RIF	Rifampicin
ROC	Receiver operator characteristic
SAHPRA	South Africa Health Products Regulatory Authority
TB	Tuberculosis
TBP	Tuberculous pericardial effusion
TN	True negative
TP	True positive
WHO	World Health Organization

TABLE SUMARISING THE DEFINITIONS OF THE VARIOUS TB SUB-POPULATIONS THAT ARE BEING REFERRED TO IN THIS THESIS

	Host response	**Symptoms and/or signs**	**Sputum *and/or* radiological *and/or* other pathological evidence for *M tb***	**Infectious**	**Smear status**	**Diagnostic strategy likely to detect patients (passive vs. active case finding)**
M tb infection	**+**	**-**	**-**	**-**	**-**	**ACF**
Sub-clinical tuberculosis (also referred to as asymptomatic TB)	**+**	**-**	**+**	**+/-**	**+/-**	**ACF**
Minimally symptomatic patients	**+**	**+**	**+**	**+/-**	**-(+)**	**ACF**
Ambulatory clinical tuberculosis disease (also referred to as active tuberculosis)	**+**	**++**	**-/+**	**+/-**	**+/-**	**PCF**
Hospitalised patients with clinical tuberculosis disease	**+**	**+++**	**-/+**	**+/-**	**+/-**	**PCF**

Legend: +: present; ++/+++: prominent symptoms and/or signs present; (+) can be positive rarely; +/-: may or may not be positive; ?+: unknown but could be present; ACF: active case finding; PCF: passive case finding.

PART 1: INTRODUCTION AND LITERATURE REVIEW

1.1: Key research questions addressed in this thesis and their context, background, coherence, and significance.

Background: In 2022 an estimated 10.6 million people became newly ill with active TB[16]. Alarmingly, for the second consecutive year the incidence of TB has continued to increase. This increase is mainly attributed to the decrease in the TB case detection rates during the COVID-19 pandemic. The current understanding about pulmonary TB is that it is a spectrum of diseasing ranging from asymptomatic disease on one end of the spectrum to overt smear positive TB on the other end of the spectrum. This spectrum of sub-clinical, minimally symptomatic disease and overt clinical TB results in numerous sub-populations with differential performance of the programmatically available TB diagnostic tests. it is estimated that, of the ~10 million patients with TB, one third (~3 million) patients were undetected or unreported contributing substantially to the ~1.3 million deaths attributable to TB[16]. Although, these patients would fall into the 'less-severe' minimally symptomatic disease spectrum, it is noteworthy that ~2 in every 5 TB patients in this sub-population remain undiagnosed or undetected[16]. The significance of these undetected TB cases is that they continue to propagate the TB epidemic through ongoing community-based transmission[17,18]. However, our primary diagnostic test for TB in South Africa, i.e., GeneXpert MTB RIF (Xpert) manufactured by Cepheid, is centralised in laboratories attached to large hospitals and diagnostic centers. Thus, Xpert testing is not a point-of-care test for TB in the high burden communities of South Africa[19]. Furthermore, the diagnostic performance of Xpert in low burden and paucibacillary

disease, as is the case in minimally symptomatic or asymptomatic community-based patients, is poor with sensitivity of only ~50-70%[20,21]. Therefore, without addressing these logistical and technical hurdles, we cannot hope to meet the goals outlined in the End TB strategy 2017-2025.

On the other end of the severity spectrum of TB disease are hospitalised HIV-infected patients who also have paucibacillary disease. This sub-population of very sick TB patients have rapid progression of disease and an extremely high mortality rate[11]. In this sub-population, the problem of paucibacillary disease that is typically associated with HIV-infected individuals, is further compounded by the patient's inability to provide a good quality sputum for diagnostic testing on account of critical illness. Thus, highlighting the necessity for using approaches that incorporate both sputum and non-sputum based diagnostic tests (e.g., urinary LAM test) in clinical testing algorithms. Although the WHO has recommended both Xpert (Cepheid) and Determine-TB LAM Ag test (Abbott) for use in this sub-population, the guidelines are unclear on the exact manner in which LAM and Xpert should be integrated in clinical algorithms for hospitalised HIV-infected patients and whether these tests should be performed sequentially or concurrently to achieve the highest yield in diagnosis in the most cost-efficient manner.

Patients with extra-pulmonary TB form yet another important sub-population of patients with paucibacillary disease. However, the diagnostic challenges in this group of patients are amplified by the difficulty in obtaining a diagnostic sample from the affected extra pulmonary compartment. The commonest types of EPTB are pleural TB and TB adenitis. However, TB meningitis and TB pericarditis, although less common, represent the more

severe sub-types of EPTB. The sensitivity of *sputum* culture in patients with EPTB seems to vary by the site of EPTB but ranges from 5-25%[22]. The yield of TB culture from pericardial and pleural fluid compartments (TB serositis) is also sub-optimal approaching a sensitivity of only ~50%[23,24] compared to a composite reference standard whilst the yield of TB culture in cerebrospinal fluid (CSF) is only ~26% even in cases where the volume of CSF was deemed to be adequate (i.e., ~6 mL)[25]. Alternative approaches to making a diagnosis of TB serositis that integrate fluid biochemical tests (e.g., adenosine deaminase [ADA]), clinical scoring tools (e.g., Tygerberg diagnostic index), and DNA-based diagnostic tests (e.g., Xpert) also perform poorly with Xpert sensitivity ranging from 30-40% in pleural and pericardial TB[26,27]. These data clearly highlight that EPTB is yet another sub-population of TB with paucibacillary disease state where even our 'gold-standard' microbiological test i.e., TB culture, performs poorly. Thus, there is a critical unmet need for developing more sensitive and potentially microbiologically independent diagnostic strategy for the diagnosis of extra-pulmonary TB sub-population.

Give the background above, the overarching aim of my PhD thesis is to describe the deficiencies in the performance of currently available first-line diagnostic tests in special sub-populations and to offer alternative approaches, including novel tests, for the diagnosis of TB in these sub-populations. Thus, 4 themes and relevant sub-populations arise and are outlined below:

Theme 1: Investigating the optimal diagnostic strategy/algorithm for TB by integrating sputum Xpert and urinary LAM testing in HIV-infected hospitalised patients.

Research Question 1: Is GeneXpert sufficient to diagnose TB in hospitalized sputum expectorating HIV-infected patients? Does urinary LAM testing add any value in sputum expectorating HIV -infected hospitalised patients? How should these two validated tests (i.e., Xpert and LAM) be integrated into clinical practice algorithms in the most cost-effective manner- which test should be performed first?

Relevant sub-population under research: Hospitalised HIV-infected patients

Significance: Diagnosis of TB in HIV-infected and hospitalized patients in challenging. Both Xpert Ultra and Urinary LAM have been approved for use in this sub-population. However, the manner of integrating these tests optimally is unclear.

Relevant publication: An optimal diagnostic strategy for TB in hospitalized HIV-infected patients Using GeneXpert MTB/RIF and Alere Determine TB LAM Ag (PMID: 32727831; J Clin Microbiol; 2020).

Theme 2: Independent confirmation of the higher sensitivity of Xpert Ultra in the smear negative TB sub-population and interrogating the epidemiology of trace readouts.

Research Question 2: is the GeneXpert MTB/RIF Ultra (Xpert Ultra) more sensitive compared to the GeneXpert MTB/RIF (G4) in a HIV/TB endemic setting and what is the significance of Xpert Ultra trace read-outs?

Relevant sub-population under research: Smear negative (paucibacillary) disease

Significance: Xpert Ultra assay has been designed to improve the sensitivity of the GeneXpert MTB/RIF. However, at the time, there were limited data about the performance of Xpert Ultra in significant sub-populations e.g., in persons living with HIV

(PLHIV) and patients with a previous history of TB. Furthermore, the epidemiology of trace-readouts is poorly understood with regards to whether these readouts correspond to active TB. Could the more sensitive Xpert Ultra be used for detection of TB in other paucibacillary sub-populations (e.g., in community-based patients with minimally symptomatic TB- evaluated in theme 3; in patients with extra-pulmonary TB- evaluated in theme 4 and theme 5).

Relevant publication: Comparison of Xpert MTB/RIF (G4) and Xpert Ultra, including trace readouts, for the diagnosis of pulmonary tuberculosis in a TB and HIV endemic setting. (PMID: 32247825; Int J Infect Dis; 2020).

Theme 3: Decentralising the more sensitive Xpert Ultra for use as a point-of-care test to detect TB in an 'at-risk' community-based patient sub-population including those who are likely infectious.

Research Question 3: Research Question 3: What is the diagnostic performance of the more sensitive Xpert Ultra performed at point-of-care (Xpert Edge) in detecting community-based participants (who are minimally symptomatic) with active TB and will it detect all the likely infectious cases to halt transmission of TB from the index patient?

Relevant sub-population under research: At-risk community-based patients with minimal symptoms and/or HIV-infection

Significance: The performance of Xpert Ultra in community-based participants has not been established and its impact on interrupting transmission is unknown. (XACT-2 study)

Publication: Comparison of two diagnostic intervention packages for community-based active case finding for tuberculosis: an open-label randomized controlled trial (PMID: 36894651; Nature Medicine; 2023).

Theme 4: Comparison of the more sensitive Xpert Ultra, TB culture and ADA with a novel immunodiagnostic test (IRISA-TB) for the diagnosis of TB pericarditis and TB pleuritis (extra-pulmonary sub-population)

Relevant sub-population under research: Patients with extrapulmonary tuberculosis (i) Patients with suspected TB pericardial effusion (Chapter 4); (ii) Patients with suspected pleural effusion (Chapter 5)

Research Question 4: What is the comparative performance of currently available same-day diagnostic tests (Xpert Ultra and ADA) in pericardial and pleural TB? How does the diagnostic performance of IRISA-TB (a novel test measuring unstimulated IFN-gamma) compare to conventional tests for the diagnosis of TB pericardial and pleural effusion

Significance: ~25% of the TB burden is extra-pulmonary. TB pleural effusion is one of the most common forms of EPTB. TB Pericardial effusion, although less prevalent compared to pleural effusion, is a more severe form of EPTB with high associated morbidity and mortality. Currently available diagnostic tests, including Xpert Ultra, perform poorly in TB pleuritis. However, there are no data from well conducted studies on the diagnostic performance of Xpert Ultra in TB pericarditis. Both pleural and pericardial fluid are pauci-bacillary compartments where even TB culture, which theoretically only needs 1 cfu/mL, only has a sensitivity of ~50%. This highlights the need for investigating the diagnostic

performance of a novel immunodiagnostic assay (IRISA-TB) in this sub-population of patients with EPTB.

Publication within this theme (chapter 4): Comparison of GeneXpert MTB/RIF Ultra versus unstimulated interferon gamma (IRISA-TB) for the diagnosis of tuberculous pericarditis in a TB endemic setting.

(Accepted for publication in Open Forum Infectious Diseases; DOI: 10.1093/ofid/ofae021, February 2024 or see proof copy).

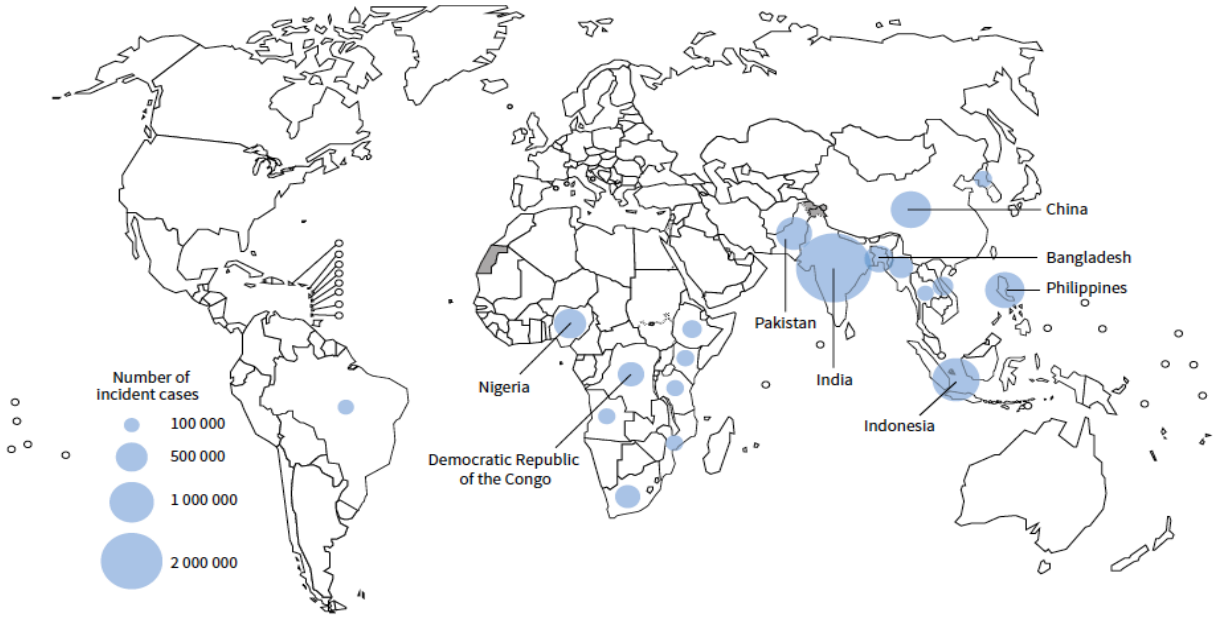
Publication within this theme (chapter 5): Diagnostic performance of unstimulated IFN-γ (IRISA-TBTM) for pleural tuberculosis: a prospective study in South Africa and India.

(submitted for peer review and publication to Open Forum Infectious Disease; February 2024; see proof of submission).

1.2: Detailed introduction and review of literature

1.2.1 Global epidemiology of TB

In 2022 an estimated 10.6 million people became newly ill with active TB [16]. Almost two thirds of this burden was concentrated in the 8 high burden countries situated in Africa and Asia (figure 1). Additionally, for the second consecutive year the incidence of TB has continued to increase. This is mainly attributed to the shortfall in the case detection rate during the COVID-19 pandemic (figure 2). Furthermore, it is estimated that of the ~10 million patients with TB, one third (~3 million) patients were undetected or unreported contributing substantially to the ~1.3 million deaths attributable to TB in 2022. Thus, it is noteworthy that ~2 in every 5 TB cases remain undiagnosed or undetected. The significance of these undetected TB cases is that they continue to propagate the TB epidemic through ongoing community-based transmission[18]. This highlights the importance of mass screening and prompt treatment (of those who screen positive) of high-risk groups to curtail transmission. In fact, more recently, community-based surveys have shockingly revealed that most of the TB burden in our high burden communities is, in fact, asymptomatic i.e., sub-clinical (figure 3) [18,28,29]. Thus, traditional tools such as symptom screening in their current form will be inadequate to detect a large proportion of undiagnosed TB in our communities[18]. Affordable, accurate and rapid (point-of-care) diagnostic tests are therefore required to identify patients with TB and to allow for the prompt initiation of TB treatment to prevent morbidity and limit transmission.



[a] The eight countries ranked in order from first to last in terms of numbers of cases, and that accounted for about two thirds of global cases in 2022, are India, Indonesia, China, the Philippines, Pakistan, Nigeria, Bangladesh and the Democratic Republic of the Congo.

Figure 1. Estimated number of incident TB cases in 2022 (Global TB report 2023).

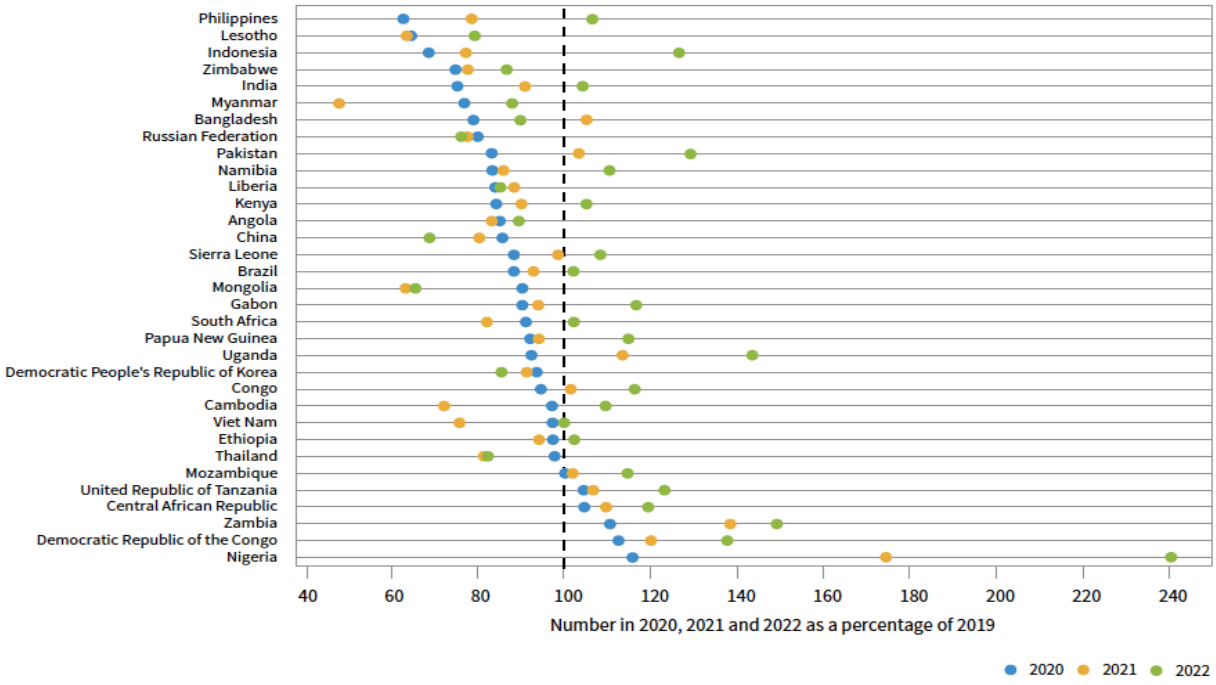
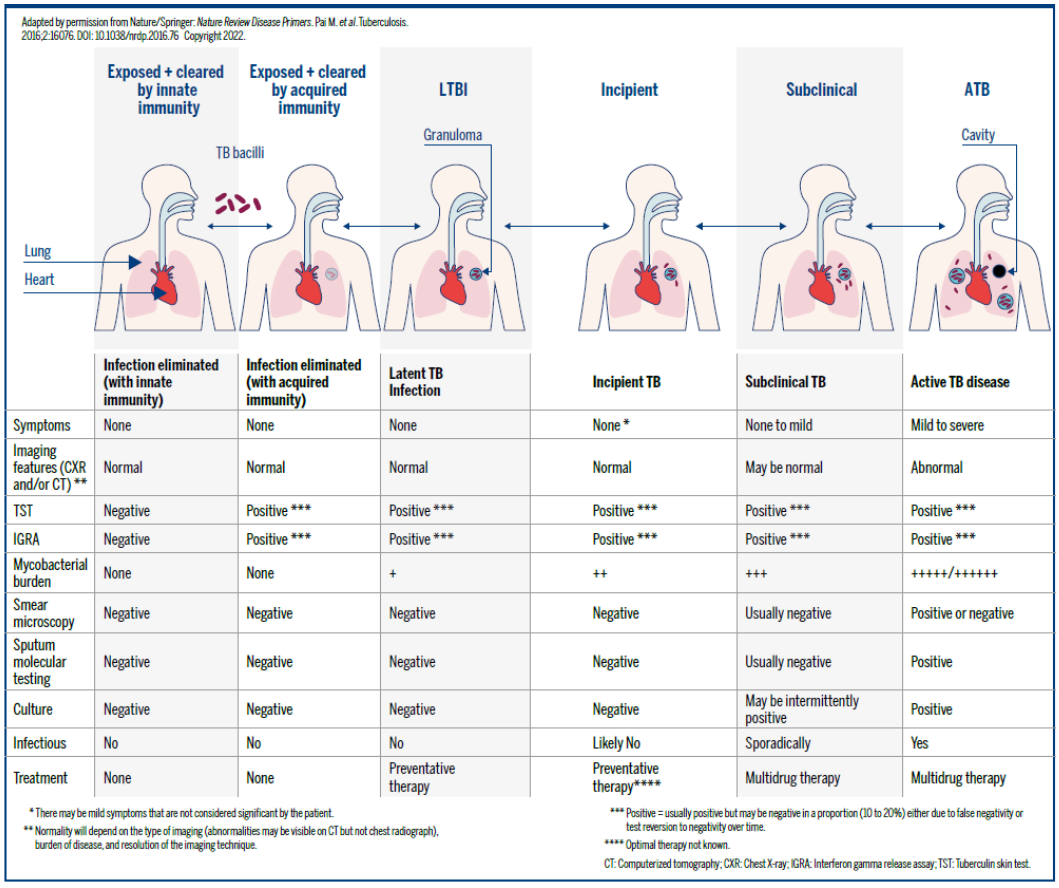


Figure 2. Case notification rates of newly diagnosed TB in 2020, 2021 and 2022 compared to 2019 represented as a dashed vertical line. (Global TB report 2023).

The paucibacillary nature of TB in various sub-populations (e.g., in minimally symptomatic patients, smear negative TB, patients with HIV co-infection and patients with extra-pulmonary TB) and the lack of availability of a representative sputum sample (e.g., from critically ill patients with HIV) compromise the diagnostic performance of our current frontline sputum-based TB tests (e.g., GeneXpert) which rely on a minimum threshold of mycobacterial load and a good quality sputum. This highlights the importance of developing non-sputum based complementary diagnostic tests.

1.2.2 Pathogenesis and transmission of tuberculosis

It is estimated that approximately 2 billion people, (a quarter of the world's population), are infected with *M tuberculosis*[30]. Humans are the natural reservoir for *M. tuberculosis* with very limited survival outside the human host[31]. The main modality for transmission of TB is through aerosol transmission from one human host to another [32]. Our understanding of the immunopathogenesis of TB continues to evolve. More recently, TB is thought to comprise of a spectrum of clinical and subclinical presentations (figure 3) with exposure to *M tuberculosis* on one end of the spectrum and development of active TB disease on the other [33]. Interestingly, the categories depicted in figure 3 are not static and individuals can switch between the categories depending on the mycobacterial bacterial and host dynamics.



	Infection eliminated (with innate immunity)	Infection eliminated (with acquired immunity)	Latent TB Infection	Incipient TB	Subclinical TB	Active TB disease
Symptoms	None	None	None	None *	None to mild	Mild to severe
Imaging features (CXR and/or CT) **	Normal	Normal	Normal	Normal	May be normal	Abnormal
TST	Negative	Positive ***	Positive ***	Positive ***	Positive ***	Positive ***
IGRA	Negative	Positive ***	Positive ***	Positive ***	Positive ***	Positive ***
Mycobacterial burden	None	None	+	++	+++	+++++/++++++
Smear microscopy	Negative	Negative	Negative	Negative	Usually negative	Positive or negative
Sputum molecular testing	Negative	Negative	Negative	Negative	Usually negative	Positive
Culture	Negative	Negative	Negative	Negative	May be intermittently positive	Positive
Infectious	No	No	No	Likely No	Sporadically	Yes
Treatment	None	None	Preventative therapy	Preventative therapy****	Multidrug therapy	Multidrug therapy

* There may be mild symptoms that are not considered significant by the patient.
** Normality will depend on the type of imaging (abnormalities may be visible on CT but not chest radiograph), burden of disease, and resolution of the imaging technique.
*** Positive = usually positive but may be negative in a proportion (10 to 20%) either due to false negativity or test reversion to negativity over time.
**** Optimal therapy not known.
CT: Computerized tomography; CXR: Chest X-ray; IGRA: Interferon gamma release assay; TST: Tuberculin skin test.

Figure 3 Mycobacterium tuberculosis (*Mtb*) infection to tuberculosis disease spectrum.

In fact, immunity against *M tuberculosis* may fluctuate in an individual patient over time depending on complex interplay between the human host, exposure to mycobacterium tuberculosis, presence of comorbidity (e.g., HIV) and environmental factors. It is therefore possible for some individuals to become infected even with minimal exposure whilst others may not be infected despite sustained exposure to tuberculosis[34].

Recent large country-specific community surveys have revealed that a very large burden of undiagnosed TB (~50-60%) in high prevalence countries is in fact sub-clinical (TB culture positive with minimal or no symptoms and abnormalities on chest x-ray) [28,29].

These are individuals who have answered no to all WHO symptom screen for TB but may still volunteer symptoms if probed in more details. It is estimated that ~25-50% of these patients have smear positive disease, it is likely that patients with sub-clinical TB plays an important role in TB transmission [28]. In fact, data from hose-hold contact and modelling studies suggest that, on aggregate, subclinical TB is more infectious than clinical TB and is likely responsible for 68% of the *M tb* transmission globally [35]. This is mainly attributed to the sheer burden of sub-clinical TB and the prolonged time lag (~6-7 months) before the development of symptoms which often then prompts the initiation of effective treatment.

The transmission of TB is determined by the complex interactions between the patient with sub-clinical or active TB, the mycobacteria, and the susceptible exposed host[36]. The main factors involved in facilitating the transmission of TB include the presence of comorbidity in the host and index patient (e.g., HIV, diabetes mellitus, presence of immunosuppressive conditions or treatment), ambulatory status of the index patient and burden of disease of the index patients (e.g., smear-positive TB). Host factors such as genetic susceptibly, duration and intimacy of exposure and the infective dose may also play an important role in effecting transmission. Environmental factors like ventilation can play a critical role in transmission given that TB is spread mainly by infective aerosols. Lastly, mycobacterial strains may play some role in influencing transmission [37], however, this may be less important based on recent insights [38].

1.2.3 Diagnostic strategies and tests for TB

Pulmonary TB is the commonest form of tuberculosis and is usually diagnosed by demonstrating microbiological proof of *M. tuberculosis* from a sputum sample [37]. Sputum smear microscopy, TB culture and nucleic acid amplification tests (NAATs) are approved methods for achieving a diagnosis. Given the drawbacks associated with TB culture (~4-8-week time delay and infrastructure costs) and challenges with smear microscopy (low sensitivity; see below), the frontline test for the diagnosis of active TB in South Africa currently is a format of NAAT called GeneXpert MTB/RIF or Xpert (Cepheid, Sunnyvale, CA). However, the WHO has recently approved several other platforms for use in TB endemic countries which will make NAATs more accessible across the African continent.

Triage/screening tests for tuberculosis.

In general, there are two diagnostic strategies for testing TB with considerable overlap. The first strategy is to develop diagnostic tests for triage or screening purposes. These tests are usually designed to have a very high sensitivity but can have moderate specificity. The specificity of the triage test will determine its cost effectiveness as it determines individuals from a high-risk population who do not require confirmatory testing. [4]. Importantly, community-based triage testing is more challenging than facility-based (health centre based) testing as pre-test probabilities of TB are lower in the community (reducing positive predictive value) due to patients having earlier-stage and potentially paucibacillary disease. The main goal of a triage test is to identify potential TB disease in a pool of at-risk people (such individuals may be symptomatic or

asymptomatic) and to select patients out for further TB investigations usually with a confirmatory test. Therefore, a positive triage result increases the probability of TB, and these selected individuals will then go on to have the more expensive confirmatory tests [39]. This strategy is important for two main reasons (i) triage tests are usually 'cheaper' and designed for mass screening at point-of-care and (ii) confirmatory testing capacity is unevenly distributed and is usually centralised [40]. Thus, a good triage/screening test has the capacity of greatly increase efficiency and reducing costs.

Symptom screening (e.g., the WHO 4 symptom screening tool) is the commonest form of triaging patients and is undertaken as part of the national TB program's mandate. This form of triage is subjective and misses asymptomatic or minimally symptomatic cases which comprise the bulk of our TB in high burden communities [41]. Chest x-ray screening is another triage strategy that is being used by the national TB programs of many TB endemic countries. Traditional chest x-rays require significant infrastructure and human readers, which detracts from its use as a triage test. However, with recent advances in the development of ultra-portable low-dose imaging systems, paired with computer-aided detection (CAD) software (e.g., CAD4TB, qXR and Lunit), have enabled us to rapidly and cost effectively mass screen high-risk patients in our communities. The artificial intelligence (AI) that is used to interpret the chest x-rays is constantly evolving and, in some circumstances, seem to perform at par or better than human readers [42-44]. Lastly, for the triage test to be maximally effective, it is essential for it to be implemented at the community-level (as opposed to the clinic level) to ensure that TB is detected as early as possible to curtail transmission [45,46].

Rule-in or confirmatory diagnostic testing

Rule-in tests like the Xpert Ultra (Cepheid), Truenat MTB (Truenat) or Mycobacteria Growth Indicator Tube 960 TB culture are usually centralised and not suitable for mass community screening. However, thanks to recent technological advancement in molecular diagnostics (e.g., Xpert Edge), this is set to change (see chapter 3). In general, a rule-in or confirmatory testing strategy is used after a triage test has identified an 'at-risk' individual who potentially may have TB. Therefore, a confirmatory test is used to confirm the presence of TB disease and justify the initiation of TB treatment in the at-risk individual. However, in practice, this results in long delays to treatment initiation primary because the confirmatory tests are located far away from POC where the patient is seen. Thus, for the confirmatory tests to assert their impact on the TB epidemiology, they need to be decentralised to ensure rapid confirmation of TB and prompt treatment initiation [19]. A key drawback of the currently available confirmatory tests is that their performance in non-sputum samples remains inadequate [8,24,47]. Thus, given these challenges of the sub-optimal performance of TB diagnostic tests, clinicians often initiate empiric TB treatment based on clinical features, logistical challenges (potential for lost to follow up), and practical considerations [48].

WHO's Target Product Profiles

WHO's target product profiles (TPPs) are now over a decade old. These are global benchmarks and guidelines that researchers use while developing triage or confirmatory diagnostic tests. It is very interesting that there are currently no diagnostic tests on the market that meet the 'optimal' threshold of the TPP for a triage or a confirmatory test

across all sub-populations of TB [49,50]. However, there are several experimental products on the market currently that at least meet the 'minimum' thresholds TPP. Given the heterogenous nature of TB disease (paucibacillary disease, EPTB etc.), reliance on the quality of sample, human and mycobacterial genetic diversity and co-morbidity it is difficult for a test to be subjected to universal TPPs across the full spectrum of TB disease. It would be more feasible and achievable if the TPPs were designed so that the thresholds would be different depending on the specific sub-population for whom the test is being designed. Lastly the WHO TPPs need to accommodate recent tools such, e.g., chest x-ray paired with CAD, to ensure that TPPs are defined for current and future non-specimen-based tests.

Technology and tests that meet the minimal requirements for the WHO's TPP

Technology class	Products included in the evaluation
	Xpert® MTB/RIF and Xpert® MTB/RIF Ultra (Cepheid)[a]
	Truenat™ (Molbio)[a]
Moderate complexity automated NAATs for detection of TB and resistance to rifampicin and isoniazid	Abbott RealTime MTB and Abbott RealTime MTB RIF/INH (Abbott) BD MAX™ MDR-TB (Becton Dickinson) cobas® MTB and cobas MTB-RIF/INH (Roche) FluoroType® MTBDR and FluoroType® MTB (Hain Lifescience/Bruker)
	TB-LAMP (Eiken)[a]
Antigen detection in a lateral flow format (biomarker-based detection)	Alere Determine™ TB LAM Ag (Alere)
Low complexity automated NAATs for the detection of resistance to isoniazid and second-line anti-TB agents	Xpert® MTB/XDR (Cepheid)
LPAs	GenoType® MTBDRplus v1 and v2, and GenoType® MTBDRsl (Hain Lifescience/Bruker) Genoscholar™ NTM+MDRTB II and Genoscholar™ PZA-TB II (Nipro)
Targeted NGS	Deeplex® Myc-TB test (Genoscreen) AmPORE TB test (Oxford Nanopore Diagnostics) TBseq® test (Hangzhou ShengTing Medical Technology Co)

LPA: line probe assay; NAAT: nucleic acid amplification test; NGS: next-generation sequencing; TB: tuberculosis.

[a] These recommendations are currently product specific but will be changed to class-based to align with the other recommendations.

Point-of-care (POC) tests for the detection of tuberculosis

A POC test that is highly sensitive (and at least moderately specific, i.e., specificity of at least 70%) test that is the holy grail of TB diagnostic research. An ideal POC test can be defined as a test that yields a diagnostic result in the same-day and from a single clinical encounter, enabling a healthcare worker to make a prompt clinical decision to potentially initiate TB treatment. We currently do not have an 'ideal' POC test (i.e., test meeting the optimal WHO TPP threshold). This is discussed in chapter 3 of my thesis where I leveraged the recently launched POC-Xpert (Xpert Edge) platform in ACF strategies for community-based screening using a mobile lab.

Table 1. Summary of recent conventional and novel TB diagnostic tests

UPDATE ON TB DIAGNOSTICS				
TECHNOLOGY	**PRINCIPAL COMMERCIAL TEST**	**SAMPLE TYPE**	**ADVANTAGES**	**DISADVANTAGES**
SPUTUM AND NON-SPUTUM BASED ASSAYS				
Traditional tests -Smear microscopy -TB culture (liquid) paired with phenotypic DST	Smear:Non-commercial	Smear: Sputum	Smear: Inexpensive, simple, rapid.	Smear: Cannot differentiate NTM and *M. tb*, low sensitivity in paucibacillary states. No resistance information
	Culture: Bactec MGIT 960 system	Culture: Sputum/ Non-sputum (blood, serosal fluid, CSF, tissue)	Culture: Gold standard Resistance information	Culture: Long time to results (6-12 weeks)
Automated nucleic acid amplification tests	GeneXpert MTB/RIF Ultra and GeneXpert MTB/XDR (Cepheid) Trunat MTB (used in India) BD MAX MDR-TB (Becton Dickson)	Sputum/ Non-sputum (blood, serosal fluid, CSF, tissue)	Rapid (results in < 2 hours) Resistance information	Lower sensitivity in paucibacillary TB (e.g., EPTB); trace readouts lead to false positives.
Semi-automated nucleic acid amplification tests	Line probe assay MTBDR-Plus (Hain); INNO LiPA-RIF TB; MTBDRsl (Hain)	Sputum (rarely other sample types)	Rapid (results in < 2 hours) Resistance information	Expensive, logistically more challenging, low sensitivity for some drugs (EMB).
Non-automated nucleic acid amplification tests	BD Thermofisher	Sputum (rarely other sample types)	Rapid (results in < 2 hours) Resistance information	Logistically more challenging in high burden settings
Sequencing-based testing	Whole genome sequencing	Performed on sputum culture isolate	Simultaneous readout on multiple drugs Resistance information possible*	Expensive, cannot sequence directly from sputum (delays diagnosis), real-world impact uncertain.
	Targeted next generation sequencing (e.g., Genoscreen)	Can be performed on direct sputum (SM+) or on culture isolate	Simultaneous readout on multiple drugs Resistance information possible* Can be performed directly on SM+ samples	Successful readouts obtained from ~20% of SM- cases
NON-SPUTUM-BASED ASSAYS				
Immunodiagnostic tests	Commercial: IRISA-TB (measured unstimulated IFN-gamma) Non-commercial: ADA (adenosine deaminase)	Extrapulmonary fluid samples (CSF, Pleural, pericardial, peritoneal)	Rapid, high sensitivity for EPTB in endemic settings, IRISA-TB has a high specificity.	Difficulty in acquiring EPTB samples, low specificity with ADA No resistance information
Antigen testing	Urine LAM (Abbott Determine-TB LAM); Fuji SILVAMP TB LAM	Urine	Rapid, point-of-care, inexpensive, 2nd and 3rd generation tests have improved sensitivity (but not commercially available)	Low specificity (cross reacts with NTM), no info on resistance, poor sensitivity in HIV-uninfected patients. No resistance information
Host transcriptomic analysis (targeted molecular screening)	Screening: Cepheid Xpert *M. tb* Host Response (MTB-HR) prototype cartridge system	Blood	Quantifies mRNA expression of differential TB-associated genes in blood. Rapid, potential screening test.	Not validated, real-world impact uncertain. No resistance information
	Rule-in test: Biomereux blood transcriptomic signature ISIT-TB			
Chest x-ray paired with CAD	CAD software: qXR (Qure-AI), CAD4TB, Lunit. Ultraportable chest x-ray units: Delft, Fuji	Digital chest X-ray	Rapid, potentially suitable for screening in endemic countries, cost-efficient when paired with ultra-portable x-ray machines for community-based screening.	Poor specificity, non-microbiological readout. No resistance information
Oral/tongue swabs (paired with POC-NAAT)	Commercial: any POC-NAAT can be used e.g., Xpert Xpress paired with various types of oral swabs	Oral swabs (Tongue, buccal mucosa, gingival etc.). Tongue swabs seems to be better since they collect a larger biomass	Rapid, (when paired with POC-NAAT), ease of sample acquisition, reasonably high specificity ~90%	Sensitivity is sub-optimal various between 36-90% in adults (5-41% in peadiatric population).
Cough analysers for TB (paired with artificial intelligence/machine learning)	Commercial software: Hyfe, Health Acoustic Representations (HeAR), Swaasa AI, Tbscreen, Timbre (technology not yet externally validated)	Cough characteristics including sounds, frequency severity and duration measured over time.	High sensitivity may be achieved when combined with clinical characteristic (internal validation), suitable for screening	Needs external validation in real world situations, requirement for smart phone may be limiting in resource limited setting
Exhaled breath analysis (detection of TB specific antigens or Mtb-independent molecules (including VOCs), peptides or metabolites	Commercial: R-tubes for collection of exhaled breath sample, Aeonose device for direct chemometric analysis of VOCs. Non-commercial: gas chromatography-mass spectrometry and various chemometric techniques	Exhaled breath condensate (TB peptides, lipids) or volatile organic compounds (chemical signature)	ease of sample collection, can be paired with NAAT	Needs validation, variable sensitivity ranging from 60%-85% in symptomatic patients, currently costly, need special equipment which makes it unsuitable for POC use.

Table 1 references: Smear microscopy [1,2], Nucleic acid amplification tests [3-5], Sequencing-based testing [6,7], Immunodiagnostic tests [8-10], Antigen testing [11], Chest x-ray-CAD [12], transcriptomic analysis [13], Oral/tongue swabs [14], Cough analyzers [15] and Exhaled breath technologies [15]

Traditionally, the gold standard for comparing new tuberculosis (TB) diagnostic tests, particularly nucleic acid amplification tests (NAATs), has been microbiological confirmation through TB culture methods. Although this strategy allows for the accurate determination of specificity, the sensitivity of TB culture is sub-optimal in various sub-populations of TB (as discussed in this thesis). Thus, this so called "gold standard" in several TB sub-populations e.g., extra-pulmonary-TB, paucibacillary disease, hospitalised patients with TB and HIV-infected patients, remains inadequate. This necessitates the use of a composite reference standard to accurately evaluate the performance of newer tests. This composite reference standard may include one or more of the following parameters depending on the TB sub-population concerned: sputum and/or aspirated fluid and/or tissue TB culture, sputum and/or aspirated fluid and/or tissue NAAT/PCR, pathological assessment of fluid and/or tissue (cytology and/or histology), sputum, fluid or tissue smear microscopy, clinical symptoms suggestive of TB, response to TB treatment (symptomatic, microbiological and/or radiological) and ancillary evidence of TB e.g., the use of protein and differential cell count in TB meningitis. In general, based on these results patients are classified as definite TB (usually microbiological or pathological confirmation of TB), probable TB (when a clinician initiates TB treatment without microbiological proof of TB with a documented response to TB treatment), non-TB (when the microbiological/pathological tests are negative and TB treatment is not empirically initiated) and unclassifiable (e.g., when test information is not available to accurately classify TB). However, caution must be exercised as it has been well established that NAAT can be falsely positive, especially in patients with previous TB and possibly in patients with low burden disease at the detection threshold of the assay (e.g., those with trace readouts on Xpert ultra).

Nucleic acid amplification tests

There are various types of nucleic acid amplification tests (NAAT) which include the line probe assays, isothermal amplification platforms and cartridge-based automated systems. The limit of detection of NAAT platforms ranges from ~20 to 150 colony forming units per ml [3-5]. This test can be performed on an expectorated sputum sample and other biological samples (table 1). A popular NAAT in Africa is the GeneXpert MTB/RIF (Xpert). Being a cartridge-based system, it is easier to implement in programmatic settings compared to TB culture, it has the potential to rapidly yield a result within ~120 minutes of test initiation in the laboratory and it can also provide a readout on the presence of rifampicin resistance. However, given its sub-optimal performance in smear-negative TB (~67%) [5], Cepheid has recently introduced the Xpert MTB/RIF Ultra (Xpert Ultra) which has an improved sensitivity of 78.9% in smear negative TB [51] and the test also seemed to have better performance in PLHIV with a sensitivity of 87.5% compared with 68.6% attributed to the older version (G4) of GeneXpert MTB/RIF. The improved sensitivity is achieved by the addition of 2-multicopy PCR amplification targets (IS1810 and IS6110) to the standard single-copy *rpo*B gene target and using a larger PCR reaction chamber [51-53]. However, the improved sensitivity comes at the cost of reduced specificity [54], mainly when the Xpert Ultra yields a 'Trace' result. 'Trace' is a semiquantitative readout unique to the Xpert Ultra assay which indicates the detection of a very small amount of mycobacterial DNA that is insufficient for the assay to detect the *rpo*B gene (which is why rifampicin resistance status is always indeterminate during a 'Trace' readout) but sufficient to detect one or both of the multi-copy IS1810 and/or IS6110 genes.

There are hardly any industry independent data about the Utility of Xpert Ultra in paucibacillary TB disease states e.g., in PLHIV and in those with a history of previous where the predominant use of this test is likely to occur. It has been suggested that patients with a trace readout may represent true or false-positive results (detection of nonviable bacilli or analytical error) and the optimal management of such patients is unclear.

Sputum smear-microscopy

For decades sputum smear microscopy was the primary test for the detection of TB. However only 50% of patients are smear positive in the context of passive case finding.[1] It may still be useful for identifying patients who are likely infectious [2,55]. Thus, it may have utility in as a biomarker for infectiousness. However, patients with smear negative TB may also contribute to substantial community transmission [56]. Smear microscopy offers several advantages for diagnosing tuberculosis (TB), particularly in resource-limited settings. It is significantly cheaper than advanced methods like molecular tests and cultures, making it accessible where resources are scarce [57,58]. The technique provides rapid results within hours, enabling prompt diagnosis and treatment initiation compared to cultures that can take weeks [59]. Its simplicity allows trained personnel to perform it without complex equipment. Additionally, it can complement other tests to confirm results and monitor treatment response [60].

Mycobacterial culture using sputum or other biological samples.

This is often regarded as gold standard for TB diagnosis. Automated liquid medium culture systems are now routinely used in endemic countries to ease workflow in the laboratory. However, these systems have a number of drawbacks including the prolonged lead time to obtain a result, high associated costs, requirement of advanced laboratory infrastructure and trained personnel to implement. Importantly, once growth is detected by the automated systems, speciation tests must be performed to confirm the presence of M.tb. Commonly used confirmatory tests for culture isolate are mycobacterial-specific NAATs (e.g., Xpert or Hain line-probe assays) and immunochromatographic assays (e.g., TBc ID, Becton Dickinson, Sparks, MD) which detects MPT64 antigen, a protein secreted by *M.tb* during culture[61].

Urine tests for tuberculosis

Urine antigen tests detect lipoarabinomannan (mycobacterial cell wall protein) using polyclonal antibodies on a lateral flow assay. This enables a point-of-care diagnosis of TB in ~30 minutes [62]. Presently, urinary LAM testing is only recommended in HIV-infected persons, especially in those with a CD4 count <200 cell/mm^3. The sensitivity of Alere LAM test is ~30% in HIV-infected hospitalised patients or PLHIV with a CD4 couth <200 cell/mm^3 [11]. The sensitivity seems to increase with decreasing CD4 count such that the sensitivity approaches ~50% in patients with CD4 count of ≤50 cell/mm^3 [62-65]. Urinary LAM when used as a screening test for TB in HIV-infected hospitalised patients has proven mortality benefit [11]. Furthermore, urinary LAM antigen testing may also be used as a screening strategy for TB in severely immunocompromised ambulatory patients

where it identifies patients with TB who are at a higher risk for mortality and allows for prompt initiation of TB treatment [66]. However, the performance of urinary LAM antigen testing for screening TB in ambulatory and HIV-infected patients is variable and is related to the level of immunosuppression with the highest yield in patients with CD4 count ≤ 200 (~25% vs. 12% in patients with CD4 >200) and the back ground prevalence of TB [67]. More sensitive tests are being developed and may be available in future, however, there are technical and inter-reader variability challenges that still need to be addressed. [68]

1.2.4 Summary of the challenges in the rapid diagnosis TB in special patient populations.

The WHO currently endorses NAAT like Xpert MTB/RIF and Xpert Ultra (Cepheid, Sunnyvale, CA) as initial diagnostic tests for the diagnosis of pulmonary TB [16]. These tests have very good sensitivities and specificities of ~85% and 99% for Xpert MTB/RIF and 90% and 96% for Xpert Ultra in smear-positive TB, respectively [16]. However, their sensitivity seems to decrease to <70% in smear-negative TB. This population represents over 50% of all patients with tuberculosis (mainly community-based minimally symptomatic or asymptomatic patients/ sub-clinical TB) [21,69]. Furthermore, Xpert currently is available at reference laboratories and centralised making it unsuitable for use as a point-of-care test for TB in the high burden communities where the TB burden is concentrated [19]. Therefore, without addressing these logistical and technical hurdles, we will not be able to achieve TB control (Chapter 3 addresses this challenge).

As alluded to earlier in this introduction that TB is a spectrum of disease and perhaps the most vulnerable sub-population of TB are patients who are critically ill who require

hospitalisation, especially if they also have HIV co-infection. This sub-population of very sick TB patients have rapid progression of disease and are at an extremely high risk for death [11]. In this sub-population, the problem of paucibacillary disease that is typically associated with HIV-infected individuals, is further compounded by the patient's inability to provide a good quality sputum for diagnostic testing on account of critical illness. Thus, highlighting the necessity for using approaches that incorporate both sputum and non-sputum based diagnostic tests (e.g., urinary LAM test) in clinical testing algorithms. Although the WHO has recommended both Xpert (Cepheid) and Determine-TB LAM Ag test (Abbott) for use in this sub-population, the guidelines are unclear on the exact manner in which LAM and Xpert should be integrated in clinical algorithms for hospitalised HIV-infected patients and whether these tests should be performed sequentially or concurrently to achieve the highest yield in diagnosis in the most cost-efficient manner (Chapter 1 addresses this challenge).

Patients with extra-pulmonary TB form yet another important sub-population of patients with paucibacillary disease. However, the diagnostic challenges in this group of patients are amplified by the difficulty in obtaining a diagnostic sample from the affected extra pulmonary compartment. The yield of TB culture from pericardial and pleural fluid compartments (TB serositis) is also sub-optimal approaching a sensitivity of only ~50%[23,24] whilst the yield of TB culture in cerebrospinal fluid (CSF) is only ~26% even in cases where the volume of CSF was deemed to be adequate (i.e., ~6 mL)[25]. Alternative approaches to making a diagnosis of TB serositis that integrate fluid biochemical tests (e.g., adenosine deaminase [ADA]), clinical scoring tools (e.g., Tygerberg diagnostic index), and DNA-based diagnostic tests (e.g., Xpert) also perform poorly with Xpert sensitivity ranging from 30-40% in pleural and pericardial TB[26,27]. These data clearly

highlight that EPTB is a paucibacillary disease state where even our most “sensitive” microbiological test (even more so than Xpert because Xpert requires ~20 cfu/mL whereas culture theoretically only requires 1 cfu/mL), performs poorly. There is a critical unmet need for developing more sensitive and potentially microbiologically independent diagnostic strategy for extra-pulmonary TB sub-populations (Chapters 4 & 5 address this challenge).

1.2.5 Importance of active case finding.

Transmission is most likely to occur in community-based settings compared to households [70,71]. However, the main modality for detecting TB in endemic countries is one of passive case finding (PCF). This strategy relies on patients self-reporting to health care facilities after the onset of significant symptoms and undergoing diagnostic testing.[37]. Given the large burden of the TB in our high prevalence communities is sub-clinical[28,29] and likely plays a major role in perpetuating transmission[35], PCF strategy is clearly inadequate to achieve TB control. Active case-finding (ACF) on the other hand involves taking sample collection processes and diagnostics tests out of laboratories and clinics and into the communities where TB is rife (TB hotspots). This strategy allows for the screening of both symptomatic and asymptomatic high-risk patient profile to detect both clinical and sub-clinical TB.

Figure 4 (left): Set up of a community-based active case finding strategy using a mobile van equipped with POC-Xpert

A: Example of a low-cost panel van that can navigate the roads around a peri-urban informal settlement.

B: Set up of the mobile van (lab) with GeneXpert Edge

C: Typical example of a densely populated peri-urban informal settlement

D: Active case finding activities in the community.

E: GeneXpert Edge mobile platform

F: GeneXpert MTB/RIF Ultra cartridge

Modern ACF strategies utilise mobile-phone Application to perform symptoms screening (e.g., WHO 4 symptom screen), targeted screening of high-risk group of patients (e.g., patients with HIV, diabetes or an index TB contact) attending health care facilities for unrelated reasons, door-to-door screening of symptomatic and high-risk patients and innovative strategies leveraging mobile-laboratories, some equipped with molecular diagnostics[18,72-76]. Unlike PCF strategies, ACF strategies have proven benefit on reducing the transmission and consequently reducing the burden of TB in communities. However, this benefit is not consistent across all settings, and seems to be dependent on the intensity of ACF strategies used and consistency with which the interventions were repeated over time [77-79]

Key strategies for community-based active case finding and their impact on prevalence of TB

The World Health Organization (WHO) characterizes community-based Active Case Finding (ACF) as a systematic approach to screening for tuberculosis (TB) among at-risk populations outside traditional healthcare settings[80]. The primary objective of ACF is to facilitate earlier diagnosis and treatment for individuals at risk, which can lead to a reduction in morbidity and mortality, lower the community prevalence of infectious TB, and ultimately decrease TB transmission rates. This, in turn, may result in a long-term decline in TB incidence.

ACF initiatives are often accompanied by other supporting TB-focused programs, including health education, improvements in laboratory services, the introduction of new diagnostic tools, and TB preventative therapy. Thus, it is challenging to isolate the specific effects of ACF[72]. Additionally, due to insufficient detection and the complex progression of TB, direct measurement of incidence is not feasible; therefore, proxy measures, such as case notification rates and prevalence surveys, are utilized.

The WHO recommends systematic ACF screening for populations where the prevalence of undiagnosed TB exceeds 0.5%[80]. This revised recommendation underscores the lingering uncertainty regarding the effectiveness of ACF as conducting robust evaluations remains a significant challenge. A systematic review of nine studies that evaluated the impact of ACF on TB prevalence (including only two cluster randomised

trials) utilised a variety of implementation strategies that complicated the meta-analysis and making its interpretation on the impact of ACF uncertain (the non-randomised trials had significant methodological limitations)[72]. Notably, the ZAMSTAR study (using symptoms and smear microscopy) found no significant effect on TB prevalence[81], whereas the ACT3 (Vietnam; including repeated rounds of TB screening with sputum Xpert testing for all able to produce a sample) showed a 44% relative reduction TB prevalence[82]. Both studies attempted to assess TB transmission by examining sentinel school children; however, neither demonstrated a significant impact on Mycobacterium tuberculosis (*Mtb*) immunoreactivity, apart from a post hoc analysis in the ACT3 study indicating a 50% reduction in Mtb immunoreactivity among children exposed to the ACF intervention. Similarly, the DETECTB trial screened ~100,000 participants randomising them to community-based screening using mobile van vs. door-to-door screening and using smear microscopy as a confirmatory test in both arms. This trial showed that repeated rounds of ACF (6 rounds of screening at 6 monthly intervals) almost halved the prevalence of TB from ~6% to 3% per 1000 adults. In contrast the TREATS study, offered 4 years of repeated rounds of door-to-door systematic symptom screening and chest X-ray with analysis by computer- aided diagnosis (CAD) software, followed by Xpert testing (HIV-positive) or smear microscopy (HIV-negative) for those symptomatic or with a high CAD score (≥ 50)[78,79]. This trial found no significant differences in TB prevalence or Mtb immunoreactivity. Meanwhile, the SCALE study conducted in Malawi used door-to-door symptom enquiry for TB, followed by sputum microscopy for those with cough ≥ 2 weeks [75]. The study was underpowered to detect the impact of ACF on TB prevalence. Thus, the primary outcome was redefined to be a comparison of case notification rates, rather

than prevalence. Despite the redefinition of the primary endpoint no increase in tuberculosis notifications were noted.

In summary, several large cluster randomized trials including the ACT3 trial and DETECTB trial[83] provide evidence that community-based ACF programs are effective in decreasing transmission provided the ACF strategy incorporates repeated rounds of high-intensity screening in communities with a high burden of undiagnosed TB (>0.5%)

NEW TB TREATMENT REGIMENS				
KEY TRIAL AND DESIGN	INTERVENTIONAL REGIMEN: DRUGS AND DURATION	PERFORMANCE OF INTERVENTIONAL REGIMEN COMPARED TO SOC		COMMENTS
		EFFICACY	SAFETY	
TRIALS ON RIF-SENSITIVE TB				
ACTG (HIV-) & TBTC (HIV+) RCT; Non-inferiority of 6.6 %; 13 countries Dorman, NEJM, 2021 & Pettit, CID, 2023	RPT, MFX, INH, PZA: 4 months	Non-inferior	Similar rate of Grade 3 & 4 AEs.	RPT & MFX regimens associated with higher cost
SHINE RCT; 1204 children ≤ 16 years. Non-inferiority trial Turkova, NEJM.	RHZE: 2 months RH: 2 months (4 months total)	Non inferior at 72 week follow up.	Similar rate of Grade 3 & 4 AEs.	Included smear negative TB (low burden)
TRUNCATE-TB: RCT; 647 adults Adaptive non-inferiority (12-point margin) Paton, NI; NEJM; 2023	BDQ, LZD, INH, PZA and EMB: 2 months	Non-inferior	Similar rate of Grade 3 & 4 AEs.	HIV excluded
INH mono-resistant TB WHO guidelines 2019	RIF, EMB, PZA, LVF	NA	NA	INH may be included for ease since FDC with RIF, EMB, PZA is not available
TRIALS ON RIF-RESISTANT TB				
NExT RCT- 111 Patients, open label, RCT Esmai & Dheda, AJRCCM, 2021	BDQ, LZD, LVF, EMB, PZA, (HD-INH or ETO)	Odds ratio of 2.2 favouring intervention arm,	Higher ates of treatment interruption in SOC but higher rates of Grade 3 AE in intervention arm (LZD)	First RCT on MDR-TB treatment shortening High rates of kanamycin induced HF- hearing loss (subclinical)
Nix-TB & ZeNix (BPAL): 109 patients, Open-label, single group. Conradie, F; NEJM; 2020 (Nix), 2023 (ZeNix)	BDQ, LZD, PTM (BPaL): 6 months	Treatment success rate: ~90%	Drug interruption/ dose adjusted due to peripheral neuropathy (80%), myelosuppression (50%)	-Trial design limits generalisability. -High LZD-related AE rate challenging for programmatic decentralised implementation. -Optimal LZD dose: 600mg daily for 26 weeks.
TB-PRACTECAL: RCT; 301 patients Non-inferiority (12-point margin) Ngang'wa, NEJM, 2022	BDQ, LZD, PTM, MXF (BPaL-M): 6 months	Unfavourable treatment rate: BPaL-M: 11% SOC: 48%.	BPaL-M: fewer Grade 3 & 4 AEs.	-Most common AEs: hepatic disorders, prolonged QT, LZD-related AEs. -BPaL-M had better AE profile than SOC. -WHO 2022 update endorses 6-month BPaL-M.
SA DOH Updated guidelines (BPaL-L & BPaL), August 2023	BDQ, LZD, PTM, ±LVF : 6-9 months BPaL: FQ resistant TB BPaL-L: FQ sensitive TB	Programmatic data collection on efficacy and safety underway		-BPaL can be used for 6 months in FQ resistance. -Regimen not recommended for severe EPTB -Regimen for HIV-positive or negative patients.

Table 2. Summary of recent advances in the treatment of drug-sensitive (DSTB) and drug-resistant TB (DR-TB)

1.2.6 Shortening drug-sensitive and drug-resistant TB treatment.

The current duration of DS-TB treatment anything but short. The standard treatment for pulmonary TB requires 4 drugs (rifampicin, isoniazid, pyrazinamide and ethambutol) for

an intensive phase of 2-months followed by 2-drugs (rifampicin and isoniazid) for the continuation phase consisting of 2-months. Although previous attempts at shortening TB treatment had failed, more recently a rifapentine and moxifloxacin based regime has shown that shortening this duration to 4-months is possible [84]. The TRUNCATE-TB trial [85] was even more ambitious. It leveraged newer drugs (WHO group A), that are traditionally reserved for DR-TB treatment, to shorten DS-TB treatment down to 2-months.

The NExT RCT[86] (lead by myself and Prof. Dheda; see table above) was the first RCT that demonstrated the possibility of shortening treatment duration for rifampicin resistant TB (RR-TB) using a bedaquiline and linezolid based all-oral 6-month regimen. However, our overall treatment success rates were still sub-optimal. Subsequently, the TB-PRACTECAL trial [87], confirmed that that a 6-month course of TB treatment using the BPAL-M (bedaquiline, linezolid, pretomanid and moxifloxacin) regimen is safe and highly effective at curing rifampicin resistant TB (RR-TB) in patients who have sensitivity to fluoroquinolones. This is now the first-line regimen for RR-TB in South Africa except that the moxifloxacin has been substituted by levofloxacin to limit QT-prolongation (see table above). For rifampicin and fluoroquinolone resistant TB, a three-drug regimen consisting of bedaquiline, linezolid and pretomanid is recommended for a duration of 6-months based on the Nix and ZeNix trials [88].

1.2.7 Prevention of tuberculosis

Effective prevention of TB requires a multifaceted approach that starts by addressing the current challenges in the diagnosis of TB, especially in the sub-populations of TB that are at the centre of this thesis (i.e., patients with paucibacillary disease, patients who are unable to provide a good quality sputum and community-based patients with sub-clinical or minimally symptomatic disease). Furthermore, improved diagnostic strategies must be accompanied by short and effective TB preventative and treatment regimens. BCG was our only semi-effective TB vaccine with proven benefit against severe forms of childhood TB, however, more recently there has been renewed hope for TB vaccines as several candidates are now entering phase 3 trials.

Modelling studies have shown that addressing sub-clinical TB is more effective than improving access to high quality diagnostics and treatment for the control of TB [89]. There are currently two main methods of addressing the burden of sub-clinical TB (i) via instituting effective community-based active case-finding strategies and (ii) via the use of TB preventive therapies. Models of population-level TB transmission studies suggest that TPT is generally more effective than ACF. However, in reality we would likely need a combination of the two strategies to optimise the impact of these intervention [90]

TB PREVENTATIVE THERAPY		
SEMINAL TRIAL OR GUIDANCE DOCUMENT	**REGIMEN, DOSE**	**COMMENTS**
Guidelines for the treatment of LTBI: Recommendations from NTCA and CDC; 2020; Sterling TR et al.	RIF 10mg/kg daily for 4 months (max 600mg) **[4R]**	Better completion rates and less toxicity (relative to INH monotherapy) Odds ratios for risk of TB was 0.25
Trial of three regimens to prevent TB in HIV-infected adults; NEJM; 1997; Whalen cc et al	INH 5mg/kg (max 300mg) and RIF 10mg/kg (max 600mg) (daily for 3 months) **[3HR]**	Better completion rates and less toxicity (relative to isoniazid monotherapy) Provides ~60% protection: Odds ratios for risk of TB was 0.33. Meta-analysis: safety and efficacy similar to 9-month daily INH regimen
Short-course therapy with rifampicin plus isoniazid (meta-analysis); CID; 2005; Ena J et al.		
Three months of rifapentine and isoniazid for latent tuberculosis infection; NEJM; 2011; Sterling TR et al.	INH 15mg/kg (max 900mg) and RPT weight-based (max 900mg) (weekly: 3 months) **[3HP]**	RPT: Rifamycin derivative, long half-life, greater in vitro potency against *M. tb* than RIF. 3HP (weekly via DOT): Non-inferior to 9 months of daily INH (self-administered) in a RCT of >7700 predominantly HIV-negative individuals at high risk for progression. Hepatotoxicity seems less, better completion rates than with 9-month INH monotherapy. Main AE: hypersensitivity or flu-like symptoms occur in ~3.5% (serious reaction rate for weekly dosing was 0.3% vs 0.5% with daily INH). INH peripheral neuropathy in predisposed individuals (DM, malnutrition, HIV co-infection etc.): prescribe pyridoxine Odds ratios for risk of TB for patients on 3HP was 0.36
Prevent study: Flu-like and Other Systemic Drug Reactions Among Persons Receiving Weekly Rifapentine Plus Isoniazid or Daily Isoniazid for Treatment of LTBI; CID; 2015; Sterling TR		
1 HP		
Clinical practice. Latent tuberculosis infection; NEJM; 2002; Jasmer RM at al.	**INH** 5mg/kg daily for 6 or 9 months **OR** **INH** 15mg/kg twice weekly for 6 or 9 months	-Fewer drug interactions (relative to rifamycin-based regimens) -Daily INH for 9 months is preferred given its efficacy but adherence is variable (25-90%) -Daily INH for 6 months (6H) an option in situations of adherence difficulty (OR of ~0.4) -Better adherence with daily INH vs twice weekly regimen (which must be given as DOT) -Patients with fibrotic lesions on chest radiograph should receive a 9-month regimen
Isoniazid for prevention in non-HIV infected persons; 2000; Cochrane Database Syst Rev; Smieja MJ.		
Systematic Review, Meta-analysis, and Cost-effectiveness of Treatment of Latent Tuberculosis to Reduce Progression to Multidrug-Resistant Tuberculosis; CID; 2017; Marks SM et al.	**MFX** (400mg daily) **or** **LFX** (750-1000mg daily) **alone or with either** EMB or ETA for 6-12 months	-Source case isolate must be fluoroquinolone susceptible -Avoid using pyrazinamide in the regimen due additive hepatoxic effect. -Longer duration is warranted in patients with immunosuppression, <5 years old, or in individuals with high risk of progression. -Clinical monitoring (sputum and chest x-ray) may be suitable in those with fluoroquinolone resistance whose treatment options are limited due to the resistance pattern of their contacts.
Tuberculosis Preventive Therapy for Individuals Exposed to Drug-resistant Tuberculosis; CID; 2020; Malik AA et al.		
Risk Factors for Adverse Events in Household Contacts Prescribed Preventive Treatment for Drug-resistant Tuberculosis Exposure; CID; 2021; Malik AA et al.		
Treatment for LTBI in contacts of MDR-TB patients; IJTLD;2014; Bamrah S et al.		

Table 3. Summary of the recent advances in TB preventive therapy.

PROMISING TB VACCINE CANDIDATES CURRENTLY IN CLINICAL TRIALS			
VACCINE NAME	**TYPE OF VACCINE**	**SPONSOR**	**COMMENTS/KEY PHASE I/II RESULTS**
PHASE II VACCINES			
ID93/GLA-SE (QTP101) (Sagawa, Z; Nature Comm; 2023)	Protein/adjuvant	Quratis Inc.	Safe and immunogenic in phase I trial Thermostable version of the vaccine was more immunogenic than the non-thermostable version.
H56:IC3 (SSI website- not peer-reviewed)		Statens Serum Institut	Well tolerated, demonstrated an immune response compared to placebo in participants who had previously had TB. Primary analysis showed no protection against TB recurrence (recurrence rate in vaccine group 5.8% vs. 3.4% in placebo)
RUTI (Nell, A; PLOS ONE, 2014)	Inactivated mycobacterial vaccine (*M. indicus pranii*)	Archivel Farma S.L.	Safety, tolerability and immunogenicity confirmed in phase II trial. Trial included HIV-infected individuals. Can be used as a therapeutic vaccine alongside TB treatment.
BNT164b1	mRNA	BioNTech SE	Well-tolerated and immunogenic in phase I trial.
PHASE III VACCINES			
M72/AS01E (Tait, D et al, NEJM, 2019)	Protein /adjuvant	Bill & Melinda Gates Medical Research Institute	Vaccine efficacy at 36 months was ~50% in phase 2b trial
GamTBVac (Tkachuk, A; Vaccines; 2020)		Gamaleya Research Institute of Epidemiology and Microbiology, Health Ministry of the Russian Federation	Safety and immunogenicity demonstrated in phase 2a trial
MTBVAC (Tameris; LRM; 2019)	Live attenuated Mycobacterial vaccine (*M. tb* with deletions of virulence genes phoP, fadD26)	Biofabri, S.L	Phase 1b/2a in patients with LTBI in South Africa (including in newborn babies) confirmed safety and immunogenicity
VPM1002 (Cotton M; LID; 2022)	Live attenuated Mycobacterial vaccine (*M. bovis* BCG-Prague)	Indian Council of Medical research (ICMR)	Safety, immunogenicity confirmed in South African neonates. Immunogenicity and reactogenicity were lower compared to BCG.
MIP (Immuvac) Sharma, Sci Rep, 2017	Inactivated mycobacterial vaccine (*M. indicus pranii*)	Indian Council of Medical research (ICMR)	safety and efficacy as an adjunctive therapy
BCG ReVAC (C-040-404) NEJM	Live attenuated mycobacterial vaccine (BCG)	Aeras	BCG vaccine reduced the rate of sustained QFT conversion, with an efficacy of 45.4% (P=0.03)

Table 4. Summary of promising candidates for TB vaccines entering phase 2 and 3 trials

1.2.7 Summary on the current TB landscape

Despite recent strides in the development of TB diagnostics, several obstacles still remain including the detection of TB from paucibacillary TB states which is a key pillar of TB control strategies. To combat the TB epidemic, new diagnostic tools and strategies are constantly being developed. However, presently we do not have a 'magic bullet' to address all the deficiencies that are highlighted in this thesis. The contents of this PhD thesis contribute to overcoming some of the current challenges. These findings are complementary with several promising technologies on the horizon (see discussion section) that are being researched and which will add to our repertoire of diagnostic tests

in the future. Lastly, improvement in strategies to detect TB early is but one important facet of TB control (it is a key component of End TB strategy). However, this must form part of a larger and global concerted effort that includes addressing poverty, policy and political will, infection control, better and shorter treatment for TB, focussed measures to prevent TB (TPT and vaccines) and addressing TB associated co-morbidity.

1.3.2. Coherence, chronology, and structure of this thesis.

The work described in this PhD thesis was conducted at the Centre for Lung Infection and Immunity (CLII), University of Cape Town Lung Institute in South Africa, under the supervision of Professor Keertan Dheda. The unit has a strong footprint in developing and validating TB diagnostic tests. All data and sample collection were performed either at the TB clinics, within high burden peri-urban communities within the Cape Town Metro or from secondary and tertiary level hospital after appropriate approvals.

This thesis is broadly divided into three parts. part 1 consists of two sub-sections. The first sub-section 1.1 includes a summary that highlights the rationale of this thesis followed by the main themes, key questions and their significance. The second subsection 1.2 describes a more extensive literature review and the current global TB landscape. This is followed by part 2 that contains the 5 chapters. Each chapter represents a manuscript which are all preceded by a cover page providing background details of each of the five projects. PART 3 focusses on a unified discussion that highlights how the findings from all of my published manuscripts fit into the current established framework of tuberculosis. It goes on to highlight the impact of my findings on patients, policies and finally concludes with providing an outlook to the future of TB diagnostics. Part 4 is a collection of supplementary materials associated with some of the published manuscripts and also has a PDF of all published manuscripts for reference.

The overarching aim of this thesis was to describe the deficiencies of current frontline diagnostic tools for the detection of TB in special populations, and to offer innovative alternative strategies, including a novel diagnostic test, to address this concern.

I enrolled for my PhD in 2019 and at the time, Cepheid had just launched its more sensitive Ultra cartridge (GeneXpert MTB/RIF Ultra). This was very exciting for me because this new test was regarded as a solution for the detection of TB in paucibacillary (smear-negative) TB [4]. However, at the time, there were limited independent data about its performance in patients with HIV co-infection and in patients with a previous history of TB. The Xpert Ultra also had an additional 'trace' semiquantitative readout. It was unclear at the time whether patients who yielded a 'trace' result, in fact had active TB? To independently verify the performance of Xpert Ultra, I was privileged to have access to the CLII's well characterized sputum biorepository (as the head of the clinical trials unit, I was leading a team of health care workers who collected these samples). I specifically selected negative sputum and performed spiking experiments using the standard strain H37RV (with assistance from my laboratory colleagues) to determine the limit of detection of the Xpert Ultra, which was indeed significantly lower compared to the older version of Xpert. I subsequently used smear-negative TB samples to evaluate the performance of Xpert Ultra (compared to the G4 Xpert), specifically to interrogate 'trace' readout (Chapter 2). This body of work provided me (a full-on clinician) with a unique opportunity to learn how to design, learn new skills and perform laboratory experiments under the supervision of experienced microbiologists.

The clinical question that served as a motivation for the publication included in chapter 1 of my thesis came from my clinical practice as a hospital-based physician in a high TB/HIV setting. The question was: Given that Xpert and LAM are both available to me for use in hospitalized HIV-infected patients, what test should I be doing first and why? The prevailing practice at the time was ill defined but many of my colleagues were performing Xpert first (in sputum expectorating patients) because it had the advantage of providing a readout for rifampicin-resistance, in addition to confirming the diagnosis of TB. However, waiting for the results at the time took 2-3 days, and only then would a urinary LAM test be requested if Xpert was negative (sighting cost as a potential reason for this sequential testing). I was uncomfortable with this delay because the recently published LAM-RCT [11] confirmed that this sub-group of patients, i.e., hospitalized and HIV-infected, had very rapid progression and were associated with a very high mortality within days of admission. Thus, to answer this research question, I performed a *post-hoc* analysis of data that was collected as part of the LAM-RCT [11]. In 2020, I selected out a sub-group of sputum expectorating patients to answer this question and perform a cost-consequence analysis to see how much each strategy i.e., Xpert followed by LAM, LAM followed and LAM+Xpert performed concurrently, would cost (Chapter 1). My findings surprised me in that both Xpert and LAM detected individual patients that were missed by the other test. However, the most efficient and cost-effective strategy was to perform both tests concurrently.

Given the confirmation of the Xpert Ultra's superior sensitivity in my own evaluation (chapter 2), I was intrigued and wanted to determine if this higher sensitivity can be leveraged to detect TB in other paucibacillary states. Specifically, if the more sensitive Xpert Ultra would be able to detect TB in minimally symptomatic or high-risk (HIV-

infected) patients in our communities (where the greatest burden of undiagnosed TB is located). I was ideally placed to interrogate this question because, at the time, I was leading a large ACF RCT trial (XACT-2). The main aim of this project was to develop a scalable low-cost strategy for community-based active case finding using molecular tools. Recent advances in technology at the time had just enabled the development of mobile platforms of Xpert testing (Xpert Edge precursor). I immediately leveraged this technology and incorporated it into the diagnostic algorithms for the XACT-2 trial (chapter 3). The findings of this trial (chapter 3) were novel and highlighted for me, for the first time, that Xpert Ultra, although more sensitive, was not going to be a panacea that solves the problem of detecting TB in this sub-population and that more sensitive sputum and non-sputum-based tests were required.

Once again, the confirmation of the higher sensitivity of Xpert Ultra from my own evaluations (chapter 1) also sparked my enthusiasm for leveraging the more sensitive Xpert Ultra test for the detection of TB in patients with extra-pulmonary disease. As a pulmonologist, I was very familiar with the deficiencies of current frontline diagnostic tests for TB in this sub-population[91]. In fact, I was also part of two previous publications that evaluated the performance of front-line diagnostic tests for EPTB[8,92]. The CLII in conjunction with a University of Cape Town owned start-up company, Antrum Biotech, have been involved in developing a novel immunodiagnostic diagnostic test for EPTB (IRISA-TB). This test was measuring unstimulated interferon gamma in fluid obtained from EPTB compartments (pleural, pericardial and cerebrospinal fluid). In early evaluations, this test had showed great promise. However, its performance needed to be validated in larger and multi-country cohorts with varying prevalence of HIV (IRISA-TB being an immunodiagnostic could be affected by HIV). To address this requirement, in

2019, I partnered with my colleague, a fellow pulmonologist from Vellore, India to write a successful grant to the NIH (via CRDF Global and RePORT consortium) to request for funding to enable the evaluation of IRISA-TB in South Africa (high HIV prevalence) and in India (lower HIV prevalence). The recruitment of patients for this project was delayed due to the logistical challenges imposed by the COVID-19 pandemic and the requirement for me to divert my attention towards front-line clinical services and COVID-19 vaccine development. Despite the delays, we were able to complete recruitment for this project in 2023. A unique aspect of this project was that the IRISA-TB test was outsourced and performed as part of routine workflows in busy laboratories in India confirming its feasibility and integration into the existing national TB program framework (Chapter 5).

In 2020, I once again leveraged the CLII's existing collaboration with cardiology colleagues working at my hospital (Groote Schuur Hospital, Cape Town) to evaluate the performance of IRISA-TB test (using a different previously validated cut-point). At the time, there were also no data on the performance of the more sensitive Xpert Ultra on pericardial fluid for the diagnosis of tuberculous pericarditis (this was in fact one of the largest cohorts that was evaluating the performance Xpert Ultra for tuberculous pericardial effusion). (Chapter 4). Both Chapter 4 & 5 are extremely important because they have contributed to the development of a novel test for the diagnosis of TB serositis. Given the findings of chapter 4 & 5, I (as the principal investigator) have now obtained a substantive grant from the EU Horizon project to conduct the validation of IRISA-TB in 3 African countries (the project is already recruiting participants in two of the three countries) as one of the key outputs emanating from the work presented in this thesis.

PART 2: EMPIRICAL RESEARCH PAPERS

Chapter 1: Optimal diagnostic strategies for TB diagnosis in hospitalised HIV-infected populations.

Theme: To investigate an ideal diagnostic strategy including sputum and non-sputum tests for tuberculosis in HIV-infected hospitalised patients with suspected tuberculosis.

Sub-population of TB under investigation: Hospitalised HIV-infected patients who are suspected of suffering from tuberculosis

Publication: **Esmail A**, Pooran A, Sabur NF, Fadul M, Brar MS, Oelofse S, Tomasicchio M, Dheda K. An optimal diagnostic strategy for tuberculosis in hospitalized HIV-Infected patients using Xpert and LAM. ***Journal of Clinical Microbiology***. 2020 Jul 29.

My contributions to this body of work: My supervisor and I (together with some of the personel in our unit who are co-authors) conceived and perfected the study idea. I, with the help from my supervisor, designed the main study plan and lead all the work involved in this project. I performed the main data-analysis and the interpretation of the analysed data. Lastly, I lead the team in drafting the manuscript and submitted it for peer-review. I was also sucessfully provided the responses to reviewer comments to their satisfaction leading to the acceptance of the manuscript. Lastly, I assisted our resident expert with the cost consequent analysis and gained insight in this important research tool.

Summary of the main findings in this publication: In sputum-expectorating hospitalized patients with advanced HIV and access to both tests, concurrent testing with Xpert and LAM may be the best strategy for diagnosing TB.

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An optimal diagnostic strategy for tuberculosis in hospitalized HIV-Infected patients using Xpert and LAM.

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Running Head: Diagnosis of HIV-infected patients with Xpert and LAM.

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ABSTRACT

Background: The diagnosis of TB in HIV-infected patients is challenging. Both urinary lipoarabinomannan (Alere TB™ LAM) and GeneXpert-MTB/RIF (Xpert) are useful for the diagnosis of TB. However, how to optimally integrate Xpert and LAM into clinical practice algorithms remain unclear.

Methods: We performed a post-hoc analysis of 561 HIV-infected sputum expectorating patients (median CD4 count of 130 cells/ml) from a previously published randomised controlled trial evaluating LAM in hospitalized HIV-infected patients with suspected TB. We evaluated 5 different diagnostic strategies using sputum culture as a reference standard [Xpert alone, LAM alone, sequential Xpert followed by LAM and *vice versa* (LAM in Xpert-ve; Xpert in LAM-ve) or performing both tests concurrently (LAM + Xpert)]. A cost-consequence analysis was performed.

Results: Strategy-specific sensitivity and specificity, using culture as a reference, was similar with the Xpert-only and sequential/concurrent strategies. However, when using any positive TB-specific test as a reference, the incremental yield of LAM over Xpert was 29.6% (45/152) and Xpert over LAM 75% (84/112). The incremental yield of LAM increased with decreasing CD4 count. The cost per TB case were similar for the sequential and concurrent strategies ($1617 to $1626).

Conclusion: In sputum-expectorating hospitalised patients with advanced HIV and access to both tests, concurrent testing with Xpert and LAM may be the best strategy for diagnosing TB. These data inform clinical practice in TB and HIV endemic settings.

INTRODUCTION

In sub-Saharan Africa, the co-epidemics of tuberculosis (TB) and HIV are out of control[1]. Approximately 70% of all incident TB cases in HIV-infected persons occur in Africa and 17% of the total TB deaths are associated with HIV [2]. The high case-fatality rate from HIV-TB co-infection is related to disseminated and extra-pulmonary forms of TB (EPTB) in patients with advanced immunosuppression [3] where the diagnosis is often missed or delayed. Indeed, post-mortem studies demonstrate that TB is the cause of death in 40-60% of hospitalized HIV patients [4-6] with the diagnosis missed in over 45% of patients [4-6].

However, two tests have recently been shown to be effective for TB diagnosis in the setting of advanced HIV co-infection. The Xpert MTB/RIF test (Xpert) is an automated, sputum-based, real-time polymerase chain reaction assay that permits rapid identification of *M. tuberculosis* and determination of rifampicin resistance [7]. However, whilst access to Xpert testing has been proven to reduce time-to-treatment initiation, [8,9] it performs sub-optimally in HIV-infected patients who often have sputum smear-negative disease [10]. The Alere Determine™ TB LAM Ag lateral flow strip test is a bedside urine-based test that identifies lipoarabinomannan (LAM), a glycolipid component of the mycobacterial cell wall, and provides a result in 25 minutes [11]. It is a WHO-approved test in HIV-infected patients with advanced immunosuppression, [12,13] and has been shown to reduce mortality in hospitalized HIV-positive patients when used to guide TB treatment initiation [14,15]. Urine LAM has obvious advantages as it is a point-of-care test facilitating rapid treatment initiation, is easy to collect, and does not have the same infection control concerns as sputum.

Current WHO guidelines recommend urine LAM testing in all HIV-infected hospitalised patients with advanced immunosuppression (CD4< 100 cells/ml or stage 3/ 4 disease) or who are seriously ill, irrespective of signs or symptoms of TB [16]. Thus, LAM should be used as a screening test in this population. In hospitalised HIV-infected persons with a CD4 > 100 cells/ml LAM is also indicated in those with signs and symptoms of TB.

However, the guidelines are unclear on how newer tests like Xpert and LAM should be integrated into clinical practice in high HIV prevalence settings. This is a critical unanswered question in TB and HIV endemic settings where often, either only one or both tests are available. Thus, it is unclear whether, in individual test-specific settings, whether performing testing sequentially or concurrently may be more useful; the cost implications thereof remain unstudied. To address this question, we evaluated the test performance and cost consequence (per TB case diagnosed) of 5 testing strategies [individual (Xpert or urine LAM alone), sequential (Xpert in LAM-negative patients OR urine LAM in Xpert-negative patients) or concurrent testing] in a large cohort of sputum expectorating hospitalized HIV-infected patients that formed part of a larger parent study, the results of which are reported elsewhere [14].

METHODS

Study design, conduct and oversight

This study represents a post hoc sub-group analysis of data from a pragmatic, randomized, parallel arm, multicenter study with stratified randomization by country. The primary analysis has already been reported [14]. The study was approved by the appropriate national regulatory authorities and by the ethics committee at each site. All patients provided informed consent in their first language. This trial was registered with Clinicaltrials.gov (https://clinicaltrials.gov/ct2/show/NCT01770730).

Study patients, enrolment criteria and randomization

Patients admitted to ten urban or peri-urban hospitals in South Africa, Tanzania, Zambia and Zimbabwe were screened for study inclusion. A detailed description of hospital care, HIV and TB prevalence, routine clinical care, and the diagnostics infrastructure at each hospital have been previously reported [14]. The inclusion criteria included: i) HIV-infection; ii) at least one of the following symptoms: current fever or cough, drenching night sweats, or self-reported loss-of-weight; iii) illness severe enough to necessitate hospitalization; iv) age ≥18 years; v) granting of informed consent. The exclusion criteria included: i) patients receiving any anti-TB medication in the 60 days prior to testing, and ii) unable to provide at least 30 ml urine. Eligible patients were randomized to receive standard available TB diagnostics at each centre or standard TB diagnostics plus

adjunctive LAM, using centralized computer-generated allocation lists, stratified by country. The patients and the study team were not masked to both allocation and test results.

Procedures and standard clinical management

In addition to a urine specimen, patients were asked to expectorate a minimum of two sputa for routine TB diagnosis. For patients, unable to self-expectorate, sputum induction was employed. Clinical assessment by the attending physicians and chest x-ray facilities were available in most cases, however additional radiology and non-sputum sampling was differentially available in study hospitals and requesting these investigations was at the discretion of the attending clinical team. The Xpert MTB/RIF assay was performed on ~1ml of sputum, according to manufacturer's instructions, at all study sites. The urine based Alere Determine™ TB LAM Ag lateral flow strip test was performed at the bedside, according to manufacturer's instructions. A grade 2 cut-off point or higher was deemed as a positive urine LAM result. The attending clinical team made all decisions regarding patient therapy and initiation of anti-TB treatment and the timing thereof, including acting upon the LAM test results. The WHO guidelines for the treatment of smear-negative tuberculosis were routinely used at study hospitals.

Patient selection for post hoc analysis

For this analysis, only those patients randomized to the LAM group and in whom Xpert MTB/RIF and sputum culture results were available were included (561 patients of the 1257 patients randomized to LAM, see Figure 1).

Outcomes and statistical analysis

The goal of this analysis was to assess the utility of single or sequential TB testing strategies incorporating urine LAM and/or Xpert MTB/RIF in HIV-infected patients admitted for suspected TB. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) was calculated for 5 different strategies using sputum culture positivity as a reference standard: (i) performing Xpert MTB/RIF alone (Xpert only); (ii) performing urine LAM alone (LAM only); (iii) performing Xpert MTB/RIF only in patients with an initial LAM negative result (Xpert in LAM-ve), (iv) performing urine LAM only in patients with an initial negative Xpert MTB/RIF result (LAM in Xpert-ve) and (v) performing Xpert and LAM concurrently (LAM+Xpert).

In addition, the incremental yield of urine LAM and Xpert MTB/RIF in the sequential testing strategies (Xpert in LAM-ve and LAM in Xpert-ve) was determined using either sputum culture positivity or any positive TB test (LAM, Xpert and/or sputum culture) as a reference standard. An *a priori* subgroup analysis was performed on those with a CD4 count ≤200 and ≤50 cells/mm^3, as the diagnostic performance of urine LAM improves with lower CD4 counts. [17] All analyses were performed using Stata V13 (StataCorp). Exact binomial 95% confidence intervals were calculated for proportions and differences in

proportions were calculated (with 95% confidence intervals using a normal approximation) for comparisons between diagnostic approaches.

Economic evaluation (cost analysis)

A cost-consequence analysis was performed from the South African healthcare provider perspective to evaluate the different diagnostic strategies (i)-(v) mentioned above. Costs were expressed in US$2018at an exchange rate of ZAR13.20 to US$1 (http://wdi.worldbank.org/table/4.16). Costs were subsequently inflated to the year of analysis where appropriate using the World Bank Consumer Price Index for South Africa (https://data.worldbank.org/indicator/FP.CPI.TOTL?locations=ZA). The unit cost of Xpert MTB/RIF was obtained from the National Health Laboratory Service (NHLS) and represents the actual costs incurred by the South African National TB Program. Such estimates have been used in other health economic studies [18-20] The unit cost of the urine LAM test was calculated using an ingredients approach and included the cost of the assay (provided by Alere), staff and other consumables. The cost of anti-TB treatment for 6 months was obtained from the WHO South African tuberculosis finance profile [21]. No discount rate was applied due to the short timeframe of the analysis. Model probabilities were calculated based on test sensitivities and specificities reported in Table 2. Outcomes were normalised to 1000 patients screened per strategy. The cost-effectiveness of each strategy was reported as the cost per culture positive case diagnosed and initiated on treatment (per 1000 patients screened). Other effectiveness measures were also considered (see online supplement). A univariate analysis was performed to determine the effect of varying specific parameter inputs for each strategy.

Details of all costs, model parameters and model assumptions are provided in the online supplement.

RESULTS

Of the 1257 patients randomized to the LAM study arm, 561 patients were selected for analysis based on availability of sputum culture results and Xpert MTB/RIF test results available for analysis (Figure 1). The baseline patient characteristics are summarized in Table 1. The reference standard for the diagnosis of TB in the primary analysis was a positive sputum culture, which was reported in 31.7% of this cohort (178/561). Any positive TB-specific test (Xpert MTB/RIF, urine LAM or sputum culture) was used as a reference standard in secondary analyses.

Test characteristics of single and sequential/concurrent testing strategies incorporating urine LAM and Xpert MTB/RIF in hospitalized patients irrespective of CD4 counts

The sensitivity, specificity, PPV and NPV (95% CI) of the different strategies were as follows (Table 2): Xpert only [74.7% (67.7-80.9), 95.1% (92.4-97.0), 87.5% (81.2-92.3) and 89.0% (85.6-91.9, respectively)]; LAM only [38.2% (30.9-45.7), 88.3% (84.6 – 91.3), 60.2% (50.5-69.3) and 75.5% (71.3-79.4, respectively)] and sequential (Xpert in LAM-ve; LAM in Xpert-ve) /concurrent (LAM+Xpert) testing [78.1% (71.3-83.9), 85.1 (81.2-88.5), 70.9 (65.5-75.8) and 89.3 (86.3-91.7, respectively)]. When the sequential/concurrent testing strategies was compared to Xpert only, there was a significant decrease in specificity

[10.0% difference (95% CI 6.2-14.6); p<0.0001] and PPV [16.6% difference (95% CI 9.1-25.7); p=0.001]. However, no difference in sensitivity [-3.4% difference (95% CI -12.2-5.4); p=0.22] and NPV was observed [-0.3% difference (95% CI -4.6-4.0); p=0.45].

Test characteristics of single and sequential/concurrent testing strategies incorporating urine LAM and Xpert MTB/RIF in hospitalized patients with CD4 ≤50 and ≤200 cells/mm^3

When test performance was assessed in patients with CD4 counts ≤50 cells/mm^3, the LAM only strategy showed improved sensitivity and PPV [60.0% (47.1 – 72.0) and 67.2% (53.7 – 79.0), respectively; Table 3] but a slightly reduced specificity and NPV [81.7% (72.9-88.6) and 77.0% (68.1 – 84.4), respectively], when compared to the entire cohort (not stratified by CD4 count). A similar pattern was observed in the Xpert only strategy with a sensitivity, specificity, PPV and NPV of 8plays% (71.3-89.2), 94.3% (88.1-97.9), 89.2% (79.8-95.2) and 89.9% (83.8-94.2), respectively (Table 3). However, sequential/concurrent testing did not show a significant difference in sensitivity when compared to Xpert only [89.2% (79.1-95.6); p=0.28] in this patient group. A similar pattern, albeit to a lesser extent, was observed when patients with CD4 counts ≤200 cells/mm^3 were analysed (Table 3).

Incremental yield of urine LAM and Xpert MTB/RIF in sequential testing strategies

The incremental yield of urine LAM testing in the LAM in Xpert-ve strategy was 4.5% (6 urine LAM positive/132 Xpert MTB/RIF positive; Table 4); while the incremental yield of Xpert MTB/RIF testing in the Xpert in LAM-ve strategy was 104% (71 Xpert MTB/RIF positive/68 urine LAM positive). When the analysis was repeated using any positive TB microbiological test (urine LAM, Xpert MTB/RIF or sputum culture) as a reference standard for the diagnosis of TB (as is the case in clinical practice), the incremental yield of LAM in the LAM in Xpert -ve strategy increased from 4.5% (when using sputum culture as a reference) to 29.6% (Table 4). The incremental yield of Xpert or LAM, using any positive microbiological test for TB as a reference standard, was also dependent on CD4 count (Figure 2).

Economic evaluation

The total cost of the sequential (LAM in Xpert-ve and Xpert in LAM-ve) and concurrent (LAM+Xpert) testing strategies were similar, ranging from $400,656 to $402,909 per 1000 patients screened (Table 5). Costs associated with unnecessary treatment were higher in the sequential and concurrent testing strategies ($186,228-$187,235 per 1000 patients screened) as more individuals with a false positive test result were initiated on treatment. LAM only was the cheapest strategy followed by Xpert only ($305,927 and $355,358 per 1000 patients screened, respectively). The higher cost of sequential/concurrent testing was a consequence of more individuals being correctly diagnosed and subsequently initiated on treatment (248 patients in each strategy) compared to LAM only (121 patients) and Xpert only (237 patients). However, sequential/concurrent testing also had the fewest missed culture-positive TB cases (49

patients in each strategy) and empirically treated patients (195 patients in each strategy). The cost per culture positive case diagnosed and initiated on treatment for the sequential and concurrent testing strategies were very similar (LAM in Xpert-ve, Xpert in LAM-ve and LAM+Xpert was $1617, $1621 and $1625, respectively) but still slightly more expensive than Xpert only ($1500). LAM only remained the least cost-effective strategy ($2525; Table 5).

A univariate sensitivity analysis indicated that TB prevalence, the rate of empirical treatment and TB treatment costs were the most influential parameters on cost-effectiveness (Figure S3 in the online supplement). Subsequently, the cost per culture positive patient diagnosed and initiated on treatment of each strategy was plotted against increasing TB prevalence and empirical TB treatment rate as these tend to vary widely in different settings. The cost-effectiveness values decreased as prevalence increased but the rankings remained unchanged (Xpert alone was cheapest followed by sequential/concurrent strategies). However, the magnitude of the cost difference between the sequential/concurrent testing strategies and Xpert only decreased (Figure S4A in the online supplement). A similar reduction in the cost difference between strategies was observed when increasing the empirical treatment rate until ~70%, when the cost per outcome of the sequential/concurrent strategies became less than Xpert alone (Figure S4B in the online supplement). In both cases, the LAM only strategy was the least cost-effective.

DISCUSSION

We evaluated the benefit of using Xpert MTB/RIF (Xpert) and urine LAM testing performed either singly, sequentially, or concurrently in *sputum-expectorating,* hospitalized, HIV-infected patients with suspected TB. Our key findings were that (i) Xpert performs better than urine LAM, irrespective of CD4 count; (ii) Serial/concurrent testing using Xpert and LAM (Xpert in LAM-ve; LAM in Xpert-ve; LAM+Xpert) improves sensitivity over either test alone; (iii) LAM + Xpert may be the most appropriate strategy for diagnosing TB, especially in the setting of advanced immunosuppression (CD4 ≤ 200 cells/mm^3), since each of these tests independently diagnoses patients with active TB; moreover, the incremental yield of LAM over Xpert increases with increasing immunosuppression, and (iv) the cost per TB case diagnosed and initiated on treatment was similar for the sequential and concurrent strategies ($1617-$1625) and only ~$120 more than the Xpert only strategy.

There are few data about how to optimally use or combine different TB tests in a clinical setting. A positive result with either Xpert or LAM is considered diagnostic for TB and will prompt the clinician to treat for TB. Given this consideration, we analysed the data using 2 reference standards: (i) sputum culture positivity and (ii) any TB microbiological test positivity. Using the former (Table 4), urine LAM provided a modest incremental yield of 4.5% (8 patients) in Xpert-ve patients. By contrast, when using Xpert, sputum culture or LAM positivity (thus any microbiological test positivity) as a reference, the urine LAM incremental yield was ~30% in Xpert -ve patients. This group (Xpert-ve and LAM positive) had the lowest Karnovsky scores and *highest mortality* in our cohort (Table S2). When only including patients with CD4+ counts ≤50 cells/ul LAM sensitivity improved by 20%

and 26% when using sputum culture or any positive microbiological test as a reference, respectively. This suggests that the patients with lower CD4 counts benefit most from LAM. However, even in this group with advanced HIV, the overall sensitivity of Xpert was superior to LAM (Table 3). Thus, our data support the need for a strategy incorporating both tests, especially at lower CD4 counts (≤200 cells/mm^3), given that each test independently identifies patients with active TB (in this scenario a patient's urine and sputum samples would be taken simultaneously and both tests would be run concurrently). Our study findings are consistent with a Ugandan study, where a combination of LAM and sputum Xpert had a greater sensitivity than either test alone [22]. Importantly, the parent study [17], demonstrated survival benefit with a LAM-guided testing strategy due to more rapid treatment initiation. More recently, the STAMP trial, which assessed patient outcomes using a concurrent LAM and Xpert diagnostic strategy, demonstrated significantly reduced mortality (7.1%) in patients with CD4<100 cells/μL and detected more TB cases compared to Xpert alone (21.9% vs. 14.9%; p<0.001) [15]. Thus, in sputum expectorating patients, concurrent testing leverages the advantages of both tests i.e. LAM allows for rapid treatment initiation while awaiting Xpert results to ascertain resistance to rifampicin (Xpert results may take up to ten days in some endemic settings and has obvious relevance in settings where rifampicin resistance is common [23,24]; this strategy is also broadly cost neutral. However, there is also a reduction in specificity with sequential/concurrent testing, especially at low CD4 counts, that subsequently results in a higher number of false positives. As such, the consequences of initiating treatment unnecessarily must also be considered when choosing a testing strategy, especially when there is an increased risk of adverse drug reactions, such as those associated with second-line TB treatment. Furthermore, in patients who are

unable to expectorate sputum, LAM is the obvious first choice and our findings in this sub-group (sputum scare patients) have been reported separately [25].

There are no cost-related data to inform test selection in TB endemic settings. Our cost-consequence analysis indicated that the cost per culture-positive patient diagnosed, and initiated on treatment, for the sequential/concurrent strategies were similar ($1617-$1626) and only marginally higher than using Xpert alone ($1500). The cost similarity is explained by the LAM assay having a low unit cost and it had little contribution to the overall strategy-specific costs. Treatment costs were greater with sequential/concurrent testing because more culture-positive patients were initiated on treatment compared to the Xpert only (11 more patients) and LAM only (127 more patients) strategies. However, the additional cost of ~$120 for every TB patient diagnosed and initiated on treatment when LAM is incorporated into Xpert-based diagnostic strategies, is likely justifiable as hospitalized patients with advanced HIV immunosuppression are at higher risk for mis-diagnosis and poor outcomes if treatment is not rapidly initiated [26]. This is in agreement with other cost-effectiveness studies evaluating the addition of LAM to existing TB diagnostic tests [27], including one related to the STAMP trial [28]. Implementation costs of these strategies would also likely be affordable given the relatively low LAM unit test cost and lack of need for additional infrastructure or expertise. Furthermore, the sequential/concurrent strategies were more cost-effective than Xpert-only at high TB prevalence and high empirical treatment rates, making this a particularly economically attractive approach in such settings.

Our study has several limitations. We confined our analysis to patients who could expectorate sputum, yet LAM may be most beneficial in those who are sputum scarce or with EPTB. However, the main aim of our study was to inform diagnostic algorithms in settings where other rapid sputum-based diagnostics, such as Xpert, were available, and the findings in sputum-scarce patients were published elsewhere [25]. Furthermore, this was a post-hoc analysis, utilising a limited subset of the patients recruited into the parent study, which may limit generalisability of the findings. However, patients were recruited from 4 different countries in sub-Saharan Africa and this is the largest study to date comparing LAM and Xpert incremental yields in patients where sputum could be obtained. Thirdly, the parent study was conducted before the availability of Xpert Ultra which is a more sensitive test [29], but it is unlikely that this would have impacted our conclusions (Ultra overall has a ~5% increased sensitivity and a disadvantage of a higher false positive rate in patients with a previous history of TB) [29]. It is possible that urine LAM may provide additional diagnostic value when interpreting Xpert ULTRA trace results in high-risk populations (but not for detection of rifampicin resistance, which cannot be inferred using a trace readout). Finally, cost-effectiveness was calculated using clinical outcomes rather than single utility metric such as DALYs so we cannot explicitly state that these strategies are cost effective compared to Xpert-only in the traditional sense i.e. below the classical willingness-to-pay threshold of three times per-capita GDP per DALY averted. Furthermore, we did not incorporate other clinical outcomes such as death or additional TB cases averted due insufficient data. Given the high mortality risk in this population, it is likely, if anything, that inclusion of these effects would have improved the cost-effectiveness of the sequential/concurrent strategies.

CONCLUSION

In sputum-expectorating hospitalised patients with advanced HIV and access to both tests, concurrent testing with Xpert and LAM is the most appropriate strategy for diagnosing TB. These data inform clinical practice in TB and HIV endemic settings.

TABLES

Table 1: Demographics and clinical characteristics of study cohort

Variable	Total N=561	CD4 count ≤200mm^3 n=361	CD4 count >200mm^3 n=143	CD4 Unknown n=57	P-value (CD4 ≤200 vs. >200)
Median Age; years (95% CI)	36.0 (18, 75)	37.3 (19, 70)	37.6 (18, 75)	36.8 (19, 61)	0.31
Male:Female; n (% male)	294:267 (52.4)	190:171 (52.6)	77:66 (53.8)	27:30 (47.3)	0.71
Country of origin; n (%)					0.79
S. Africa	151	70 (27.5)	74 (29.1)	7 (13.5)	
Tanzania	193	96 (37.7)	97 (38.2)	0 (0.0)	
Zambia	217	89 (34.9)	83 (32.7)	45 (86.5)	
Median Karnofsky Score (95% CI)	60 (20, 90)	60.0 (20, 90)	60.0 (30, 90)	50.0 (50, 60)	0.91

Median CD4; cells/mm^3 (95% CI)	99.5 (1, 933)	58 (1, 199)	320 (201, 933)	- -	**0.0001**
Median CD4 in patients with any positive TB result; cells/mm^3 (95% CI)	73.0 (1, 695)	44 (1, 199)	283 (206, 695)	- -	**0.0001**
Median BMI (95% CI)	18.7 (9, 37)	18.6 (9, 27)	19.4 (10, 37)	17.1 (16.6, 19.3)	**0.002**
Positive culture results; n/N (%)	178/561 (31.7)	127/361 (35.2%)	35/143 (24.5%)	16/57 (28.1%)	**0.02**

Table 2: Diagnostic performance of sputum-based Xpert MTB/RIF and urine-based Alere Determine TB LAM Ag testing using single and sequential testing strategies in HIV-infected hospitalised patients irrespective of CD4 count and using *sputum culture positivity* as the reference standard (PPV - positive predictive value; NPV – negative predictive value; LR(+) – positive likelihood ratio; LR(-) – negative likelihood ratio).

Test strategy	**Sensitivity % (95% CI)**	**Specificity % (95% CI)**	**PPV % (95% CI)**	**NPV % (95% CI)**	**LR (+)**	**LR (-)**
Xpert MTB/RIF Only	74.7 (67.7 – 80.9)	95.1 (92.4 – 97.0)	87.5 (81.2 – 92.3)	89.0 (85.6 – 91.9)	15.1 (9.7 – 23.6)	0.27 (0.2 – 0.34)
Urine LAM only	38.2 (31.0 – 45.7)	88.3 (84.6 – 91.3)	60.2 (50.5 – 69.3)	75.5 (71.3 – 79.4)	3.3 (2.3 – 4.5)	0.7 (0.6 – 0.8)
*Sequential or concurrent testing using Xpert MTB/RIF and LAM	78.1 (71.3 – 83.9)	85.1 (81.2 – 88.5)	70.9 (65.5 - 75.8)	89.3 (86.3 – 91.7)	5.3 (4.1 – 6.8)	0.3 (0.2 – 0.34)

* Sequential testing refers to performing Xpert in LAM negative patients (Xpert in LAM-ve) or LAM in Xpert-negative patients (LAM in Xpert-ve), whilst concurrent testing refers to both tests being performed at the same time rather than on reflex testing based on the initial test result (in all 3 scenarios, the number of TB cases diagnosed remains the same and hence sensitivities and specificities are identical).

Table 3: Diagnostic performance of sputum-based Xpert MTB/RIF and urine-based Alere LAM in single, sequential, and concurrent testing strategies in HIV-infected hospitalised patients with CD4 ≤50 and ≤200 cells/mm^3 using *sputum culture* or *any microbiological test* positivity as a reference.

Reference standard	CD4 count (cells/ mm^3)	Test strategy	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
Sputum culture positivity	≤50	Xpert MTB/RIF only	81.5 (71.3 – 89.2)	94.3 (89.1 – 97.5)	89.2 (79.8 – 95.2)	89.9 (83.8 – 94.2)
		Urine LAM only	60.0 (47.1 – 72.0)	81.7 (72.9 – 88.6)	67.2 (53.7 – 79.0)	76.6 (67.6 – 84.1)
		*Sequential or concurrent testing	89.2 (79.1 – 95.6)	79.8 (70.8 – 87.0)	73.4 (62.3 -82.7)	92.2 (84.6– 96.8)
	≤200	Xpert MTB/RIF Only	80.3 (72.3 – 86.8)	94.9 (91.3 – 97.3)	89.5 (82.3 – 94.4)	90.0 (85.5 – 92.4)
		Urine LAM only	44.9 (36.1 – 54.0)	88.1 (83.3 – 92.0)	67.1 (56.0 – 76.9)	74.8 (69.3 – 79.8)
		*Sequential or concurrent testing	83.5 (75.8 – 89.5)	84.7 (79.5 – 89.1)	74.6 (66.7 -81.6)	90.5 (85.8 – 94.0)
**Any microbiological test positivity	≤50	Xpert MTB/RIF only	71.3 (60.6 – 80.5)	-	-	77.1 (70.7-82.4)
		Urine LAM only	66.7	-	-	74.3

			(55.8-76.4)			(68.3-79.6)
		*Sequential or concurrent testing	92.0 (84.1-96.7)	-	-	92.3 (85.5-96.1)
	≤200	Xpert MTB/RIF only	69.9 (62.3 – 76.9)	-	-	80.3 (76.4-83.8)
		Urine LAM only	52.2 (44.1-60.0)	-	-	71.9 (68.6-75.1)
		*Sequential or concurrent testing	87.1 (81.0-91.8)	-	-	90.5 (86.5-93.4)

* Sequential testing refers to performing Xpert in LAM negative patients (Xpert in LAM-ve) or LAM in Xpert-negative patients (LAM in Xpert-ve) and concurrent testing refers to performing both Xpert and LAM concurrently (LAM+Xpert).

** Specificity and PPV could not be determined when using any microbiological test positivity as a reference standard (as this will always equate to 100%)

Table 4: Incremental yield of urine LAM and Xpert in sequential testing strategies using different reference standards.

Testing strategy	Diagnostic reference standard	Proportion of patients diagnosed with TB with the initial test	Incremental diagnostic yield in patients who test negative with the initial test
Xpert followed by LAM in Xpert-negative patients. (LAM in Xpert-ve; Incremental yield of LAM)	TB Culture	74.7% (133/178) using Xpert as the initial test	4.5% (6/133 patients)
	Any TB positive test*	64.7% (152/235) using Xpert as the initial test	29.6% (45/152 patients)
LAM followed by Xpert in LAM negative patients. (Xpert in LAM-ve; Incremental yield of Xpert	TB Culture	38.2% (68/178) using LAM as the initial test	104% (71/ 68 patients)
	Any TB positive test*	47.6% (112/235) using LAM as the initial test	75% (84/112 patients)

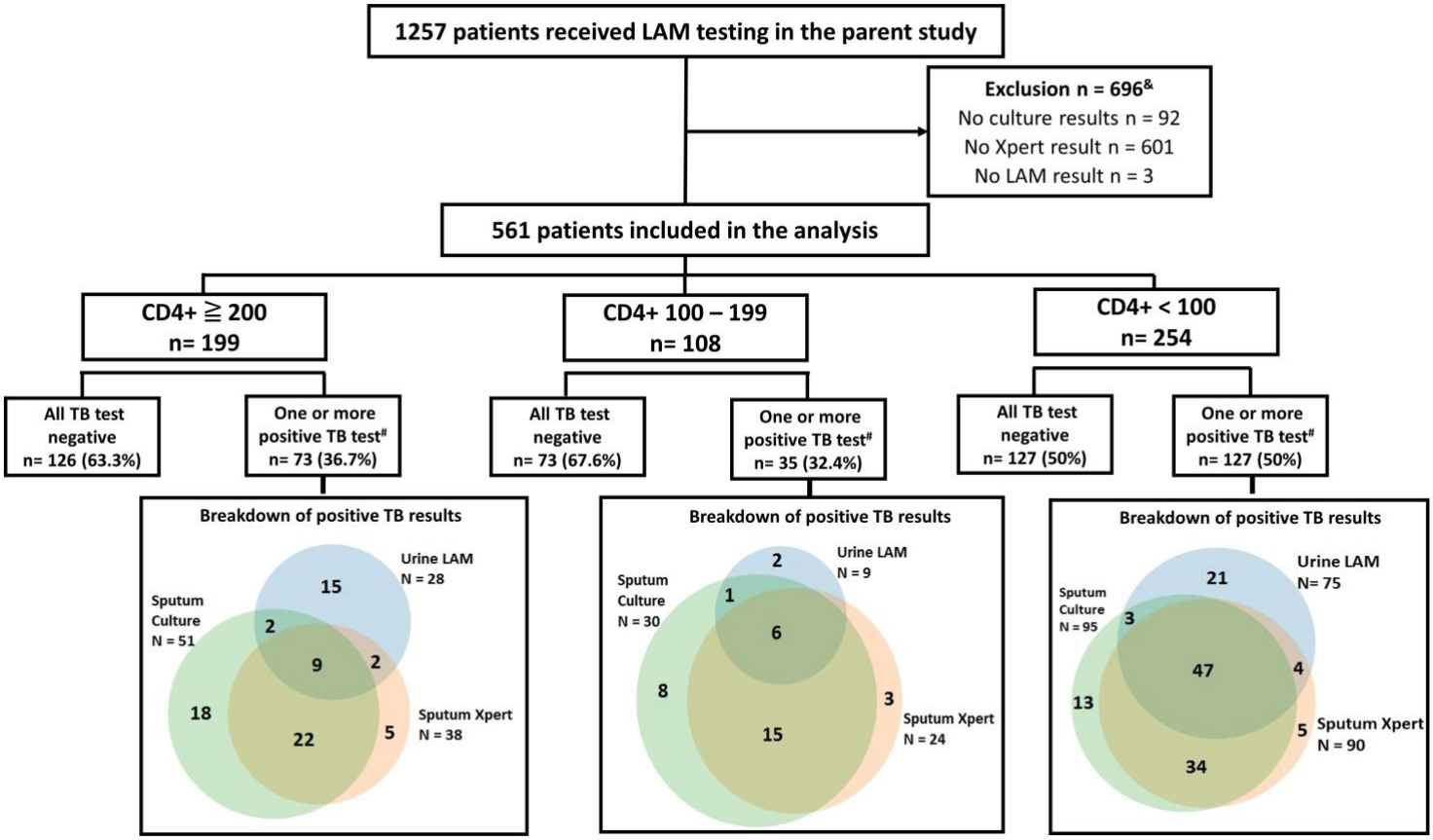
* Any positive test refers to a positive result obtained from MGIT sputum culture, Xpert MTB/RIF, or urine LM. It is acknowledged that inclusion of Xpert or LAM in the reference standard represents inclusion bias that tends to overestimate sensitivity (data are provided for purpose of comparison).

Table 5: Costs, outcomes and cost-effectiveness for single, sequential and concurrent test strategies to diagnose TB in hospitalized patients with advanced HIV using Xpert MTB/RIF and LAM urine tests. Costs are expressed in 2018 $US with 95% CI in parentheses.

	Single test strategies		**Sequential test strategies**		**Concurrent testing strategy**
	Xpert only	**LAM only**	**LAM in Xpert-ve**	**Xpert in LAM-ve**	**Xpert+LAM**
Total cost (per 1000 patients screened)					
Total cost	$355,358 (353256, 359086)	$305,927 (305271, 307949)	$400,656 (412366, 406317)	$401,476 (405139, 414150)	$402,909 (400992, 406859)
Diagnostic costs	$14,375 (14375, 14375)	$3,557 (3557, 3557)	$15,049 (14997, 15014)	$16,978 (16954, 16991)	$17,932 (17932, 17932)
Treatment costs	$340,983 (338881, 344711)	$302,370 (303714, 304392)	$385,608 (397370, 391303)	$384,499 (388185, 397160)	$384,977 (383061, 388927)

Outcomes (per 1000 patients screened)					
Number of culture positive patients correctly diagnosed and initiated on treatment	237.1 (214.9, 256.7)	121.3 (98.4,145.4)	247.9 (205.7, 288.6)	247.7 (219, 278.1)	247.9 (226.3, 266.3)
Cost-effectiveness					
Cost per culture positive patient diagnosed and initiated on treatment	$1500 (1399, 1645)	$2525 (2120, 3124)	$1617 (1429, 1976)	$1621 (1458, 1892)	$1626 (1507, 1799)
Cost difference (compared to Xpert only)	-	$1025	$117	$122	$127

FIGURES



*Excluded patients had similar demographic characteristics.

LAM and/or Xpert and/or BACTEC MGIT 960 TB-culture positive.

& approximately 27% were sputum scarce

Figure 1: Flow diagram demonstrating patient selection and distribution of TB positive results (LAM: lipoarabinomannan; Xpert: GeneXpert MTB/RIF)

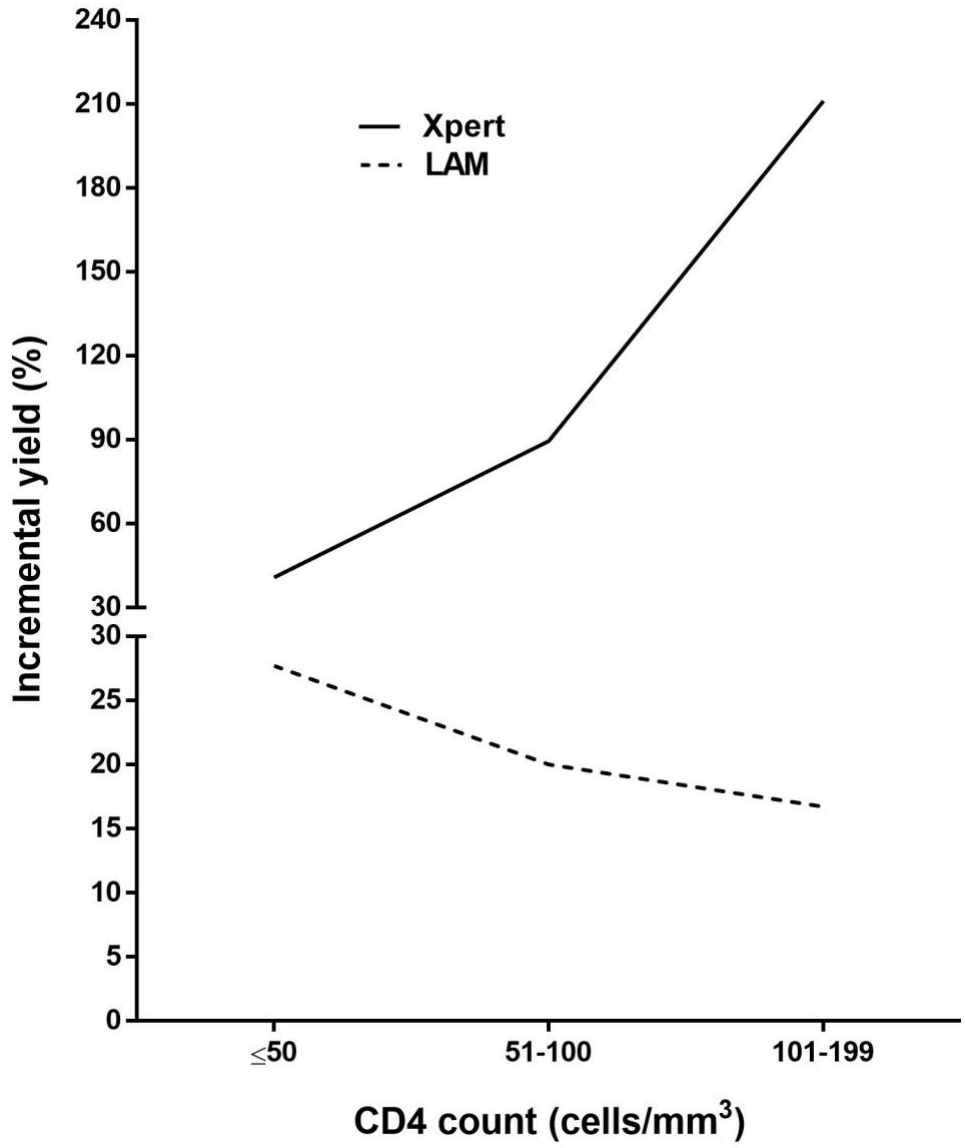


Figure 2: Incremental yield of LAM (LAM positivity in Xpert-ve patients) and Xpert (Xpert positivity in LAM-ve patients) when using any positive TB specific test as a reference standard in patients with CD4 ≤ 200.

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Additional disclosures

Prior to patient recruitment into the parent study, approval was obtained by the appropriate national regulatory authorities and by the University of Cape Town Human Research Ethics Committee (HREC 007/2012). Informed written consent was obtained for each participant prior to enrolment into the parent study. This trial is registered with Clinicaltrials.gov, number NCT01770730.

The dataset used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

AE, AP, NS and KD were involved in the conception of the study. NS, AE, MB, and KD were involved in study design. AE, NS, AP, SO and MB did the analysis. AE, NS, AP, MB, SO, MT and KD interpreted the data. All authors read and approved the manuscript.

Competing interests

KD has obtained speaker fees at industry-sponsored symposia and non-financial support from Alere in the form of kits and test strips, outside the submitted work. No other authors declare competing interests.

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Chapter 2: Independent confirmation of the higher sensitivity of Xpert Ultra in the smear negative TB sub-population and interrogating the epidemiology and significance of trace readouts.

Sub-population of TB under investigation: Smear-negative (paucibacillary/low burden) TB

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My contributions to this body of work: My supervisor and I (together with some of the personel in our unit who are co-authors) conceived study idea. I, with the help from my supervisor, designed the main study plan and lead all the work involved in this project. I performed the data-analysis and the interpretation of the analysed data. Lastly, I lead the team in drafting the manuscript and submitted it for peer-review. I assisted the microbiologist in the laboratory the serial dilution experiments and learnt laboratory techniques in the process.

Summary of the main findings in this publication: Xpert Ultra had a lower limit of detection (serial dilution experiments). < 5% of the samples with 'trace' readout in our study were likley false positive

Comparison of Xpert MTB/RIF (G4) and Xpert Ultra, including trace readouts, for the diagnosis of pulmonary tuberculosis in a TB and HIV endemic setting.

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ABSTRACT.

Background. There are limited data about Xpert-Ultra performance in different settings, in HIV-infected persons, in those with a history previous TB, and with trace readouts.

Methods. We evaluated the comparative accuracy of Xpert-MTB/RIF and Xpert-Ultra in 272 selected but well-characterised archived sputum samples. Of these 168 were culture-positive (64/168 smear-positive and 104/168 smear-negative) and 104 were culture-negative (102/104 from patients with previous TB and 2/104 from patients without a TB history). Assay-specific limit-of-detection (LOD) experiments were conducted using serial dilutions of H37RV.

Results. Overall sensitivity (95%CI) in smear-negative culture-positive samples for Xpert-MTB/RIF and Xpert-Ultra were 71.2% (62.5-79.9) and 77% (68.9-85.1), respectively (and in HIV-infected persons: 63.5 (50-76.1) and 73.1% (61.1-85.2), respectively). The LOD for Xpert-Ultra was lower (9 versus 184 CFU/ml). There were a total of 9/272 (3.3%) Xpert Ultra trace readouts (6/104 [5.8%]) in smear-negative culture-positive persons, and 3/102 (3%) in culture-negative non-TB persons with a history of previous TB).

Conclusions. Xpert-Ultra had a lower LOD compared to Xpert-MTB/RIF-G4. A small proportion of samples (< 5%) from patients who were culture-negative but with a history of previous TB had a likely false-positive trace readout. These data inform the management of patients with suspected TB in endemic settings.

198/200

Keywords: Xpert Ultra, trace results, tuberculosis, performance, HIV infected, smear-negative

1. INTRODUCTION.

The development of rapid and accurate diagnostic tests for tuberculosis (TB), which decreases the time of treatment initiation is an important strategy to control the TB epidemic. The WHO recommended Xpert MTB/RIF assay (Cepheid, USA) is an automated cartridge-based, real-time polymerase chain reaction (PCR) assay that has been proven to reduce time to treatment initiation in TB patients [1,2] by detecting the presence of TB and drug resistance to rifampicin (RIF^R) in sputum samples within 2 hrs . [3] It has been shown to have better sensitivity (98%) at diagnosing TB over sputum smear microscopy (20%-60%; [4]). However, it has suboptimal sensitivity in smear-negative sputum (67%; [4]), which is often the case in HIV-infected patients. [3] To improve the sensitivity Xpert Ultra was introduced, which has enhanced assay design and uses high resolution melt analysis. [5]

The reported sensitivity for Xpert Ultra in smear-negative sputum reached 78.9%, which is higher than Xpert MTB/RIF (66.1%; [5]) and the assay performed better in sputum from HIV-infected individuals (87.5% versus 68.6% for Xpert Ultra versus Xpert MTB/RIF, respectively; [6]). The threshold for detection with Xpert Ultra was ~1-log CFU better over Xpert MTB/RIF (12 CFU/ml versus 130 CFU/ml; [5]). However, this improvement in sensitivity comes at the cost of decreased specificity. [7]

However, there are several gaps in our knowledge about the utility of Xpert Ultra in HIV-infected persons, in those with a history previous TB, and the epidemiology of trace readouts has been poorly studied. It has been suggested that the latter may represent true or false positive results and optimal management of such patients are unclear.

There are also limited data about the comparative limit of detection of the two Xpert assays. Therefore, in the current study we sought to evaluate the diagnostic accuracy of Xpert MTB/RIF versus Xpert Ultra from selected patients in a TB and HIV endemic setting and explore the epidemiology of trace results.

2. MATERIALS AND METHODS.

2.1 Case Definitions.

The following case definitions were used for the analysis:

(A) Confirmed active TB: patients fulfilled **all** of the following criteria.

(i) Presented at the TB clinic with at least one WHO defined symptom suggestive of TB.

(ii) A **positive** sputum culture for *M.tb*.

(iii) Initiated on TB treatment.

(iv) Had resolution of their TB symptom(s) at 8 weeks of follow up.

(B) Confirmed Non-TB: patients fulfilled **all** of the following criteria.

(i) Presented at the TB clinic with at least one WHO defined symptom suggestive of TB.

(ii) A **negative** sputum culture for *M.tb*.

(iii) **Not** Initiated on TB treatment.

(iv) Had resolution of their TB symptom(s) and/or who had a confirmed alternative diagnosis at 8 weeks of follow up.

(C) **Previous TB:** patients fulfilled **all** of the following criteria.

(i) Presented at the TB clinic with at least one (new onset) WHO defined symptom suggestive of TB.

(ii) Had a history of one or more episodes of culture or Xpert confirmed TB.

(iii) Had completed TB treatment at least 6 months prior to their current onset of symptoms.

2.2. Archived sputum samples

Sputum samples (n=272) from symptomatic individuals suspected of having pulmonary TB were obtained between June 2013 and December 2015 from TB clinics in Cape Town, South Africa. The University of Cape Town Human Research Ethics Committee approved (approval # 068/2016) the current study and all patients provided written informed consent for study participation and bio-banking of clinical samples for downstream evaluation.

Xpert MTB/RIF and Xpert Ultra testing

The Xpert MTB/RIF and Xpert Ultra assays were performed as described previously (Helb et al., 2010). Briefly, sodium hydroxide and isopropanol-containing sample buffer (Cepheid, USA) was added to the sputum sample at a ratio of 2:1 and incubated for 15 minutes at room temperature with gentle intermittent agitation. Following incubation, 2 mL of sample was transferred to the Xpert MTB/RIF and/or Xpert Ultra cartridge and run on the Xpert system (Cepheid, Dx System Version 4.0c), depending on the outcome under investigation.

Limit-of-detection experiments.

Sputum samples from culture and smear-negative patients with no history of previous TB were combined, diluted in sample buffer and spiked with *M. tb* H37Rv at 1500, 750, 375, 188, 94, 47, 24, 12, 9, 5 and 0 CFUs/ml. Each dilution of spiked sputa was tested using the Xpert MTB/RIF and Xpert Ultra assay in triplicate and the results were compared at each concentration (. Dilutions were plated onto 7H9 Middlebrook agar (Sigma, Germany) and CFUs were counted to ensure that the spiked levels of CFU/ml were accurate.

The effect of serial freeze-thaw cycles on Xpert performance

Samples classified as smear-negative Xpert MTB/RIF-positive and culture-positive (n=16) were randomly selected from the biobank to evaluate whether freeze-thawing the sample has an effect on test performance. The samples underwent three complete freeze-thaw cycles, after which Xpert MTB/RIF was performed as described above. The cyclic threshold (Ct) values were compared to previously documented Xpert MTB/RIF results using a fresh sample at the same time of sample collection.

Statistical analysis

For demographic analysis, the chi-squared ($\chi 2$) test was employed for categorical variables and for continuous variables, Mann-Whitney test was used for non-parametrically distributed data (GraphPad, Version 6). Diagnostic accuracy of Xpert

MTB/RIF and Xpert Ultra including, sensitivity and specificity, with or without the trace call included for Xpert Ultra, was performed. Fishers Exact was utilised for comparison of the diagnostic variables Xpert MTB/RIF and Xpert Ultra. The diagnostic accuracy analysis was performed in Stata, Version 13. A p-value of < 0.05 was considered significant for all statistical analyses.

RESULTS

Patient clinical parameters and sputum sample characteristics

The demographic characteristics of the patients enrolled in the study are shown in Table 1. Patient subgroups were pre-selected to answer our research questions. Overall, the median age of the patients was 39 with ~60% (155/261) being males and ~40% being females (106/261).

A majority (~62% 104/168) of patients in the confirmed active-TB group were smear negative (i.e. potentially with pauci-bacillary disease) with ~35% (58/168) patients having had a previous history of TB. The confirmed non-TB group almost entirely [98% (102/104)] consisted of patients with a previous history of tuberculosis with an HIV prevalence of 33% (31/93) in this group. Xpert MTB/RIF and Xpert Ultra were positive for 82.1% and 82.7%, respectively amongst the confirmed active-TB patients.

Figure 1A shows the study plan of the patients tested for Xpert MTB/RIF and Xpert Ultra (n=272). Out of the 168 confirmed active-TB cases, 64 and 104 were smear-positive and smear-negative, respectively. For the confirmed active-TB, smear-negative sputum samples, Xpert Ultra had the ability to detect 5 and 3 more TB cases than Xpert MTB/RIF

in the HIV-infected and HIV-uninfected patients, respectively. Within the confirmed non-TB with history of previous TB sputum samples tested (n=100), Xpert Ultra could detect an additional 3 TB cases above Xpert MTB/RIF in the HIV-infected patients and an additional 2 in the HIV-uninfected patients.

Limit-of-detection (LOD) of Xpert MTB/RIF versus Xpert Ultra.

The LOD for Xpert MTB/RIF and Xpert Ultra are shown in Figure 1B. Culture-negative, Xpert MTB/RIF-negative sputum samples from individuals without a history of TB, and who did not receive TB treatment with resolution of their respiratory symptoms on follow up, were pooled and diluted 2:1 (lysis buffer: sputum) in lysis buffer as indicated in the materials and methods. The sputum was spiked with 1500, 750, 375, 188, 94, 47, 24, 9, 5 and 0 CFUs/mL of *M. tb* H37RV and analysed by Xpert MTB/RIF and Xpert Ultra. The Xpert Ultra sputum samples spiked with 1500 and 750 CFU/ml had similar average *rpoB* Ct values of approximately 28. The average *rpoB* Ct values increased to approximately 29 when the Xpert Ultra sputum samples were spiked with 187 CFU/ml. Xpert MTB/RIF could not detect TB at a CFU/ml below 187 and Xpert Ultra was less reproducible below 94 CFU/ml (Figure 1B), where one or more of the triplicates were negative for TB or trace. The LOD for Xpert Ultra was 9 CFU/ml.

Sensitivity of Xpert MTB/RIF and Xpert Ultra in smear-negative sputum samples from HIV-infected and HIV-uninfected patients with definite TB

The sensitivity for Xpert MTB/RIF and Xpert Ultra for the HIV-infected and HIV-uninfected sputum samples are shown in Table 2. Overall the sensitivity for Xpert MTB/RIF and Xpert

Ultra were 71.2% (95% CI; 62.5%-79.9%) and 77% (68.9%-85.1%), respectively. For the sputum samples from the HIV-infected individuals the sensitivity for Xpert MTB/RIF was lower at 63.5% (50%-76.1%) compared to that of Xpert Ultra (73.1% [61.1%-85.2%]; Table 2), however this was not significant. The sensitivity for Xpert MTB/RIF and Xpert Ultra were 73% (58.7%-87.3%) and 78.4% (65.1%-91.7%), respectively for the sputum samples from HIV-uninfected individuals.

Accuracy of Xpert MTB/RIF and Xpert Ultra in sputum samples from non-TB patients with previous history TB (false positivity rate).

The diagnostic accuracy of Xpert MTB/RIF and Xpert Ultra for non-TB patients with previous history of TB is shown in Table 3. The sensitivity for Xpert MTB/RIF and Xpert Ultra were 12.7% [7%-20.8%; p=0.470] and 11.8% [6.2%-19.6%], respectively. In the HIV infected individuals versus the HIV uninfected individuals the sensitivity of Xpert MTB/RIF was 11.4% [3.2%-26.7%] versus 13.8% [6.5%-24.7%]; p=0.732), respectively and Xpert Ultra was 17.1% [6.6%-33.6%] versus 9.2% [3.5%-19%; p=0.246], respectively.

The significance of Xpert Ultra trace readouts

In smear negative active TB group (Table 2): When the trace results for Xpert Ultra were excluded from the analysis, the sensitivity did not change for both the HIV-infected group (63.5% [49%-76.4%] and the HIV-uninfected groups (75.7% [58.8%-88.2%]) (Table 2). The Xpert Ultra trace readouts appeared higher in the HIV infected (9.6% [3.2%-21%]; p=0.199) versus the HIV uninfected individuals (2.7% [0%-14.2%]), but this was not significant.

In non-TB group with a previous history of TB (Table 3): When the trace results were excluded for Xpert Ultra the sensitivity in the HIV infected versus the HIV uninfected individuals were similar at 11.4% (3.2%-26.7%; p=0.534) and 7.7% (2.5%-17%), respectively.

Positive predictive values (PPV) and negative predictive values (NPV) of Xpert MTB/RIF and Xpert Ultra overall and when stratified to HIV-infected and smear-negative samples.

The PPV and NPV values for the study cohort overall and when stratified according to HIV-infected and smear-negative sputum samples is shown in Table S1 (supplementary material). Overall the PPV and NPV did not change for Xpert MTB/RIF versus Xpert Ultra (91.4% [55.9%-89.3%] vs. 92.1% [77.4%-100%] and 75.2% [60.6%-92.3%] vs. 76% [61.3%-93.3%], respectively). When stratified to HIV-infected sputum samples, the NPV appeared to decrease for both Xpert MTB/RIF (75.2% [60.6%-92.3%] vs. 62% [42.1%-88%]) and Xpert Ultra (76% [61.3%-93.3%] vs. 61.7% [41.3%-88.6%]), but this was not significant. The PPV and NPV did not change for both tests overall compared to smear-negative sputum samples.

The effect of Freeze-thaw cycles on the performance of Xpert MTB/RIF

The effect of freeze/thawing sputum samples on Xpert MTB/RIF performance is shown in Figure 2. Eight pairs of culture-positive, Xpert MTB-RIF-positive sputum samples were randomised and either remained fresh or were subjected to 3 freeze/thaw cycles prior to Xpert MTB/RIF. On average the Ct values for the freeze/thawed sputum samples (Ct=23;

p=0.078) were similar to the fresh sputum samples (Ct=26), indicating that freeze/thawing sputum samples does not affect Xpert MTB/RIF performance (Figure 2).

Distribution of test positive results for culture, Xpert MTB/RIF and Xpert Ultra.

The relationship between test positivity for culture, Xpert MTB/RIF and Xpert Ultra is shown in Figure 3. Culture had the ability to detect TB in an additional 19 sputum samples above Xpert MTB/RIF and Xpert Ultra. Both Xpert MTB/RIF and Xpert Ultra performed equally by detecting TB in an additional 6 and 5 sputum samples, respectively.

DISCUSSION.

We evaluated the diagnostic accuracy of Xpert MTB/RIF and Xpert Ultra using selected archived sputum samples from patients with suspected TB in a high TB and HIV prevalence setting. The three key findings in our study were: (i) Xpert Ultra had lower LOD for *M.tb* compared to Xpert MTB/RIF, (ii) trace results with Xpert Ultra were relatively infrequent, and even in the group of patients who have a predisposition for trace false positive readouts (i.e. non-TB with a previous history of TB who turned out to be culture negative and remained well on follow up), Xpert Ultra positivity was ~3%, (iii) Xpert Ultra sensitivity was consistently lower in HIV-infected persons and trace readouts were higher in this group, and (iv) overall, Xpert Ultra was 6% more sensitive than Xpert MTB/RIF in smear-negative samples although this did not reach statistical significance.

Overall, a minority of samples (3%) comprised trace readouts. This was less than the ~10% overall trace readout found by Berhanu and co-workers in South Africa. [6] In that study reclassification of trace results resulted in a loss of sensitivity of 5.6% in the smear-

negative group. [6] In the study by Dorman and colleagues the reduction in sensitivity from excluding the trace readout was almost 9% in smear-negative culture-positive persons. [8] In our study this figure was less than 6% in the smear-negative group though this increased to almost 10% in HIV-infected persons. This may be due to several factors including using a convenience sample set, patient classification based on follow up, differential disease burden, and different TB strains. The interpretation of trace readouts on the Xpert Ultra semi-quantitative scale is controversial. On the one hand it may represent a true positive, i.e. detection of *M. tb* DNA where the culture result is falsely negative. This could be due to a variety of factors including sub-clinical TB, differentially culturable mycobacteria that do not optimally grow on conventional culture media [9], sampling error (sequential paired samples collected in the field are known to be discordant due random sampling error), or alternatively samples may be falsely culture-negative due to sample preparation, death of mycobacteria during transport to the laboratory, or overgrowth of mouth flora. Indeed, in the Dorman study longer term follow-up uncovered Xpert Ultra-positive culture-negative patients who subsequently turned out to be culture-positive [8], and in the Berhanu report there were samples that became culture-positive well beyond the 42-day culture threshold limit. [6] On the other hand, trace readouts may be false positive due to technical factors including detection of DNA artefacts (e.g. primer dimers) and inherent noise at the limit of detection of the fluorescence signal (false positive signal at very low-level fluorescence). A drawback of our study was that we did not sequence the amplicons from the Xpert Ultra cartridges, which could inform on the issue of technical artefacts. Dorman and co-workers sequenced amplicons obtained from 14 cartridges (samples from 14 participants that

were Xpert Ultra-positive but culture-negative); in 12 of the 14 participants detection of *M. tb* DNA was confirmed. [8]

How trace readouts should be handled in clinical practice remains unclear and there are implications for missing a TB diagnosis versus erroneously prescribing potentially toxic treatment. The current WHO guidelines suggest that trace readouts should signal TB treatment in paucibacillary disease (e.g. HIV co-infection, extra-pulmonary TB etc.), whilst in other contexts repeat testing should be performed though this may not be feasible in endemic setting where almost ~10% of readouts are trace. [8] A prior modelling study has shown that Xpert Ultra, despite lower specificity (but higher sensitivity), could have mortality benefit in TB and HIV hyper-endemic settings whilst over-treatment of false positive cases will likely occur in low prevalence regions. [10]

Our study highlights that trace-positive results in those with a previous history of TB should be carefully considered before TB treatment is commenced. Prospective studies in patients with trace readouts will be required to provide more guidance on how to optimally manage such patients in different clinical settings. However, we also quantified the magnitude of trace readouts in those who were culture-negative but with a prior history of TB. Our cohort was particularly well-characterised, and, in such patients, we had follow-up of at least 2 months with resolution of symptoms suggesting that Xpert Ultra in this context was detecting 'old' residual DNA from a prior episode. Indeed, detection of DNA in patients with prior TB is well-described and Xpert Ultra cannot distinguish between the DNA from viable organisms and those that have demised. [11,12] We also saw the well-described phenomenon of improved sensitivity with

a trace readout, but reduced specificity as outlined by others. [6,8] In our study trace readouts occurred mostly in patients with a history of TB treatment within 2 years of their most recent TB episode (Figure 4). In this group of patients, analysing the data by re-classifying trace calls as "negative" improves the specificity from 62% to 77%.

There are few data about Xpert Ultra performance in HIV-infected persons including those with a previous history of TB. Our results suggest that Xpert Ultra sensitivity (irrespective of the version) was lower in HIV-infected participants than HIV-uninfected participants, probably likely related to the more paucibacillary nature of the disease (at least in sputum samples) in such patients. The contribution of trace readouts to improving sensitivity was higher in HIV-infected than uninfected persons. Given the low burden of mycobacteria in the sputum of HIV-infected patients, the higher mortality seen in such patients, and inherent difficulties in diagnosis, the WHO has recommended that trace readouts in such patients should signal initiation of TB treatment. [13] Our data showed that a history of previous TB had minimal impact in HIV-infected compared to uninfected persons.

There are limited data comparing performance between Xpert Ultra and Xpert MTB/RIF. [6,8] However, overall though our limited sample sizes did not reach significance, we did confirm the higher sensitivity of Xpert Ultra compared to Xpert MTB/RIF. In addition, we provide laboratory evidence that Xpert Ultra performed better in *in vitro* studies using serial dilutions of *M. tb*. This is concordant with the findings of Chakravorty and colleagues (LOD for Xpert Ultra was 15.6 CFU/ml versus 112.6 CFU/ml for Xpert MTB/RIF). [5] An interesting finding is that there were also individuals that were Xpert

MTB/RIF cartridge positive but Xpert Ultra negative. This probably represents sampling error and discordance between sequentially obtained samples is well recognised. [5]

Our study has a number of limitations. First, the small sample sizes limited our power to make intergroup sensitivity and specificity comparisons. However, we were limited by the size of our biobank, and our key aim was to perform a preliminary interrogation of trace readouts and to gain more information about performance in HIV-infected persons. Second, we used bio-banked rather than fresh samples, which may have impacted our results. However, similar trends and results were shown in the Berhanu and Dorman reports. We also undertook experiments showing that freeze-thaw probably had a negligible effect on the study findings (though again sample sizes were limited and, if anything, freeze-thaw improved performance). Third, we did not perform sequencing of the cartridge amplicons. However, we were limited by resource constraints and this would have enabled us to consolidate technical false positives but would have not corrected misclassification bias in those with previous TB. Fourth, the lower than predicted proportion of trace readouts compared to previous reports [8] may have been due to suboptimal sample volume in some of the samples (not strictly recorded prior to running the Ultra assay) although our biobanking protocols stipulated collection of at least a 1ml volume of sputum.

In conclusion, Xpert Ultra had a lower limit of detection compared than Xpert MTB/RIF. Moreover, we confirmed that a significant minority of samples (<5%) comprised trace

readouts, and this may represent a false-positive signal in those with previous TB. Prospective studies are required on how to optimally manage such patients.

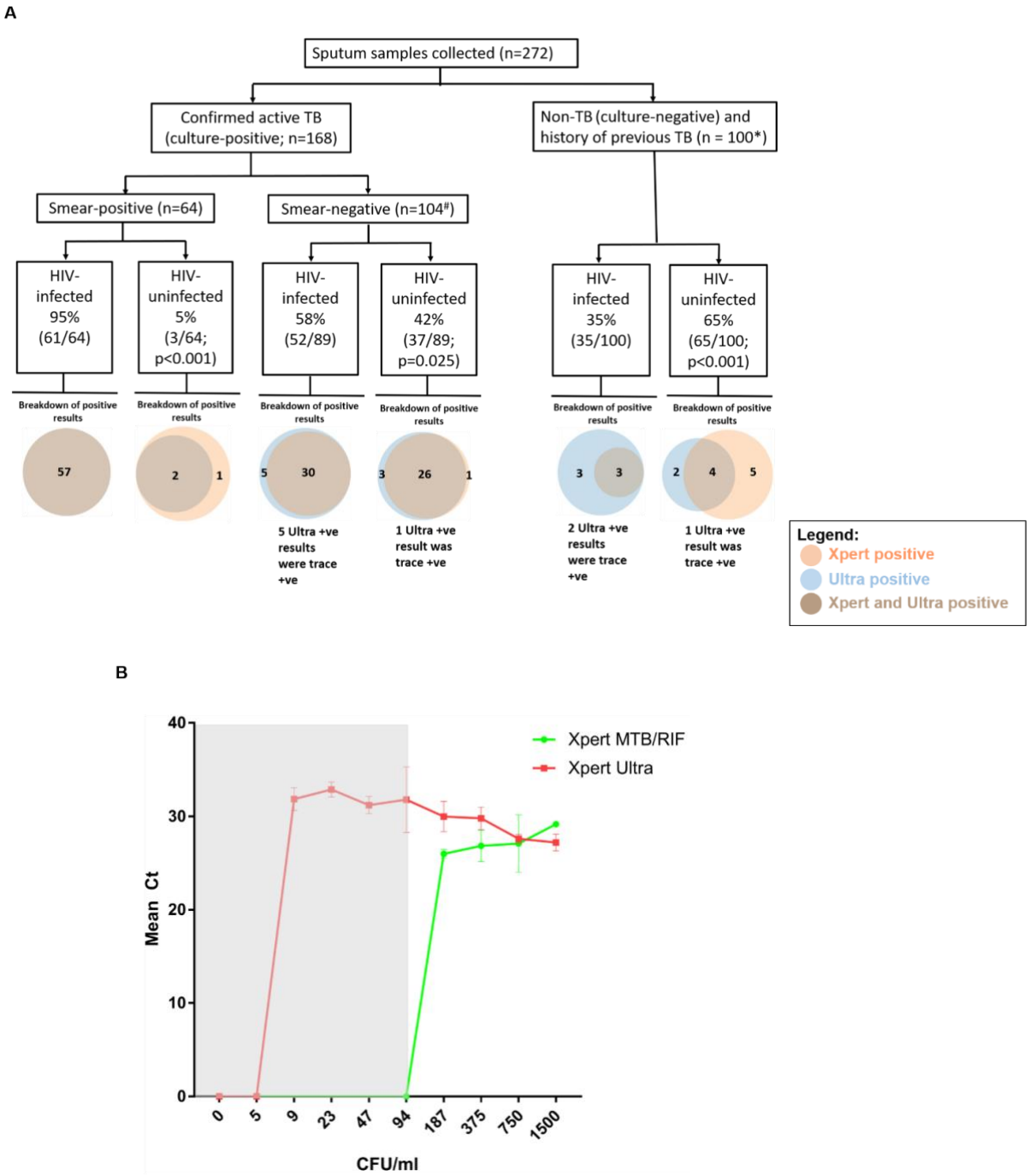

Figure 1. Study flow and LOD for Xpert MTB/RIF and Xpert Ultra. (**A**) Study flow showing the number of sputum samples analysed with Xpert MTB/RIF and Xpert Ultra. #Fifteen samples were of HIV unknown status. *Two patients had no history of previous TB and two HIV results were of unknown status. p-values compare the HIV-infected versus uninfected status. **(B)** LOD for Xpert MTB/RIF and Xpert Ultra. Culture-negative, smear-negative pooled sputum samples were diluted 1:2 in lysis buffer. The diluted sputum was spiked with H37*Rv* at 1500, 750, 375, 188, 94, 47, 24, 9, 5 and 0 CFUs/mL.

Each dilution was analysed by Xpert MTB/RIF and Xpert Ultra in triplicate. The shaded area indicates the relative CFU/ml where one or more of the replicates were either Xpert Ultra negative or trace. Xpert = Xpert MTB/RIF, Ultra = Xpert Ultra.

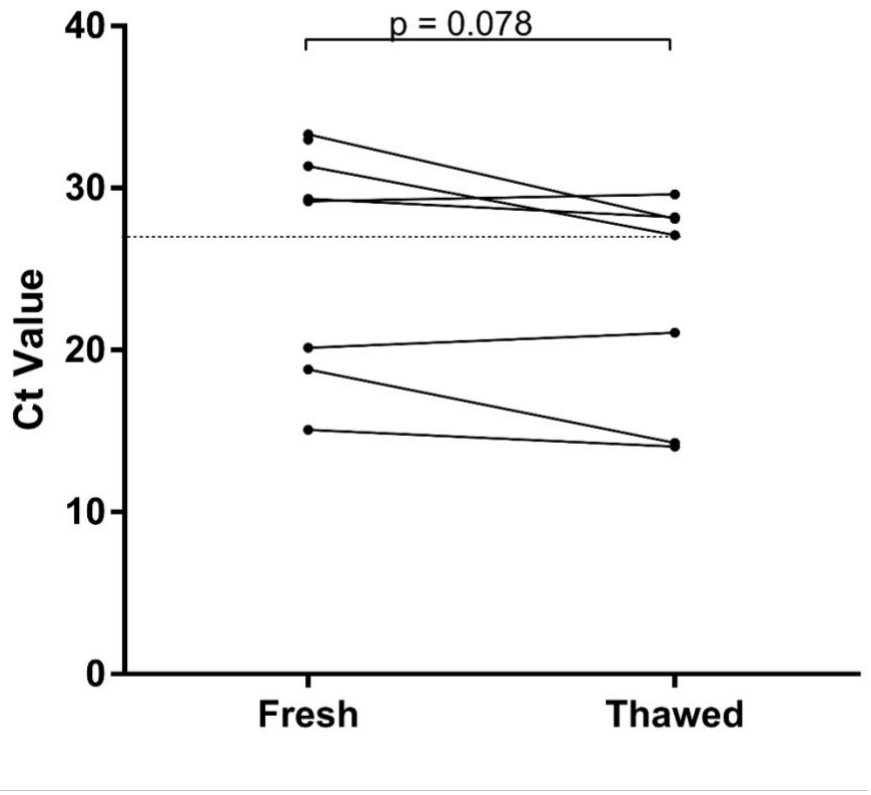

Figure 2. Ct values of fresh or freeze-thawed (3 cycles) sputum samples (16 sputum samples; 8 pairs of samples). Smear-negative culture-positive Xpert MTB/RIF-positive sputum samples were subjected to three rounds of freeze/thaw prior to repeat Xpert MTB/RIF. The dotted line represents the median Ct value=27.

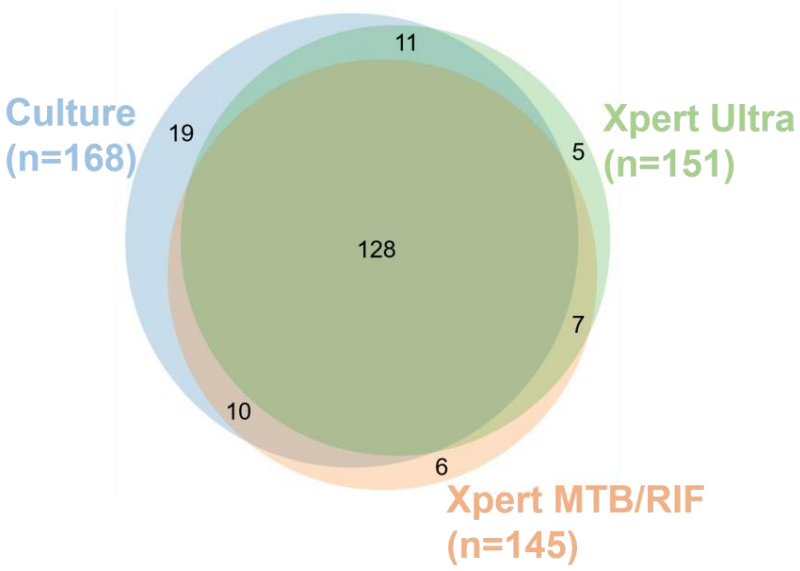

Figure 3. Venn diagram showing the relationship between test positivity for Xpert MTB/RIF (n=145), Xpert Ultra (n=151), and culture (n=168).

Tables.

Table 1. Demographic characteristics of the sub-groups (data are n (%) unless otherwise stated).

Demographic data	All samples (%) (n = 272)	Confirmed active-TB (%) (n = 168)	Non-TB (%) (n = 104)	p-value
Age				
****Median years (range)**	39 (19-68)	39 (19-65)	39 (26-68)	
Gender				0.1289
Male	164 (60.3)	94 (56)	70 (67.3)	
Female	108 (39.7)	74 (44)	34 (32.7)	
HIV-infected				< 0.0001
Yes	148 (54.4)	113 (67.2)	35 (33.7)	
No	107 (39.3)	40 (23.8)	67 (64.4)	
Not determined	17 (6.5)	15 (8.9)	2 (1.9)	
CD4 count (cells/ml) (range)#	235 (6-788) $^{\Psi}$	235 (6-788)$^{\omega}$	235 (25-681)$^{\phi}$	0.1494

Smear status				<0.0001
Smear-negative	191 (70.2)	104 (61.9)	87 (83.7)	
Smear-positive	68 (25)	64 (38.1)	4 (3.8)	
Unknown	13 (4.7)	–	13 (12.5)	
Previous TB				<0.0001
Yes	160 (58.8)	58 (34.5)	100 (98.0)	
< 5 years ago	61	24	37	
5 ≤ 10 years ago	68	22	46	
> 10 years	56	14	42	
No	111 (40.8)	109 (64.9)	2 (1.9)	
Unknown	1 (0.4)	1 (0.6)	–	
Xpert MTB/RIF				< 0.0001
Positive	151 (55.5)	138 (82.1)	13 (12.5)	
Negative	121 (44.5)	30 (17.9)	91 (87.5)	
Xpert Ultra				< 0.0001
Positive	151 (55.5)	139 (82.7)	12 (11.5)	
Negative	121 (44.5)	29 (17.3)	92 (88.5)	
Trace	9/272	6/168	2.9 (3/104)	

** Median (range)

[#]Performed if HIV-infected. There was no CD4 count data for 5^{Ψ}, 3^{ω} and 2^{ϕ} patients, respectfully.

Table 2. Sensitivity of Xpert MTB/RIF and Xpert Ultra in smear-negative culture positive samples stratified according to HIV status.

	Confirmed smear-negative TB (n=104♠)		
	Overall (n=104)	**HIV-infected (n=52)**	**HIV-uninfected (n=37)**
	Positive (%, 95%CI, n/N, p-value)	**Positive (%, 95%CI, n/N, p-value)**	**Positive (%, 95%CI, n/N, p-value)**
Xpert MTB/RIF	**71.2%**, 62.5%-79.9%, 74/104	**63.5%**, 50%-76.1%, 33/52	**73.1%**, 58.7%-87.3%, 27/37 #p=0.345
Xpert Ultra (with trace)	**77%**, 68.9%-85.1%, 80/104, p=0.343	**73.1%**, 61.1%-85.2%, 38/52, *p=0.292	**78.4%**, 65.1%-91.7%, 29/37, *p=0.588 #p=0.568
Xpert Ultra (without trace)	**71.2**%, 61.4%-79.6%, 74/104, p=0.484	**63.5%**, 49%-76.4%, 33/52,	**75.7%**, 58.8%-88.2%, 28/37, *p=0.790 #p=0.221
Xpert Ultra (trace readout)	**5.8%** 2.1%-12.1% 6/104	**9.6%** 3.2%-21% 5/52	**2.7%** 0%-14.2% 1/37 #p=0.199

♠Fifteen patients had an unknow HIV status.
*P values are for comparison between Xpert MTB/RIF and Xpert Ultra.
#P values are for comparison between HIV-infected and uninfected.

Table 3. False positive rates (specificity) of Xpert MTB/RIF and Xpert Ultra for the detection of TB in sputum samples from non-TB patients with previous history of TB.

	Non-TB with history of previous TB (n=102♠)		
	Overall (n=102)	**HIV infected (n=35)**	**HIV uninfected (n=65)**
	Positive (%, 95%CI, n/N, p-value)	**Positive (%, 95%CI, n/N, p-value)**	**Positive (%, 95%CI, n/N, p-value)**
Xpert MTB/RIF	**12.7%**, 7%-20.8%, 13/102	**11.4%** 3.2%-26.7% 4/35	**13.8%** 6.5%-24.7% 9/65 #p=0.732
Xpert Ultra (with trace)	**11.8%**, 6.2%-19.6%, 12/102, p=0.470	**17.1%** 6.6%-33.6% 6/35 *p=0.495	**9.2%** 3.5%-19% 6/65 *p=0.410 #p=0.246
Xpert Ultra (without trace)	**8.8%**, 4.1%-16.1%, 9/102, p=0.70	**11.4%** 3.2%-26.7% 4/35	**7.7%** 2.5%-17% 5/65 *p=0.258 #p=0.534

♠Two patients had an unknow HIV status.
*P values are for comparison between Xpert MTB/RIF and Xpert Ultra.
#P values are for comparison between HIV status.

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Chapter 3: The diagnostic impact of POC-Xpert Ultra in community-based active case finding strategies.

Theme: (i) Diagnostic impact of the more sensitive Xpert Ultra assay when used at **point-of-care** for the detection of TB in community-based (minimally symptomatic or HIV-infected) participants. (ii) Diagnostic impact of Xpert Ultra in detecting potentially infectious TB patients

Sub-population of TB under investigation: Community-based participants with minimal symptoms and/or HIV-infection

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My contributions to this body of work: My supervisor and I conceived the idea and designed this project. I was also intricately involved in applying for grant funding to conduct the project. I led the team conducting this trial, interpreted clinical results, performed the data-analysis and interpreted the analysed data. Lastly, I lead the team in drafting the manuscript and submitted it for peer-review. And saw it through to publication.

Summary of the main findings in this publication: performing Xpert Ultra at POC as part of a scalable strategy for community-based active case finding using mobile labs is feasible. Although, Xpert Ultra missed ~50% of culture positive TB, it detected almost all potentially infectious patients.

nature medicine

Comparison of two diagnostic intervention packages for community-based active case finding for tuberculosis: an open-label randomized controlled trial.

ABSTRACT

In 2020 it was estimated that 2 in every 5 patients with active tuberculosis (TB) remained undiagnosed or unreported. Therefore community-based active case-finding strategies require urgent implementation. However, whether point-of-care (POC) portable battery-operated molecular diagnostic tools deployed at community level, compared to POC smear-microscopy, can shorten time-to-treatment initiation , thus potentially curtailing transmission, remains unclear. To clarify this issue, we performed an open-labelled randomised controlled trial in two peri-urban informal settlements in Cape Town, South Africa, where we screened 5274 individuals using a community-based scalable mobile clinic. 584 individuals with HIV infection or symptoms of TB were randomised (1:1) to same-day smear-microscopy (n=296) or onsite DNA-based molecular diagnosis (n=288; Xpert). TB infectiousness was inferred in participants with at least one of the following criteria: culture positive status on quantitative cough aerosol sampling, presence of cavitation on chest radiographs, and/or smear-positive status. 9.9% (58/584) of participants who underwent targeted screening had culture confirmed TB. Time-to-treatment initiation (the primary outcome) occurred significantly earlier in the Xpert versus the smear-microscopy arm [8 versus 41 days, p=0.02; restricted-mean-time-lost

ratio 2.4 (95%CI 1.3-4.5), p=0.008]. However, Xpert detected only 52% (30/58) of those with culture-positive TB. Importantly, Xpert detected almost all of the likely infectious patients compared to smear-microscopy [16/17 (94.1%) versus 4/17 (23.5%); p=<0.001], Xpert was associated with a shorter median time to treatment of likely infectious patients [7 versus 24 days; p=0.02], and a greater proportion of infectious patients were on treatment at 60 days compared to the likely-non-infectious patients [76.5% (13/17) versus 38.2% (13/34); p<0.01]. Overall, a greater proportion of POC Xpert-positive participants were on treatment at 60 days compared all culture-positive participants [100% (16/16) versus 46.5% (27/58); p<0.01]. These findings challenge the traditional paradigm of a passive case-finding public health strategy and argues for the implementation of portable DNA-based diagnosis as a community-orientated transmission-interruption strategy. Collectively, these findings inform improved active case-finding strategies in TB endemic settings, and emphasizes the need to improve linkage to care in such models.

South African Clinical Trials Registry (SANCTR): DOH-27-0317-5367

ClinicalTrials.gov: NCT03168945.

Main text

Tuberculosis (TB) is now the world's second leading cause of death by an infectious disease contributing to ~1·5 million deaths annually in 2020 after COVID-19 [1]. Despite advances in TB diagnostics, ~4 million (almost 2 in 5) patients remain undiagnosed or unreported globally, the majority of whom reside in peri-urban informal settlements of large cities in Africa and Asia [1,2]. The COVID-19 epidemic has worsened this situation with case detection plummeting by ~ 30 to 50% [3]. The 'missing' patients are important since they serve as a reservoir for transmission of drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis* [4]. Indeed, modelling studies have indicated that substantial reduction in transmission, and hence disease burden and mortality, will require community-based active case finding (ACF; health care worker seeks, identifies, and procures samples for TB testing in the community) rather than passive case-finding (patient-driven self-presentation at a health care facility), which detects cases after most transmission has already occurred [2,5-7].

There are several approaches to performing ACF in high prevalence settings including targeted screening of high-risk groups (e.g. close contacts) [8-10], community-based door-to-door screening [5,10-12], and community-based screening using a mobile unit or clinic [13,14]. Door-to-door ACF using *laboratory-based* molecular tools like the Cepheid GeneXpert® system (Sunnyvale, USA), which has recently shown to have favorably impacted disease burden in the wider community [5]. However, the door-to-door ACF strategy is labour intensive and may not be affordable in many settings. Corbett *et al*, prior to the availability of automated molecular diagnostic tools, showed that both door-

to-door and a mobile unit-based screening strategy favorably impacted disease burden at community level but the latter was more efficient [13]. Advancing these findings we showed, in a randomised controlled trial, that a mobile clinic/unit-based approach using point-of-care (POC) molecular tools was feasible and effective using a high-cost truck equipped with generator-powered GeneXpert [15]. Two subsequent cross-sectional studies showed that mobile clinic-based ACF approaches using molecular tools were feasible, with a number-needed-to-screen (NNS) of between 15-20 [14,16]. Notably, these studies were personnel intensive [staffed by doctors [14,16], nurses [14,16], laboratory technicians [16], and radiographers [14,16]] and used relatively high-cost non-scalable approaches including electricity-driven mobile laboratories with capacity to perform on-site smear microscopy, molecular testing and/ or chest radiography [16]. However, such models are resource-intensive and not easily scalable, limiting their applicability in TB-endemic settings. In summary, controlled trials to evaluate the utility of molecular tools at primary care level, have hitherto, not been undertaken. Indeed, the recent WHO guidance on systematic screening [17] and a recent systematic review [18], have underscored the need for well-designed studies to elucidate which ACF delivery methods and diagnostic strategies are most effective.

More recently, a portable battery-operated version of GeneXpert has become available (GeneXpert Edge) that is ideally suited as a POC diagnostic that could be incorporated into a scalable and affordable ACF model. We evaluated the feasibility of such a model (designated XACT) incorporating a 2-person mobile clinic using a low-cost multi-purpose vehicle (MPV) minivan (< USD $12 000) equipped with GeneXpert Edge. An imperative of

any such approach is not only to detect all the TB patients but also to detect all the *infectious* patients that drive transmission. We therefore prioritised evaluating the model's efficacy to detect infectious patients, which we identified using a combination novel cough aerosol sampling technology, imaging characteristics, and smear microscopy status. Given this imperative we compared the molecular strategy to a same-day smear microscopy strategy for several reasons. Firstly, and most importantly, at the time of study design smear microscopy was the standard of care in primary health settings in TB endemic countries, and this remains so in many African countries. More recent WHO guidance (2021) recommends Xpert for active case finding in HIV un-infected persons at the discretion of the health care provider, as the available evidence is of very low quality and based on a small dataset [17], and the use of symptom screening with smear-microscopy is endorsed in resource-poor settings [19]. Second, at the time the study was planned we hypothesised that the smear-microscopy would detect the infectious patients to the same extent as GeneXpert (given the much higher mycobacterial burden in presumed infectious patients, most of whom would likely be smear positive). Third, although Xpert has clearly been shown to be more sensitive that smear-microscopy in numerous large clinic and hospital-based studies [20,21], there are hardly any data from paucibaciliary populations (e.g. with minimal disease burden as is commonly seen in the context of ACF). In such a sub-population Xpert testing might miss a substantial proportion of low burden disease (mycobacterial load below the detection limit of the assay) thus significantly negating its intuitively higher sensitivity or beneficial effect [22] . Fourth, Xpert's utility might be negated by false positive readouts in those with previous TB (~20% of those presenting with symptoms in many endemic settings), and finally that that Xpert-positive persons (because they are likely to be minimally

symptomatic) are more likely to decline or fail to continue with treatment. Collectively, the rationale and equipoise underpinned by these reasons mandated why same-day smear microscopy was used as the comparator in our trial. Thus, in summary, the primary aim was to determine whether Xpert led to significantly shorter time to treatment initiation, and the key secondary aims were to determine whether Xpert identified a higher proportion of TB (especially infectious TB), and whether the scalable XACT model using advanced portable genomic technology was feasible in a TB endemic setting. Our findings confirmed that Xpert was associated with a more rapid time to treatment initiation, detected a higher proportion of patients (albeit only 50% of the total burden) but detected almost all the likely infectious cases, and was feasible in a resource-poor endemic setting.

Online Methods

More details about the mobile clinic setup, randomization, testing procedures and timeframes, treatment referral protocols, and statistical methods can be found in the online supplement

Trial design and participants

This parallel group single centre open-labelled randomised control trial was performed in the peri-urban resource-poor Mitchel's Plain and Klipfontein sub-districts of Cape Town, South Africa. These sites were selected because they have a high density of informal settlements (~9000 persons per km^2). A research nurse and a community health care worker screened potential participants at the mobile clinic (vehicle equipped with a portable awning providing shelter, fold up tables, HIV lateral flow testing capability, a small portable fold-up cubicle for privacy during sputum acquisition, and which securely housed the portable GeneXpert Edge system). The initial rapid screen at the mobile clinic took ~5 minutes, and comprised 5 questions related to the presence of TB symptoms and HIV status. Participants who provided informed consent (over the age of 18 years) were randomised if they had at least one TB symptom for ≥ 2 weeks or were HIV-infected irrespective of symptoms (positive HIV test onsite or were known to be HIV-infected). Participants who had attended a TB clinic for their symptoms or those who were on TB treatment were excluded. Screening was clustered around community congregate settings e.g., outside community centres, shopping malls, and churches. The study was registered with the South African Clinical Trials Registry (Application ID 4367; DOH-27-

0317-5367) and ClinicalTrials.gov (NCT03168945). The registration with SANCTR occurred prior to the recruitment of the first patient.

Randomisation

Participants that were eligible were randomised (1:1) to undergo either sputum smear microscopy (smear arm) at a nearby microscopy centre (within ~5 km radius) or POC Xpert (Xpert arm) using a block size of 4. The clinical staff had access to TB culture results for all participants but only had access to POC-Xpert results for participants in the Xpert arm, and smear microscopy results for participants in the smear arm. The laboratory staff were blinded to the POC-Xpert results and clinical details of the participants. Laboratory based Xpert testing for all participant (in addition to the POC Xpert testing) was performed using biobanked samples [23].

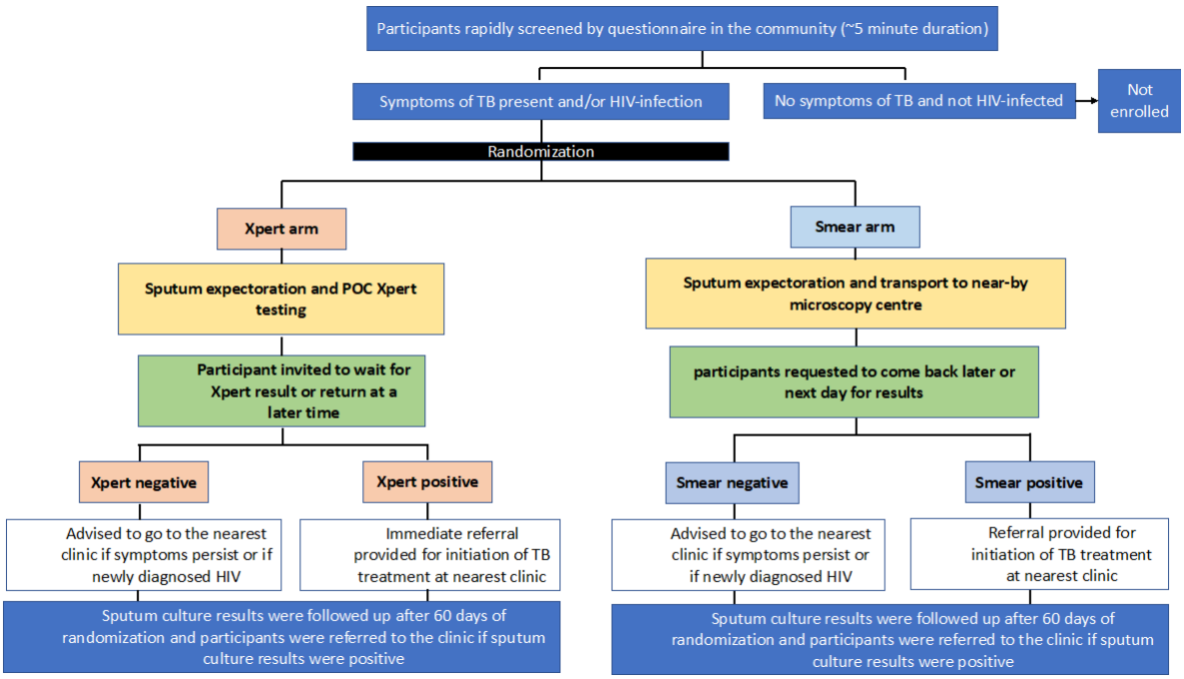

Figure OM 1 (of online methods). Overview of the recruitment activities and procedures for the XACT-II study.

Procedures.

All specimen collection was performed at POC. Xpert testing was performed by the trained research nurse/community health worker at POC using GeneXpert Edge and Xpert Ultra cartridges. Sputum for smear microscopy (auramine staining) was dispatched to a near-by microscopy centre. POC-Xpert results were interpreted by the personnel performing the test while smear microscopy results were emailed by the microscopy centre to a designated clinical staff member as soon as they were available. A second sputum was collected for TB culture. A further sputum sample was bio banked in both arms of the study (all sputum samples were collected over a period of 60 mins). If participants could not produce sputum, sputum production was induced using a standard protocol, which was also available as part of the mobile unit. If patients were

diagnosed with TB, they were referred to the nearest clinic for treatment (within a 5km radius) using a standardised referral template. Participants who tested positive in the Xpert arm of the study were referred to the TB clinic during the same visit while participants in the smear arm of the study were contacted telephonically to return to the mobile clinic and subsequently referred to the TB clinic to initiate treatment (the referral letter contained the Xpert and smear microscopy results and participants were informed and advised about the nature of the disease and importance of initiating treatment). Sputum culture results were only evaluated 60 days after randomization for all participants (see Figure 1 above). Participants who tested positive for TB on sputum culture were traced and given a referral letter to initiate TB treatment at the nearest TB clinic.

Feasibility of implementing the XACT ACF model: Xpert testing was performed on paired sputum samples (one test performed in the mobile van at POC and the other in a research laboratory at the end of the study) to assess the competency of the health care workers that had no prior laboratory experience or training. The research nurse/community health care workers were trained on Xpert testing using a hybrid model (didactic lecture of ~1 hour and a 2 hour hands-on session). The trained staff were then paired with an experienced 'Xpert tester' in the field for a duration of 4-6 weeks to obtain hands-on experience. User appraisal questionnaires were also administered to assess staff competency in performing Xpert in the community at baseline (day of training), 4 weeks and 8 weeks. The number of 'days lost' due to technical (e.g. equipment, staffing and vehicle) or environmental factors (e.g. weather and socio-political factors) were also

recorded. A ‘day lost’ was defined by the inability of the ACF team to perform any screening activities.

Determination of infectious patients: Infectious patients were defined as those who were expectorating culture positive aerosol (< 10uM) using a cough aerosol sampling system (CASS) and/ or who had acid-fast bacilli detectable on sputum smear microscopy and/or those with cavitary disease evident on chest x-ray. Smear microscopy samples were processed in real time for participants in both arms of the study. However, the smear results for participants in the Xpert arm were withheld by the laboratory staff (i.e., clinical staff and recruitment staff were blinded) up until the last participant had achieved the primary endpoint.

Cough aerosol sampling system (CASS) assessment: All patients with positive results on sputum Xpert, sputum smear-microscopy and/or sputum culture (when culture only resulted in treatment initiation) were evaluated with CASS at a separate facility prior to, or within 48 hours of starting treatment. This validated technique quantifies and size-classifies cough aerosol particles that contain culturable *M. tuberculosis.* Patients enter the cubicle and cough into the mouthpiece. B) The cough particles flow, with the aid of a vacuum pump, to a chamber holding an Andersen six-stage cascade impactor (A). Air is filtered and the CASS chamber is sterilized between each use. C) shows the cough aerosol capture plates following incubation. Aerosols from each stage of the impactor are deposited on to plate holding selective culture media mirroring the position of the filter apertures; each colony represents a footprint of likely one M. tuberculosis-

containing infectious particle. Colony forming units (CFUs) on each plate were enumerated.

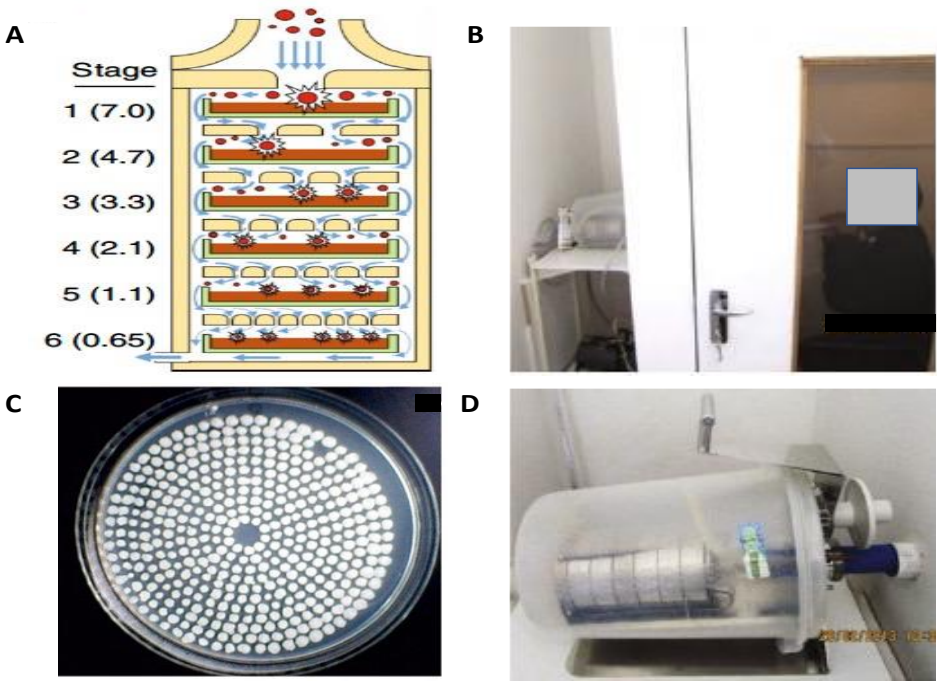

Figure OM 2 (online methods). Cough Aerosol Sampling System (CASS) used to measure culturable M. tuberculosis in cough aerosol droplets as a surrogate for infectiousness. The CASS system consisting of a six-stage Anderson cascade impactor (ACI) showing the median expected droplet diameter (μm) for each stage (Panel A).Patient sitting in a negative pressure cubical while coughing into a mouth piece that is attached to the ACI (Panel B).Culture plate that enables CFUs from individual aerosol droplets to be isolated (Panel C) The ACI was horizontally contained in a 10 litre autoclavable chamber (panel D).

Chest X-ray assessment: All patients with a positive result on sputum Xpert, sputum-smear microscopy and/or sputum culture underwent a single standard posterior-anterior projection chest x-ray assessment prior to/or within 72 hours of treatment.

Outcomes

The primary endpoint was time-to-treatment in patients with *M. tb* culture positive sputum who initiated therapy within 60 days of diagnostic testing. This time threshold was chosen to allow for the results of sputum TB culture to become available. Secondary outcomes included (i) feasibility of the XACT ACF model (evaluated by comparing performance of Xpert at POC versus in the laboratory; user appraisal questionnaires and determination of days lost); (ii) detection efficacy of potentially infectious patients; (iii) time to treatment of likely infectious patients; (iv) the proportion of culture-positive TB patients initiating TB treatment in each study arm; (v) the number needed to screen to detect a single patient with TB.

Statistical analysis

We reasoned that a total of 34 culture-positive participants (17 in each arm) would be required to demonstrate a 3.5-fold reduction in the time-to-treatment initiation assuming 95% confidence and 80% power i.e. assuming a risk ratio of 3·5 (patients were likely to start treatment 3·5 times sooner in the Xpert versus smear arm based on the findings of the XACT 1 study). We calculated that 500 at risk participants would require targeted screening (i.e., participants who were HIV infected or had at least one symptom of TB) after rapidly screening ~5,200 participants to detect at least 40 culture-positive TB patients (20 in each arm) taking into account a 10% loss to follow-up, a TB prevalence of

8%, and rapid screen to targeted screen ratio of 1:10). The probability of an event in the Xpert group relative to the smear group per unit time was expressed as a hazards ratio, or mean restricted time lost ratio when the hazard assumption was not met. Full details about sample size derivations and statistical methods are provided in the online supplement.

Results

Trial population

A total of 5274 participants were screened between November 2016 to February 2019. The demographic characteristics of the participants are shown in Table 1.

Figure 1 outlines the consort diagram, which includes 596 participants. 295 and 301 participants were randomised into the Xpert and smears arms, respectively. Of the participants who underwent randomisation, 7 were excluded from the Xpert group while 5 were excluded from the smear group because of lack of symptoms (1 patient prom each group) or being sputum scarce despite sputum induction (6 patients in the Xpert arm and 4 in the smear arm). The remaining 288 participants in the Xpert group and 296 participants in the smear group were included in the analysis. 11.1% (32/288) of participants in the Xpert group and 8.8% (26/296) in the smear group had detectable growth on M. tuberculosis cultures from their sputum specimen. TB treatment was initiated in 56.3% (18/32) and 34.6% (9/26) in the Xpert and smear groups, respectively (including culture-only based treatment initiation)

Patients randomised to the Xpert group initiated treatment sooner than patients randomised to the smear group

Absolute time-to-treatment initiation [95% CI (IQR)] was significantly shorter in the Xpert compared to the smear group [8 (4.3-29.5) vs. 41 (24.0-71.5) days; P=0.002]; Table 2. Comparatively, Xpert was associated with a more rapid time to treatment initiation within 60 days of testing (RMTL ratio: 2.4 (95% CI 1.3-4.5 p=0.008; hazard ratio: 2.3 (95% CI 1.0-5.1 p=0.04 (Figure 2A). This shorter time to treatment initiation was irrespective of whether start of therapy was based on the same day test result alone or combination of same day and culture result (Figure 2). Although the proportional hazards assumption was violated, the hazard ratio very closely approximated the RMTL ratio, and both were significant (Figure 2A). The RMTL ratio indicates that the mean treatment time gained over 60 days was ~2.4 times greater in the Xpert compared to the smear arm. Proportionally, Xpert detected more TB patients than smear microscopy (50.0% [16/32] versus 11.5% [3/26]; p=0.03). A greater proportion of participants needed to be screened in the smear group (n=98.6) versus the Xpert group (n=18.0) to detect one patient with TB. 235/288 (81.6) of participants in the Xpert arm and only 42/296 (14.2) of participants in the smear arm received their results on the same day; p < 0.01. Overall, only 27/58 (46.5%) of those with microbiologically proven TB initiated treatment (including culture-only-based treatment initiation) with in 60 days [Xpert arm 18/32 (56.3) vs. smear microscopy arm 9/26 (34.6); p=0.10]

Xpert detects more infectious patients than smear microscopy.

The proportion of likely infectious and likely non-infectious culture-positive patients are shown in Figure 3 and Table 3· Of all the culture-positive patients (n=58), only 51 participants had all three diagnostic tests portending infectiousness (cough aerosol sampling system [CASS] and chest x-ray and smear status). 17/51 participants were deemed infectious (positive by one or more of the 3 criteria) and 34 were deemed likely non-infectious (negative by all 3 criteria; Figure 3). Overall, Xpert detected more infectious TB patients than smear microscopy 94.1% [16/17] versus 23.5% [4/17]; p=0.004; Table 3). This pattern remained consistent when defining infectiousness using different combinations and permutations including by CASS and cavitary status alone (see Table 3). Notably, the median time to treatment initiation for infectious patients was significantly earlier in the Xpert arm (7 versus 24 days; p= 0.02; i.e. 17 days earlier in the Xpert arm; Table 2).

Feasibility of implementing the XACT model for active case finding.

The diagnostic accuracy of POC Xpert performed by minimally trained health care workers was comparable to Xpert performed by a qualified technician in a research laboratory (sensitivity 0.52 vs. 0.61 and specificity of 0.98 for both; p=0.46 see Table S1). A total of 36/494 (7.3%) days were lost due to logistical challenges (breakdowns, inclement weather etc.); mechanical problems with the vehicle was the most common reason of the lost screening days (see table S2).

Discussion

To our knowledge this is the first controlled trial to show that an ACF strategy using a mini-mobile clinic-based scalable intervention package, incorporating a low-cost minivan and portable battery-operated Xpert (i.e. the XACT model), was feasible and more importantly detected the majority of infectious TB patients. Importantly, these were patients who did not self-report to health care facilities. The number needed to screen was 18 for Xpert versus 99 for smear-microscopy. Furthermore, POC Xpert detected a higher proportion of culture-positive TB patients initiated on treatment within the first 60 days post-testing, and significantly reduced time-to-treatment initiation compared to same-day smear microscopy performed at a near-by microscopy centre (within a 5 km radius).

There are recent reports confirming the feasibility of using the Xpert Edge system at POC for the diagnosis of TB [24,25] . However, we have now established the feasibility of using Xpert Edge as part of a standardised intervention package for community-based ACF. We demonstrated that community based ACF using the XACT model was feasible with good concordance between Xpert performed at POC by minimally trained health care workers versus Xpert performed at a centralised laboratory (see supplementary material; Table S1; p=0.52). Indeed, the XACT ACF model was well accepted within communities, with a vast majority (~98%) of the invited eligible participants being recruited into the study. Furthermore, we showed that minimally trained health care workers can correctly perform Xpert at POC and trouble shoot problems with the Xpert machine software. Only 7.3% (36/494) of days were “lost” due to non-screening, which were attributable to

problems with the vehicle, bad weather, community political protests, problems with the Xpert platform and staffing issues (see supplementary material; Table S2). Importantly, the model is scalable (more easily reproducible because of better affordability) compared to more expensive and retro-fitted larger trucks that are manned by more personnel, use a generator, and use larger Xpert modules etc. Furthermore, such vehicles are less suited to densely populated shanty towns with narrow roads and lanes. Thus, in summary, the XACT model is feasible in endemic settings and reflects real world conditions.

Xpert performed at point-of-care identified significantly more patients with culture-positive TB, reduced time-to-treatment initiation (8 vs 41 days) and enabled the initiation of treatment within 60 days of testing in a significantly larger proportion of culture-positive patients compared to same-day smear microscopy. This is mainly attributable to Xpert's higher detection rate for culture positive TB and its ability to facilitate prompt referral for treatment initiation during the same interaction (reducing pre-treatment loss-to-follow up). Delays in obtaining a same day result (higher proportion in the smear group received results the following day) contributed negligibly to this effect. The overall case detection amongst participants with HIV infection and/or symptoms suggestive of TB (targeted screened group) was high at ~10%. This rate is relatively higher than that found in the Philippines where POC Xpert was associated with a 6% TB rate in targeted screened prisoners, whilst in Nepal a minivan-based POC Xpert was associated with a 5% rate of confirmed TB detection (n = 1239) in those that were targeted screened [14,26]. In the latter study, the yield of TB was highest in HIV-infected persons at 6%. The higher

yield shown in our study probably reflects a combination HIV endemicity (~20% of the population) and higher rates of socioeconomic deprivation. However, despite the superior performance of Xpert, its over-all sensitivity in detecting culture-positive TB was only ~51% whilst retaining a high specificity of 98%. This may be attributed to the pauci-bacillary nature of disease in this community-based cohort. Indeed, Lawn and colleagues have previously characterised this group of patients (Xpert-negative culture-positive) as having less severe disease (less symptomatic but higher CD4 count, body mass index, and haemoglobin level) [27]. There were several reasons for selecting a study design using smear microscopy as a comparator and the suboptimal sensitivity of Xpert (compared to culture) was expected based on our previous experience [15]. Firstly, at the time of study design symptom screen and smear microscopy was the standard practice in most TB endemic countries and this remains the case in many resource limited countries [19,28]. More recently (2021) the WHO issued a conditional recommendation (at the discretion of the provider and the patient) for use of Xpert in the context of systematic screening given the very poor quality of evidence and scanty available data [17], and the use of symptom screening and smear-microscopy is endorsed for ACF in resource-poor settings [19]. Second, we had reasoned *a priori* that smear may well have picked up all the infectious patients, third that Xpert may have performed even more poorly (pick up was only 50%) thus negating its intuitively higher sensitivity (in the context of low burden disease), fourth that Xpert's utility might be negated by false positive readouts in those with previous TB [29], and finally that that Xpert-positive persons (because they are likely to be minimally symptomatic) are more likely to decline or fail to continue with treatment (further negating possible benefits of Xpert). Collectively, all these reasons mandated that the study design and that smear microscopy be the comparator arm. Our findings also

highlight the need to develop more sensitive user-friendly rule-in tests for community-based ACF [experimental approaches have included urine biomarker-based assays [30], exhaled breath-based assays[31], genomic [5] and serum-based assays [32], and computer aided chest x-ray diagnosis (CAD) [33]]. Indeed, in our study, ~90% of patients who were Xpert-negative and culture-positive had chest x-ray abnormality (see supplementary material; Table S3).

Significantly, although detecting only 50% of culture-positive patients, Xpert was highly effective in detecting a majority of potentially infectious patients (~90% compared to 25% with smear microscopy; Table 3), and the median time to treatment of these likely infectious patients was 17 days sooner than in the smear arm. This is a critical point as the overarching purpose of ACF is to interrupt transmission and should hence detect most infectious patients. Xpert succeeds over smear microscopy in this respect. However, the definition of infectiousness is controversial as although cavitary disease is associated with transmission and smear positivity [34,35], ~15% of all tuberculosis transmission within the community occurs in smear-negative persons [36,37], and only a minority (~ one third) of smear-positive persons have detectable culture-positive cough aerosol (culturable bacilli in cough microdroplets less than 10μm in size) [38]. Notably, in the latter study, CASS positivity was associated with clinically less well persons with minimal symptoms yet stronger cough strength, highlighting that those with minimal symptoms may be highly infectious [38]. As there is no clear-cut definition of what constitutes infectiousness, we used a flexible and non-redundant approach incorporating the presence of smear positivity and/ or cough aerosol positivity, and /or cavitary disease. Indeed, CASS is the only measure of infectiousness that correlates with clinically meaningful endpoints i.e. TST conversion and development of active TB within

2 years [39,40], and over 50% of CASS positive persons or those who had cavitary disease were smear-negative. Given the equipoise around the definition of infectiousness we analysed our data using several definitions including combinations and permutation of CASS, smear and cavitary disease (using a validated assessment tool and 2 independent readers). Our conclusions remained unchanged despite the use of different definitions (Table 3).

Importantly, only 46.5% of culture +ve participants (though 100% of Xpert +ve persons) were on TB treatment at 60 days. This was due to a combination of factors including disease stigmatisation, social and health system factors that may have modulated access to treatment (e.g. transport costs, impact on work, etc.), migration of individuals (significant in areas with high rates of informal housing as in the study population), and the presence of minimal symptoms (or no symptoms in HIV infected persons) that may have made individuals more likely not to initiate or continue with treatment. The latter factor is likely playing a significant role as we found that a high proportion of the patients who were likely infectious (higher mycobacterial load, more symptoms, and higher rates of cavitory disease) were on treatment compared to those who were likely non-infectious. The pragmatic trial design meant that patients were provided with a single TB education session and a referral letter (with the results) linking them to care. Given these considerations, future studies and programmatic roll-out of ACF packages should include appropriate intensive follow-up and incentives to ensure that patients are properly linked to care. In a significant proportion of patients, addresses and contact telephone numbers had changed (despite taking details of a friend and relative) making further follow-up problematic. This also speaks to the issue of using national

identifiers and alternative strategies so that patients can be appropriately tracked and followed up.

Our study had several limitations. First, we only screened symptomatic patients and may have missed asymptomatic patients with active TB. However, interrogating asymptomatic persons would have required a much larger study, which was beyond the mandate of our funding, though our study did include asymptomatic HIV-infected participants. Additionally, the broader objective of our study was to validate a low-cost and scalable screening model, and WHO-endorsed symptom screening has a high negative predictive value [41]. Second, the definition for infectiousness is contentious. However, we defined infectiousness using a composite of robust and previously validated measures incorporating CASS positivity and/or smear positivity and/or the presence of cavitary disease. Analysing the data incorporating these variables in different combination and permutations, including with other measures of bacillary burden like TTP (time-to-positivity) did not substantially change our findings (see Table 3). Third, our study had a limited sample size and may have limited generalisability (single centre study in an HIV endemic setting). Nevertheless, we screened over 5200 participants and our study, as proof-of-concept, is the first controlled study to confirm the feasibility and impact of a scalable ACF strategy using a mobile clinic / unit equipped with POC Xpert and staffed by three minimally trained health care workers (XACT model). Our sample size and design did not allow for assessing impact on disease burden and mortality, and although systematic reviews of ACF showed limited impact on these variables [42], these studies pre-dated the molecular diagnostics era where Xpert-orientated ACF has been shown to impact disease burden [5]. Fourth, we used smear

microscopy, and not Xpert performed at a centralised laboratory, as the comparator arm. However, we have justified the rationale for this in detail (paragraph 3 above), including that we first needed to establish with certainty the superiority of Xpert in a community setting where low burden disease predominates, and a different trial (NCT04303104) evaluating optimal placement of the Xpert platform (POC versus centralised) is currently recruiting ~20,000 participants and will report its results in three years' time. Fifth, the potential impact of this active case finding strategy on TB prevalence may be limited by low TB treatment initiation and completion rates. However, there is an opportunity to harmonise this ACF strategy with existing TB programmatic initiatives to maximize its impact. Sixth, we did not investigate the utility of the XACT model for the diagnosis of sub-clinical TB, and identification infectious patients in this sub-group. However, our aim here was to focus on a targeted screening strategy that would be less resource-intensive and the sub-clinical TB question is being addressed in the XACT-19 study that is screening ~80,000 asymptomatic community-based participants (NCT04303104). Finally, we did not report the health system and patient level cost-effectiveness (per case diagnosed) of each strategy. However, these data have been collected, are being submitted as part of separate manuscript, and is beyond the remit of the current report.

In conclusion, community-based ACF using a scalable mobile health intervention package incorporating portable molecular diagnostics is feasible and detects the majority of community-based likely infectious TB patients. These data define a new standard of care for community based ACF and inform ACF strategies in TB endemic settings.

Contributions

KD, AE, PR and GC conceived the trial and KD is the principal investigator. KD, AE, PR, SO, MT, AP, RM, EM and LM designed and performed the experiments. KD, AE, SM, PR, SO, MT and AP analysed the data and wrote the paper. All authors critically reviewed and approved the final version.

Declaration of interests.

All the authors declare that they have no conflict of interests. All authors have submitted an ICMJE form indicating that they have no conflict of interest.

Data sharing

Individual participant data will be made available to researchers who provide methodologically sound proposals beginning 3 months and ending 5 years following publication. Data sharing requests should be directed to keertan.dheda@uct.ac.za. A data access agreement will need to be concluded.

Patient and public involvement statement

The study was discussed with community leaders and the community advisory board. Lessons learnt from patients during the conduct of the XACT-1 study were implemented in the design of this study to ensure greater acceptance of our ACF model.

Acknowledgements

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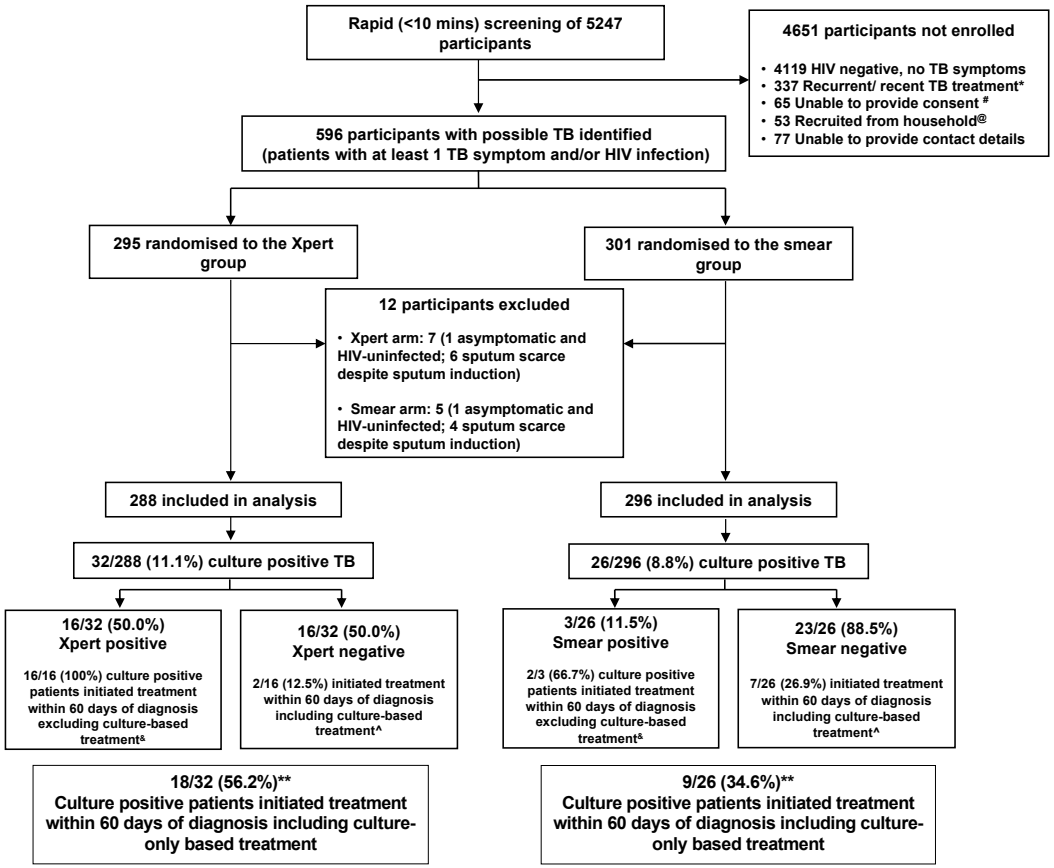


* Patients previously self-presented to a tuberculosis community clinic in the past 60 days, or who had received treatment in the past 60 days.

Unable to consent (impaired/underage) or withdrew consent.

@ Participants were recruited from their informal housing units due to an inability to walk to the mobile clinic on account of illness.

[&]Only the diagnostic test (and not culture) signalled treatment initiation.

^ Empiric or culture signalled treatment initiation

**Both the diagnostic test and/ or culture signalled treatment initiation.

Figure 1. Consort schematic summarising the recruitment strategy and overall finding

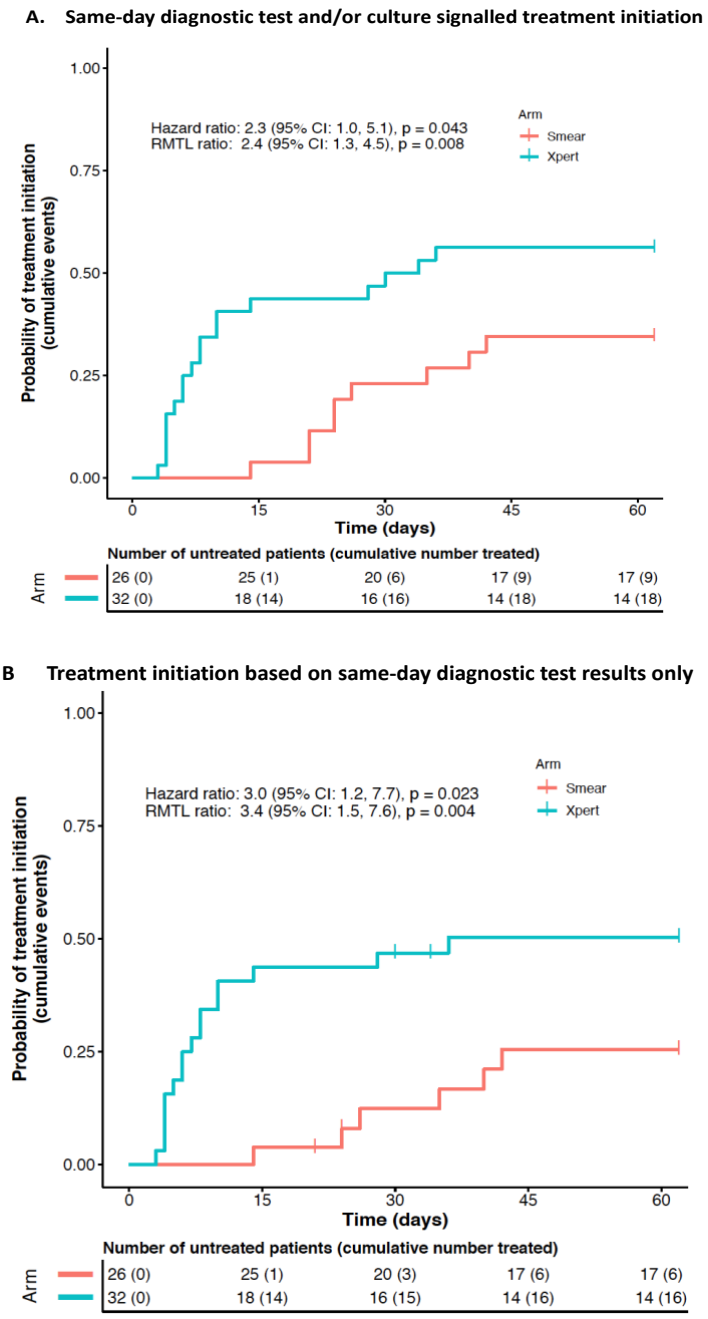
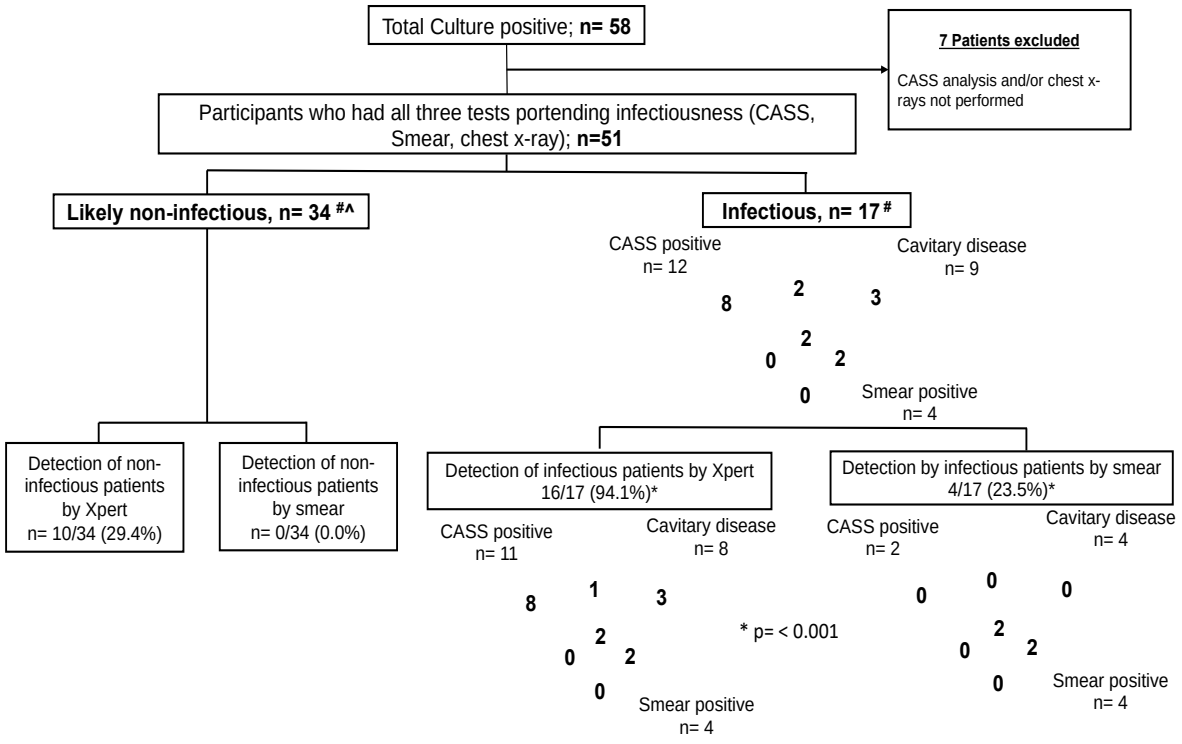


Figure 2. Kaplan-Meier estimates of time-to-treatment initiation amongst culture-positive patients (A) including same-day diagnostic test (Xpert and smear microscopy result) and/or culture-signalled treatment initiation and (B) Including only same-day diagnostic test (Xpert and smear microscopy result) signalled treatment initiation (but excluding culture-only-based treatment initiation). The Schoenfeld test results indicated

that the proportional hazards assumption was not met for both A (p=0.009) and B (p=0.004), however the hazards ratio for both A (2.3: 95% CI 1.1-4.9 p=0.03) and B (3.0: 95% CI 1.2-6.9 p=0.02) closely approximated the RMTL ratio. The censor dashes in B represent patients that were initiated on treatment based on culture results.



Proportion of patients on treatment at 60 days was significantly greater in the infectious group compared to the likely non-infectious group [13/17 (76.5%) vs. 13/34 (38.2%); p=0.007].

^ Given the limitation of current tools, a minority of these patients (< 10%) may still have transmission potential.

Figure 3. Proportion of likely infectious tuberculosis patients identified by Xpert and smear microscopy (only patients with a valid smear, chest x-ray and cough aerosol sampling (CASS) results were included i.e. n=51)

TABLES.

Table 1. Overall baseline characteristics and comparison by participant study group

	Overall **n= 584**	**Smear** **n= 296**	**Xpert** **n= 288**
Median age in years (IQR)	38·8 (30.8-48.0)	38·9 (31.5-48.0)	39·4 (30.0-47.9)
Sex			
Male (%)	203 (34.8)	111 (37.5%)	92 (31.9)
Female (%)	381 (65.2)	185 (62.5%)	196 (68.0)
HIV parameters			
HIV positive (%)	287 (49.1)	143 (48.3)	144 (50.0)
Median CD4 count (IQR)	424 (265-605)	407 (265-611)	434 (262-605)
Presence of TB Symptoms at screening			
Fever (%)	222 (38.0)	112 (37.8)	110 (38.2)
Cough (%)	521 (89.2)	262 (88.5)	259 (89.9)
Weight loss (%)	370 (63.4)	197 (66.6)	173 (60.0)
Fatigue (%)	400 (68.5)	201 (67.9)	199 (69.1)
Night sweats (%)	475 (81.3)	244 (82.4)	221 (76.7)
Participants with more than three TB Symptoms (%)	379 (64.9)	193 (65.2)	186 (64.6)
Patients who underwent induced sputum sampling	52/584 (8.9)	23/296 (7.7)	26/288 (9.0)
Proportion of culture-positive TB overall, and in each arm (n, %)	58/584 (9.9)	26/296 (8.8)	32/288 (11.1)
Xpert positivity (data in the smear arm was derived by performing Xpert in bio-banked samples)**	30/58 (51.7)**	14/26 (53.8)**	16/32 (50)

Median time to culture positivity (IQR)	12 (8.0-16.25)	12 (8.0-15.3)	12 (8.3-18.8)
Cavitary disease in culture-positive TB (%)	9/51 (17.6)	4/23 (17.4)	5/28 (17.9)
Culture-positive patients who were on treatment at 60 days (including same-day and culture-only signalled treatment initiation)*	27/58 (46.5)	9/26 (34.6)	18/32 (56.3)
Same day i.e. POC-test-positive patients initiating treatment within 60 days[#] (excludes those detected by culture only)	18/19 (94.7)	2/3 (66.6)	16/16 (100)

**Xpert data for the smear arm (and the overall performance) was derived by delayed testing (> 60 days after diagnosis) of bio-banked samples in the laboratory.

* An additional 2/14 (14.3) patients in the Xpert arm and 7/17 (41.2) in the smear arm initiated treatment between day 60 and day 120; The proportion of participants starting treatment with in 120 days in the Xpert vs. the smear arm was [20/32 (62.5) vs. 16/28 (61.5); p=0.94).

The denominator refers to all patients testing positive for Xpert and smear-microscopy in the respective arms of this study.

Table 2. Tuberculosis diagnosis and treatment associated performance outcomes per group.

Table 2A. Tuberculosis diagnosis and treatment associated performance outcomes (Data presented as % unless otherwise specified)

Time-to-treatment initiation within 60 days of testing	**Hazards ratio (95% CI)**	**Smear arm RMTL (95% CI)**	**Xpert arm RMTL (95% CI)**	**RMTL-ratio (95% CI)**
Same-day and/or culture-based treatment initiation	2.3 (1.0-5.1) p=0.04	11.3 (5.0, 17.6)	26.8 (18.2, 35.5)	2.4 (1.3-4.5) p=0.008
Only same-day diagnostic test signalled treatment initiation (i.e. excluding culture-only-based treatment initiation)*	3.0 (1.2-7.7) p=0.02	7.5 (2.0, 13.0)	25.2 (16.2, 34.2)	3.4 (1.5-7.6 p=0.004

Table 2B. Microbiological and clinical performance outcomes

Microbiological/ other clinical parameters	Smear arm	Xpert arm	Odds ratio (95% CI)	Risk ratio (95% CI)	Risk difference	p-value
Median time to treatment initiation in days (IQR)	41 (24.0-71.5)	8 (4.3-29.5)	NA	NA	NA	**0.002**
Number-needed-to-screen (NNS) per tuberculosis case detection	98.6	18.0	6.8 (2.0, 23.2)	6.5 (1.9, 21.5)	5.3 (2.4, 8.3)	**0.0001**
Culture-positive patients initiating treatment within 60 days of testing [excluding culture-based treatment i.e. only the same day diagnostic test (and not culture) signalled treatment initiation (%)]*	6/26 (23.0%)	16/32 (50.0%)	3.3 (1.1, 10.5)	2.0 (1.0, 4.3)	27 (3.1, 49.9)	**0.04**
Culture-positive patients initiating treatment within 60 days of testing but including culture-only signalled treatment i.e. both same-day diagnostic test and/ or culture signalled treatment initiation (%)*	9/26 (34.6%)	18/32 (56.2%)	2.4 (0.8, 7.1)	1.6 (0.9, 3.0)	21.6 (-2.6, 46.3)	0.10
Culture-negative patients initiating treatment within 60 days of testing (%) i.e., empiric treatment rate	3/270 (1.1%)	6/256 (2.3%)	1.5 (0.4, 5.4)	1.5 (0.4, 5.2)	1.2 (-1.5, 3.0)	0.75
Proportion of potentially infectious patients detected i.e., CASS	4/17 (23.8%)	16/17 (94.1%)	44.2	3.4	64.4	**< 0.001**

and/or cavitary disease and/or smear positive patients (%)			(4.6, 425.8)	(1.6, 7.2)	(43.4,89.9)	
Median time to treatment initiation in patients that were deemed to be *infectious* (in days) i.e. CASS and/or cavitary disease and/or smear positivity (IQR)	24 (21.0-67.0)	7 (4.0-21.0)	NA	NA	NA	**0.02**

*This is a time-point specific analysis i.e., at the 30 or 60 day time-point and not a cumulative time to event analysis as illustrated in Figure 2; 2/6 participants were initiated in treatment based on same-day smear microscopy results, 4/6 smear negative participants had empiric treatment initiation prior to the availability of TB culture results.

CASS: Cough aerosol sampling; RMTL: restricted mean time lost; NA: Not applicable; IQR: interquartile range

Table 3. The performance of Xpert and smear in identifying likely infectious patients [only patients with availability of all four results i.e., cough aerosol sampling (CASS), smear status, chest x-ray result and time-to-culture positivity have been included in this analysis; n=51].

Definition of infectiousness	Smear arm (% positivity)	Xpert arm (% positivity)	Odds ratio (95% CI)	Risk ratio (95% CI)	Risk difference	p-value*
CASS positivity only	2/12 (16.6)	10/12 (83.3)	33.0 (2.9, 374.3)	3.7 (1.4, 9.9)	66.7 (37.6, 95.7)	**< 0.001**
Presence of cavitary disease only	5/9 (55.6)	8/9 (88.8)	6.4 (0.6, 74.9)	1.6 (0.9, 3.0)	33.3 (-5.1, 71.8)	0.11
Smear positivity only[@]	4/4 (100)	4/4 (100)	NA	NA	NA	N/A
CASS positivity and/or cavitary disease[%]	4/17 (23.5)	16/17 (94.1)	44.2 (4.6, 425.8)	3.4 (1.6, 7.2)	64.7 (43.4, 89.9)	**<0.001**
CASS positivity **and/or** smear positivity **and/or** presence of cavitary disease[$]	4/17 (23.5)	16/17 (94.1)	44.2 (4.6, 425.8)	3.4 (1.6, 7.2)	64.7 (43.4, 89.9)	**< 0.001**
CASS positivity **and/or** smear positivity **and/or** presence of cavitary disease **and/or** TTP ≤ 7 days^	4/20 (20.0)	17/20 (77.2)	11.6 (2.8, 47.4)	3.4 (1.5, 7.6)	57.2 (29.8, 79.3)	**< 0.001**
CASS positivity **and/or** smear positivity **and/or** presence of cavitary disease **and/or** TTP ≤ 14 days[#]	4/23 (17.4)	20/23 (86.9)	8.5 (2.3, 30.9)	3.4 (1.5, 7.8)	69.6 (23.9, 72.1)	**< 0.001**

NA: Not applicable; TTP: time-to-sputum culture positivity in days

[@] smear was performed on separate dedicated samples from participants in both groups.

[%] Infectiousness defined by the presence of at least one of 2 criteria (CASS positivity AND/OR presence of cavitary disease)

[$] infectiousness defined by the presence of any one or more of 3 criteria (smear positivity; CASS positivity; presence of cavitary disease)

^ infectiousness defined by any one or more of 4 criteria (smear positivity; CASS positivity; presence of cavitary disease and time-to-sputum positivity ≤7 days on sputum culture)

[#] Infectiousness defined by any one or more of 4 criteria (smear positivity; CASS positivity; presence of cavitary disease and time-to-sputum positivity ≤14 days on sputum culture)

* p-values compare the proportion of infectious patients detected by Xpert to the proportion of infectious patients detected by smear microscopy using Z-scores.

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Chapter 4: Comparison of currently available diagnostic tests for tuberculous pericarditis including Xpert Ultra to a novel test measuring unstimulated interferon gamma assay (IRISA-TB) in pericardial fluid

Theme: Comparative diagnostic performance of the more sensitive Xpert Ultra for the diagnosis of tuberculous pericarditis compared to a novel immunodiagnostic test (IRISA-TB)

Sub-population of TB under investigation: Extra-pulmonary TB (EPTB) i.e., Tuberculous pericarditis

Publication: Randall P*, **Esmail A*,** Wilson L, Makambwa E, Pooran A, Tomasicchio M, Dheda K, Ntsekhe M **(*contributed equally as first authors).** Comparison of GeneXpert MTB/RIF Ultra versus unstimulated interferon gamma (IRISA-TB) for the diagnosis of tuberculous pericarditis in a TB endemic setting. (Manuscript DOI: 10.1093/ofid/ofae021; **currently in-press and has been accepted for publication in Open Forum Infectious Diseases- see proof of acceptance in the appendix)**

My contributions to this body of work: I am a co-first author for this project. My supervisor and I conceived and perfected the plan with help from the senior author of this manuscript. I lead all the clinical work performed in this project including the classification of patients into categories for analysis. I lead the the data-analysis and the interpretation of the analysed data. Lastly, I led the drafting of the manuscript and submission for peer-review. Note: my collegue and co-first author PR is a laboratory scientist & employee of Antrum Biotec who played a key role in the invention of the IRISA-TB test that was used in this project. She provided laboratory support and training for this study (see COI statement in the manuscript).

Main findings: Unstimulated interferon-gamma (IRISA-TB) was significantly more sensitive than Xpert Ultra for the diagnosis of TB pericarditis in a TB endemic resource poor setting.

COMPARISON OF GENEXPERT MTB/RIF ULTRA VERSUS UNSTIMULATED INTERFERON GAMMA (IRISA-TB) FOR THE DIAGNOSIS OF TUBERCULOUS PERICARDITIS IN A TB-ENDEMIC SETTING

Open Forum Infectious Diseases

Running title: Diagnosis of TB pericarditis

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Tuberculous pericarditis, interferon gamma, GeneXpert Ultra, diagnosis, accuracy

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ABSTRACT

BACKGROUND: Tuberculosis pericarditis (TBP) is a paucibacillary disease, where host biomarkers like unstimulated interferon-gamma (IRISA-TB) have high diagnostic accuracy. However, recently, DNA-based diagnostic tests (GeneXpert-Ultra), more sensitive than an earlier versions, have become available. Given that the diagnosis of TBP is challenging we performed a comparative diagnostic accuracy study comparing both assays.

METHODS: We recruited 99 consecutive patients with suspected TBP in Cape Town, South Africa. Definite TBP was defined by microbiological confirmation of tuberculosis on pericardial fluid culture or an alternative PCR-based test [GeneXpert MTB/RIF], or when using sputum (PCR or culture). Probable TBP was defined as a high clinical suspicion TB accompanied by anti-TB treatment, whilst non-TBP was defined as negative microbiological tests for TB without initiation of TB treatment and/or the presence of an alternative diagnosis.

RESULTS: There were 39 patients with definite TBP, 35 with probable TBP and 23 with non-TBP. ~70% of participants who received TB treatment were HIV co-infected. Overall, IRISA-TB (95% CI) was more sensitive than Xpert Ultra [88.6% (74.1;95.5) versus 71.5% (55.0;83.7); n=53], and significantly more sensitive in HIV-uninfected participants (100% [72.3;100.0] versus 60% [31.3;83.2]; P=0.03). In patients with definite and probable TBP combined (n=84) sensitivity was significantly higher with IRISA-TB [77.3 (65.9;85.8)

versus 37.9 (27.2;50.0); P<0.0001]. A similar pattern was seen in HIV-uninfected persons [88.3% versus 35.3%; P=0.002]. Specificity was high for both assays (>95%).

CONCLUSION: Unstimulated interferon-gamma (IRISA-TB) was significantly more sensitive than Xpert Ultra for the diagnosis of TB pericarditis in a TB endemic resource poor setting.

INTRODUCTION

Globally, TB remains out of control with an estimated ~11 million incident cases of tuberculosis and 1.5 million deaths in 2022 [1]. ~15 to 20% of newly ill individuals had extrapulmonary tuberculosis (EPTB). TB serositis (specifically pleural TB) is one of the most common forms of EPTB globally and the often the dominant form of EPTB seen in TB endemic countries[2]. Although TBP makes up a smaller proportion of TB serositis and EPTB, the associated 6-month mortality remains high (20 to 40%), accounting for between 5 and 10% of hospital admissions for acute heart failure in Africa [3]. Despite the significant burden of disease and the high mortality, the diagnosis of TBP remains challenging. Smear microscopy and culture have a sensitivity of ~5% and 50% respectively [4]. Alternative approaches to making a diagnosis of TBP that integrate pericardial fluid biochemical tests (e.g., adenosine deaminase [ADA]), clinical scoring tools (e.g., Tygerberg diagnostic index), and DNA-based diagnostic tests (e.g., Gene Xpert MTB/RIF) also perform poorly. [5,6]. Although Xpert MTB/RIF is endorsed by WHO as a first line test in many forms for TB, studies focussed on its accuracy in TBP have been limited by small numbers of patients, [7-10], and in a recent meta-analyses of studies using culture and pericardial biopsy as a reference standard its sensitivity was reported to be suboptimal [< 60%] [11].

More recently the manufacturer, Cepheid, has developed a cartridge for the detection of TB designated GeneXpert MTB/RIF Ultra [12]. Compared to the GeneXpert MTB/RIF assay, this assay was ~5% more sensitive in smear-positive PTB, 17% more sensitive in smear-negative PTB [12], and the level of detection in sputum improved from ~115 colony-forming units (CFU) per ml to ~15 CFU per ml[13]. Given these considerations, and the absence of

published data about the performance of Xpert Ultra in TBP, we performed a prospective study evaluating Xpert Ultra against a host biomarker, unstimulated interferon gamma (IRISA-TB; Antrum Biotech). Unstimulated interferon gamma was chosen as a comparator because it has been shown to be a promising biomarker for the diagnosis of TB serositis in general [8,14,15] and for the diagnosis TBP specifically [16]. This is in contradistinction to interferon gamma release assays (IGRA) which requires overnight stimulation and are not recommended by the WHO for diagnosis of TBP [17]. Indeed, a recent meta-analysis of studies assessing the diagnostic accuracy of unstimulated interferon gamma for TBP, confirmed its high diagnostic accuracy with a pooled sensitivity and specificity of 0.97 (95%CI: 0.87-0.99] and 0.99 (95%CI: 0.74-1.00), respectively[18].

METHODS

Patient recruitment, categorization, and routine laboratory testing

Patients with suspected pericardial TB (large pericardial effusion; defined as echo-free space around the heart >1cm in diastole) were prospectively recruited from Groote Schuur Hospital in Cape Town, South Africa. The University of Cape Town Human Research Ethics Committee approved the study (HREC 370/2015). All patients provided informed consent for study participation. Pericardial fluid was collected by ultrasound-guided pericardiocentesis. Pericardial fluid samples were subjected to routine biochemical and cytological analysis by the National Health Laboratory Services (NHLS). This included protein, albumin, ADA (cut-off 30IU/L), glucose, cell differentials, cytology, concentrated smear microscopy, and liquid culture for *M. tuberculosis* using the MGIT 960 (Becton, Dickinson, Sparks, MD). The remaining fluid was placed in a biobank, frozen

at -20°C, and subsequently used for Xpert Ultra, and IRISA-TB analyses. HIV testing was performed in consenting patients.

Due to the limitations of a single pericardial fluid TB culture for confirming a diagnosis (~40% sensitivity), a composite micro-pathological reference standard was used for patient categorization (this reference standard was used in all analyses presented). Patients were categorized as (i) definite TB, i.e., patients with one positive *M. tuberculosis* culture (pericardial fluid, biopsy specimen, and/or sputum) and/or caseating granulomatous inflammation suggestive of TB on histological examination and improvement on anti-TB treatment (all patients in this category received anti-TB treatment), (ii) probable TB, i.e., patients not meeting the micro-pathological criteria for definite TB but with clinical indicators suggestive of TB and who were initiated on anti-TB treatment based on high clinical suspicion of having TB (all patients in this category received anti-TB treatment based on clinical and laboratory criteria), and (iii) non-TB, i.e., patients that do not fit the criteria for definite and probable TB, with no microbiological or histological evidence of *M. tuberculosis* and/or for whom an alternative diagnosis was available. These patients did not receive anti-TB treatment either at presentation or on follow-up. Patients who did not fall into any of these categories remained unclassified.

Patient Consent Statement

Written informed consent was obtained from each patient and this study was approved by the University of Cape Town Human Research Ethics Committee (HREC REF 370/2015).

Unstimulated IFNγ measurement (IRISA-TB).

Of the 97 enrolled participants, 84 had sufficient pericardial fluid to measure interferon gamma concentrations using the IRISA-TB assay (Antrum Biotech Pty Ltd., Cape Town, South Africa) according to the manufacturer's instructions. The assay was performed in duplicate and the average values reported. Pericardial fluid supernatant was prepared by centrifuging 1 ml of pleural fluid at 12 000 RCF for 90 seconds.

Cepheid GeneXpert MTB/RIF and GeneXpert Ultra assays.

Both the Xpert Ultra and Xpert MTB/RIF assays were performed using 1ml of pericardial fluid diluted with 2 ml of Xpert sample buffer, followed by vigorous mixing. Xpert Ultra and Xpert MTB/RIF cartridges were run on a GeneXpert 4-module machine (Cepheid, Dx System Version 4.7b). Of the 97 enrolled participants Xpert Ultra was performed on the pericardial fluid of 84.

Definitions and micro-pathological reference standard used in this study

-Definite TB pericardial effusion (presence of ANY one or more of the following criteria):

(i) Microbiologically (Xpert *and/or* smear *and/or* culture) confirmed pericardial effusion.

(ii) The presence of necrotising granulomatous inflammation (with or without acid fast bacilli) on pathological evaluation of fluid (cytology) or pericardial tissue sample (where available).

(iii) Concurrent microbiologically confirmed sputum for TB (Xpert and/or culture) within 4 weeks (28 days) of sampling the pericardial effusion.

-Non-TB pericardial effusion (must meet ALL of the following criteria):

(i) Microbiologically (Xpert *and* smear *and* culture) negative pericardial effusion for TB

(ii) Absence of necrotising granulomatous inflammation on pathological evaluation of fluid or tissue (where available); with or without a suggestion for an alternative diagnosis

(iii) Patient not initiated on TB treatment

-Probable pericardial effusion (must meet ALL of the following criteria)

(i) Patient presenting with a clinical syndrome suggestive of chronic pericardial effusion (clinically suspected TBP)

(ii) Patients not meeting criteria outlined for 'definite'

(iii) Empiric TB treatment initiation by the clinician based on suspicion of TB

(iv) Documented clinical response to TB treatment at 2-4 month follow up in the opinion of the treating clinician.

(v) absence of a more plausible alternative diagnosis for TBP

-Unclassifiable

(i) patients not falling into the three groups defined above (e.g., patients with non-diagnostic pericardial fluid sample who are lost-to-follow-up or who die before treatment could be initiated)

Sample size estimations.

Assuming a sensitivity of Xpert Ultra of ~60% for TBP[19] and of unstimulated IFN-gamma of ~95%[18], and at 80% power and 95% confidence, a total of ~30 definite TB patients

would be required to determine a statistically significant difference between Xpert Ultra and IRISA-TB after accounting for a 5% loss-to-follow-up.

Statistical analysis.

Diagnostic accuracy, including 95% confidence intervals (95% CIs), was assessed using sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the receiver operator characteristic curve (AUROC) in definite TB and non-TB groups. Unpaired and paired categorical variables were compared using the χ^2 and McNemar tests, respectively. Continuous variables were compared using Student's *t* test where appropriate. The Mann-Whitney and Wilcoxon rank sum test was used for unpaired and paired nonparametric continuous variables, respectively. Statistical analyses were performed using GraphPad Prism (version 6.0), Medcalc, version 18.6, and Microsoft Excel.

RESULTS

Clinical and demographic data.

A total of 99 patients were recruited into the study; 39 subjects had definite TB, 23 were classified as non-TB and, 35 subjects were classified as probable TB. Those with non-TB effusions had a spectrum of malignant and non-malignant diagnoses, including lymphoma, adenocarcinoma, bacterial pericarditis and hypothyroidism. Two patients had insufficient clinical data to be categorized in the above-described groups and were subsequently excluded.

Definite and probable TB groups had higher HIV infection rates and NYHA Class I – II and III – IV comparable to the non-TB group (p-value: < 0.0001, 0.0031 and 0.0031, respectively). Diastolic blood pressure, whole cell counts (serum) and creatinine levels were significantly lower in the definite and probable TB groups (p-value: 0.0237, 0.0048 and 0.0480, respectively).

A study overview is provided in Figure. 1. Demographic and clinical data are summarized in Table 1.

Performance outcomes of IRISA-TB (definite and non-TB).

The median (IQR) IFN-γ levels (*N* = 53; 9 patients did not have remnant pericardial fluid for evaluation) were significantly higher in definite TB than non-TB pericardial effusions (93.7 pg/ml [26.1 to 325.1] versus 0.0 pg/ml [0.0 to 0.0], p-value: < 0.0001) (Fig. 2A). Using definite and non-TB groups, a ROC curve-derived rule-in cut point of 10 pg/ml (the sensitivity, specificity, PPV, and NPV of IRISA-TB [all with 95% CI] were 88.6% [74.1 to 95.5], 94.5% [74.3 to 99.1], 96.9% [84.3 to 99.5], and 81% [60 to 92.4], respectively) (Fig. 2B). Table 2 compares the diagnostic accuracy of IRISA-TB with Xpert Ultra assay in the definite TB versus non-TB groups. The sensitivity of IRISA-TB was superior in comparison to Xpert Ultra MTB/RIF assay in HIV infected individuals (100% versus 60%; p-value: 0.0293) (Table 2).

Performance outcomes of IRISA-TB (definite TB and probable TB and non-TB).

The median (IQR) IFNγ levels (*N* = 84) were significantly higher in definite and probable TB than non-TB pericardial effusions (62 pg/ml [10.43 to 223.6] versus 0.0 pg/ml [0.0 to 0.0], p-value: < 0.0001) (Fig. 2A). Using definite TB, probable TB and non-TB groups at a rule-in

cut-point of 10pg/ml a sensitivity, specificity, PPV, and NPV of IRISA-TB [all with 95% CI] of 77.3% [65.9 to 85.8], 94.5% [74.3 to 99.1], 98.1% [89.9 to 99.7], and 53.2% [36.5 to 69.2], respectively was observed (Fig. 2B). Table 3 compares the diagnostic accuracy of IRISA-TB with Xpert Ultra in the definite TB and probable TB versus non-TB groups. The sensitivity of IRISA-TB was superior in comparison to Xpert (77.3% versus 37.9%; p-value: < 0.0001) and this significance was carried through to both HIV infected (73.5% versus 38.8%; p-value: 0.0006) and uninfected individuals (88.3% versus 35.3%; p-value: 0.0017) (Table 3). In addition, the NPV of IRISA-TB was significantly better than that of Xpert Ultra (53.2% versus 30.6%; p-value: 0.0431), and this significance is also observed in HIV uninfected persons (86.7% versus 54.2%; p-value: 0.0386) (Table 3).

Performance outcome of Xpert Ultra assay.

The sensitivity, specificity, PPV and NPV (95% CI) of Xpert Ultra (*N = 53*) in definite and non-TB was 71.5% [55 to 83.7], 100% [82.5 to 100], 100% [86.7 to 100], and 64.3% [45.9 to 79.3], respectively (Table 2). The sensitivity, specificity, PPV and NPV (95% CI) of Xpert Ultra (*N = 84*) in definite, probable and non-TB was 37.9% [27.2 to 50], 100% [82.5 to 100], 100% [86.7 to 100], and 30.6% [20.3 to 43.2], respectively (Table 3).

DISCUSSION

There are two key findings from this study. First, unstimulated interferon gamma (IRISATB) had superior performance to Xpert Ultra in patients with definite TB and definite/probable TB combined, when compared to those with non-TB pericarditis. Second, despite its improved sensitivity relative to other nucleic acid amplification tests (NAATS), the negative predictive value for Xpert Ultra remained very low.

Although Xpert Ultra is more sensitive than Xpert MTB/RIF, the chronic and paucibacillary nature of the disease is below the detection limit of the assay. This is not surprising given that the sensitivity of culture for confirmed TB pericarditis is only ~30% to 50% [4]. Thus, in patients with TB pericarditis, and other paucibacillary forms of TB such as TB meningitis and TB serositis (pleural, peritoneal TB), conventional microbiological tests such as culture and nucleic acid amplification tests (NAATS) often have sub-optimal sensitivity. We have previously shown that LAM antigen levels in the pericardium were low and had poor sensitivity for the diagnosis of a TB aetiology [8]. Nevertheless, in this and other compartments there are potent Th-1 pro-inflammatory responses with physiological 'trapping' of interferon gamma, the mechanics and pathophysiology of which, is poorly understood. Indeed, several studies have now shown the superior utility of host biomarkers in TB serositis and TB meningitis [9,14,15,20]. We have demonstrated for the first time that despite the higher sensitivity of Xpert Ultra compared to Xpert MTB/RIF, it still fails to out-perform host biomarkers for this category of disease and may be too low to reliably rule out TB pericarditis in practice. On the other hand IRISA-TB is now available as a single or multiple use test marketed by a South African-based company

(https://antrumbiotech.co/innovations/) and we performed an independent evaluation of this assay.

Diagnostic evaluation in the face of an imperfect reference standard is tricky. Given that conventional diagnostics including culture and Xpert MTB/RIF only have sensitivities of between 50% and 60%, in the absence of pericardial biopsy, inferring a definitive diagnosis of TB is challenging. To maximise the accuracy of our reference standard, we used a combination of culture, and to avoid confirmation bias, used an alternative NAAT (i.e. Xpert MTB/RIF) compared to the one being evaluated (Xpert Ultra). However, given the inadequacy of existing microbiological tools, we may have still missed some true positive cases of TB pericarditis. We therefore also performed an alternative analysis including both definite and probable TB; the latter was defined as a high clinical likelihood of TB pericarditis such that TB treatment was administered by the expert clinician presiding over the case (this is likely to provide a more realistic indication of sensitivity). When using this approach, the sensitivity of IRISA-TB was significantly higher. Given that the specificity of both tests remained high (95 to 100%) even when this approach was used (combination definite and probable TB) suggests that the rate of non-TB patients (false positives) included in this analysis was likely very low.

The sensitivity of Xpert Ultra was limited and similar to that of the Xpert MTB/RIF assay [15]. This is likely because of the paucibacillary nature of the disease and limited antigen levels in serosal fluid below the detection limit, even of the more sensitive Ultra assay. This is confirmed by the similar Ct values with the older Xpert MTB/RIF and the newer Xpert Ultra assays [15]. Would using a higher pericardial fluid volume together with

centrifugation have increased sensitivity? We have previously shown that using a higher volume with centrifugation of the sample failed to increase sensitivity of the Xpert MTB/RIF assay in TB pericarditis [8].

There are some limitations of our findings. This study was conducted in high TB and HIV setting, which may limit the generalisability of the findings. Nevertheless, TB pericarditis is a major problem in sub-Saharan Africa where HIV co-infection rates are high. Furthermore, we found that IRISA-TB performed even better in HIV uninfected persons likely because of more potent and robust Th1 responses, at the site of disease, in such patients. The sample size of the study was limited resulting in wider than desired sensitivity confidence intervals. Nevertheless, this remains the only and largest study evaluating Xpert Ultra for pericardial TB. A Cochrane meta-analysis published in 2021 identified only 1 study with more than 20 positive TB pericarditis participants that evaluated the Xpert MTB/RIF assay [19]. Finally, we did not use pericardial biopsy, which could have strengthened the reference standard. However, pericardial biopsy is hardly ever used to confirm the diagnosis except in unusual or challenging cases and is inaccessible in most TB endemic countries. Thus, to strengthen the reference standard, in addition to culture, we used an alternative nucleic acid amplification test to avoid confirmation bias (we could not include Xpert Ultra in the reference standard for this reason). This approach also avoided significant misclassification bias when using culture only, as a reference standard.

In conclusion, despite the higher reported sensitivity of Xpert Ultra compared to Xpert MTB/RIF in sputum samples, in patients with suspected TB pericarditis, the sensitivity

and negative predictive value of Xpert Ultra for TB in pericardial fluid was low. By way of comparison, the sensitivity and overall diagnostic performance of unstimulated interferon gamma (IRISATB) was significantly higher than Xpert Ultra, with excellent accuracy values consistent with those reported in the setting of TB pericarditis in a recent meta-analysis. [16]

These data inform the selection of diagnostic tests in TB endemic countries and underscores the precept that in some types of extrapulmonary TB characterised by TB serositis or the accumulation of fluid, host biomarkers out-perform traditional microbiological tools. Interestingly, a similar trend is being seen in pulmonary TB where host biomarkers (blood-based gene transcripts) have excellent sensitivity for the diagnosis of pulmonary TB [21] and may be useful especially in patients with sputum scarce TB.

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	Definite TB (n = 39)	Non-TB (n = 23)	p-value Definite versus Non-TB	Probable TB (n = 35)	Definite and Probable TB (n = 74)	p-value Definite and Probable versus Non-TB
Males [no. (%)]	23 (59%)	11 (47.8%)	ns	23 (65.7%)	46 (62.2%)	ns
Age [yr (IQR)]	41 (34; 50)	58 (51; 63)	**0.0004**	38 (31; 48)	40 (33; 49)	**< 0.0001**
HIV infected [no. (%)]	27 (69.2%)	5 (21.7%)	**0.0003**	27 (77.1%)	54 (73%)	**< 0.0001**
HIV uninfected [no. (%)]	12 (30.8%)	18 (78.3%)	**0.0003**	8 (22.9%)	20 (27%)	**< 0.0001**
CD4 count [cells/ml (IQR)]	129.5 (77; 187.5)	168 (100.5; 382)	ns	176 (122; 381)	158 (83.5; 222)	ns
ARV therapy	10 (37%)	3 (60%)	ns	15 (55.6%)	25 (33.8%)	ns
Previous TB [no. (%)]	11 (28.2%)	5 (21.7%)	ns	9 (25.7%)	20 (27%)	ns
NYHA Class I – II [no. (%)]	24 (61.5%)	9 (39.1%)	ns (0.0903)	30 (85.7%)	54 (73%)	**0.0031**
NYHA Class III – IV [no. (%)]	15 (38.5%)	14 (60.9%)	ns (0.0903)	5 (14.3%)	20 (27%)	**0.0031**
Pulse [beat/min (IQR)]	110 (102; 125)	103 (90; 130)	ns	106 (100; 120)	108.5 (100; 123)	ns
Blood pressure systolic [mmHg (IQR)]	113 (105; 121)	121 (112; 129)	ns	115 (107; 127)	114 (106; 124)	ns
Blood pressure diastolic [mmHg (IQR)]	70 (61; 77)	77 (68; 90)	**0.0241**	70 (66; 80)	70 (61.75; 78.25)	**0.0237**
Serum total WCC [X109/L (IQR)]	6.32 (4.58; 8.39)	9.96 (7.04; 15.98)	**0.0016**	6.41 (4.71; 8.79)	6.39 (4.65; 8.65)	**0.0048**
Serum creatinine [umol/L (IQR)]	66.5 (54.5; 83.75)	74 (62; 98.5)	ns (0.0671)	61 (49; 76)	64 (52; 82)	**0.048**
Serum haemoglobin [g/L (IQR)]	10.75 (9.2; 11.45)	10.55 (9.58; 12.23)	ns	9.4 (8.6; 11.2)	10.3 (8.8; 11.3)	ns
Global effusion [no. (%)]	35 (89.8%)	20 (87%)	ns	28 (80%)	63 (85.1%)	ns
Other effusion [no. (%)]	2 (5.1%)	0	ns	4 (11.4%)	6 (8.1%)	ns

Unknown [no. (%)]	2 (5.1%)	3 (13%)	ns	3 (8.6%)	5 (6.8%)	ns
Tamponade [no. (%)]	27 (69.2%)	15 (65.2%)	ns	23 (65.7%)	50 (67.6%)	ns
ADA [IU (IQR)]	56.65 (49.88; 78.15)	22.2 (15.08; 26.5)	**< 0.0001**	43.15 (35.98; 54.53)	51.85 (40.05; 65.93)	**0.0001**
Total protein [g/L (IQR)]	59 (51; 70)	59 (50.5; 65.75)	ns	66 (58; 70.25)	65 (52; 70)	ns
LDH [IU (IQR)]	970 (642; 1599)	1039 (283; 1996)	ns	603 (456; 703.3)	708 (499; 1050)	ns
Lymphocyte count [medium (IQR)]	1.26 (0.54; 2.27)	1.88 (0.56; 3.52)	ns	1.51 (0.68; 2.6)	1.37 (0.6; 2.45)	ns
Neutrophil count [medium (IQR)]	0.63 (0.47; 1.22)	0.99 (0.09; 1.67)	ns	0.39 (0.15; 1.48)	0.59 (0.26; 1.29)	ns
IFN-γ [pg/ml (IQR)]	93.7 (26.1; 325.1)	0 (0; 0)	**< 0.0001**	54.6 (0; 150.4)	62 (10.43; 223.6)	**< 0.0001**
TTP [days (IQR)]	12 (6; 13)	(-)	(-)	(-)	12 (6; 13)	(-)
CT [unit (IQR)]	26 (24; 28)	(-)	(-)	(-)	26 (24; 28)	(-)

Table 1 Baseline characteristics (demographic, clinical, serum, ECG and pericardial fluid analysis) of definite, probable and non-TB groups. Continuous data were analysed by unpaired *t* test and categorical data were analysed by chi-square test. p-value = 0.05

		Sensitivity	Specificity	PPV	NPV	AUC	PLR	NLR	DOR	NNT
IRISA-TB Definite TB vs Non-TB (cut-off 10pg/ml)	Test performance (N = 53) (CIs; n/N)	88.6%[b] (74.1, 95.5) 31/35	94.5% (74.3, 99.1) 17/18	96.9% (84.3, 99.5) 31/32	81% (60, 92.4) 17/21	0.96 (0.90, 1.01)	15.94	0.121	131.8 (13.61; 1276)	3.1
	Test performance HIV infected individuals (N = 30) (CIs; n/N)	84% (65.4, 93.6) 21/25	80% (37.6, 96.4) 4/5	95.5% (78.3, 99.2) 21/22	50% (21.6, 78.5) 4/8	0.88 (0.74, 1.02)	4.20	0.200	21 (1.83; 240.7)	2.8
	Test performance HIV uninfected individuals (N = 23) (CIs; n/N)	100%[a] (72.3, 100) 10/10	100% (77.2, 100) 13/13	100% (72.3, 100) 10/10	100%[a] (77.2, 100) 13/13	1.00 (1.00, 1.00)	(-)	0	567 (10.35; 31051)	3.8
Xpert Ultra Definite TB vs Non-TB	Test performance (N = 53) (CIs; n/N)	71.5%[b] (55, 83.7) 25/35	100% (82.5, 100) 18/18	100% (86.7, 100) 25/25	64.3% (45.9, 79.3) 18/28	(-)	(-)	0.285	89.86 (4.94; 1633)	3.9
	Test performance HIV infected individuals (N = 30) (CIs; n/N)	76% (56.6, 88.6) 19/25	100% (56.6, 100) 5/5	100% (83.2, 100) 19/19	45.5% (21.3, 72) 5/11	(-)	(-)	0.24	33 (1.60; 682.2)	3.1
	Test performance HIV uninfected individuals (N = 23) (CIs; n/N)	60%[a] (31.3, 83.2) 6/10	100% (77.2, 100) 13/13	100% (61, 100) 6/6	76.5%[a] (52.8, 90.5) 13/17	(-)	(-)	0.40	39.00 (1.81; 839)	6.3
p-value (<0.5)		[a]0.0293 [b]0.0755			[a]0.0649					

Table 2: Accuracy of Xpert Ultra and IRISA™-TB for the diagnosis pericardial tuberculosis in definite and non-TB patients. Positive *M. tuberculosis* pericardial fluid and/or sputum culture and/or histology in

keeping with *M. tuberculosis* infection was used as a reference for definite TB. No microbiological or histological evidence of *M. tuberculosis* and/or an alternative diagnosis being available was defined as non-TB. NNT [(number needed to test): Cohort / number of positive tests] denotes the number of individuals that need to be tested before one identifies a positive case.

		Sensitivity	Specificity	PPV	NPV	AUC	PLR	NLR	DOR
IRISA-TB Definite and Probable TB vs Non-TB (cut-off 10pg/ml)	Test performance (N = 84) (CIs; n/N)	77.3%[a] (65.9, 85.8) 51/66	94.5% (74.3, 99.1) 17/18	98.1% (89.9, 99.7) 51/52	53.2%[a] (36.5, 69.2) 17/32	0.89 (0.82, 0.96)	13.91	0.240	57.8 (7.09; 471)
	Test performance HIV infected individuals (N = 54) (CIs; n/N)	73.5%[b] (59.8, 83.8) 36/49	80% (37.6, 96.4) 4/5	97.3% (86.2, 99.6) 36/37	23.6% (9.6, 47.3) 4/17	0.81 (0.67, 0.95)	3.67	0.331	11.08 (1.13; 108.5)
	Test performance HIV uninfected individuals (N = 30) (CIs; n/N)	88.3%[c] (65.7, 96.8) 15/17	100% (77.2, 100) 13/13	100% (79.7, 100) 15/15	86.7%[b] (62.2, 96.3) 13/15	0.94 (0.85, 1.04)	(-)	0.117	167.4 (7.37; 3805)
Xpert Ultra Definite and Probable TB vs Non-TB	Test performance (N = 84) (CIs; n/N)	37.9%[a] (27.2, 50) 25/66	100% (82.5, 100) 18/18	100% (86.7, 100) 25/25	30.6%[a] (20.3, 43.2) 18/59	(-)	(-)	0.621	22.73 (1.31; 394.1)
	Test performance HIV infected individuals (N = 54) (CIs; n/N)	38.8%[b] (26.5, 52.8) 19/49	100% (56.6, 100) 5/5	100% (83.2, 100) 19/19	14.3% (6.3, 29.4) 5/35	(-)	(-)	0.612	7.03 (0.37; 134.5)
	Test performance HIV uninfected individuals (N = 30) (CIs; n/N)	35.3%[c] (17.4, 58.7) 6/17	100% (77.2, 100) 13/13	100% (61, 100) 6/6	54.2%[b] (35.1, 72.2) 13/24	(-)	(-)	0.647	15.26 (0.77; 301.3)

		[a] < 0.0001 [b]0.0006 [c]0.0017			[a]0.0431 [b]0.0386				

Table 3: Accuracy of Xpert Ultra and IRISA™-TB for the diagnosis pericardial tuberculosis in definite and probable versus non-TB patients

Figures

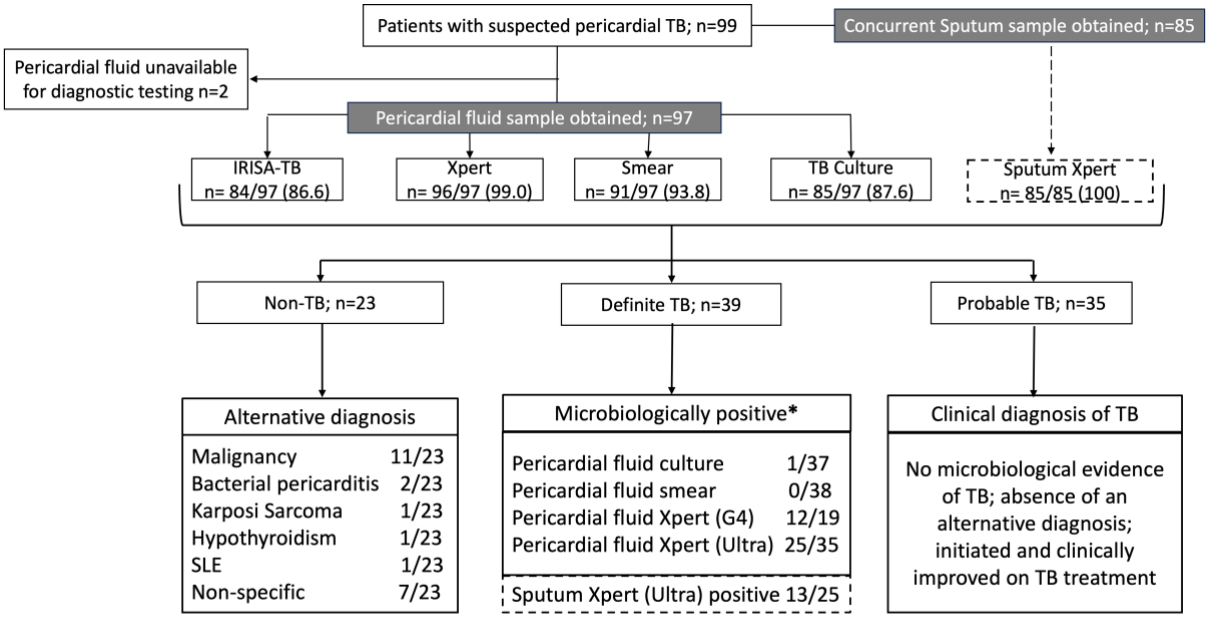

Figure 1: Study overview of patient groups, investigations performed, and tests undertaken.

* Patients tested positive on multiple diagnostic tests causing the tally of positive tests to exceed the number of patients in this group.

Legend- SLE: Systemic lupus erythematosus.

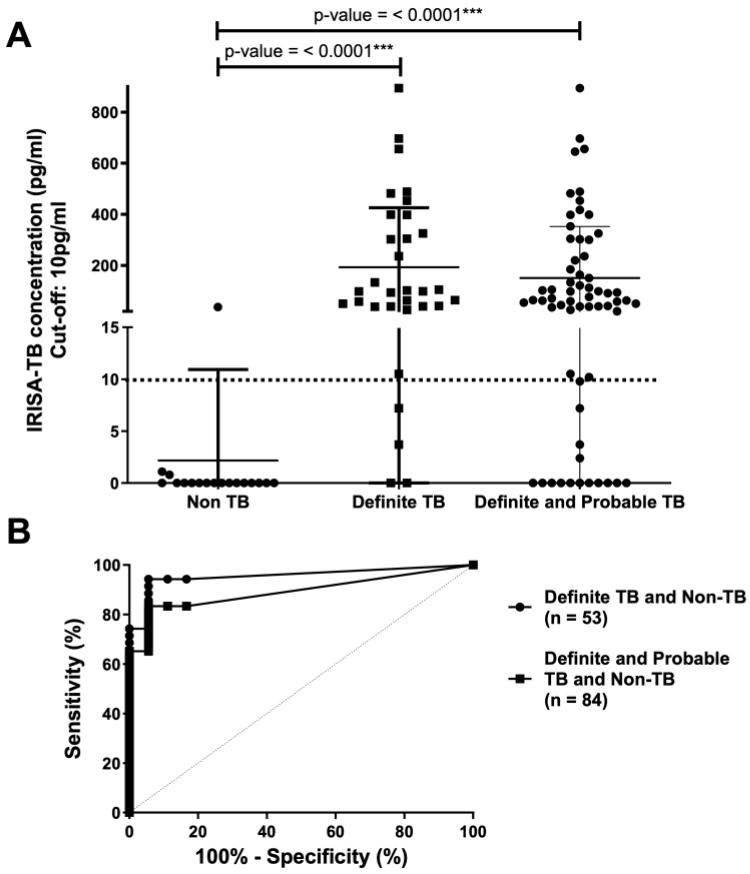

Figure 2: Performance of IRISA-TB in differentiating Definite TB and/or Probable TB from non-TB, using pericardial fluid from patients with suspected pericardial TB. A: Scatter plot of IFN-γ levels measured by IRISA-TB (p-value determined by Mann-Whitney test). Receiver operator characteristic (ROC)-derived cut point of 10pg/ml IFN-γ for IRISA-TB is indicated by black dotted line. B: Area under the ROC curves for IRISA-TB using the definite TB and non-TB groups (black graph) and definite and probable TB versus non-TB group (grey graph). Areas under the curve were 0.96 (Definite TB versus Non-TB) and 0.89 (Definite and Probable TB versus Non-TB). No significant difference was observed between the two ROC curves.

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Chapter 5: Comparing pleural fluid Xpert Ultra, ADA and the novel IRISA-TB test measuring unstimulated interferon gamma for the diagnosis of pleural tuberculosis.

Theme: Comparative diagnostic performance of the more sensitive Xpert Ultra for the diagnosis of Pleural TB compared to a novel immunodiagnostic test (IRISA-TB)

Sub-population of TB under investigation: Extra-pulmonary TB (EPTB) i.e., Pleural tuberculosis

Publication: Diagnostic performance of unstimulated IFN-γ (IRISA-TBTM) for pleural tuberculosis: a prospective study in South Africa and India. **Aliasgar Esmail*,** Devasahayam J. Christophera*, Alex J. Scott, Lindsay Wilson, Philippa Randallb, Balamugesh Thangakunama, Deepa Shankara, Sekar Rajasekara, Jeremi Swanepoel, Louié Kühnb, Tahlia Perumal, Anil Pooran, Suzette Oelofse, Keertan Dheda. **(*contributed equally as first authors). (Manuscript is currently undergoing peer review- see proof of submission to Open Forum Infectious Diseases in the appendix)**

My contributions to this body of work: I am a co-first author for this project. My supervisor and I conceived and perfected the research plan. We then approached the India-based collaborator (first co-author) to apply for the grant (NIH). I lead all the clinical work performed in this project in south Africa including the classification of patients into categories for analysis. I also did the protocol training for the site in India. I lead the overall data-analysis and the interpretation of the analysed data. Lastly, I lead the team in drafting the manuscript and submitted it for peer-review. Note: my colleague and co-first author DJC is the study lead based at Christian Medical College, Vellore, India. **Co-authorship is warranted because study took place in two countries.**

Main findings: IRISA-TB demonstrated markedly better sensitivity and NPV than Xpert-Ultra, and excellent specificity for the diagnosis of TPE in TB-endemic settings. These data have implications for clinical practice in TB-endemic settings.

Diagnostic performance of unstimulated IFN-γ (IRISA-TB™) for pleural tuberculosis: a prospective study in South Africa and India (submitted for publication)

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Keywords

Mycobacterium tuberculosis, tuberculous pleural effusion, unstimulated IFN-γ, diagnostics, diagnostic accuracy

Running title

Unstimulated IFN-γ (IRISA-TB™) for pleural tuberculosis

Summary

With the urgent need for improved diagnostic approaches to detect extrapulmonary tuberculosis, unstimulated IFN-γ (IRISA-TB™) demonstrated a high sensitivity and NPV (rule-out value) for the diagnosis of pleural tuberculosis and was superior in performance to both Xpert Ultra and ADA.

Abstract

Background: Tuberculous pleural effusion (TPE) is the most common form of extrapulmonary tuberculosis in many settings. The diagnostic performance of the frontline PCR-based GeneXpert®-MTB/RIF-Ultra (Xpert-Ultra) remains sub-optimal (sensitivity of ~30%) but data are limited. Improved diagnostic approaches are urgently needed to detect EPTB in TB-endemic settings.

Methods: This multicentre, observational study evaluated the diagnostic performance of a rapid (same-day) interferon-gamma-rapid-immunosuspension-assay (IRISA-TB™) in patients with presumed TPE from South Africa and India. Participants underwent pleural biopsy and testing with other available same-day diagnostic assays (adenosine deaminase [ADA], Xpert-Ultra, and IRISA-TB™) were concurrently undertaken. The reference standard for TB was microbiological and/or histopathological confirmation using pleural fluid and/or pleural biopsy samples.

Results: A total of 217 participants with suspected TPE were recruited (106 from South Africa; 111 from India). The sensitivity of IRISA-TB™ (cut-point 20.5pg/ml) was significantly better than Xpert-Ultra (81.8% [70.4-90.2] vs 32.9% [22.1-45.1], $p<0.001$) and ADA at the 40 IU/ml cut-point used in India (81.8% [70.4-90.2] vs 53.8% [41.0-66.3], $p=0.002$). Compared to ADA at the 30 IU/ml cut-point used in South Africa, IRISA-TB™ had a higher specificity (96.6% [90.3-99.3] vs 87.1% [78.6-93.2]) and higher PPV (94.7% [85.5-97.3] vs 81.8% [72.4-88.5]). The NPV (rule-out value) of IRISA-TB™ was significantly

better than Xpert-Ultra (87.5% [83.2-93.0] vs 64.9% [61.1-68.6], $p<0.001$) and ADA at the 40 IU/ml cut-point (87.5% [83.2-93.0] vs 74.1% [68.7-79.0], $p<0.001$).

Conclusions: IRISA-TB™ demonstrated markedly better sensitivity and NPV than Xpert-Ultra, and excellent specificity for the diagnosis of TPE in TB-endemic settings. These data have implications for clinical practice in TB-endemic settings.

Introduction

Tuberculosis (TB) is the most common fatal infectious disease globally, now annually surpassing deaths due to COVID-19 [1, 2]. In 2022, there were an estimated 10.6 million newly diagnosed individuals with TB and ~1.3 million deaths [1]. A significant minority of these (~20%) were due to extrapulmonary TB (EPTB; ~2 million newly diagnosed persons). However, EPTB accounts for almost a third of all TB in many HIV-endemic settings [3]. In several endemic settings the most common manifestation of EPTB is TB pleural effusion (TPE) [3, 4]. The differential diagnosis is wide and may include acute lower respiratory tract infection, malignancy, left ventricular failure, chronic renal failure, pulmonary embolism, autoimmune disease, and drug reactions. For the diagnosis of TPE, a pleural fluid sample is usually obtained by needle aspiration (sputum is usually not obtainable or negative). However, making the diagnosis is problematic and the current conventional diagnostic tools perform sub-optimally in TPE (e.g., smear microscopy and culture have sensitivities of ~2% and 40%, respectively) and results of culture are delayed for several weeks. TB antigen detection (lipoarabinomannan) in pleural and pericardial effusion has a sensitivity of <20% [5, 6].

The diagnostic performance of frontline nucleic acid amplification tests (NAAT), such as the GeneXpert®, for TPE has been poor. An earlier version (GeneXpert® MTB/RIF) had a sensitivity of ~30% using pleural biopsy as a reference standard and ~50% using fluid culture, which tends to overestimate performance [7]. More recently, a new ultra-sensitive cartridge was developed (GeneXpert® MTB/RIF Ultra [Xpert Ultra]) with an ~15% better sensitivity in sputum smear-negative TB [8, 9]. However, there are limited data using Xpert Ultra for the diagnosis of TPE [10]. Histopathological analysis and culture of pleural biopsy

material has an excellent yield, but biopsy sampling is often not performed due to resource constraints given the overburden in TB-endemic countries.

Given these shortcomings and the paucibacillary nature of TPE, an immunodiagnostic approach has traditionally been used by measuring adenosine deaminase levels (ADA: an enzyme produced by mononuclear cells) in pleural fluid [11]. However, there are several challenges and hurdles with this approach. Firstly, specificity was shown to be sub-optimal (60%-70%) in several studies, when using pleural biopsy as a reference standard, and the negative predictive value (rule-out value) was also sub-optimal at ~70%, meaning that it was a poor tool to portend an alternative diagnosis and hence the need for pleural biopsy [7,10]. Secondly, different ADA cut-points have been used in different parts of the world (e.g., South Africa uses the 30 IU/ml cut-point, whereas the 40 IU/ml cut-point is used in India, which improves specificity, but sensitivity is considerably lower at ~50%) [7].

More recently, and to circumvent these shortcomings, a same-day clinically validated single/multiple-use assay to detect unstimulated interferon gamma (IFN-γ) and pleural fluid was developed. This assay (IRISA-TB™) showed excellent sensitivity of ~90%-95% for the diagnosis of TPE (and other forms of TB serositis), and a specificity of ~95% [5,7,10,12]. Prior systematic reviews and meta-analyses has also shown that unstimulated IFN-γ was an excellent diagnostic test for TPE [13], but until now a standardised clinically validated assay is unavailable. It is important to note that IRISA-TB™ uses unprocessed and unstimulated *de novo* pleural fluid and must be distinguished from interferon gamma release assays (IGRAs: e.g., QuantiFERON®-TB Gold In-Tube and T-SPOT.TB), which

require overnight stimulation and co-culture with TB antigens. Thus, IRISA-TB™ is not an IGRA [14]. However, it uses bespoke and clinically validated antibody pairs that have better sensitivity in TB serositis, which presents its own specific technical challenges.

Given the limited data on the performance of IRISA-TB™ versus Xpert Ultra in different settings, we undertook a multicentre, cross-sectional study with IRISA-TB™, in patients with presumed TPE using pleural biopsy and microbiology as a composite reference standard.

Methods

Study design and patient recruitment

Patients with presumed TPE (any TB symptoms, including cough, fever, night sweats, loss of weight, haemoptysis, chest pain, and/or shortness of breath, and a chest x-ray [CXR] showing features of a pleural effusion) were prospectively recruited from Groote Schuur Hospital in Cape Town, South Africa, and Christian Medical Centre in Vellore, India. All patients provided informed consent for study participation.

Sample collection and routine laboratory tests

Pleural fluid was collected by ultrasound-guided pleurocentesis. Pleural fluid samples were subjected to routine biochemical and cytological analyses in accordance with local standard-of-care. This included the measurement of total protein, albumin, glucose, differential cell counts, cytology, ADA, Xpert Ultra, and liquid culture for *M. tuberculosis* using the MGIT 960 (Becton, Dickinson, Sparks, MD). Pleural fluid ADA levels of >30 IU/ml and >40 IU/ml were reported as suggestive of pleural TB in accordance with national

guidelines in South Africa and India, respectively.[7,93] The remaining fluid was placed in a biobank, frozen at -80°C, and subsequently used for IRISA-TB™ testing (measuring unstimulated IFN-γ). To aid in achieving a final diagnosis for the patient, all individuals who had a non-diagnostic pleurocentesis (e.g., if pleural fluid Xpert or cytological analysis were negative for TB or malignancy, respectively) underwent either a 'closed' (Abrahams Needle) or thoracoscopic pleural biopsy according to routine practice and expertise at the local centre. Pleural biopsy samples were sent to local laboratories for histology, Xpert Ultra, and liquid TB culture as part of routine care. Sputum was also concurrently collected for routine smear microscopy and liquid TB culture. HIV testing was performed in all consenting patients

Study definitions and patient categorisation

Due to the limitations imposed by the lack of a 'perfect' gold standard for the diagnosis of TPE, a composite reference standard was used for patient categorisation (this reference standard was used in all analyses presented). Patients were categorised as (i) Definite TPE: patients with a microbiological test specific for *M. tuberculosis* (Xpert Ultra and/or TB culture positivity in pleural fluid, biopsy specimen, and/or concurrent sputum), and/or the presence of caseating or necrotising granulomatous inflammation with acid fast bacilli (AFBs) suggestive of TB on histological examination of pleural biopsy tissue and with improvement on anti-TB treatment (all patients in this category received anti-TB treatment), (ii) Probable TPE: patients not meeting the criteria for Definite TPE, but with clinical and radiological indicators suggestive of TB and who were initiated on and responded to anti-TB treatment (all patients in this category received a complete course of anti-TB treatment), (iii) Non-TPE: patients with no microbiological or histological evidence of *M. tuberculosis* and/or for whom an alternative diagnosis was available

(these patients did not receive anti-TB treatment either at presentation or on follow-up), and (iv) Unclassifiable: patients who could not be subjected to the composite reference standard, were lost-to-follow-up, or died before the assessment of TB treatment response or obtaining a final diagnosis.

Interferon gamma (IFN-γ) measurement using IRISA-TB™

IFN-γ concentrations were measured in pleural fluid supernatants using the IRISA-TB™ assay (Antrum Biotech Pty Ltd., Cape Town, South Africa) according to manufacturer instructions. Pleural fluid supernatant was prepared by centrifuging 1 ml of pleural fluid at 3,000 g for 15 min. The assay was performed in duplicate and the average values reported.

Xpert MTB/RIF Ultra

Xpert Ultra assay was performed using 2 ml of pleural fluid diluted with 2 ml of Xpert sample buffer, followed by vigorous mixing. Xpert Ultra cartridges were run on a GeneXpert® 4-module system (Dx System, version 4.7b; Cepheid, Sunnyvale, USA) as per manufacturer instruction for a sputum sample.

Statistical analysis

Diagnostic accuracy, including 95% confidence intervals (95% CIs), were assessed using sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the receiver operator characteristic curve (AUROC) in Definite TPE and Non-TPE groups. Unpaired and paired categorical variables were compared using the Chi-squared and McNemar test, respectively. Continuous variables were compared using Student's t test, where appropriate. The Mann-Whitney and Wilcoxon rank sum

tests were used for unpaired and paired non-parametric continuous variables, respectively. Statistical analyses were performed using SPSS Statistics (Version 28.0. Armonk, NY: IBM Corp.) and GraphPad Prism (Version 10.1.2. Boston, MA: GraphPad Software).

Results

The study overview is illustrated in Figure 1. A total of 217 patients were recruited into the study (106/217 [48.8%] from South Africa and 111/217 [51.2%] from India). After 11 patients were excluded due to having incomplete critical data (unclassifiable) that hindered their correct classification, 206 participants were included in the analysis. 80 participants had Definite TPE, 102 were classified as Non-TPE, and 24 participants were classified as Probable TPE. Those with non-TB effusions had a spectrum of malignant and non-malignant diagnoses, including lymphoma, non-small cell lung cancer, small-cell lung cancer, and parapneumonic effusion.

Demographic and clinical data

Demographic and clinical data are summarised in Table 1. The study population had a median (IQR) age of 52 (38-65), with individuals with Definite TPE significantly younger compared to Non- and Probable TPE groups (42 [31-56] versus 62 [49-70] and 45 [38-66], respectively; $p<0.001$). There were more males than females in our study (135/206 [65.5%] versus 71 [34.5%]). A total of 19/206 (9.2%) individuals were living with HIV with a significantly higher proportion in the Definite TPE group compared to Non- and Probable TPE groups (13/80 [16.3%] versus 5/102 [4.9%] and 1/24 [4.2%], respectively; $p=0.021$). The most reported symptoms across all groups were cough and weight loss.

Demographic and clinical characteristics stratified by country are presented in Supplementary table S1, whereas microbiological and radiological characteristics are described in Supplementary table S2.

Performance outcomes of IRISA-TB™

As shown in Figure 2, the median (IQR) IFN-γ levels were significantly higher in Definite TPE than Non-TPE (52.3 pg/ml [22.2-112.4] versus 8.8 pg/ml [4.5-10.4]; $p<0.001$). Using Definite and Non-TPE groups, at an ROC curve-derived rule-in cut-point of 20.5 pg/ml against the composite reference standard, the sensitivity and specificity (95% CI) of IRISA-TB™ was 81.8% (70.4-90.2) and 96.6% [90.3-99.3], respectively. The PPV and NPV (95% CI) was 94.7% (85.5-97.3) and 87.5 (83.2-93.0), respectively. The AUROC (95% CI) for IRISA-TB™ was 87.6 (80.5-94.8: Figure 3).

Performance outcomes of pleural fluid Xpert MTB/RIF Ultra

Against the composite reference standard, the sensitivity, specificity, PPV, and NPV (all with 95% CI) of Xpert Ultra were 32.9% (22.1 - 45.1), 100.0% (95.9-100.0), 100.0% (85.2-100.0), and 64.9% (61.1-68.6), respectively.

Performance outcomes of ADA

As illustrated in Figure 2, median (IQR) ADA levels were significantly higher in Definite TPE than Non-TPE (41.0 IU/ml [33.0-55.0] versus 12.2 UI/ml [8.1-19.5]; $p<0.001$)). Against the composite reference standard and using a clinical cut-point of 30 IU/ml (used in South Africa), the sensitivity, specificity, PPV, and NPV (all with 95% CI) of ADA were 83.1% (71.7-91.2), 87.1% (78.6-93.2), 81.8% (72.4-88.5), and 88.0% (81.0-92.7), respectively.

However, when using a cut-point of 40 IU/ml (as is the case for India), the sensitivity decreased to 53.8% (41.0-66.3) whilst the specificity improved to 92.5% (85.1-96.9). The PPV and NPV were 83.3% (70.3-91.4) and 74.1% (68.7-79.0), respectively. The AUROC (95% CI) for ADA was 92.6 (88.0-97.3: Figure 3).

Comparison of IRISA-TB™ with Xpert MTB/RIF Ultra and ADA

Table 2 compares the diagnostic accuracy of IRISA-TB™ with that of other same-day diagnostics (Xpert Ultra and ADA) in the Definite TPE versus Non-TPE groups. The sensitivity of IRISA-TB™ (cut-point 20.5pg/ml) was significantly better than Xpert Ultra (81.8% [70.4-90.2] vs 32.9% [22.1-45.1], $p<0.001$) and ADA at the 40 IU/ml cut-point used in India (81.8% [70.4-90.2] vs 53.8 [41.0-66.3], $p=0.002$). The specificity of IRISA-TB™ was higher than ADA at the 30 IU/ml cut-point used in South Africa (96.6% [90.3-99.3] vs 87.1% [78.6-93.2]). The PPV of IRISA-TB™ (94.7% [85.5-97.3]) was higher than that of ADA (81.8% [72.4-88.5]). The NPV of IRISA-TB™ was significantly better than Xpert Ultra (87.5 [83.2-93.0] vs 64.9 [61.1-68.6], $p<0.001$) and ADA (87.5 [83.2-93.0] vs 74.1 [68.7-79.0], $p<0.001$) at the 40 IU/ml cut-point.

Stratifying by country (Table 2), sensitivity and specificity of IRISA-TB™ and ADA were generally comparable. However, Xpert Ultra positivity was significantly higher in individuals with Definite TPE from South Africa as compared to India (52.4% (29.8-74.3) versus 24.5% (13.3-38.9); $p=0.023$).

Discussion

This study evaluated the performance of unstimulated IFN-γ (IRISA-TB™) compared to the newer and more sensitive GeneXpert® MTB/RIF Ultra for the diagnosis of TPE, where pleural biopsy and pleural fluid microbiological confirmation served as the composite reference standard. The key findings of the study were that: (i) IRISA-TB™ was significantly more sensitive than Xpert Ultra (~82% versus 33%) with both assays displaying excellent specificity; (ii) IRISA-TB™ was also significantly more sensitive than ADA at the 40 IU/ml cut-point that is used routinely in India; (iii) IRISA-TB™ specificity was better than ADA at the 30 IU/ml cut-point used in South Africa (~97% versus 87%); (iv) the PPV of IRISA-TB™ was much higher than ADA (~95% versus 83%) at either the 30 or 40 IU/ml cut-point (implying that ~20% of all positive ADA tests were falsely positive); and (v) the NPV or rule-out value of IRISA-TB™ (~88%) was significantly better than that of Xpert Ultra or ADA, which provides the magnitude of probability that TB is unlikely, thus prompting the need for pleural biopsy to confirm an alternative diagnosis.

IRISA-TB™ showed substantially better sensitivity than Xpert Ultra (~250% increased sensitivity). TPE is a paucibacillary disease and although Xpert Ultra has a detection limit of ~20 organisms/ml [9], the mycobacterial burden in TPE remains below the detection limit of the assay. In such a situation, an immunodiagnostic approach using host biomarkers makes more sense. Such an approach has traditionally used pleural fluid ADA, an enzyme used by mononuclear cells. Indeed, blood-based host immunodiagnostic biomarkers to both predict the development of active TB in the short term (also called incipient TB) [16], and for the diagnosis of active TB [17,18], show great utility, and are being developed as immunodiagnostic blood-based tests. It is therefore not

surprising that a host biomarker like unstimulated IFN-γ has a high discriminatory value for the diagnosis of TPE.

TB drives a potent Th1 response and Th1 cytokines and chemokines are 'trapped' within the pleural compartment [19]. The reasons are poorly understood but are related to complex transport mechanisms that govern movement of molecules into and out of compartments. As such, they have excellent diagnostic value as indicated by systematic reviews and meta-analyses [13]. However, whilst several research enzyme-linked immunosorbent assay (ELISA) kits exist for the detection of unstimulated IFN-γ, these run approximately four-hour ELISA protocols, sensitivity is variable, these assays have not been clinically validated, are prohibitively expensive (~$500 to $600 per kit), and do not accommodate single patient testing (another reason making it cost prohibitive). Moreover, the antibody pairs required to detect IFN-γ in the serosal compartments are not the same as those that optimally detect IFN-γ in blood given Ph and chemistry considerations, and the heterophile effect (high concentration of molecules and antibodies in serosal compartments that prevent optimal antigen and antibody binding). Antrum Biotech, a University of Cape Town start-up, after several years of foundational research developed a single/multiple use, low-cost assay (IRISA-TB™) that uses a two-hour protocol, that has been validated for detection of IFN-γ in several compartments [7,10,20]. Several studies have shown IRISA-TB™ to be highly sensitive and specific for the diagnosis of pleural TB and considerably higher than that of Xpert in rigorous studies using pleural biopsy and fluid microbiology as the reference standard [7,10]. The same was shown for pericardial TB [20].

IRISA-TB™ also previously showed improved performance compared to ADA (default assay used in many TB-endemic settings as pleural biopsy is often not accessible). In the South African setting where the 30 IU/ml cut-point is used, the specificity of IRISA-TB™ was better than ADA, which results in significant misclassification bias such that ~1 in 7 to 8 persons would be erroneously diagnosed with TB when they did not have TPE. This results in huge additional cost to the healthcare system and avoidable anxiety and adverse events to individuals, which may be fatal (not to mention absence from schoolwork and the anxiety due to social stigmatisation). In the South African setting where there are ~80,000 newly diagnosed patients with EPTB annually (and assuming ~40,000 have pleural TB and hence ~400,000 patients with presumed pleural TB would need to be screened with ADA) [21], given the lower specificity of ADA, we estimate this would conservatively result in ~53,000 false treatment starts for TPE. Indeed, a recently published cost-effectiveness study on IRISA-TB™ in South Africa showed that the assay was highly cost-effective as it prevented false positive treatment starts due to its high specificity [21]. Cost savings amounted to ~US$ 45 million per annum in the South African setting. In the Indian setting where an ADA cut-point of 40 IU/ml is used (resulting in higher specificity thus avoiding the false treatment starts), sensitivity is only ~50 to 60% thus missing a large proportion of individuals with TPE. The NPV is also important in the context of a presumed TPE as it provides the probability of ruling out TB, thus prompting the search for alternative diagnoses through a pleural biopsy. IRISA-TB™ NPV was considerably higher than that of Xpert Ultra, thus providing clinical value as it is helpful to guide further investigation. The PPV of ADA at either cut-point (30 or 40 IU/ml) was only ~80% (i.e., of all positive tests 20% don't have TB), while for IRISA-TB™ and Xpert it was

~95%-100%. Given these considerations, it is not surprising that IRISA-TB™ was more cost-effective than ADA [21].

IRISA-TB™ uses unprocessed pleural fluid (unfiltered, unstimulated, and not antigen challenged) unlike an IGRA which uses highly processed material, overnight stimulation, and the biological material undergoes challenge with TB specific antigens. We have previously evaluated IGRA for the diagnosis of pleural TB [14]. Although it has some value, a substantial proportion of assays are indeterminate, limiting its utility. Furthermore, the cost and additional incubation/stimulation step makes this assay redundant because the same (antigen stimulation of Th1 lymphocytes) has already happened *in vivo* within the pleural compartment where IFN-γ is effectively trapped and is present in high concentrations. Thus, further processing of the sample is unnecessary. We had previously evaluated other Th1 biomarkers like IP10 and pleural fluid, but sensitivity was lower than that of IFN-γ [6].

There are several strengths to our study. Firstly, pleural biopsy and pleural fluid microbiology was undertaken in all individuals thus substantially reducing misclassification bias that other studies were prone to. Most studies use only microbiologically positive fluid sample (culture positive), which overestimates PCR test performance, and it further excludes the majority of paucibacillary samples. Second, we evaluated ADA at both commonly used cut-points (30 IU/ml the high sensitivity cut-point and 40 IU/ml the high specificity cut-point). Third, we compared results across different settings and in different sub-populations including in people living with HIV (PLWH). However, our study also had several limitations. Firstly, it would have been beneficial to

have a larger sample size and thus tighter confidence intervals to our estimates. However, this is one of the largest published studies on pleural fluid TB diagnostics. Second, our study was unable to obtain good estimates of performance of IRISA-TB™ in PLWH. However, previous studies of IRISA-TB™ showed excellent performance in the PLWH sub-group and better than in HIV-negative persons [7,10]. Third, the IRISA-TB™ assay is laboratory-based and not point-of-care. Nevertheless, a simple ELISA plate reader is found in almost all basic laboratories in TB-endemic countries and a point-of-care version (in lateral flow format) is already in the advanced stages of development and testing. Finally, the impact on treatment outcomes using this technology has not been ascertained. However, a large multicentric European Union-funded study across four African countries is currently underway to address this limitation (EDCTP ref. RIA101103281) [22].

In conclusion, IRISA-TB™ has a high sensitivity and NPV (rule-out value) for the diagnosis of pleural TB and is thus superior in performance to both Xpert Ultra and ADA.

Patient consent statement and ethical approval

Written informed consent was obtained from all participants. This study was ethically approved by the University of Cape Town Human Research Ethics Committee (HREC refs. 421/2006 and 919/2014) and the Christian Medical College Office of Research Institutional Review Board (IRB: 10377 [DIAGNO], dated 27 June 2018).

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data, and were blinded to the patient's clinical diagnosis and classification of patient sub-groups. PR is an employee of Antrum Biotech, the company that developed the IRISA-TB™ test. She provided training to laboratory technicians who performed the assay for this project and assisted with the performance of quality checks on the kits.

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Data availability

Deidentified data from this study is available upon reasonable request to the corresponding author.

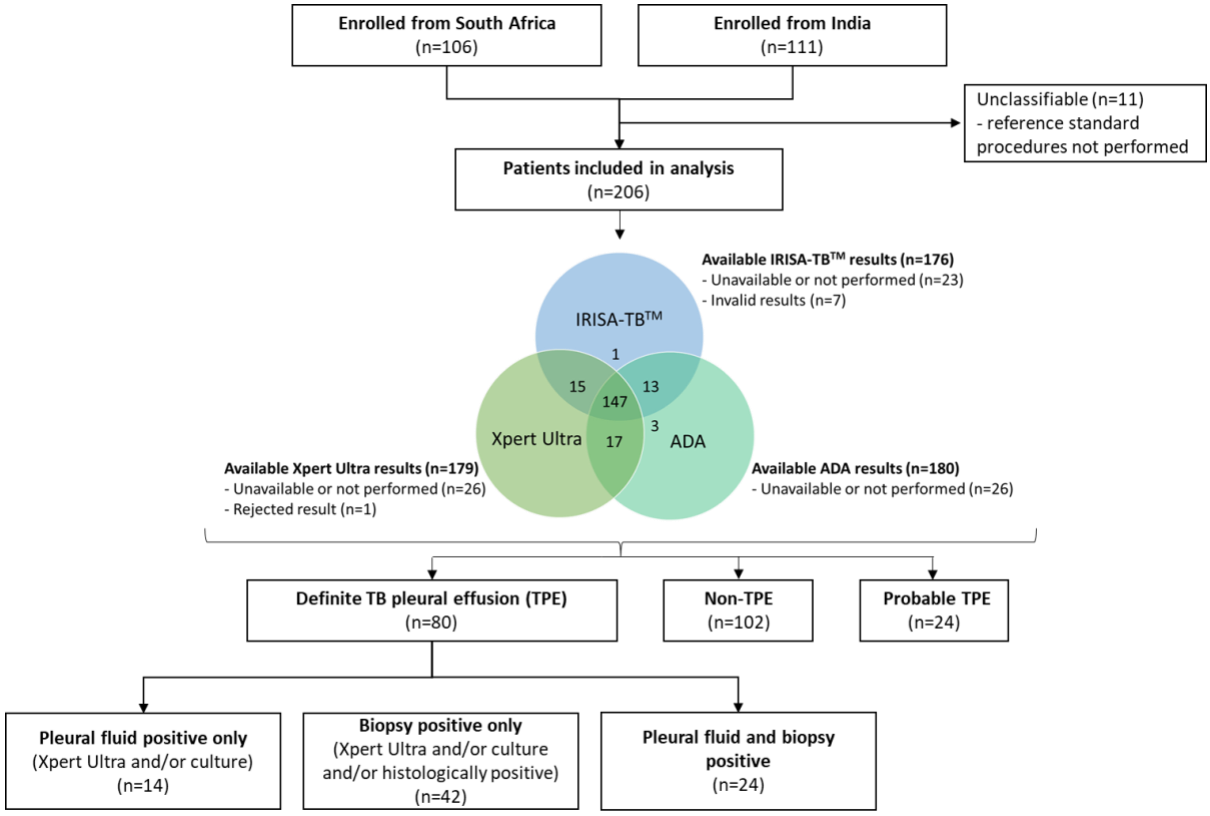

Figure 1. Study overview of patient groups. Participant classification: Definite TPE, (i) at least one positive *M. tuberculosis* Xpert Ultra and/or culture on pleural fluid and/or biopsy specimen; and/or (ii) granulomatous inflammation with caseation and/or necrosis on histological examination of pleural biopsy tissue; and/or (iii) acid fast bacilli on histological examination of pleural biopsy tissue. Non-TPE, patients with no microbiological or histological evidence of *M. tuberculosis* and/or an available alternative diagnosis (not started on TB treatment). Probable TPE, not meeting the criteria for definite or non-TPE but initiated on TB treatment. ADA, adenosine deaminase. TB, tuberculosis. TPE, tuberculous pleural effusion. Xpert Ultra, GeneXpert® MTB/RIF Ultra.

Table 1. Demographic and clinical characteristics

	Total (n=206)	**Definite TPE** (n=80)	**Non-TPE** (n=102)	**Probable TPE** (n=24)
Age - median (IQR)[a]	52 (38-65)	42 (31-56)	62 (49-70)	45 (38-66)
Sex				
Male	135 (65.5)	58 (72.5)	61 (59.2)	16 (66.7)
Female	71 (34.5)	22 (27.5)	41 (40.2)	8 (33.3)
Current smoker	42 (20.4)	14 (17.5)	25 (24.5)	3 (12.5)
PLWH[b]	19 (9.2)	13 (16.3)	5 (4.9)	1 (4.2)
History of previous TB	18 (8.7)	9 (11.3)	7 (6.9)	2 (8.3)
Symptoms				
Cough	130 (63.1)	53 (66.3)	59 (57.8)	18 (75.0)
Fever[c]	75 (36.4)	47 (58.8)	21 (20.6)	7 (29.2)
Weight loss	143 (69.4)	55 (68.8)	68 (66.7)	20 (83.3)
Night sweats	26 (12.6)	10 (12.5)	10 (9.8)	6 (25.0)

All numbers are n (%) unless otherwise indicated. Superscript letters indicate statistical significance between groups: [a]$p<0.001$, [b]$p=0.021$, [c]$p<0.001$. IQR, interquartile range. PLWH, people living with HIV. TB, tuberculosis. TPE, tuberculous pleural effusion. Xpert Ultra, GeneXpert® MTB/RIF Ultra.

Table 2. Diagnostic accuracy of IRISA-TB™, Xpert Ultra, and ADA for the diagnosis of TPE[a]

Assay[b]	TP	FP	TN	FN	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Positive LR (95% CI)	Negative LR (95% CI)
IRISA-TB™ (cut-point: 20.5 pg/ml)										
Total (n=153)	54	3	84	12	81.8 (70.4-90.2)[c,d]	96.6 (90.3-99.3)	94.7 (85.5-97.3)	87.5 (83.2-93.0)[f,g]	23.7 (7.8-72.6)	0.2 (0.1-0.3)
South Africa (n=69)	15	1	49	4	78.9 (54.4-94.0)	98.0 (89.4-99.9)	93.8 (68.0-99.1)	92.5 (83.7-96.7)	39.5 (5.6-278.6)	0.2 (0.1-0.5)
India (n=84)	39	2	35	8	83.0 (69.2-92.4)	94.6 (81.8-99.3)	95.1 (83.4-98.7)	81.4 (69.9-89.2)	15.4 (4.0-59.5)	0.2 (0.1-0.3)
Xpert MTB/RIF Ultra										
Total (n=157)	23	0	87	47	32.9 (22.1-45.1)[c]	100.0 (95.9-100.0)	100.0 (85.2-100.0)	64.9 (61.1-68.6)[f]	-	0.7 (0.6-0.8)
South Africa (n=68)	11	0	47	10	52.4(29.8-74.3)[e]	100.0 (92.5-100.0)	100.0 (71.5-100.0)	82.5 (75.0-88.0)[h]	-	0.5 (0.3-0.8)
India (n=89)	12	0	40	37	24.5(13.3-38.9)[e]	100.0 (91.2-100.0)	100.0 (73.5-100.0)	51.9 (48.0-55.9)[h]	-	0.8 (0.6-0.9)
ADA (cut-point: 30 IU/ml)										
Total (n=158)	54	12	81	11	83.1 (71.7-91.2)	87.1 (78.6-93.2)	81.8 (72.4-88.5)	88.0 (81.0-92.7)	6.4 (3.8-11.0)	0.2 (0.1-0.3)
South Africa (n=78)	19	6	50	3	86.4 (65.1-97.1)	89.3 (78.1-96.0)	76.0 (59.4-87.3)	94.3 (85.3-98.0)	8.1 (3.7-17.5)	0.2 (0.1-0.4)
India (n=80)	35	6	31	8	81.4 (66.6-91.6)	83.8 (68.0-93.8)	85.4 (73.5-92.5)	79.5 (67.1-88.0)	5.0 (2.4-10.6)	0.2 (0.1-0.4)
ADA (cut-point: 40 IU/ml)										

Total (n=158)	35	7	86	30	53.8 (41.0-66.3)[d]	92.5 (85.1-96.9)	83.3 (70.3-91.4)	74.1 (68.7-79.0)[g]	7.2 (3.4-15.1)	0.5 (0.4-0.7)
South Africa (n=78)	14	3	53	8	63.6 (40.7-82.8)	94.6 (85.1.-98.9)	82.4 (59.8-93.6)	86.9 (79.2-92.0)	11.9 (3.8-37.3)	0.4 (0.2-0.7)
India (n=80)	21	4	33	22	48.8 (33.3-64.5)	89.2 (74.6-97.0)	84.0 (66.5-93.3)	60.0 (52.3-67.2)	4.5 (1.7-12.0)	0.6 (0.4-0.8)

[a]Reference standards for accuracy analysis: Definite TPE, (i) at least one positive *M. tuberculosis* Xpert Ultra and/or culture on pleural fluid and/or biopsy specimen; and/or (ii) granulomatous inflammation with caseation and/or necrosis on histological examination of pleural biopsy tissue; and/or (iii) acid fast bacilli on histological examination of pleural biopsy tissue. Non-TB, patients with no microbiological or histological evidence of *M. tuberculosis* and/or an available alternative diagnosis. [b]Available results for participants with Definite TPE and Non-TPE: IRISA-TB™ (n=153); Xpert Ultra (n=157); ADA (n=158). Superscript letters indicate statistical significance: [c]$p<0.001$, [d]$p=0.002$, [e]$p=0.023$, [f]$p<0.001$, [g]$p<0.001$, [h]$p<0.001$. ADA, adenosine deaminase. CI, confidence interval. LR, likelihood ratio. NPV, negative predictive value. PPV, positive predictive value. TB, tuberculosis. TPE, tuberculous pleural effusion. Xpert Ultra, GeneXpert® MTB/RIF Ultra.

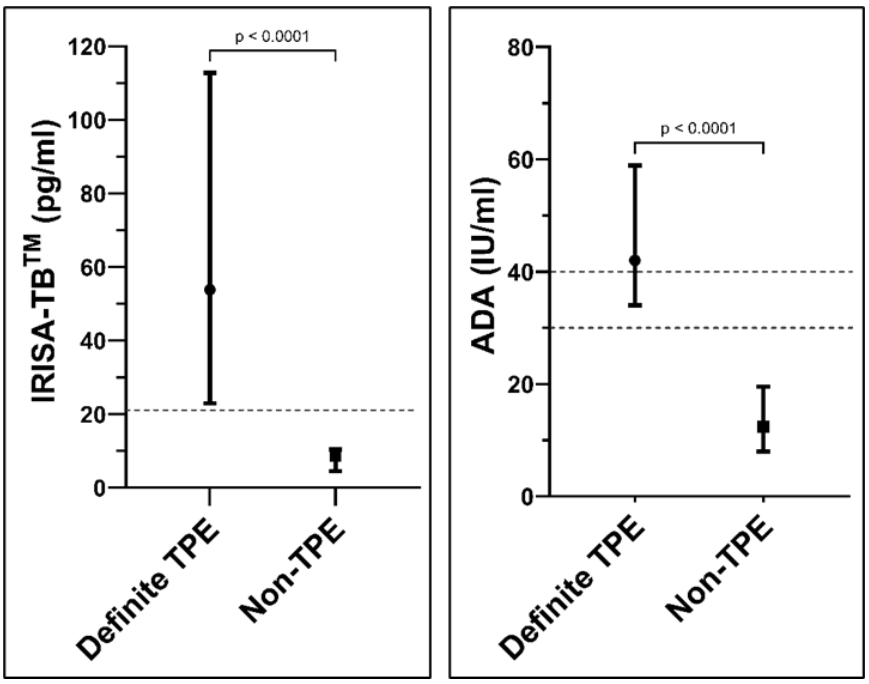

Figure 2. Box-plot depicting median (IQR) IFN-γ levels of IRISA-TB™ (left) and ADA (right) using pleural fluid from patients with Definite TPE and Non-TPE. Dotted lines represent cut-points (IRISA-TB™, 20.5 pg/ml; ADA, 30 IU/ml and 40 IU/ml). ADA, adenosine deaminase. IQR, interquartile range. Interferon gamma, IFN-γ. TB, tuberculosis. TPE, tuberculous pleural effusion.

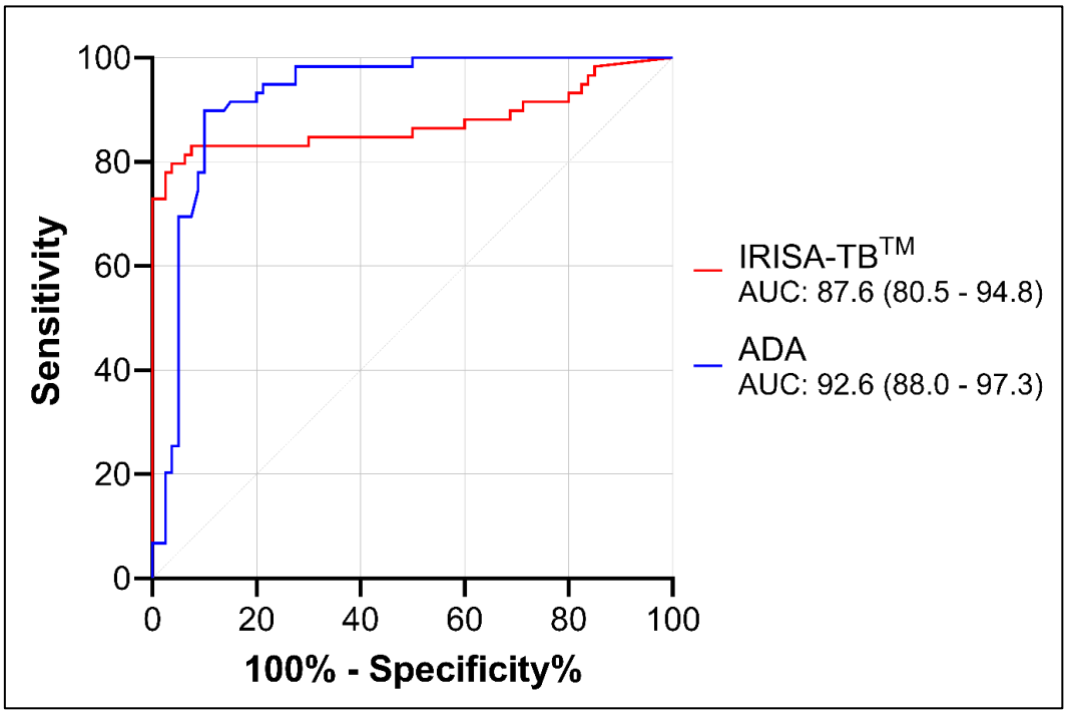

Figure 3. Area under the receiver operator characteristic curves (AUROC) for IRISA-TB™ and ADA. ADA, adenosine deaminase. CI, confidence interval. TB, tuberculosis. TPE, tuberculous pleural effusion.

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PART 3: DISCUSSION AND CONCLUSIONS

Chapter 6: CONSOLIDATED DISCUSSION

6.1: Introduction to the discussion

Tuberculosis is a leading cause of death from an infectious disease globally [94]. South Africa bears one of the highest burden of TB globally with an estimated prevalence of 852 cases per 100,000 population [94]. The prevalence of TB in HIV-infected persons is more than double that of HIV-negative individuals with an estimated prevalence of 1734 cases per 100,000, despite the extensive coverage of HIV prevention and treatment programs in South Africa.

The global benchmark that are used for developing TB diagnostic tests are the WHO's target product profile (TPP). These TPP are now a decade old and no diagnostic screening test on the market currently has met the 'optimal' TPP thresholds for tuberculosis. An important reason for this is the heterogenous nature of TB disease in various sub-populations of TB (e.g., paucibacillary disease, in minimally symptomatic community-based patients, patients with EPTB, etc.), poor quality of sample (e.g., sputum) from patients who are critically ill, human, and mycobacterial genetic diversity and the presence of co-morbidity like HIV. Thus, it is the case of 'one-size' not addressing the unique needs of all the important sub-populations of TB.

The diagnosis of TB in HIV co-infected sub-population is challenging due to the paucibacillary nature of pulmonary TB and the higher prevalence of extra-pulmonary tuberculosis (EPTB) in PLHIV (persons living with HIV) [95]. The challenge in establishing a diagnosis of TB in *hospitalised* HIV-infected patients is even greater due to patient's inability to provide a good quality sputum on account of their critical illness, which further compounds the problem of paucibacillary TB.

This sub-population is at risk of rapid progression of disease and exceptionally high mortality rates [11].

In addition to HIV, there are several other pauci-bacillary sub-populations in whom the frontline diagnostics, such as GeneXpert, do not perform well. One such sub-population is that of patients with EPTB where the diagnosis of TB is especially challenging, not only because of the paucibacillary disease state associated with TB in an extra-pulmonary compartment but also by the difficulty in obtaining a diagnostic sample from the affected extra pulmonary site. These challenges are associated with delayed treatment initiation with consequent morbidity and mortality [96]. The commonest types of EPTB are pleural TB and TB adenitis. However, TB meningitis and TB pericarditis, although less common, represent the more severe sub-types of EPTB associated with higher mortality. The sensitivity of *sputum* culture in patients with EPTB seems to vary by the site of EPTB: 28%-50% for abdominal TB, 10%-11% for tuberculous pericarditis, 24%-29% for tuberculous meningitis, and 5%-14% for tuberculous lymphadenitis [22]. The yield of TB culture from EPTB compartments such as pericardial and pleural fluid compartments (TB serositis) is also sub-optimal approaching a sensitivity of only ~50% [23,24] whilst the yield of TB culture in cerebrospinal fluid (CSF) is only ~26% even in cases where the volume of CSF was deemed to be adequate (i.e., ~6 mL)[25]. Alternative approaches to making a diagnosis of TB serositis and meningitis that integrate fluid biochemical tests (e.g., adenosine deaminase [ADA]), clinical scoring tools (e.g., Tygerberg diagnostic index), and DNA-based diagnostic tests (e.g., Xpert) also perform poorly [26,27]. This clearly highlights that EPTB is a paucibacillary disease state where even “gold standard” microbiological test i.e., TB culture, performs poorly. This underscores the critical need for developing more sensitive and potentially microbiologically independent diagnostic strategy for EPTB.

Lastly, a sub-population of TB that is important but often neglected, is that of minimally symptomatic and smear negative individuals who form an enormous proportion of our undiagnosed TB burden [29]. This sub-population, on account of potentially early disease, also represents a group of patients who have paucibacillary disease with milder symptoms and resultant delays in seeking care. This delay likely contributes markedly to the transmission and perpetuation of the TB epidemic [94]. The feasibility and impact of scalable strategies that incorporate the highly sensitive and point-of-care molecular diagnostic tools, such as Xpert has, hereto, had not been evaluated. In summary, the sub-populations alluded to in this introduction above form a significant proportion of the overall TB burden. However, diagnostic tools like Xpert, sputum smear and TB culture are inadequate to effectively detect TB in these sub-population. Therefore, I have selected my PhD thesis to focus on the diagnosis of tuberculosis in these specific but important sub-populations. Addressing the diagnostic challenges in these sub-populations are critical for us to avert deaths, prevent suffering and interrupt the transmission of TB in endemic African countries.

6.2: Summary of the key findings in this thesis

Overall: The performance of newer diagnostic tools varied in different subpopulations due to differential mycobacterial load, the compartment interrogated, patient phenotype (e.g. HIV-infected), testing strategy, and clinical context (e.g. hospitalized versus community-based). Main findings from each chapter as summarized as follows:

Chapter 1: Optimal diagnostic strategy for the diagnosis of TB in sputum expectorating hospitalized HIV-infected patients.

Key findings: the incremental yield of LAM over Xpert was 29.6% (45/152) and that of Xpert over LAM was 75% (84/11). The incremental yield of LAM increased with decreasing CD4 count. The costs per TB case diagnosed were similar for the sequential and concurrent strategies ($1,617 to $1,626). Thus, concurrent testing with Xpert and LAM may be the best strategy for diagnosing TB in this group of patients who may not be able to expectorate a good sputum sample.

Chapter 2: Confirmation of the higher sensitivity of Xpert Ultra and significance of trace readouts

Key findings: The sensitivity of Xpert Ultra was confirmed to be significantly higher than that of Xpert MTB/RIF G4 in smear negative TB (confirmed in limit-of-detection (LOD) experiments, 9 vs. 184 cfu/mL). However, this increased sensitivity of Ultra is achieved at a cost of slightly decreased specificity (<5%) in patients with a previous history of TB. Trace results are associated with culture positive and negative samples (66.7 vs. 33.3). Thus, clinical judgement needs to be applied when initiating treatment in a patient with a trace readout.

Chapter 3: Impact of Xpert Ultra at POC for community-based active case finding.

Key findings: Xpert Ultra performed at POC as part of a scalable strategy for community-based active case finding using mobile labs is feasible. Although, Xpert Ultra missed ~50% of culture positive TB, it detected almost all potentially infectious patients.

Chapter 4: Comparative diagnostic performance of the more sensitive Xpert Ultra for the diagnosis of tuberculous pericarditis compared to a novel immunodiagnostic test (IRISA-TB)

Key findings: Unstimulated interferon-gamma (IRISA-TB) was significantly more sensitive than Xpert Ultra for the diagnosis of TB pericarditis in a TB endemic resource poor setting.

Chapter 5: Comparative diagnostic performance of the more sensitive Xpert Ultra for the diagnosis of Pleural TB compared to a novel immunodiagnostic test (IRISA-TB)

Key findings: IRISA-TB demonstrated markedly better sensitivity and NPV than Xpert-Ultra, and excellent specificity for the diagnosis of TPE in TB-endemic settings. These data have implications for clinical practice in TB-endemic settings.

6.3: Diagnosis of TB in hospitalized HIV-infected patients

TB is a leading cause of death in HIV-infected individuals [95,97]. A major contributor to the high mortality is attributed to the difficulty in establishing a diagnosis of TB timeously, particularly in HIV-infected hospitalised patients who have the highest mortality rates [95,97]. Achieving a timely diagnosis of TB in this sub-population is critical due to their rapid progression to severe disease and exceedingly high mortality [11,98]. However, this is easier-said-than-done since this sub-population poses significant diagnostic challenges due to the patient's inability to provide a good quality sputum, higher rates of smear-negative (paucibacillary) TB attributed to the immune dysregulation in the HIV-infected host and having a higher prevalence of extra-pulmonary disease. It is therefore not surprising that the frontline rapid sputum-based diagnostic tests such as smear microscopy, TB culture and nucleic acid amplification tests (e.g., GeneXpert MTB/RIF/ Xpert) perform sub-optimally in this sub-population compared to ambulant HIV-negative patients. Sputum TB culture is often regarded as the 'gold standard' for the diagnosis of TB in this group of patients. However, despite its higher sensitivity compared to NAAT (since theoretically TB culture only requires only 1 viable cfu/mL to achieve a diagnosis of TB compared to ~20 cfu/mL with Xpert Ultra), it is associated with an unacceptable time-to-result delay of between 2-6 weeks in a patient sub-population that progresses to death rapidly over days [11].

Thus, to enable a timely diagnosis of TB in hospitalised patients, a novel approach is required that incorporates both sputum and non-sputum diagnostic strategies to secure the diagnosis of TB. Several years ago, a seminal RCT from our research group demonstrated the utility and mortality benefit conferred by a point-of-care urinary LAM test (AlereTM Lipoarabinomannan) in this patient sub-population[11]. Since then, both GeneXpert MTB/RIF and Alere LAMTM are

tests that have been recommended by the WHO for the rapid diagnosis of TB in hospitalised HIV-infected patients. The sensitivity of sputum Xpert is ~60% [99] in smear negative TB whereas urinary LAM achieves a sensitivity of ~30-45% [63,100] in this patient sub-population. However, the WHO guidelines are unclear on the integration LAM and Xpert testing in clinical algorithms for hospitalised HIV-infected sub-population, and whether these tests should be performed sequentially or concurrently to achieve the highest yield in diagnosis in the most cost-efficient manner. To address this, we performed a post hoc analysis of 561 HIV-infected sputum-expectorating hospitalized HIV-infected patients with suspected TB [100] (Chapter 1). We demonstrated that a strategy of concurrent LAM and Xpert testing was the optimal approach to diagnosing TB in HIV-infected hospitalised patients. Furthermore, this publication also highlights the importance of performing a non-sputum based diagnostic tests (LAM), even in sputum expectorating hospitalised HIV-infected patients. We further demonstrated that the diagnostic performance of LAM, in contrast to the diagnostic performance of Xpert, increased with a decrease in CD4 counts. Finally, using a cost-consequence analysis, we highlighted, that the cost per TB case diagnosed were similar between the concurrent and sequential testing strategy (USD 1,626 vs. 1,617). Our conclusion emanating from this piece of work is that in hospitalised patients, whether sputum expectorating or not, the optimal strategy to diagnose TB is to perform both LAM and Xpert concurrently. These data have informed the South African LAM guidelines [101]. However, despite the incremental yield of LAM in this patient population, the overall sensitivity of LAM remains sub-optimal at ~30-60% in this patient population.

6.4: Improving the sensitivity of diagnostic tests (LAM and Xpert) for detection of TB in paucibacillary states.

One potential approach is to improve the sensitivity of diagnostic tests such as LAM is by lowering their detection threshold [102]. The Abbott Determine-TB LAM has a detection threshold for LAM of ~300-500 pg/mL. Second generation tests (e.g., SILVAMP TB-LAM also known as FujiLAM) have reduced the detection threshold for LAM even further using high affinity monoclonal antibodies that can detect LAM concentrations down to the level of ~50 pg/mL while the more novel third generation tests, using sensitive electrochemiluminescence assays, are capable of detecting concentrations of LAM in the region of ~10pg/mL [103,104]. The prospect of the improved sensitivity in detecting urinary LAM offered by the second and third generation assays may, in the future, facilitate a more broader use of urinary LAM detection including in HIV-negative patients [105], in ambulatory HIV-positive patients without advanced immune suppression [103,106], in patients with EPTB [107] in children and in the context of community-based active case finding since urine is much easier to obtain than sputum from these groups of minimally symptomatic or asymptomatic patients. However, despite their initial promise [106], the enthusiasm for SILVAMP TB-LAM (second generation) has waned due to the wide lot-to-lot variability [108,109]. Given these findings, the manufacturer has retracted this test from the market in 2022 and it is my sincere hope that the technological challenges associated with this will be overcome in the near future. Thus, to date, Abbott Determine TB LAM test remains the only LAM antigen test with evidence from a randomised controlled trial of a mortality benefit[11].

The performance of the urinary lipoarabinomannan (LAM) test for diagnosing tuberculosis (TB) can exhibit variations influenced by several factors. Broadly speaking, these factors they are divided into (i) patient factors: including the patient population being tested (hospitalised vs. non-hospitalised) and stage of HIV (advanced HIV with disseminated TB and low CD4 counts

vs. non-disseminated TB, preserved CD4 count and on treatment with antiretroviral agents); (ii) operator factors: (experienced vs. non experienced, testing SOPs according to the manufacturer's instructions, interpretation of results in natural light vs. artificial light); (iii) deficiencies with the LAM assay itself (including batch to batch variability in the performance of the antibodies contained, poor storage conditions in which the LFA is stored); (iv) other contributing environmental factors such as the time of the day when the test is performed and prevalence of non-tuberculous mycobacterial infection (leading to false positivity) in the particular geographical location etc. Thus, the currently approved Abbott Urinary LAM antigen test should be interpreted with caution in the context of individual patient characteristics and local epidemiology

Similarly, Cepheid more recently have developed a more sensitive version of GeneXpert called GeneXpert MTB/RIF Ultra (Xpert Ultra). Xpert Ultra has a reportedly improved sensitivity of ~12% in smear negative TB compared to the G4 version of the GeneXpert MTB/RIF test[51]. The test also seemed to have a better performance in PLHIV with a sensitivity of 87.5% compared with 68.6% attributed to the older version of GeneXpert MTB/RIF. The improved sensitivity of Xpert Ultra is achieved by the addition of 2-multicopy PCR amplification targets (IS1810 and IS6110) to the standard single-copy rpoB gene target and using a larger PCR reaction chamber [51-53]. However, the improved sensitivity comes at the cost of reduced specificity[54], mainly when the Xpert Ultra yields a 'Trace' readout. 'Trace' is a semiquantitative readout unique to the Xpert Ultra assay which indicates the detection of a very small amount of mycobacterial DNA that is insufficient for the assay to detect the rpoB gene (which is why rifampicin resistance status is always indeterminate during a 'Trace' readout) but sufficient to detect one or both of the multi-copy IS1810 and/or IS6110 genes. There were hardly any industry independent data

about the Utility of Xpert Ultra in paucibacillary TB disease states at the time e.g., in PLHIV and in those with a history of previous TB where the predominant use of this test is likely to occur. Additionally, the epidemiology of trace readouts has been poorly studied. There were limited data at the time about the comparative limit of detection of the two Xpert assays. In Chapter 2, Using assay-specific limit of detection experiments performed using serial dilutions of M.tb H37Rv, we confirmed that the limit-of-detection for Xpert Ultra was indeed lower at 9 compared to 184 CFU/ml with the G4 Xpert MTB/RIF [110]. Overall, in our study, Xpert Ultra was ~6% more sensitive compared to Xpert MTB/RIF (G4) [77% (68.9–85.1) vs. 71.2% (62.5–79.9)] in well characterised and archived smear-negative sputum samples. Although this was not significant due to the small sample size, we conclusively confirmed this higher sensitivity using the limit-of-detection (LOD) experiments. Importantly, ~3.3% (9/272) of all samples in our study were trace positive with Xpert Ultra. Of these, 6/9 (66.7%) were likely true-positives (TB culture positive) whilst 3/9 (33.3%) were false-positive (TB culture negative). In the sub-group of non-TB patients with a previous history of TB treatment (this group was chosen to accentuate the trace readouts), analysing the data by re-classifying trace calls as “negative” improves the specificity from 62% to 77%, suggesting that a proportion of patients with a trace readout may not active culture positive TB disease. The recent WHO guidance regarding Xpert Ultra “trace readouts” recommend that consideration be given for initiating TB treatment in HIV-negative adults who do not have a prior history of TB with in the last 5 years, In HIV-infected patients, in children and in patients with trace readout from EPTB samples[111]. However, this guidance is unhelpful for many TB endemic settings, for example, a recent study from Zambia conducted in primary health care clinics showed that only ~10% of patients with a trace readout were in fact culture positive despite majority ≥60% of these patients having symptoms of TB, an elevated CRP and/or an abnormal chest x-ray[112]. Similarly, a recent study from Uganda also showed that although ~50% of Xpert Ultra positive results are trace readouts during

community-based ACF, only ~25% had any microbiological confirmation of TB [113]. In patients with trace readouts without any microbiological evidence of TB, ~50% of patients had abnormality on chest CT, with most having non-specific findings such as nodules, septal thickening and coarse bronchovascular changes. Only 10% of this group had abnormalities suggestive of cavitation on CT compared to 30% in the group of patients with trace readouts and microbiologically proved TB. Thus, these finding together suggest that in the context of passive case finding, substantial proportion of trace results may be false positive (when using TB culture as a reference) but may also represent TB that is non-replicating. There may also be challenges with the transport and storage of the sputum sample which may contribute to the attrition in TB growth.

In summary, sputum Xpert Ultra trace call results, which indicate a minimal TB burden, are commonly encountered in high TB-HIV burden settings and present new clinical challenges in interpretation, especially among previously treated patients. Where possible sending another sample and requesting adjunct investigations such as CXRs and CRP levels may help to guide optimized management of presumptive TB patients with Ultra trace call results. Trace readouts from extra-pulmonary fluid samples would often urge the clinician to initiate TB treatment especially when dealing with severe forms of EPTB like suspected TB meningitis or pericarditis. However, recent emerging evidence is challenging this practice. Our study [110] together with data from various other publications [91,112,113] suggest that the implications for missing a TB diagnosis must be balanced with erroneously prescribing potentially toxic treatment. Therefore, trace-positive results of TB should be carefully considered in the clinical context (history of previous TB, duration since the last episode of TB, clinical symptoms, severity of illness and presence of extrapulmonary TB) before a decision to initiate TB treatment is taken.

6.5: Use of more sensitive Xpert Ultra as a point-of-care test for detecting TB, including potentially infectious patients in the community

There are approximately 3 million patients who remain undiagnosed or unreported with TB globally, most reside in informal settlements surrounding major cities. The missing patients with undiagnosed TB in the community are an important sub-population to detect and treat, and hence crucial for TB control, because they serve as a potential reservoir for transmission of drug-sensitive and drug-resistant strains of Mycobacterium tuberculosis [114]. Xpert Ultra with its improved sensitivity in paucibacillary states was hailed as a potential game changer in detecting the missing cases of TB especially when paired with battery-operated portable Xpert platforms. However, simply having improved molecular diagnostics (such as Xpert ultra) is not sufficient as one must consider the ideal strategy along with a package of interventions to screen for TB in high burden communities.

A door-to-door screening strategy using Xpert has recently been shown to impact disease burden in the wider community [82]. However, such as strategy is labour intensive and expensive making it unsuitable for use in many resource-limited settings. Screening using a mobile unit-based strategy (without the use of molecular diagnostics) has also previously been shown to favorably impacted TB prevalence in the community at a better cost efficiency compared to the door-to-door strategy [74]. Advancing these findings, we (and others) have previously successfully confirmed the feasibility and impact of incorporating molecular diagnostics tools as part of a mobile clinic-based approach [73,115,116]. However, these strategies were personnel intensive (staffed by doctors, nurses, laboratory technicians and radiographers) and were associated with relatively high-cost on account of using electricity-driven mobile laboratories

with capacity to perform on-site smear microscopy, molecular testing, and chest x-ray. Furthermore, although GeneXpert has a proven track record for detecting TB in numerous large clinic- and hospital-based studies [60,117], there are limited data about its performance from paucibacillary populations (for example, in patients with minimal disease burden as is commonly seen in the context of ACF). In such a subpopulation, GeneXpert testing might miss a substantial proportion of low-burden disease (mycobacterial load below the detection limit of the assay), thus significantly negating its intuitively higher sensitivity[118]. Furthermore, GeneXpert's utility might be negated by false-positive readouts within those with previous TB (~20% of those presenting with symptoms in many endemic settings).

Thus, to answer these critical questions, we leveraged the recently launched portable battery-operated version of GeneXpert, to developed a scalable and affordable ACF model (XACT model) consisting of a two-person low-cost minivan which we subsequently evaluated in a RCT (Chapter 3) [20]. Our intention was to develop a model that would not only detect TB within our communities but to also identify potentially infectious patients who perpetuate the transmission of TB. This posed a unique challenge in that, the definition of potentially infectious patients is contentious. We therefore used a combination of well-regarded and proven markers of infectiousness (cough aerosol sampling positivity and/or presence of cavities on chest x-rays and/or smear positivity) to identify potentially infectious patients (GeneXpert Ct-values and TB culture days-to-positivity did not add significantly to the identification of infectious patients). Our findings revealed for the first time in a controlled trial that an ACF strategy using a mini-mobile, clinic-based, scalable intervention package, incorporating a low-cost minivan and portable battery-operated Xpert (that is, the XACT model), was feasible and more importantly detected almost all infectious TB patients[20].

Significantly, these were patients who did not self-report to healthcare facilities. Furthermore, it was shocking for us to learn that Xpert Ultra missed ~50% of patients with culture positive TB (i.e., sensitivity of ~50%). This finding has massive implications for using molecular diagnostic tools for ACF. Our study glaringly highlights the need for more sensitive tools to be incorporated in ACF strategies. In fact, data from this trial has already informed two other large multi-country ACF RCTs which are already underway that are also incorporating chest x-rays (which is a highly sensitive but only moderately specific screening tool) performed using ultra-portable machines and paired with computer assisted diagnosis (CAD) in ACF models. A key limitation of our trial was that we only recruited patients who were either symptomatic and/or had HIV-infection. More recently, the door-to-door population based surveys using chest x-ray screening have revealed that most (~60%) of our culture-positive TB burden in communities is in fact in asymptomatic patients [29].

Thus, more recent ACF trials including our own trials are now screening a broader group of individuals in the community with risk factors such as HIV-infection, presence of diabetes mellitus, having a recent/current close TB contact and/or taking immunosuppressive medications. However, there are several challenges with such strategies that incorporated Xpert Ultra. Firstly, we and others have shown that the performance of Xpert in ACF is sub-optimal ranging from ~50-70% [112,119] and TB culture results, although more sensitive, are associated with an unacceptable delay in diagnosis leading to the patient becoming lost-to-follow up and failing to initiate treatment. Thus, more sensitive rapid, point-of-care sputum and non-sputum tests are urgently required to address this unmet need (one such example is using chest x-rays paired with CAD). Secondly, given that these patients have paucibacillary disease and are asymptomatic, they will likely have a higher rate of trace positive results (low levels TB

DNA). This will undoubtedly lead to endless confusion on which patients require TB treatment. Indeed, a recent door-to-door TB survey from Uganda, suggests that over-all, ~50% of the Ultra positive results were in fact trace readouts in this paucibacillary population. Notably, only 14% of these trace calls were in fact culture confirmed TB[21] suggesting that most patients with a trace readout, in this context, may not actually have active TB.

In summary, community-based active case finding (ACF) for tuberculosis (TB) attempts to screen at-risk populations for TB often integrating health promotion behaviours and screening for other diseases like HIV and sexually transmitted diseases (diseases for which there is a POC test available). The WHO's recent TB screening guidelines conditionally advocate for a broader implementation of TB ACF strategies in communities where the prevalence of undiagnosed pulmonary TB exceeds 0.5% among adults. Subclinical TB (this entity was not included in Chapter 3 of my thesis) is believed to significantly contribute to TB transmission[18], and incorporating chest x-rays paired with CAD in ACF strategies may facilitate earlier diagnoses of sub-clinical TB. However, the evidence regarding the overall effectiveness of ACF at the population level is inconsistent. For example, the ZAMSTAR study (using symptoms and smear microscopy) found no significant effect on TB prevalence[81], whereas the ACT3 (Vietnam; including repeated rounds of TB screening with sputum Xpert testing for all able to produce a sample) showed a 44% relative reduction TB prevalence[82]. Similarly, the TREATS study, offered 4 years of repeated rounds of door-to-door systematic symptom screening and chest X-ray with analysis by computer- aided diagnosis (CAD) software, followed by Xpert testing (HIV-positive) or smear microscopy (HIV-negative) for those symptomatic or with a high CAD score (≥ 50)[78,79]. This trial also found no significant differences in TB prevalence or *M tb* immunoreactivity. Thus, the success of ACF strategy largely depends on the screening

methods used, the intensity of ACF implementation, and the level of participation from both the community and health systems. National TB program managers and public health practitioners must carefully evaluate the potential benefits and drawbacks of ACF programs for both populations and individuals and ensure that they select the correct strategy for their bespoke setting. It is my firm opinion that with meticulous planning and significant investment, community-based ACF can serve as a powerful strategy to expedite the elimination of TB in high-burden settings. It is also important to ensure that investment in ACF strategies should not come at the cost of equitable access to responsive and accessible primary care services for everyone.

Lastly, there are no data on the natural history of asymptomatic TB nor how to best treat such patients. We also do not know whether the asymptomatic patients would agree to initiating and completing a potentially toxic 6-month TB treatment regimen which may cause adverse reactions. Anecdotal data from our large-scale active case finding trials (XACT 3 & 19) are suggesting that only ~50% of patients do in fact complete TB treatment for asymptomatic culture positive TB. Thus, urgent research is required to clarify the natural history and treatment regimens in asymptomatic TB. Given the paucibacillary nature of disease, this phenotype of TB disease may be ideally suited for treatment using shorter duration of TB treatment, for example, using the 2-,month regimen that was successfully used in the TRUNCATE-TB trial (bedaquiline, linezolid, isoniazid and pyrazinamide) [120].

6.6: Novel tests for the diagnosis TB serositis (extra-pulmonary TB)

It is estimated that ~20-25% of the overall burden of TB is extra-pulmonary. This means that of the ~10 million new cases of TB, ~2 million would have extra pulmonary TB. This is an enormous burden despite accounting for the fact that some patients with EPTB (~10-20%) would also have concurrent pulmonary TB [121]. Two of the most common forms of TB serositis are TB pleuritis and TB pericarditis. TB pleuritis is one of the commonest forms of EPTB and accounts for almost a third of all TB in HIV-endemic settings [122,123]. TB pericarditis although less common compared to TB pleuritis, is a more severe form of TB serositis associated with ~20-40% 6-month mortality rates and accounts for up to 10% of hospital admissions for acute heart failure in Africa [124]. Diagnosis of TB serositis is challenging due to the difficulty in obtaining fluid for diagnostic testing and due to the paucibacillary nature of fluid. The performance of NAAT-based tests (e.g., Xpert Ultra) is poor with sensitivity of ~30-40% in TB pleuritis and Pericarditis [47]. Furthermore, 'trace' readouts for Xpert Ultra are common in EPTB accounting for ~25% for all Xpert positive results[125]. However, only ~70% of these patients with trace readouts in fact had culture confirmed TB. It is therefore uncertain whether the remainder ~30% of patients with trace-positive and culture negative results, had TB. Thus, highlighting the importance of sequencing the remnant product from the trace-positive MTB/RIF Ultra cartridge to confirm the presence or absence of TB DNA to study this group of patients better. Even TB culture, a test that often takes up to 6 weeks to yield a result, and which is used as part of the 'gold standard' for the diagnosis of TB serositis, has a sensitivity of only ~40-50% when using a composite reference standard of tissue histopathology, fluid and tissue culture [47]. This clearly illustrates that fluid obtained from the pleural and pericardial space is paucibacillary.

Given these shortcomings an immunodiagnostic approach has traditionally been used by measuring adenosine deaminase levels (ADA: an enzyme produced by mononuclear cells) in pleural and pericardial fluid.[126] However, there are several challenges and hurdles with this approach. Mainly, specificity of ADA was shown to be sub-optimal (60%-70%) in several studies, when using pleural biopsy as a reference standard [8,127], and the negative predictive value (rule-out value) was also sub-optimal at ~70-80%, meaning that it was a poor tool to portend an alternative diagnosis and hence the need for pleural biopsy [8,127]. More recently, unstimulated IFN-γ has been shown to be an excellent immunological biomarker for the diagnosis of pleural and pericardial TB in systematic reviews and meta-analysis [128-130]. However, a standardised clinically validated assay to measure unstimulated IFN-γ is unavailable. To address this unmet need, a University of Cape Town owned start-up company called Antrum Biotech developed the novel IRISA-TB test using bespoke and clinically validated antibody-pairs for detecting IFN-γ to enable the diagnosis of TB serositis. This test, in smaller single centre studies, has showed an excellent sensitivity of ~80-90% with a specificity of >90%, positioning itself as the best same-day diagnostic test for TB serositis [8,127,131,132].

As part of my PhD, I lead two separate projects to validate the performance of the IRISA-TB test in TB pericarditis (Chapter 4 and TB pleuritis Chapter 5). The main advancement for the broader clinician and scientific community with these two studies is that we used a commercially available kit (IRISA-TB) that requires very basic laboratory equipment and can be implemented rapidly in all resources limited settings. Furthermore, for the validation of IRISA-TB test in TB pericarditis (Chapter 4), our study enrolled the largest cohort of patients with pericardial TB who were very comprehensively evaluated with Xpert Ultra, pericardial TB fluid culture, pericardial biopsy histopathology, ADA and IRISA-TB. Similarly, the evaluation and validation

of IRISA-TB test for Pleural TB (Chapter 5) was also performed in a large study (n=~200) that recruited patients from two geographically different areas (i.e., South Africa and India) with very different HIV prevalence. This allowed us to not only comprehensively evaluate the performance of the test in both low (India) and high (south Africa) HIV-prevalence settings but also outsource the performance of the IRISA-TB test to a busy public health laboratory based at the Christian Medical Centre in Vellore, Chennai, India which provides data on the external validity of this test when incorporated in the workflow of busy clinical laboratories. Collectively, the results from both these studies confirm that IRISA-TB™ demonstrated markedly better sensitivity and NPV compared to Xpert-Ultra, and excellent specificity (better than ADA) for the diagnosis of TB serositis in TB-endemic settings. Lastly, as part of a separate publication where I was the senior author, we showed using a cost-consequence analysis that IRISA-TB was more cost-effective compared to ADA for the diagnosis of pleural TB with a cost saving of ~5000 USD compared to ADA per TB patient diagnosed [133]. In summary, most patients with TB serositis are likely to be assessed in hospitals where a diagnostic tap is normally performed. Thus, given the high sensitivity and specificity of IRISA-TB, it has the potential to provide a result during the same consultation which would then enable the attending clinician to initiate or withhold TB treatment as appropriate to limit morbidity and prevent the patient from getting lost-to-follow up.

6.7: Impact of my PhD work

The work contained in my PhD thesis at its core highlights the deficiency in the performance of frontline diagnostic tests in these significant sub-populations, and the potential solutions and strategies that can be leveraged to address these deficiencies. Many of the sub-populations described in my PhD, e.g., hospitalised HIV-infected patients and patients with EPTB, are associated with high rates of poor outcomes. Thus, the work contained in this thesis directly addresses the morbidity and mortality associated with these sub-populations. The work contained in my PhD has direct relevance to clinicians specifically, and the National Tuberculosis Program, broadly.

Our work on the integration of LAM and Xpert testing in hospitalised patients offers direct and clear guidance to hospital-based clinicians to investigate patients with both sputum Xpert and urinary LAM tests concurrently. This body of work also informed the currently South African LAM guidelines (study was presented at the 'Think Tank' meeting) and also confirmed that the cost per TB case diagnosed with the concurrent (LAM+Xpert) testing strategy was the most favourable. Furthermore, detecting the 'missing millions' of patients with undiagnosed TB in our communities is a priority task set out by the WHO and the STOP-TB global partnership. Our Important work in developing the XACT model for active case finding to detect TB in our communities (Chapter 3; publication in Nature Medicine) provides a low-cost and efficient model that is ideally suited for detecting TB in peri-urban informal settlements, and which leverages the latest advancements in technology to incorporate point-of-care molecular diagnostic tools in the strategy. It also serves to highlight the pitfalls of relying solely on molecular diagnostics which tend to miss a significant proportion of culture positive TB.

This project also pioneers a unique method of measuring the infectiousness of patients in the community. These data directly inform strategies for ACF in high burden TB/HIV communities. The findings of this trial have directly led to the funding and initiation of two well-funded large scale community-based active case finding trials (NCT04303104; funded by the UKMRC/ Wellcome Trust and NCT05220163; Funded by European Union Horizon program) that are currently using the XACT model to evaluate TB epidemiology, diagnostic strategies and infectiousness of TB patients in three African countries. Additionally, data from this trial has been shared by the WHO and deposited in an open access data repository to inform guidelines and enable further analysis leading to a multiplier effect. Chest x-ray data from this trial has also been contributed to the WHO/FIND had have been used to validate leading CAD software (artificial intelligence to detect TB in community-based ACF)[133] for use as a screening test in our communities.

Lastly, the work contained in my PhD thesis has led to the refinement and validation of a novel test for TB serositis (Pleural and Pericardial TB). This is a substantive contribution to science and patient care because both TB pericarditis and TB pleuritis are associated with massive delays in diagnosis, increased morbidity and, in the case of TB pericarditis, increased mortality. IRISA-TB is probably the best performing commercially available same-day diagnostic test for the detection of TB in patients with suspected TB pericarditis and TB pleuritis. Importantly, the IRISA-TB test is now registered and licensed with SAHPRA (south African Health Products Registration Authority) and is also ISO certified and CE marked which further attests to the impact of the work undertaken as part of my PhD. I have leveraged the data from the TB pericarditis and TB pleuritis studies to obtain funding for a large IRISA-TB test validation trial in 4 (four) forms of EPTB (TB pericarditis, TB pleuritis, TB peritonitis and TB meningitis) which is

being performed in three African countries (South Africa, 3 sites; Zimbabwe, 1 site; Zambia, 1 site). This trial will also be evaluating the impact of the IRISA-TB test on treatment initiation and patient outcomes.

6.8 Future directions and research

Sputum is challenging to obtain in patients with EPTB, those that are critically ill patients and in patients who are minimally symptomatic or who are asymptomatic on account of early disease. This highlights the need for better, more sensitive and novel non-sputum-based diagnostic tests. These tests could be used to screen the population for TB (very high sensitivity with moderate specificity) or could be used as a rule-in test where the specificity is very high (with a reasonable sensitivity) that allows it to be used as a confirmatory test. Urine is an excellent sample to use for both a screening as well as a rule-in test because its non-invasive and easily obtainable in most patients including the critically ill. More recently, improvement in technology have allowed us to develop and validate more sensitive urinary LAM tests (e.g., Fuji SILVAMP-TB LAM). Even though this test has recently been pulled off the market due to the variability in lot-to-lot performance of the test, it still holds promise. Many other large companies e.g., Abbott (who also own the Determine TB LAM test) have now invested in developing better and more sensitive LAM-based urine assays. It is therefore very likely that we will have a useful urinary LAM test emerging in the next few years that can be used in HIV infected and uninfected persons. Additionally, technological advancement in sequencing technology is enabling novel approaches for the diagnosis of TB such as those focusing on detecting cell-free DNA in urine or in exosomes in urine [134]. These approaches although experimental hold great promise for the future.

I have been involved in studies where we have leveraged the production of volatile organic compounds (VOC) by patients with active TB either in their breaths (obtained by using an electronic 'nose') or exuded from their skin (collected using a skin plaster)[135]. These approaches generally utilise machine learning of massive data generated by either the use of mass spectrometry to identify molecules associated with active TB or the use of different types of electrochemical sensors (e.g., metal oxide) that measure the reaction of the VOCs on a particular metal oxide sensor by stimulating them with heat at various temperatures. These technologies, although nascent, may hold promise for use in the future.

A recently launched blood-based screening test by Cepheid called the MTB-HR test uses a 3-gene host signature to detect TB. This test has generated great excitement and has been shown to be achieve the minimum target product profile for a triage test [136]. We have currently applied for funding to evaluate and validate the performance of this test as a triage test to detect the 'missing' patients with TB in our communities.

In recent years, samples obtained using tongue scrapes have been subjected to Xpert Ultra with variable results. The key issue here seems to be that Xpert testing is optimised for use in sputum. However, efforts are underway to experiment various other methods of preparing the tongue scape samples to improve its performance[137].

The development of ultra-portable chest x-ray systems (e.g. Delft Ultra) allows digital chest x-rays paired with CAD, to lend themselves as ideal tools for TB screening since they have a high

sensitivity. However, its utility in community based ACF is limited due to its low specificity (~50%) in this sub-population. Despite this, there is global effort, including a trial from our own unit (NCT05220163), that is evaluating the impact of incorporating chest x-rays in screening algorithms that also include highly specific molecular tests such as POC-Xpert Ultra (in essence the strategy involves using a highly sensitive tool like chest x-rays for triage with a specific microbiological tool like Xpert used as a confirmatory test). Chest x-ray/CAD technology has the potential for rapidly screening populations at risk to identify potential patients who can undergo confirmatory testing at POC to enable prompt TB treatment initiation and halt transmission.

Extrapulmonary TB (EPTB) is often neglected as part of broad approaches to control TB, partly because it is thought to be non-transmissible. Thus, investment in developing diagnostic tests for EPTB have generally lagged behind. There are two main challenges with regards to the diagnosis of EPTB: (i) the difficulty in obtaining fluid from the affected compartment and (ii) The lack of validated highly sensitive and specific tests. These factors underscore the need for the development of tests for EPTB that use non-invasive samples like blood and urine. Although IRISA-TB addresses the need for a more accurate diagnostic test, it still requires basic laboratory infrastructure to perform and may still be a hinderance to initiating TB treatment at point-of-care. To address this, we are now developing a POC lateral flow version of the IRISA-TB test (IRISA-POC). This test will have the potential to greatly reduce morbidity and mortality by enabling prompt treatment decisions at primary/secondary level health care facilities which have capacity to sample the affected EPTB compartment. Early laboratory evaluations of the IRISA-POC have shown excellent performance comparable to that of IRISA-TB testing in the laboratory. This test will now be moving to early evaluations in the coming months.

Transcriptomic analysis of fluid from extra pulmonary compartments is yet another experimental frontier which may be used to develop host biomarker signatures for the diagnosis of active TB or to evaluate response to treatment.

Lastly, it is important to recognize that a huge burden of our TB is asymptomatic/minimally symptomatic and is concentrated in high burden communities. We have shown in our publication (Chapter 3) that this sub-population most likely is involved in the perpetuation of the TB epidemic (modelling studies attribute ~2/3 of the transmission to this sub-population of TB). Thus, special efforts need to be diverted towards addressing diagnostic challenges in this important sub-group of patients. Preliminary evidence from our ongoing active case finding trial suggests that despite the low burden of symptoms these patients have substantial burden of disease on chest x-rays and PET-CT scans including cavitary disease (data from XACT-19 trial). Thus, it is critical that these patients are detected as early as possible into their disease course to ensure that prompt treatment is initiated to limit post-TB lung disease (PTLD). Although it is now widely appreciated that PTLD is an important non-communicable disease, and that it's the most common cause of pulmonary disability in many resource-poor settings, hardly anything is known about the natural history of this disorder (i.e. who progresses to more severe disease, and to what extent, over time). However, the ideal treatment of patients with minimally symptomatic or asymptomatic TB disease is unknown. Thus, advances in TB diagnostics for this sub-population of patients must go together with the development of evidence-based, short and effective treatment strategy to ensure that these patients with minimal symptoms go on to complete prompt TB treatment to potentially stop the development of PTLD. There are some exciting new TB vaccines (see introduction section with

summary table) on the horizon that hold promise for the potential eradication of TB in the distant future when used in conjunction with other key pillars of TB control.

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PART 4. APPENDIX

Chapter 1: SUPPLEMENTARY MATERIAL

An optimal diagnostic strategy for tuberculosis in hospitalized HIV-Infected patients using GeneXpert MTB/RIF and Alere Determine LAM

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MATERIALS AND METHODS

Economic analysis

A cost-effectiveness analysis was performed from the South African healthcare provider perspective to evaluate and compare the following strategies for diagnosing tuberculosis (TB) in hospitalized patients with advanced HIV suspected of TB: (i) performing Xpert alone (Xpert only); (ii) performing urine LAM alone (LAM only); (iii) performing Xpert only in patients with an initial Xpert negative result (Xpert in LAM-ve); (iv) performing urine LAM only in patients with an initial negative Xpert result (LAM in Xpert-ve); (v) performing Xpert and LAM concurrently (LAM+Xpert). Calculations were performed using Microsoft Excel (Microsoft) and GraphPad Prism 6.0 (GraphPad).

Costs

Costs were expressed in US$2018 at an exchange rate of ZAR13.20 to US$1 (http://wdi.worldbank.org/table/4.16). Costs were subsequently inflated to the year of analysis where appropriate using the World Bank Consumer Price Index for South Africa (https://data.worldbank.org/indicator/FP.CPI.TOTL?locations=ZA). No discount rate was applied due to the short timeframe of the analysis. The unit cost of Xpert was obtained from the National Health Laboratory Service (NHLS). The NHLS is a reference lab which provides services for the public healthcare system in South Africa, so these costs represent the actual costs incurred by the South African National TB Program. Such estimates have been used in other health economic studies (1-3). The unit cost of the urine LAM test was provided by Alere, the test suppliers of the Determine™ TB LAM Ag lateral flow strip test and included laboratory consumables and staff time. The cost of anti-TB treatment for 6 months in South Africa was estimated from the per patient TB budget as reported in the

WHO South African tuberculosis finance profile (4). (Table S5).

Outcomes

Model probabilities were calculated based on test sensitivities and specificities reported in Table 2 of the main manuscript. TB prevalence for this specific population was similar to the prevalence reported in the main trial (5). The probabilities of a positive, negative, true positive, false positive, true negative and false negative test were subsequently calculated and normalised to 1000 patients screened per strategy.

Cost-effectiveness

Cost-effectiveness was expressed as the cost per culture positive case diagnosed and initiated on treatment (per 1000 patients screened) for each strategy.

Sensitivity analysis

A univariate sensitivity analysis was performed where a single parameter was changed to determine its effect on the cost per culture-positive patient diagnosed and initiated on TB treatment. Input values for probability estimates were varied based on clinical advice and on estimates from the literature. Cost estimates were either halved or doubled for the low and high input values, respectively.

Assumptions

The following assumptions were made for the analysis: (i) sputum culture was used as the gold standard TB diagnostic test. However, culture was not included in the costs as all patients were subjected to sputum culture in each strategy; (ii) any patient with a positive test in each strategy (Xpert or urine LAM) was assumed to be immediately initiated on treatment, which is in line

with current clinical practice even without the availability of sputum culture; (iii) all patients initiating treatment based on a positive test result will complete a full 6-month course of first line anti-TB therapy; (iv) we assumed 30% of patients with a negative test result (LAM and/or Xpert) will be empirically initiated on treatment. Furthermore, based on clinical advice, we assumed 70% of empirically treated patients will complete a full 6-month course of anti-TB treatment whereas 30% will complete a 3-month course; (v) although Xpert MTB/RIF can detect rifampicin resistance, we did not incorporate the costs or outcomes associated with drug resistant TB into the analysis; (vi) additional diagnostic tests e.g. chest-x-ray and drugs, including those associated with HIV, were not included as they were assumed to be equivalent in each of the strategies; (vii) treatment outcomes (cure, died, etc) were not included in the outcomes due to insufficient data.

TABLES

Table S1: Diagnostic performance of sputum-based Xpert MTB/RIF and urine-based Alere Determine TB LAM Ag testing in single and sequential testing strategies in hospitalized HIV-infected patients (stratified by CD4 count) using sputum culture positivity as the reference standard (PPV - positive predictive value; NPV – negative predictive value)

CD4 counts stratification	Test strategy	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
CD4 count >200 cells/mm^3	Xpert MTB/RIF Only	60.0 (42.1 – 30.3)	96.4 (91.0 – 99.0)	84.0 (81.3 – 93.5)	88.4 (81.3 – 93.5)
	Urine LAM only	14.3 (4.8 – 52.6)	90.1 (83.0 – 94.9)	31.3 (11.0 – 58.7)	76.9 (68.7 – 83.9)
	*Sequential/concurrent testing using Xpert MTB/RIF and LAM	62.9 (44.9 – 78.5)	86.5 (78.7 – 92.2)	59.5 (42.1 - 75.2)	88.1 (80.5 – 93.5)
CD4 count ≤200 cells/mm^3	Xpert MTB/RIF Only	80.3 (72.3 – 86.8)	94.9 (91.3 – 97.3)	89.5 (82.3 – 94.4)	90.0 (85.5 – 92.4)
	Urine LAM only	44.9 (36.1 – 54.0)	88.1 (83.3 – 92.0)	67.1 (56.0 – 76.9)	74.8 (69.3 – 79.8)
	*Sequential/concurrent using Xpert MTB/RIF and LAM	83.5 (75.8 – 89.5)	84.7 (79.5 – 89.1)	74.6 (66.7 - 81.6)	90.5 (85.8 – 94.0)
CD4 count ≤100 cells/mm^3	Xpert MTB/RIF Only	83.5 (74.6 – 90.3)	94.3 (89.4 – 97.3)	90.0 (81.9 – 95.3)	90.2 (84.6 –94.3)
	Urine LAM only	51.5 (41.2 – 61.8)	84.1 (77.4 – 89.4)	66.7 (54.8 – 77.1)	73.7 (66.7 - 80.0)
	*Sequential/concurrent testing using Xpert MTB/RIF and LAM	86.6 (78.2 – 92.7)	80.9 (73.9 – 86.7)	73.7 (64.6 - 81.5)	90.7 (84.6 - 95.0)

	Xpert MTB/RIF Only	81.5 (71.3 – 89.2)	94.3 (89.1 – 97.5)	89.2	89.9
CD4 count ≤50 cells/mm^3				(79.8 – 95.2)	(83.8 – 94.2)
	Urine LAM only	60.0 (47.1 – 72.0)	81.7 (72.9 – 88.6)	67.2 (53.7 – 79.0)	76.6 (67.6 – 84.1)
	*Sequential/concurrent testing using Xpert MTB/RIF and LAM	89.2 (79.1 – 95.6)	79.8 (70.8 – 87.0)	73.4 (62.3 - 82.7)	92.2 (84.6– 96.8)

* Sequential testing refers to performing Xpert in LAM negative patients (Xpert in LAM-ve) or LAM in Xpert negative patients (LAM in Xpert -ve). Concurrent testing refers to performing LAM and Xpert concurrently (LAM+Xpert)

Table S2: Indicators of disease severity in HIV-infected patients suspected of TB according to LAM and Xpert test result.

	LAM Pos and Xpert Neg (n = 45)	LAM Pos and Xpert Pos (n = 68)	LAM Neg and Xpert Pos (n = 84)	LAM Neg and Xpert Neg (n = 39)
Median CD4 count; cells/mm^3 (95% CI)	63.5 (30.3, 182.9)	27.0 (20.3, 40.4)	98 (68.8, 130.9)	139 (61.7, 189.1)
Median Karnofsky score (95% CI)	50 (50, 60)	60 (50, 60)	60 (50, 70)	60 (50, 70)
Mortality; n (%)	12 (28.6%) (n = 42)	14 (22.2%) (n = 63)	15 (19.2%) (n= 78)	4 (10.8%) (n = 37)
Median Weight; kg (95% CI)	48 (45.1, 53.6) (n = 41)	48.0 (46.1, 50.0) (n = 65)	50.0 (46.8, 51.9) (n = 77)	50 (48.7, 55.0) (n= 31)

Table S3: Incremental yield of LAM over Xpert (LAM positive in patients who tested negative with Xpert) stratified according to CD4

CD4 grouped	Incremental yield using any positive test as reference %		Incremental yield using TB culture as a reference %	
	Total (n)	% incremental yield	Total (n)	% incremental yield
≤ 50	90	27.7 (18/65)	68	3.3 (2/59)
51 ≤ 100	43	20.0 (6/30)	35	3.7 (1/27)
101 ≤ 200	37	16.7 (4/24)	31	4.8 (1/21)
200 ≤ 500	48	65.0 (13/20)	34	11.8 (2/17)
> 500	8	25 (2/8)	6	16.6 (1/8)

Table S4: Incremental yield of Xpert over LAM (Xpert positive in patients who tested negative with LAM) stratified according to CD4

CD4 grouped	Incremental yield using any positive test as reference %		Incremental yield using TB culture as a reference %	
	Total (n)	% incremental yield	Total (n)	% incremental yield
≤ 50	90	40.7 (24/59)	68	52.5 (21/40)
51 ≤ 100	43	89.5 (17/19)	35	115.4 (15/13)
101 ≤ 200	37	211.1 (19/9)	31	266.6 (16/6)
200 ≤ 500	48	106.2 (17/16)	34	260.0 (11/4)
> 500	8	250.0 (5/2)	6	200.0 (4/2)

Table S5: Probability estimates and costs used in the cost-consequence analysis

Probablility estimates	Value	Source
Prevalence of TB in study population	0.317	[5]
Prevalence of TB in Xpert MTB/RIF negative patients	0.110	calculated
Prevalence of TB in LAM negative patients	0.245	calculated
Xpert MTB/RIF sensitivity	0.747	Table 2 of main manuscript
Xpert MTB/RIF sensitivity in urine LAM -ve patients	0.646	calculated
Xpert MTB/RIF specificity	0.951	Table 2 of main manuscript
Xpert MTB/RIF specificity in urine LAM -ve patients	0.962	calculated
Urine LAM sensitivity	0.382	Table 2 of main manuscript
Urine LAM sensitivity in Xpert -ve patients	0.133	calculated
Urine LAM specificity	0.882	Table 2 of main manuscript
Urine LAM specificity in Xpert -ve patients	0.896	calculated
TB treatment initiated if test (urine LAM or Xpert MTB/RIF) positive	1.00	assumption
TB treatment initiated if test negative (empirical treatment)	0.30	assumption
Cost estimates	Value	Source
Xpert MTB/RIF	$14.38	National Health Laboratory Service
Urine LAM	$3.56	Alere
6month course of DOTS-based TB treatment	$746.95	[4]
cost of those initiated on empirical TB treatment (assuming 70% patients complete a full 6-month and 30% of patients complete a 3-month course)	$634.91	calculated

Table S6: Costs and outcomes associated with unnecessary and empirical treatment for single, sequential and concurrent test strategies to diagnose TB in hospitalized patients with advanced HIV using Xpert MTB/RIF and LAM urine tests. Costs are expressed in 2018 258 $US with 95% CI in parentheses.

	Single test strategies		Sequential test strategies		Concurrent testing strategy
	Xpert only	LAM only	LAM in Xpert-ve	Xpert in LAM-ve	Xpert+LAM
Total costs associated with unnecessary treatment (per 1000 patients screened)					
Total cost	$148,653 (141435, 158911)	$174,487 (163090, 188544)	$187,235 (169068, 218473)	$186,228 (168745, 218566)	$186,645 (174487, 202601)
Costs incurred by false positives initiating treatment	$249,88 (15299, 38757)	$59,665 (44366, 78533)	$76,775 (54005, 108414)	$75,424 (52203, 108642)	$75,983 (59665, 97401)
Costs incurred by true negatives initiating treatment	$123,666 (120155, 126137)	$114,823 (118724, 110012)	$110,460 (110060, 115063)	$110,805 (109924, 116543)	$110,662 (105201, 114823)
Outcomes (per 1000 patients screened)					
Number of suspected TB patients who were empirically treated	218.9 (216.9, 220.0)	239.7 (238.6, 239)	194.9 (195.1, 191.9)	195.4 (191.4, 196)	195.2 (193.1, 196.2)
Number of culture negative patients who were initiated on TB treatment	228.3 (219.2, 241.2)	260.8 (246.4, 278.5)	276.8 (248.3, 312.8)	275.5 (247.3, 311.7)	276.1 (260.8, 296.1)

Number of missed TB cases (false negatives)	56.2 (42.5, 71.8)	137.3 (120.4, 153.3)	48.6 (36.4, 61.8)	48.8 (40.8, 53.7)	48.7 (35.8, 63.8)

FIGURES

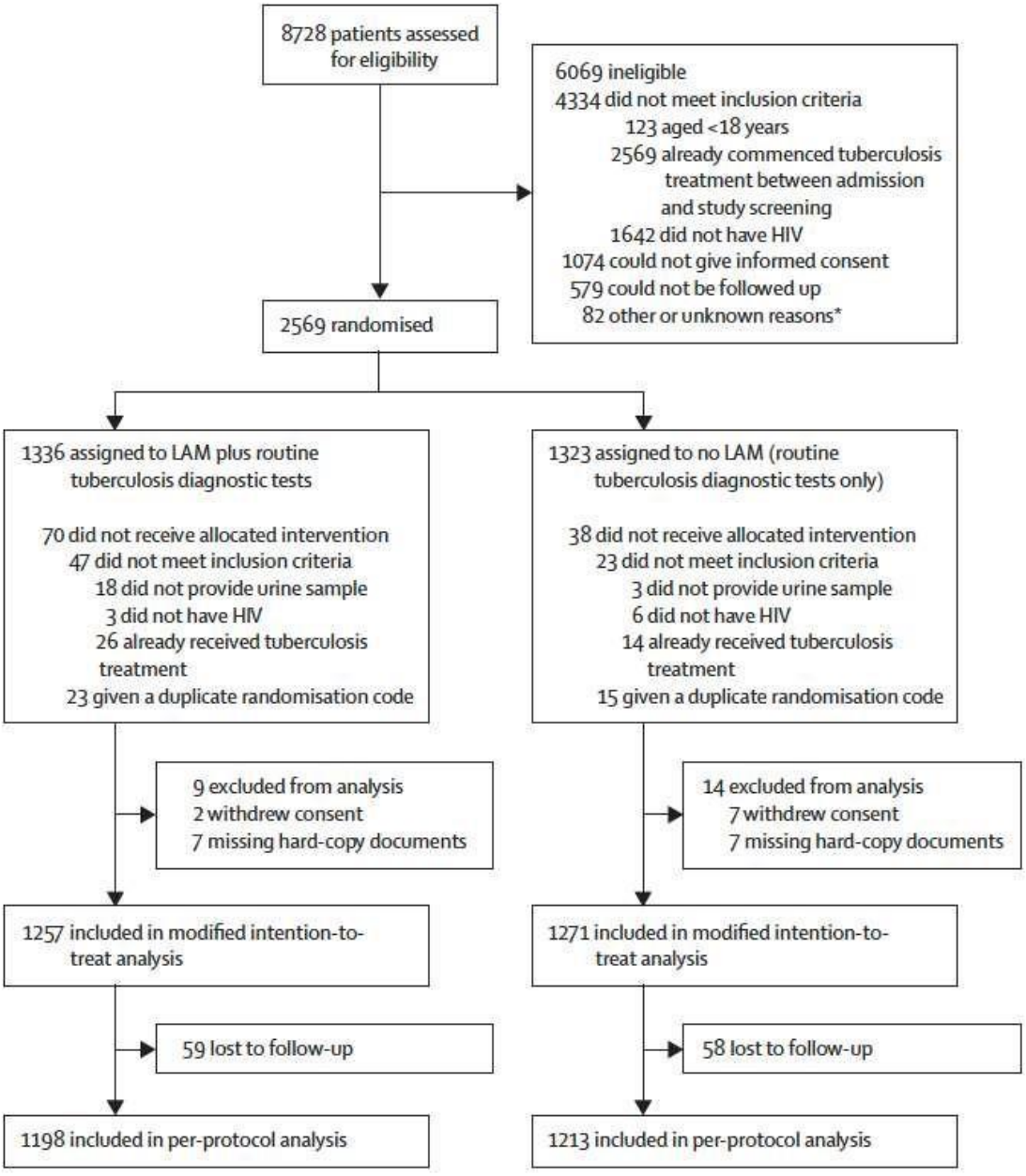

Figure S1: Study plan of the parent study (LAM RCT: [5])

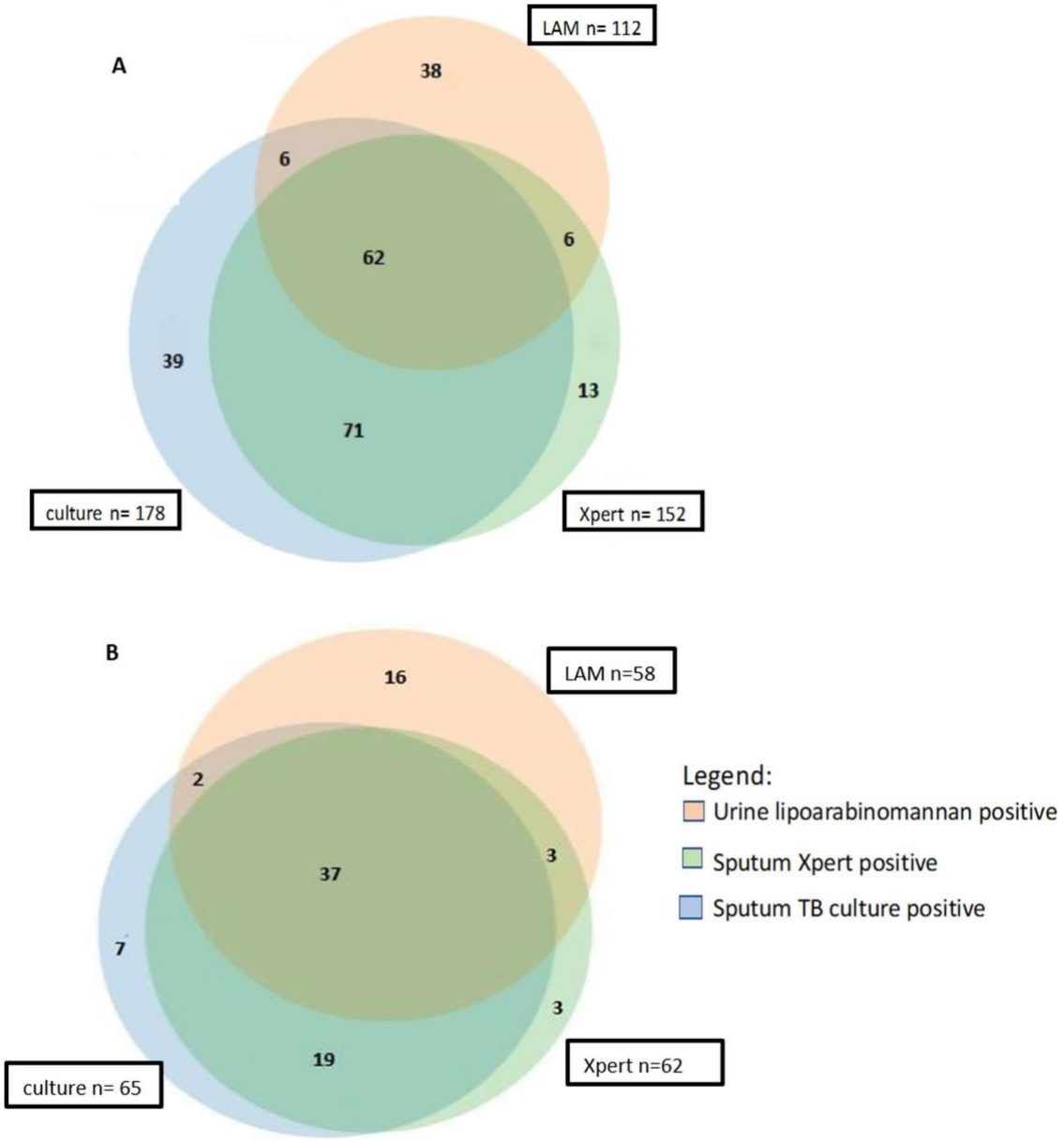

Figure S2: VENN Diagram demonstrating proportion of positive test results (sputum Xpert, sputum culture and urinary LAM) in (A) all patients (n=235) and (B) patients with CD4+ ≤ 50 cells/ul (n= 87).

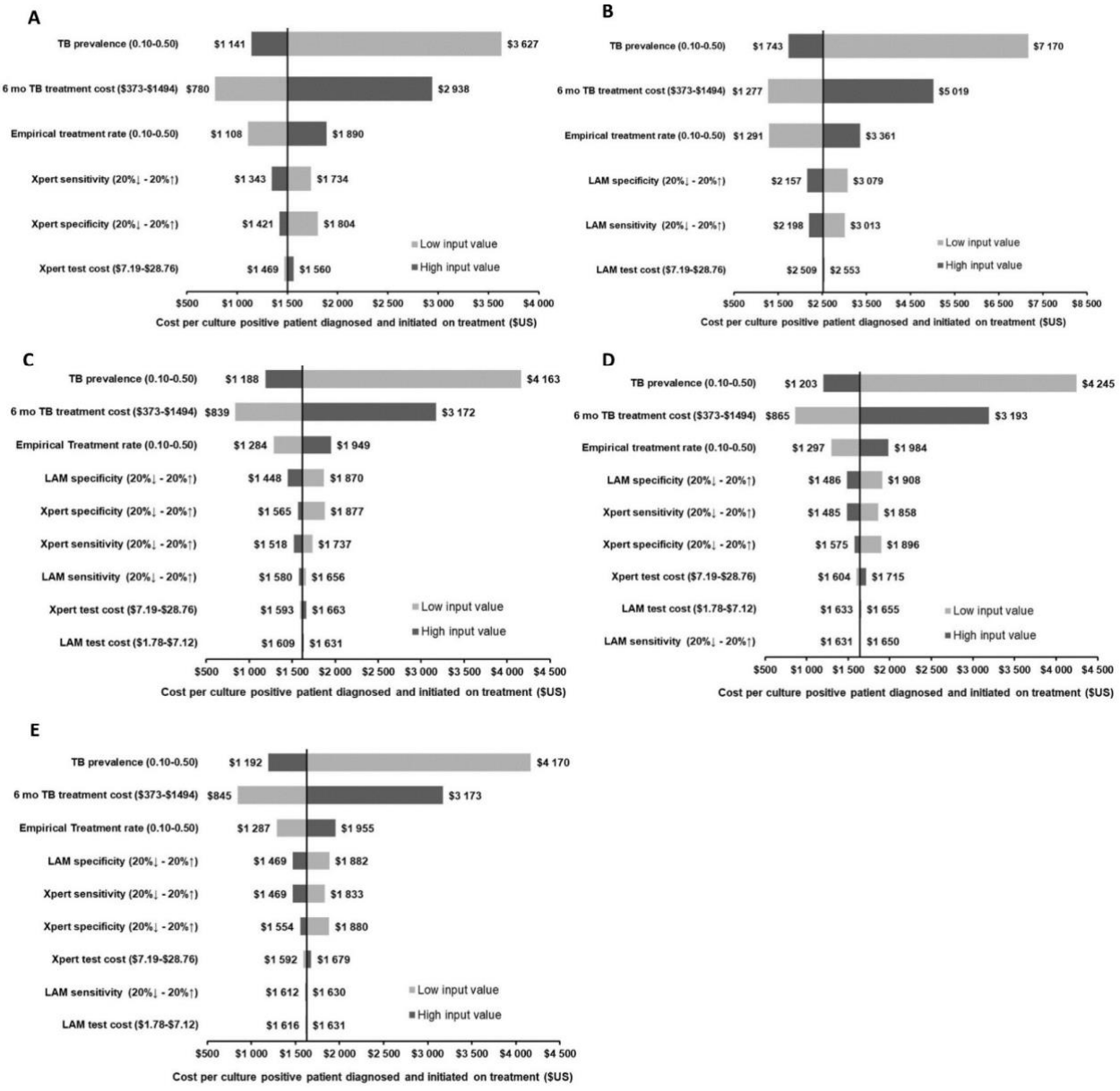

Figure S3: Univariate sensitivity analysis. Tornado diagrams outlining the effect of changing a single parameter in terms of the cost per culture positive case diagnosed and initiated on treatment for (A) Xpert only (B) LAM only (C) Xpert in LAM -ve patients and (D) LAM in Xpert -ve patients and (E) LAM+Xpert. Cost are expressed in 2018 $US

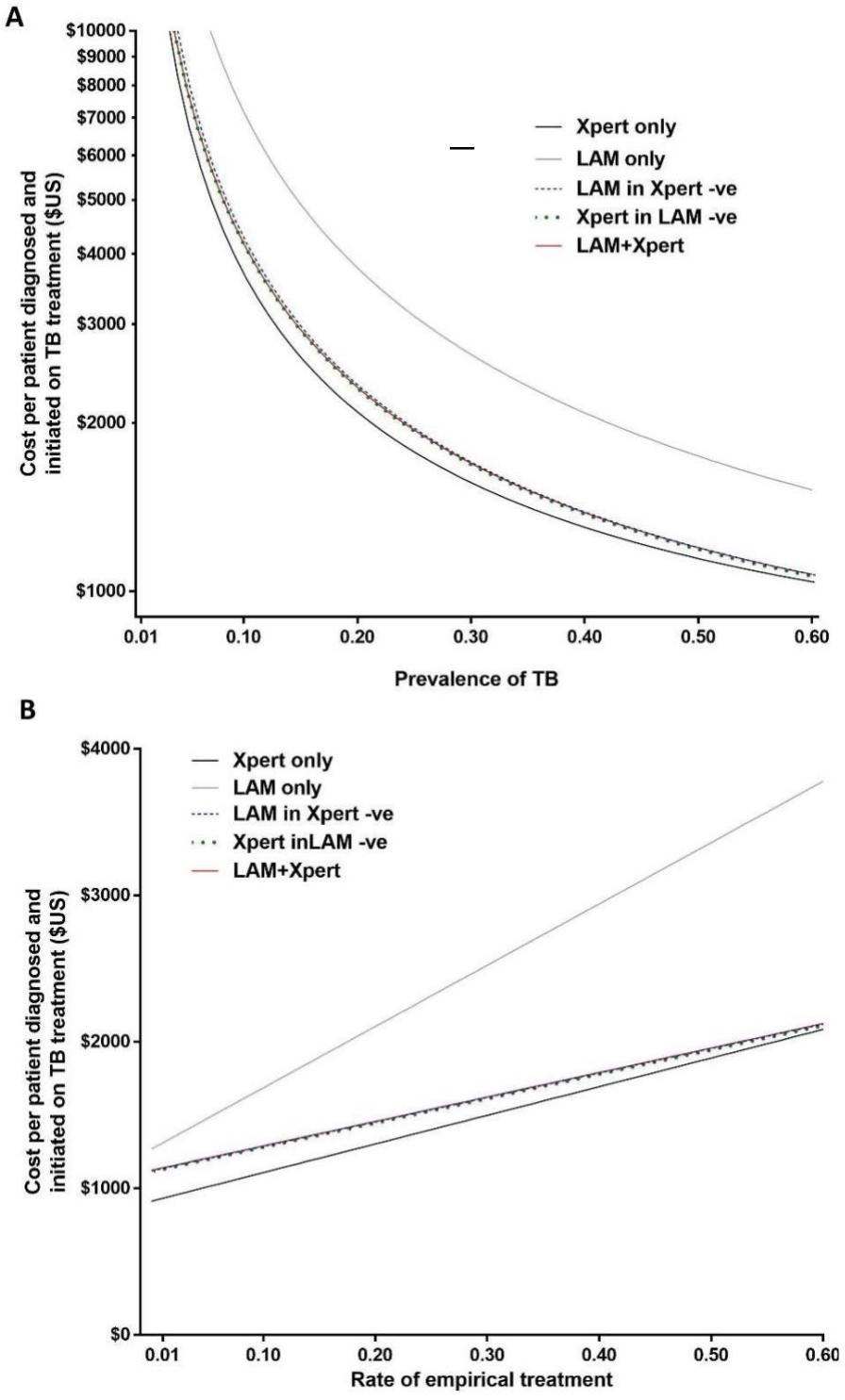

Figure S4. Effect of varying the (A) prevalence of TB and (B) rate of empirical treatment on the cost per culture positive case diagnosed and initiated on treatment.

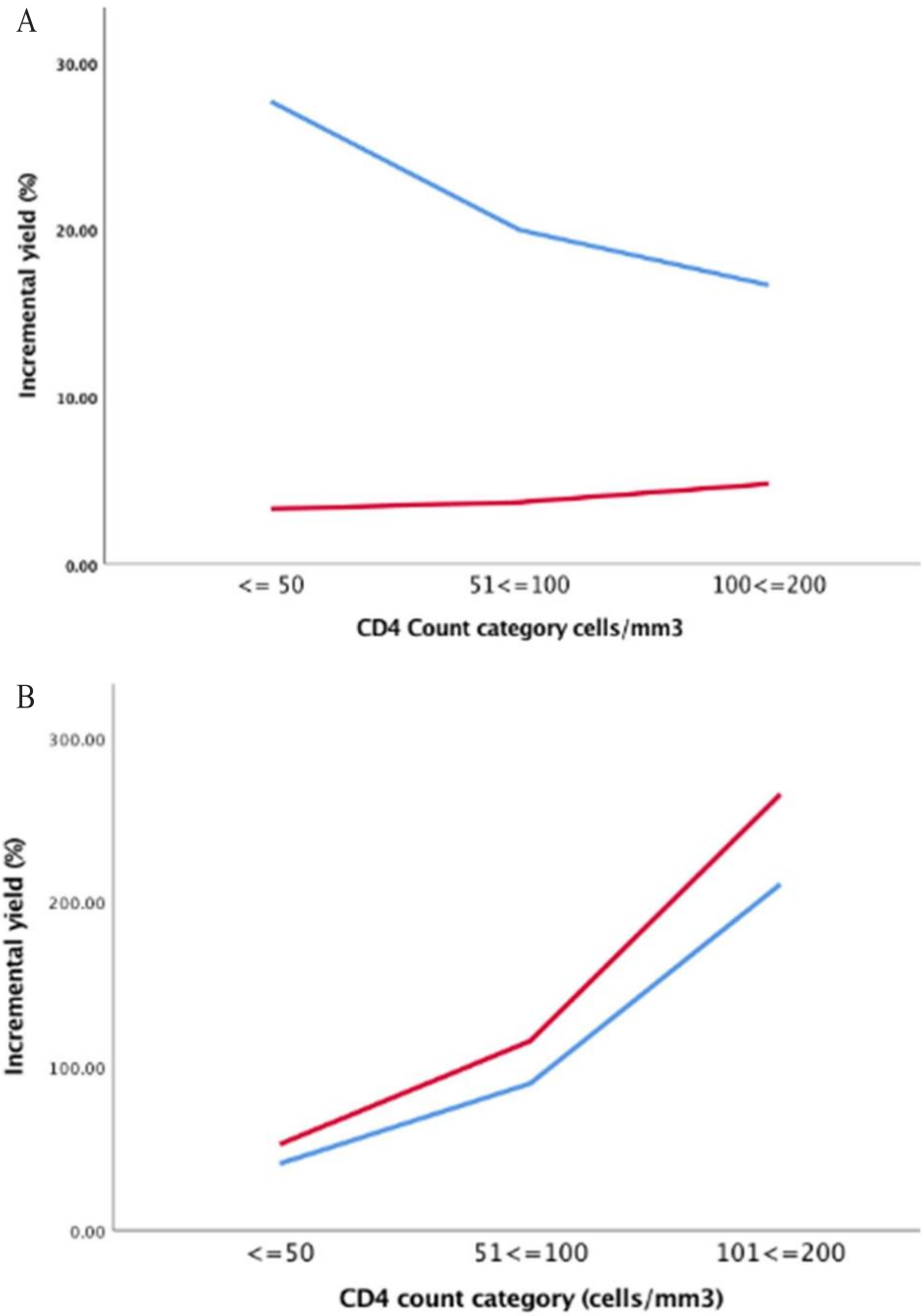

Figure S5. Incremental yield of (A) LAM (LAM positivity in Xpert-ve patients) and (B) Xpert (Xpert positivity in LAM-ve patients), stratified by CD4 count using any positive TB test (blue line) and sputum culture (red line) as a reference.

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Chapter 2 Supplementary material

Manuscript title: Comparison of Genexpert MTB/RIF (G4) and GeneXpert Ultra, including trace results, for the diagnosis of pulmonary tuberculosis in a TB and HIV endemic setting.

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METHODS.

Sample Selection.

Samples were bio-banked according to good clinical laboratory practice (GCLP) at the Centre for Lung Infection and Immunity, University of Cape Town and underwent culture and Xpert MTB/RIF at the time of sample collection. A total of 261 sputum samples were selected, based on the criteria for posthoc analysis, namely; comparative performance of Xpert MTB/RIF versus Xpert Ultra in smear-negative, culture-positive individuals, culture-negative with a history of previous TB and those who are HIV-infected.

Assay procedure.

Sodium hydroxide and isopropanol-containing sample buffer (Cepheid, USA) was added to the sputum sample at a ratio of 2:1 and incubated for 15 minutes at room temperature with gentle intermittent agitation. Following incubation, 2 mL of sample was transferred to the Xpert MTB/RIF and/or Xpert Ultra cartridge and run on the GeneXpert G4 system (Cepheid, Dx System Version 4.0c), depending on the outcome under investigation.

Freeze-thaw evaluation

Samples classified as smear-negative, Xpert MTB/RIF-positive and culture-positive (n=16) were randomly selected to evaluate whether freezing the sample has an effect on test performance. The samples underwent three freeze-thaw cycles, after which Xpert MTB/RIF was performed as described above. The cyclic threshold (Ct) values were compared to previously documented Xpert MTB/RIF results pertaining to the same sample at the time of sample collection.

RESULTS.

Patients and Samples.

The demographics of the patients enrolled in the study are shown in Table S1. The median age of the patients was 39 and they consisted of an equal number of males and females. The patients with confirmed active-TB were more likely to be HIV-infected and their median CD4 count was 235 cells/ml. The greatest proportion of patients with confirmed active-TB were smear-negative versus smear-positive and most patients had no previous history of TB. Xpert MTB/RIF and Xpert Ultra were positive for 82.1% and 82.7%, respectively amongst the confirmed active-TB patients.

Accuracy of Xpert MTB/RIF and Xpert Ultra in sputum samples from patients with non-TB, but with previous history TB.

The diagnostic accuracy of Xpert MTB/RIF and Xpert Ultra for non-TB patients with previous history of TB is shown in Table S2. The accuracy (sensitivity, specificity) for Xpert MTB/RIF and Xpert Ultra were (3.2% [0%-6.8%], 94.6% [90%-99.2%]) and (5.5% [0.8%-10.2%], 94.5% [89.9%-99.2%]), respectively.

IMPACT OF TRACE: When the trace results were excluded for Xpert Ultra the sensitivity and specificity were similar at 4.4% (0.2%-8.6%) and 95.6% (91.4%-99.8%), respectively.

Freeze/thawing sputum samples improves the performance of Xpert MTB/RIF.

The effect of freeze/thawing sputum samples on Xpert MTB/RIF performance is shown in figure S1. Eight pairs of culture-positive, Xpert MTB-RIF-positive sputum samples were randomised and either remained fresh or were subjected to 3 freeze/thaw cycles prior to Xpert MTB/RIF. On average the Ct values for the freeze/thawed sputum samples (Ct=22; p=0.039) were lower than the fresh sputum

samples (Ct=26), indicating that freeze/thawing sputum samples improves Xpert MTB/RIF performance (Figure S1).

Relationship of tests positivity for culture, Xpert MTB/RIF and Xpert Ultra.

The relationship between test positivity for culture, Xpert MTB/RIF and Xpert Ultra is shown in figure S2. Culture had the ability to detect TB in an additional 19 sputum samples above Xpert MTB/RIF and Xpert Ultra. Both Xpert MTB/RIF and Xpert Ultra performed equally by detecting TB in an additional 6 and 5 sputum samples alone.

Figures.

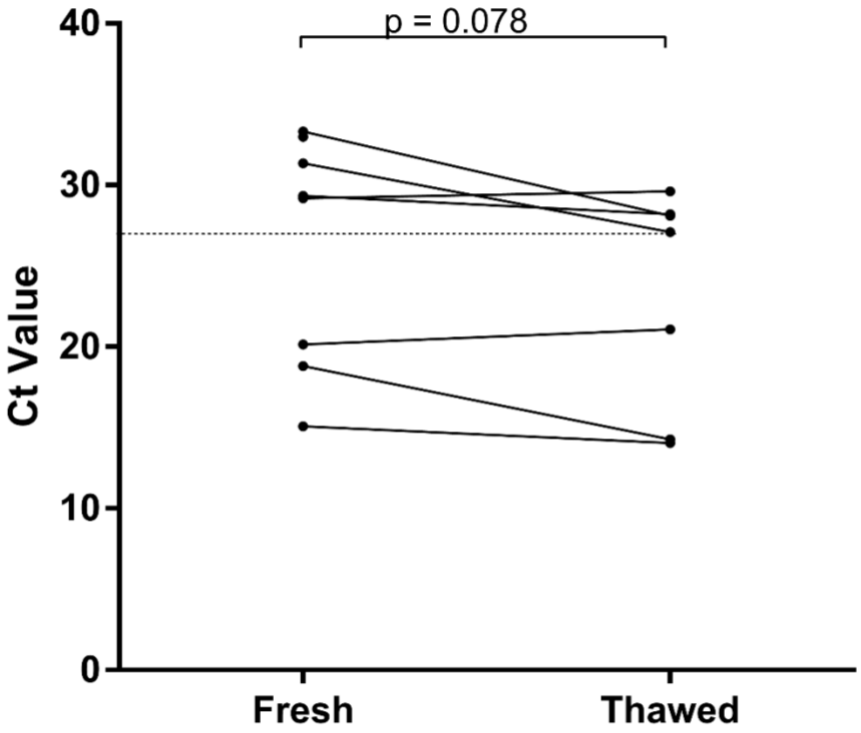

Figure S1. Three-post freeze/thaw cycles of sputum samples (16 sputum samples; 8 pairs of samples collected and randomised) improves the performance of Xpert MTB/RIF. Smear-negative, culture-positive, Xpert MTB/RIF-positive sputum samples were subjected or not subjected to three rounds of freeze/thaw prior to Xpert MTB/RIF. The dotted line represents the median Ct value=27.

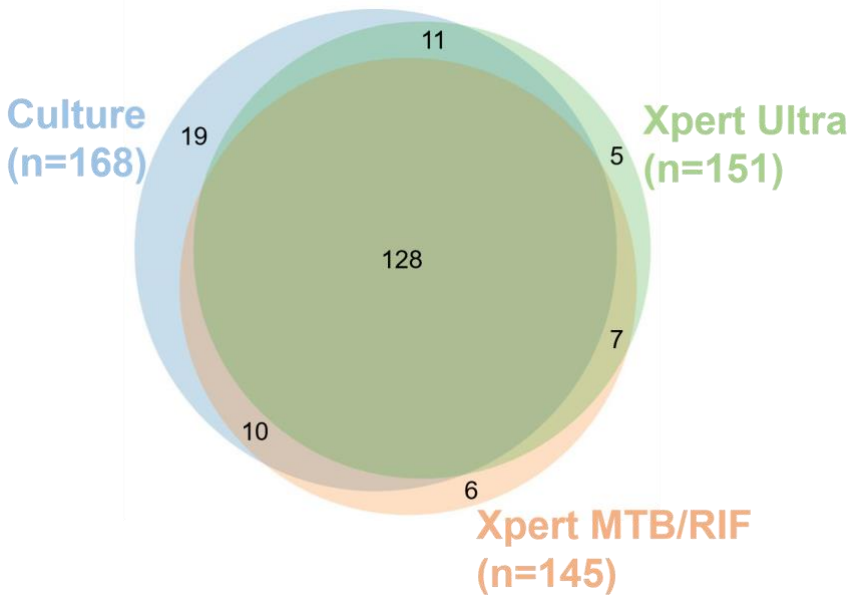

Figure S2. Venn diagram showing the relationship of test positivity for culture, Xpert MTB/RIF (n=145) and Xpert Ultra (n=151).

Tables.

Table S1. Demographic data of the cohorts used in study. Data is n (%) unless otherwise stated.

Demographic data	Study Cohort (%) (n = 261)	Confirmed active-TB (%) (n = 168)	Non-TB (%) (n = 93)	p-value
Age				
[**]Median years (range)	39 (19-68)	39 (19-65)	39 (26-68)	
Gender				0.1289
Male	155 (59.4)	94 (56)	61 (65.6)	
Female	106 (40.6)	74 (44)	32 (34.4)	
HIV-infected				< 0.0001
Yes	144 (55.2)	113 (67.2)	31 (33.3)	
No	100 (38.3)	40 (23.8)	60 (64.5)	
Not determined	17 (6.5)	15 (8.9)	2 (2.2)	
CD4 count (cells/ml) (range)[#]	235 (6-788) [Ψ]	235 (6-788)[ω]	235 (25-681)[φ]	0.1494
Smear status				<0.0001
Smear-negative	183 (70.1)	104 (61.9)	79 (84.9)	
Smear-positive	65 (24.9)	64 (38.1)	1 (1.1)	
Unknown	13 (4.9)	–	13 (14)	
Previous TB				<0.0001
Yes	149 (57.1)	58 (34.5)	102 (98.1)	
No	111 (42.5)	109 (64.9)	2 (1.9)	

Unknown	1 (0.4)	1 (0.6)	–	
Xpert MTB/RIF				< 0.0001
Positive	141 (54)	138 (82.1)	3 (3.2)	
Negative	121 (46)	30 (17.9)	90 (96.8)	
Xpert Ultra				< 0.0001
Positive	144 (55.2)	139 (82.7)	5 (5.4)	
Negative	121 (44.8)	29 (17.3)	88 (94.6)	
Trace	7	6	1	

** Median (range)
[#]Performed if HIV-infected. There was no CD4 count data for 5[Ψ], 3[ω] and 2[φ] patients, respectfully.

Table S2. False positive rates (specificity) of Xpert MTB/RIF and Xpert Ultra for the detection of TB in sputum samples from non-TB patients with previous history of TB.

	Non-TB with history of previous TB (n=91)	
	Positive (%, 95%CI, n/N, p-value)	**Negative (%, 95%CI, n/N, p-value)**
Xpert MTB/RIF	**3.2%**, 0%-6.8%, 3/91	**94.6%**, 90%-99.2%, 88/91
Xpert Ultra (with trace)	**5.5%**, 0.8%-10.2%, 5/91, p=0.470	**94.5%**, 89.8%-99.2%, 86/91, p=0.470
Xpert Ultra (without trace)	**4.4%**, 0.2%-8.6%, 4/90, p=0.70	**95.6%,** 91.4%-99.8%, 86/90, p=0.70

p-values are for comparisons between Xpert MTB/RIF and Xpert Ultra.
*History of previous TB was unavailable for 2 patients.

nature medicine

Article

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Comparison of two diagnostic intervention packages for community-based active case finding for tuberculosis: an open-label randomized controlled trial

In the format provided by the authors and unedited

Comparison of two diagnostic intervention packages for community-based active case finding for tuberculosis: an open-label randomized controlled trial.

BACKGROUND INFORMATION

Study site descriptions.

The study was conducted in peri-urban informal settlements in the Mitchells Plain and Klipfontein districts (Cape Town, South Africa). These are historically disadvantaged areas with considerable informal housing, high unemployment rates, and with a high HIV prevalence (~20%). Historically, these areas were designated for Black Africans and people of mixed race in the apartheid era. It has a high population density with a large population residing in informal housing (shacks and shanty towns). The average TB burden in the local clinics servicing these two districts is ~850 new cases per annum per TB clinic, which is one of the highest in Cape Town. Approximately 3-5% of diagnosed cases are multidrug-resistant. There are several facilities in these two districts that are designated as specialist

TB/HIV primary care clinics and operate under the Cape Town Central Health District of the Metro Region.

Since November 2012 Xpert has been rolled out as the preferred diagnostic test for all respiratory samples in the Western Cape· Through a passive case-finding process, symptomatic patients selfpresent to primary care clinics where sputum samples are collected. The samples, from the designated primary care clinics are sent to a centralised laboratory ~30 km away. Chest radiography is not available at the clinics and patients need to be referred to a nearby day hospitals or there are several mobile chest x-ray units that visit some of the larger health facilities in the area.

The location for community-based screening using the mobile minivan was selected after consulting with local community leaders. The sites were clustered around community congregate settings such as shops, hostels, meeting points, prayer hubs, entrances or thoroughfares to an informal housing cluster, or transit hubs (taxi ranks and bus stops). Seventeen such sites were identified: six were around central shopping areas, five around transit hubs, and the remainder among high-density urban slum

housing, also called ‘hostels. Recruitment and follow-up were rotated between these sites.

RESULTS

Feasibility: The diagnostic accuracy of POC Xpert performed by minimally trained health care workers was comparable to Xpert performed by a qualified technician in a research laboratory (sensitivity 0.52 vs. 0.61 and specificity of 0.98 for both; p=0.46 see table S1). A total of 36/494 (7.3%) days were lost due to logistical challenges; mechanical problems with the vehicle was the most common reason (36%) for lost screening days (see table S2).

Table S1. Diagnostic accuracy of minivan-based vs. laboratory-based Xpert. 288 participants had an Xpert performed on their sputum at the minivan-clinic; 277 participants were able to produce an extra sputum sample that was sent to the research laboratory and frozen at -20°C for later processing. Of the latter group, 2 cultures (taken at the same time) were contaminated and were excluded from the analysis.

	POC-Xpert (%)	Lab-based Xpert (%)	P-value
Sensitivity	**16/32 (50.0)**	**19/32 (59.3)**	**0·49**
Specificity	**241/243 (99.1)**	**239/241 (99.6)**	**0.56**
Error result	**2/277 (0.7)**	**4/277 (1.4)**	**0.41**

Table S2. Number of screening days lost due to logistical and environmental factors (total screening days were 494)

Reason for screening days "lost"	Number of days "lost"
Problems with vehicle	13
Community unrest/ civil protest	8
Bad weather	7
Staffing issue (illness etc.)	6
Problem with the Xpert platform (battery and software)	2
Total	36

Table S3. Description of all TB culture-positive patients in both arms of the study according to their Xpert status (n=58)

	Xpert positive n=31 (n, %)	Xpert Negative n=27 (n, %)	P value
TB symptoms			
Cough	**30/31 (96.8)**	**24/27 (88.9)**	0.14
Fever	**5/31 (16.1)**	**10/27 (37.0)**	0.07
Night sweats	**27/31 (87.1)**	**24/27 (88.8)**	0.93
Chest pain	**19/31 (61.3)**	**11/27 (40.7)**	0.15
Dyspnoea	**20/31 (65.5)**	**18/27 (66.6)**	0.86
Weight loss	**21/31 (64.7)**	**16/27 (59.2)**	0.53
HIV	**13/31 (41.9)**	**11/27 (40.7)**	0.93
Median CD4 count (IQR)	**306 (31-661)**	**180 (21-681)**	0.43
CD4 <200	**5/13 (50.0)**	**6/11 (57.1)**	0.43
Chest x-rays abnormalities			
Presence of cavities*	**8/9 (71.4)**	**1/9 (16.6)**	0.03
Chest x-ray median score; (IQR) maximum 140 points	**55 (24-90)**	**42 (15-70)**	0.22
Time to culture positivity	**11 (3-31)**	**13 (1-34)**	0.29
Smear status	**4/23**	**0/28**	0.22
Positive on Cough aerosol sampling (CASS)*	**11/12 (91.6%)**	**1/12 (8.3%)**	0.01

*Seven participants did not have an evaluation of cavities and/or CASS assessment performed and were excluded from this analysis

Table S4: Characteristics of patients who Xpert positive but sputum TB culture-negative in the intervention arm of the study.

Participant ID	MGIT sputum TB culture	CASS results	Smear status	HIV status	CXR	Previous TB	Time from last TB treatment	Initiation of TB treatment	Response to TB treatment
RN 359	Negative	Negative	Negative	Positive	Not in keeping with active TB	Yes		Yes	Improved & cured
RN 456	Negative	Not done	Negative	Positive	Not done	No	N/A	Yes (started 30-60 days)	Improved & cured
RN 568	Negative	Negative	Negative	Positive	Not in keeping with active TB	No	N/A	Yes, but patient migrated	LTFU (month 2 sputum also negative)
RN 622	Negative	Negative	Negative	Positive	Not done	Yes		Yes	Improved & cured

Table S5: Diagnostic accuracy of Xpert in the study

Xpert testing (both arms of study)	Sensitivity	Specificity	PPV	NPV
All patients	54.1 (40.9 – 66.7)	98.3 (96.0 – 99.4)	78.6 (62.7 – 89.1)	94.9 (92.6 – 96.5)
HIV-Infected	53.8 (33.7 -72.9)	98.1 (95.4 – 99.3)	73.7 (48.6 – 89.9)	95.5 (92.2 – 97.6)
CD4 ≤200 cell/uL	45·5 (18.5 – 75.4)	97.2 (83.8 – 99.8)	83.3 (36.5 – 99.1)	85.4 (70.1 – 93.9)
CD4 >200cells/uL	64.2 (35.6 – 86.0)	97.7 (94.4 – 99.1)	64.3 (35.7 – 86.0)	97.7 (94.3 – 99.1)
HIV-uninfected	54.3 (36.9 – 70.7)	99.2 (97 -99.8)	90.5 (68.7 – 98.3)	94.3 (90.7 – 96.7)
Infectious patients*	94.4 (70.6 – 99.7)	58.1* (42.2 -72.6)	48.5 (31.7 – 65.7)	96.2 (78.4 – 99.8)

* Infectious patients are classified as CASS (cough aerosol sampling) positive and/or smear-positive and/or cavitary disease, and thus specificity is calculated in non-infectious some of whom were culture positive (and hence the low specificity).

Table S6: Distribution of parameters that correlated with infectiousness in the Xpert and smear arm of the study

Variable	Over-all number	Xpert arm (%)	Smear arm (%)	p- value
CASS	Positive: 12 Negative: 39 Not done: 7	6/30 (20)	6/21 (28.6)	0.74
Smear	Positive: 4 Negative: 54	4/32 (12.5)	3/26 (11.5)	0.51
Cavitary disease	Positive: 9 Negative: 42 Not done: 7	6/29 (20.7)	4/22 (18.1)	0.70
Time to positivity ≤ 7 days	TTP ≤ 7 days: 10 TTP ≥ 7 days: 48	5/32 (15.6)	5/26 (19.2)	0.78

Scalability of the XACT active case finding model (see figure S2).

The intention of this trial was to develop a model that was scalable (i.e. a package of interventions that could be operationalised with minimally trained health care workers at a modest cost and with equipment readily available in TB endemic settings e.g. minivan type of motor vehicle, portable battery operated Xpert Edge (Cepheid, the manufacturer has agencies and distribution hubs in virtually all high burden TB countries), CD4 analyser and a battery operated nebuliser for sputum induction. Thus, the scalability of the model was defined by cost (under US$ 25,000 with all equipment purchased new at 2020 prices), no need for a generator, use of only one vehicle that could accommodate all the required equipment outlined in the table below, a minimum or 2 and a maximum of 3 health workers, not incorporating the use of a truck, and easy availability of all the required components). Thus, the XACT model could

easily be implemented in most resource constrained high TB burden settings and is distinctly different from models that use a truck accommodating several personnel and more suited to accommodating a mobile laboratory or x-ray facilities on board. The XACT model was designed to be a lower cost ACF intervention accommodating 2 to 3 health care workers and portable TB and HIV testing equipment. Not having the portable Xpert on board would reduce the cost of the model by 50%.

We are currently conducting a large RCT to determine whether addition to the model at POC is essential (versus sending the samples to a centralised laboratory).

Vehicle	Comments
Minivan vehicle; an exemplar is outlined in figure S2 (e.g., Toyota Avanza or Nissan NV 200)	Cost ≤ USD13,000; large enough to accommodate the ACF equipment
Equipment list	**Comments**
GeneXpert Edge platform	Portable battery/solar operated platform
GeneXpert MTB/RIF Ultra cartridges	Detects TB and provides the rifampicin resistance readout; results are available within ≤90 mins
HIV lateral flow assay (Alere Determine HIV 1/2 Ag/Ab combo)	Point of care test using finger prick blood
CD4 count PIMA™ analyser (Abbott/Alere)	Portable analyser using finger prick blood with results available in 20 mins
Portable and foldable sputum booth	Well ventilated (open from the top) and yet providing some privacy to participants when expectorating sputum
4 folding plastic chairs	For participants and staff to sit while the screening process is explained to them

Two foldable tables	To stabilize the GeneXpert Edge/OMNI system and to complete documentation
Battery operated nebuliser	Used for sputum induction
Disposable face mask and tubing to connect to the nebuliser	Used for sputum induction
Hypertonic (3%) saline solution	Used for sputum induction
Awning or foldable gazebo	Used to provide shelter from the sun
Personnel	Comments
Two to three registered nurses/community health care workers	Personnel are trained in using the GeneXpert, PIMA analyser, in counselling for HIV testing and performing HIV POC testing.
Driver/community health care worker	Also has the responsibility of going into an informal settlement inviting community-based participants to come to the mobile site for testing.

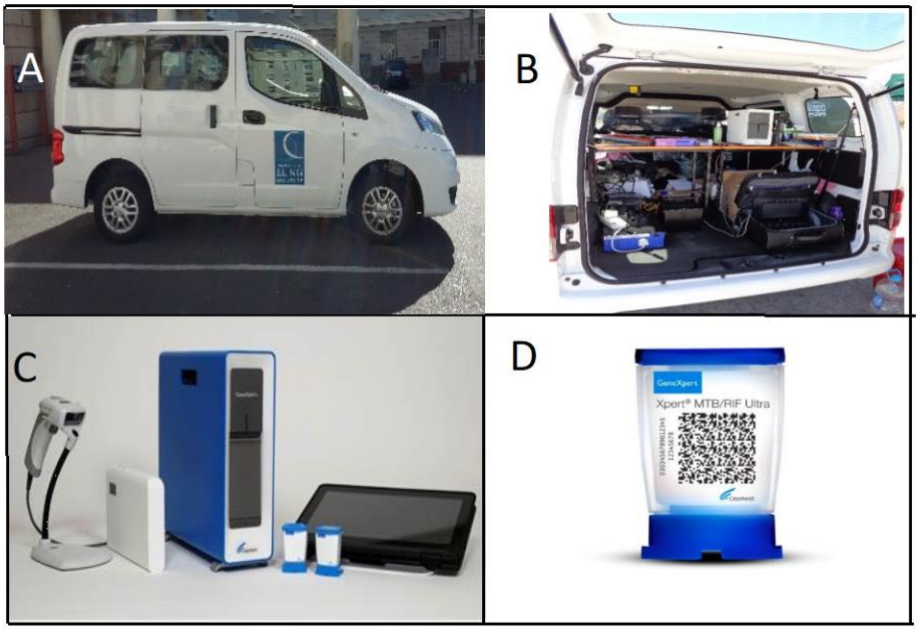

Figure S2. Illustration of the XACT active case finding model. Panel A: An exemplar of a low-cost panel van (less than USD 13,000) that may be staffed with two minimally trained health care workers for conducting active case finding and contact tracing for drug-resist tuberculosis; Panel B: Typical set up of the mobile laboratory "lab-in-a-cab" showing diagnostic tools used in an active case finding strategy; Panel C: Cepheid GeneXpert Edge setup including GeneXpert Edge, Barcode scanner, Xpert Ultra cartridges, External battery pack and tablet; Panel D: GeneXpert MTB/RIF Ultra cartridge.

Chapter 4: Proof of manuscript acceptance

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